Bile acids regulate colonic epithelial barrier function: implications for pathogenesis and therapy of inflammatory bowel disease

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Bile acids regulate colonic epithelial barrier function: implications for pathogenesis and therapy of inflammatory bowel disease

A thesis presented to the Faculty of Medicine and Health Sciences, Royal College of Surgeons in Ireland, in fulfilment of the degree of Doctor of Philosophy

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List of Abbreviations

7β-HSDH 7β-hydroxysteroid dehydrogenase
A.U Arbitrary units
ABTS 2,2'-azino-bis
AKT Protein Kinase B
ANOVA Analysis of variance
AQP Aquaporin
ASBT Apical sodium-dependent bile salt transporter
ATP Adenosine triphosphate
BAM Bile acid malabsorption
BAT Brown adipose tissue
BCS Bovine Calf Serum
BLAST Basic Local Alignment Tool
BSA bovine serum albumin
CA Cholic acid
Ca2+ Calcium ion
CaCC Calcium activate chloride channels
cAMP 3'-5'-Cyclic adenosine monophosphate
CAR coxsackie adenovirus receptor
CARD15 Caspase recruitment domain-containing protein 15
CCh Carbachol
CCR6 C-C chemokine receptor type 6
CD Crohn’s disease
CDCA Chenodeoxycholic acid
CDI Clostridium difficile infection
cDNA Complimentary DNA
CFTR Cystic fibrosis transmembrane conductance regulator
cGMP 3’5’ – cyclic mono-phosphate
Cl- Chloride ion
Cys Cystine
DCA Deoxycholic acid
DMEM Dulbecco’s Modified Eagle’s Medium
DMSO Dimethylsulphoxide
DNA Deoxyribonucleic acid
DNase Deoxyribonuclease
dNTP Deoxyribonucleotide triphosphate
DSS Dextran Sodium Sulfate
DTT Dithiothreitol
E Eosin Y
ECL enhanced chemiluminescence
EGF Epidermal growth factor
EGFR Epidermal Growth Factor Receptor
EHC Enterohepatic circulation
ELISA Enzyme-Linked Immunosorbent Assay
ENaC Epithelial sodium channel
ER Endoplasmic reticulum
ERK Extracellular signal-regulated kinases
FBS Fetal Bovine Serum
FGF19 Fibroblast growth factor intestine
FMT Faecal Microbiota Transplantation
FSK Forskolin
FXR Farnesoid X receptor
G Conductance
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GIT Gastrointestinal tract
GLP-2 glucagon-like-peptide-2
GPCR G-protein coupled receptor
GR Glucocorticoid Receptor
GW40643-[2-[2-Chloro-4-[[3-(2,6-dichlorophenyl)-5-(1-methylethyl)-4-isoxazolyl]methoxy]phenyl]ethenyl]benzoic acid
H Hemalum
<table>
<thead>
<tr>
<th>Term</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>HCl</td>
<td>Hydrochloric Acid</td>
</tr>
<tr>
<td>HNP</td>
<td>Human neutrophils peptide</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>HβD</td>
<td>Human beta defensins</td>
</tr>
<tr>
<td>IBD</td>
<td>Inflammatory bowel disease</td>
</tr>
<tr>
<td>IFNγ</td>
<td>Interferon-γ</td>
</tr>
<tr>
<td>IKK</td>
<td>IkB Kinase</td>
</tr>
<tr>
<td>IL</td>
<td>Interculin</td>
</tr>
<tr>
<td>IL-1R</td>
<td>Interleukin-1 receptor</td>
</tr>
<tr>
<td>INT77</td>
<td>6α-ethyl-23(S)-methyl-3 α,7 α,12 α -trihydroxy-5 β-cholan-24-oic acid</td>
</tr>
<tr>
<td>IP</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>IP3</td>
<td>Inositol triphosphate</td>
</tr>
<tr>
<td>Isc</td>
<td>Short circuit current</td>
</tr>
<tr>
<td>JAM-1</td>
<td>Junctional adhesion molecule 1</td>
</tr>
<tr>
<td>JNK</td>
<td>c-Jun N-terminal kinase</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>KGF</td>
<td>Keratinocyte growth factor</td>
</tr>
<tr>
<td>LBP</td>
<td>Lipopolysaccharide binding protein</td>
</tr>
<tr>
<td>LCA</td>
<td>Lithocholic acid</td>
</tr>
<tr>
<td>LDH</td>
<td>lactate dehydrogenase</td>
</tr>
<tr>
<td>LPA</td>
<td>Lysophosphatidic acid</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LRH-1</td>
<td>liver receptor homolog-1</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen-activated protein kinases</td>
</tr>
<tr>
<td>MAPKKK</td>
<td>Mitogen-activated protein kinase kinase kinase</td>
</tr>
<tr>
<td>Min</td>
<td>Minutes</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug resistance gene</td>
</tr>
<tr>
<td>MHC</td>
<td>Major histocompatibility complex</td>
</tr>
<tr>
<td>MLCK</td>
<td>Myosin light chain kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>MUC2</td>
<td>Mucin 2 gene</td>
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</table>
Na⁺ Sodium ion
Na+/K+ ATPase sodium-potassium adenosine triphosphatase
NAD⁺/NADH Nicotinamide adenine dinucleotide
NEMO NF-kappa-B essential modulator
NF–κB Nuclear factor kappa-light-chain-enhancer of activated B cells
NKCC1 Sodium-potassium co-transporter
NOD2 Nucleotide-binding oligomerization domain 2
NP40 Nonidet P40
PAGE Polyacrylamide gel electrophoresis
PAMP Pathogen-associated molecular patterns
PARP Poly (ADP-ribose) polymerase
PBC Primary Biliary Schlerosis
PBS Phosphate buffered saline
PCR Polymerase Chain Reaction
PD Potential difference
PGE2 Prostaglandin E2
PI3-K Phosphotidylinositol
PIP2 Phosphatidylinositol biphosphate
PKA Protein kinase A
PKC Protein Kinase C
PML Progressive multifocal leukoencephalopathy
PMSF Phenylmethanesulfonyl fluoride
PPARγ Peroxisome proliferative-activated receptor γ
PVDF Polyvinylidene difluoride
PXR Pregnane X receptor
q-PCR Real-time quantitative polymerase chain reaction
Raf-1 Rapidly accelerated fibrosarcoma
RNA Ribonucleic Acid
ROS Reactive Oxygen Species
RQ Relative quantification
RT Reverse transcription
RT-PCR Reverse transcriptase polymerase chain reaction
SDS Sodium dodecyl sulphate
SEM Standard error of the mean
SHP Small heterodimer partner
TAMPs Tight junction–associated marvel proteins
TB Tuberculosis
TBST Tris buffered saline with 0.1% tween
TCRS Time-controlled release system
TDCA Tourodeoxycholic acid
TEER Transepithelial Resistance
TEMED Tetramethylethylenediamine
TGF-β Transforming growth factor beta
TGR5 G protein-coupled bile acid receptor
Th T lymphocytes helper cell
TJ Tight junction
TLR Toll Like Receptor
TMB Tetramethylbenzidine
TNBS Trinitrobenzene sulfonate
TNF-α Tumor necrosis factor alpha
TUDCA Touroursodeoxycholic acid
UC Ulcerative Colitis
UDCA Ursodeoxycholic acid
VDR Vitamin D Receptor
VIP Vasoactive intestinal peptide
Vt Voltage
WGO World Gastroenterology Organization
ZO-1 Zonula occludens
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Abstract

Although inflammatory bowel disease (IBD) represents a significant health and economic burden to Western society, current therapeutic approaches often lack efficacy and can have severe side effects. While much progress has been made in the past 2 decades towards understanding its pathogenesis and diagnosis, there is still a significant need for new, safe and targeted therapies to treat IBD. Bile acids are classically known for their roles in digestion and absorption of dietary lipids but, more recently, they have become recognised as hormones that regulate many aspects of intestinal physiology. In particular, while secondary bile acids, such as deoxycholic acid (DCA), have been implicated in pathogenesis of IBD, the cytoprotective and anti-inflammatory effects of ursodeoxycholic acid (UDCA) suggest it may be of therapeutic benefit.

Mucosal wound healing and secretion of inflammation-inducing human β-defensins (HβDs) are important aspects of intestinal barrier function that are dysregulated in IBD. This thesis set out to investigate the effects of DCA and UDCA on these processes. Our studies show that DCA stimulates HβD release from colonic epithelial cells and human colonic tissues in vitro. Moreover, we found DCA to inhibit restitution in a model of wounded colonic epithelial cells. In contrast, UDCA attenuates DCA-induced effects on HβDs and restitution and exerts protective effects in a chemically-induced model of mucosal inflammation. Taken together, these data suggest that high concentrations of colonic DCA are likely to dysregulate barrier function and contribute to disease progression in IBD, while UDCA appears to have therapeutic properties. Data presented in this thesis contribute to our understanding of the importance of bile acids in regulating intestinal barrier function, provide new insights into the molecular pathways involved, and may lead to the identification of new therapeutic targets for effective treatment of patients suffering from IBD.
Acknowledgments

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Finally, I want to express my deepest gratitude to the wonderful Daniel, for believing in me and my abilities every day. Your constant support, understanding and unconditional love made this possible, for which I am truly and forever grateful.

Thank you / Dziękuje.
“Words... They're innocent, neutral, precise, standing for this, describing that, meaning the other, so if you look after them you can build bridges across incomprehension and chaos. But when they get their corners knocked off, they're no good any more... I don't think writers are sacred, but words are. They deserve respect. If you get the right ones in the right order, you can nudge the world a little or make a poem which children will speak for you when you're dead.”

— Tom Stoppard, The Real Thing: A Play

This thesis is dedicated to my parents,

Dysertację dedykuję moim ukochanym rodzicom,

Kazimiera and Czesław Łajczak
Publications, awards and communications

Publications:

1. Natalia K. Lajczak¹, Vinciane Saint-Criq¹, Magdalena S. Mroz¹, Alessia Perino², Frank Murray¹, Kristina Schoonjans² and Stephen J Keely¹ – “Ursodeoxycholic acid regulates Human β-defensin-1 and 2 secretion in colonic epithelium” (manuscript in preparation)


3. Joseph B.J. Ward¹, Orlaith B. Kelly¹, Natalia K. Lajczak¹, Aoife M. O’Dwyer, Murtaza Tambuwala², Carolina Colliva³, Silvia Spinozzi³, Joan Ní Gabhann⁴, Caroline Jefferies⁴, Aldo Roda³, and Stephen J. Keely¹ – “The secondary bile acids, ursodeoxycholic acid and lithocholic acid inhibit inflammatory responses in vitro and in vivo; implications for treatment of ulcerative colitis” (Submitted to Gut)

4. Raphael Rapetti-Mauss², Viviana Bustos¹, Aine Nolan¹, Natalia K. Lajczak¹, Warren Thomas¹, Brian J. Harvey¹ – “Beta-Catenin Regulates KCNQ1 Potassium Channel Expression in Colon Cancer Cells” (Submitted to Gastroenterology)

5. Vinciane Saint-Criq¹, Natalia K. Lajczak¹, Raphaël Rapetti-Mauss¹, Brian J. Harvey¹ – “Autophagy-independent role of LC3B in bronchial epithelial wound healing” (Submitted to BBA Mol Cell Research).

Published abstracts:


therapy of IBD.” The FASEB Journal 29.1 Supplement (2015): 854-13 (Lajczak et al., 2015b)


Awards:

- The recipient of The Meritorious Research Award from The American Physiological Society – Epithelial Transport Group. Experimental Biology, Boston, Massachusetts, USA, April 2015
- The winner of the Pfizer award at Main Physiological Society Meeting, London, UK, July 2014
- First Runner up for Donegan Medal at Royal Academy of Medicine in Ireland, Annual Meeting, Dublin, Ireland, June 2014
- The winner of the Early Investigator Award at the 6th Irish Epithelial Physiology Group Meeting in Kilkenny, Ireland, October 2013
- 3rd Prize Poster award winner at the IBD meeting, Falk Foundation, Stuttgart, Germany, June 2013

Travel Awards:

- Physiological Society Travel award to attend Experimental Biology 2015, Boston, Massachusetts, USA.
- Physiological Society Travel award to attend Physiological Society 2014, London.
- Physiological Society Travel award to attend International Union of Physiological Sciences (IUPS), July 2013, Birmingham, UK.
- United European Gastroenterology Federation Travel Award to attend Human tissue in Gastroenterology research workshop, July 2013, London, UK.
• Physiological Society Travel award to attend Physiological Edinburg (2012)

Oral Communications:

• Main Physiological Society Meeting, July 2015, Cardiff, UK. “Bile acids regulate colonic epithelial defensin secretion: implications for pathogenesis and therapy of inflammatory bowel disease”

• Experimental Biology Meeting, April 2015, Boston, Massachusetts, USA. “Bile acids regulate colonic epithelial defensin secretion: implications for pathogenesis and therapy of inflammatory bowel disease”


• Royal Academy of Medicine in Ireland Annual Meeting, June 2014, Dublin, Ireland. “Protective action of Ursodeoxycholic acids in colonic epithelia”

• Irish Epithelial Physiology Group Meeting, October 2013, Kilkenny, Ireland. “Targeting bile acids to treat inflammatory Bowel Disease”

Poster Communications:

National:


• Irish Society of Immunology (ISI), September 2013, Dublin. “Bile acids regulate innate barrier functions of colonic epithelial cells”. Natalia K. Lajczak, V. Saint-Criq, M. S. Mroz, S. J. Keely


International:

• Experimental Biology, April 2015 Boston, Massachusetts, USA. “Bile acids regulate colonic epithelial defensin secretion: implications for pathogenesis and therapy of inflammatory bowel disease.” Natalia K. Lajczak, V. Saint-Criq, M. S. Mroz, S. J. Keely

• Falk Foundation: Bile Acid Meeting, October 2014, Freiburg, Germany “Protective and anti-inflammatory action of Ursodeoxycholic acids in colonic epithelia”. Natalia K. Lajczak, V. Saint-Criq, M. S. Mroz, S. J. Keely


• International Union of Physiological Sciences (IUPS), July 2013, Birmingham, UK. “Bile acids regulate innate barrier functions of colonic epithelial cells” Natalia K. Lajczak, V. Saint-Criq, M. S. Mroz, S. J. Keely

• UEG, Human tissue in Gastroenterology research, July 2013, London, UK “Bile acids regulate innate barrier functions of colonic epithelial cells”. Natalia K. Lajczak, V. Saint-Criq, M. S. Mroz, S. J. Keely


Statement of purpose

Inflammatory Bowel Disease (IBD) is an umbrella term encompassing two main conditions: Ulcerative Colitis (UC) and Crohn’s Disease (CD), which are characterised by relapsing and remitting inflammation of the gastrointestinal tract (GIT). Symptoms of IBD include severe abdominal pain, diarrhoea, rectal bleeding, weight loss, vomiting and fever (Molodecky et al., 2012). IBD affects around 25,000 people in Ireland and 2.2 million people in Europe (Dawn, 2011). Together with rapidly increasing incidence, the cost of IBD treatment has also escalated, exceeding €5 billion per annum in Europe alone (Ghosh et al., 2014, Burisch et al., 2013). Furthermore, IBD has a wide range of associated complications, including colon cancer, bowel obstruction, ulcers, fistulas, malnutrition, arthritis, kidney stones, gallstones and osteoporosis (Rothfuss et al., 2006, Vatn, 2009). Current therapies include the use of anti-inflammatory and immunosuppressant drugs, antibiotics and, in many cases, surgical intervention (Carter et al., 2004). Despite an increased understanding of the pathways involved in the pathophysiology of IBD and the wider availability of targeted drugs, such as anti-TNF and anti-integrin antibodies, there remains a distinct lack of safe, specific and effective therapies. It is now accepted that a primary underlying factor in the pathogenesis of IBD is loss of mucosal barrier function, and consequently the potential for targeting dysregulated barrier function in disease treatment is receiving increased interest. In recent years significant advances have been made in increasing our understanding of the importance of bile acids as hormones that regulate many aspects of intestinal physiology, including barrier function. Therefore, with this in mind, the purpose of the work carried out in this thesis was to elucidate the effects of naturally-occurring bile acids on colonic epithelial barrier function and to investigate their potential as targets for the development of new approaches to treat IBD.
CHAPTER 1
General Introduction
1.1 Inflammatory Bowel Disease

IBD is characterised by uncontrolled chronic, and recurring, gastrointestinal inflammation. The symptoms of IBD vary, depending on the severity of inflammation and in which part of the GIT it occurs. The World Gastroenterology Organization (WGO) has identified the following symptoms which are associated with inflammatory damage of the digestive tract: abdominal cramping and pain, irregular bowel habits (diarrhoea and constipation), weight loss, fever, rectal bleeding, and growth retardation in children (Achleitner et al., 2012). The peak age of onset for IBD is 15 to 30 years old, with a second smaller peak occurring in individuals aged 50 to 70 years (Tsuda et al., 1988). The prevalence of IBD in Ireland is approximately 25,000, with an estimated 2.2 million people living with the disease throughout Europe (Figure 1.1) (Melgar et al., 2005, Molodecky et al., 2012). There is currently a rapid increase in disease prevalence occurring in Western societies. The incidence of UC and CD has increased in Europe from 6.0 per 100,000 person/year for UC and 1.0 per 100,000 person/year for CD in 1962, to 9.8 per 100,000 person/year and 6.3 per 100,000 person/year in 2010, respectively (Burisch et al., 2014). Closer analysis of racial and ethnic groups indicates that higher rates of IBD occur in people of Caucasian and Ashkenazic Jewish origin than in individuals from other ethnic groups (Economou et al., 2004). The incidence of IBD is also noted to be higher among more wealthy socio-economic populations and is associated with increased industrialisation (Bernstein et al., 1999). It has been hypothesised that IBD may also be diet-related, with high saturated fat and low fibre intake causing changes in the intestinal microbiota, leading to an imbalance in gastrointestinal homeostasis (Tabata, 2011).

IBD is closely associated with several diseases and co-morbidities including, mouth ulcers, kidney stones, gallstones, ocular inflammation, slow healing of wounds, and in extreme cases, gangrene. IBD is also closely associated with a chronic autoimmune condition called primary sclerosing cholangitis (PSC) which may lead to cirrhosis (Zeng et al., 2010). Patients with IBD are also at increased risk of developing colorectal cancer (Claessen et al., 2009, Bansal and Sonnenberg, 1996, Jurjus et al., 2015). It has also been reported that the severity and duration of IBD is strongly correlated with the risk of colorectal cancer (Eaden et al., 2000, Claessen et al., 2009).
IBD not only affects patients physically but it also has a large impact on psychological performance. Due to chronic pain, diarrhoea and rectal bleeding, IBD has been shown to affect the ability of patients to work; hence, causing patients to take increased periods of sick leave from the workplace. There have been studies showing strong associations between IBD and increased anxiety, depression and low self-esteem (Greenley et al., 2010, Mackner et al., 2004, Reed-Knight et al., 2014). Furthermore, there are links showing decreased fertility in IBD patients, highlighting the broad spectrum of impact that IBD can have on a patients quality of life (Mahadevan, 2006, Dejaco et al., 2001).

Figure 1.1 Incidence of IBD in European adult patients. Wide differences regarding frequency of IBD have been reported in Europe. In this figure, incidence is shown as the number of cases per 100 000 population/year, over a 3 year period. Figure sourced from (Melgar et al., 2005, Vongsa et al., 2009).
1.1.1 Ulcerative Colitis vs. Crohn’s disease

Clinical features of IBD include architectural distortions, lesions, and tissue inflammation. Based on these parameters, IBD is divided into 2 broad groups - CD and UC (Tontini et al., 2014). Although they share many similarities, there are also key differences between the 2 diseases as summarised in Table 1.1.

Table 1.1 Clinical comparisons between CD and UC. The diagnostic hallmarks, used by clinicians to differentially diagnose idiotypes of IBD (Sartor, 1995, Ponder and Long, 2013).

<table>
<thead>
<tr>
<th>Diagnostic Hallmarks</th>
<th>Crohn’s Disease</th>
<th>Ulcerative Colitis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Location</td>
<td>Inflammation may occur anywhere along the digestive tract</td>
<td>Colon and rectum</td>
</tr>
<tr>
<td>Pattern of Inflammation</td>
<td>Inflammation may occur in patches</td>
<td>Inflammation is continuous throughout affected areas</td>
</tr>
<tr>
<td>Pain</td>
<td>Pain is commonly experienced in the lower right abdomen</td>
<td>Pain is common in the lower left part of the abdomen</td>
</tr>
<tr>
<td>Appearance</td>
<td>Colon wall may be thickened and may have a rocky appearance. Ulcers along the digestive tract are deep and may extend through all layers of the wall</td>
<td>Colon wall is thinner and shows continuous inflammation. Ulcers may be present but they do not extend beyond the mucosa</td>
</tr>
<tr>
<td>Bleeding</td>
<td>Rare</td>
<td>Bleeding from the rectum during bowel movements</td>
</tr>
</tbody>
</table>
1.1.2 Pathogenesis of IBD

Although the pathogenesis of IBD is still poorly understood, it is clear that genetic traits, environmental influences, and immune-regulatory factors are all involved. Alterations in epithelial barrier function and the microbiome, together with changes in the intestinal innate and adaptive immune systems are all recognised to be critical factors in the development of IBD as represented in Figure 1.2 (Sartor, 2006).

![Figure 1.2. Interaction of a range of factors contributing to chronic intestinal inflammation.](image)

Genetic susceptibility is influenced by the luminal microbiota, which provides antigens and adjuvants that stimulate either pathogenic or protective immune responses. Environmental triggers are necessary to initiate or reactivate disease expression (Sartor, 2006).

1.1.2.1 Environmental and genetic factors in IBD

It is suspected that “westernisation” of lifestyle, such as smoking, excessive dietary fat intake, pollution, and increasing use of industrial chemicals, such as food preservatives, might have an influence in the increasing incidence of IBD cases worldwide. Improvement of personal hygiene and overuse of antibiotics has given rise to the hypothesis that increased frequency of IBD could also be credited to a lack of exposure to enteric pathogens in childhood (Bernstein et al., 1999, Bernstein and Shanahan, 2008, Shanahan and Bernstein, 2009), leading to
inappropriate immunological responses when exposed to antigens in adulthood. Risk factors such as smoking (Birrenbach and Bocker, 2004), oral contraceptive use (Godet et al., 1995), appendectomy (Koutoubakis et al., 2002), diet (Wild et al., 2007), and stress (Lerebours et al., 2007) are all implicated in IBD development. However, none of these risk factors have been fully validated by sufficient meta-analysis data. Interestingly, smoking has been shown to have a paradoxical relationship in IBD development as it is negatively associated with UC but positively associated with CD (Semlali et al., 2012). Potential mechanisms involved in these correlations include changes in gut motility and permeability, blood flow, cellular immunity, cytokine and eicosanoid levels, colonic mucus, and reactive oxygen species (ROS) (Gyires et al., 2013).

Multiple studies have demonstrated that genetic factors play a significant role in the development of IBD, with increased prevalence of disease being noted in first and second-degree relatives of patients (Hanauer, 2006). However, the presence of a mutated gene does not guarantee the development of IBD, as evidenced by studies showing that the disease is not inherited as a Mendelian trait but rather through a complex interplay of several contributing genes (Hugot et al., 2001).

Studies utilising genome wide microsatellite DNA markers isolated multiple genetic sites, which are presumed to be correlated with CD and UC pathogenesis (Mesbah-Uddin et al., 2015). One of the clearest links was located on chromosome 16 and thus led to the identification of the nucleotide-binding oligomerization domain 2 (NOD2) gene and its involvement in susceptibility to IBD (Wallace et al., 2014). The protein product of this gene, NOD2, is also known as a caspase recruitment domain-containing protein 15 (CARD15) and it has been implicated in the regulation of innate immune responses (Philpott et al., 2014). NOD2 is a conserved intracellular pattern recognition receptor which recognizes molecules containing a specific structure found in bacteria. One of the host-signalling pathways involving NOD2 is the activation of the transcription factor kappa-light-chain enhancer of activated B cells (NF-κB), a transcription factor with important roles to play in inflammatory responses (Ahmed et al., 2014a). NF-κB activation induces the release of pro-inflammatory cytokines, chemokines and growth factors, which consequently leads to tissue destruction (Ahmed et al., 2014b). A point mutation in the NOD2 gene attenuates macrophage-induced activation of NF-κB leading to altered innate immune responses. Mutated NOD2
fails to clear Salmonella from infected epithelial cells, where clearance of invasive bacteria is dependent on NF-κB activation of cell-death regulatory proteins (Peterson and Artis, 2014, Caruso et al., 2014). It is also possible that defective NOD2 can result in increased luminal bacterial populations, particularly within the colonic crypts (Strober et al., 2014). In turn, this can lead to increased susceptibility to infection and/or prevent the development of tolerance to commensal bacteria (Round and Mazmanian, 2009). An attenuated NOD2 expression in colonic epithelial cells also leads to a decreased production of chemokines, with an associated loss of innate barrier function (Eckmann et al., 1997, Bonen et al., 2003, Netea et al., 2005).

Another mutation associated with IBD is found in the multidrug resistance gene (MDR1) which encodes P-glycoprotein 170, a transporter which governs the efflux of drugs and possibly xenobiotic compounds from the cells. It can also function as an exchanger of amphipathic substrates from the inner to the outer leaf of the cell membrane. MDR1 variants have been associated with UC and CD (Hampe et al., 2007, Brant et al., 2003). MDR1 is of particular interest, as it has been associated with treatment-refractory IBD and the importance of this protein is illustrated in the MDR1−/− mouse model, where animals spontaneously develop colitis (Panwala et al., 1998, Wilk et al., 2005).

Finally, rare peroxisome proliferative-activated receptor γ (PPARγ) polymorphisms have also been found to be associated with development of CD in humans (Dubuquoy et al., 2006). PPARγ is a nuclear receptor known for its ability to target and inhibit activation of NF-κB. In patients with active UC, levels of PPARγ have been found to be significantly decreased, highlighting the importance of the balance between activation and repression of NF-κB in development of intestinal inflammation (Aloi et al., 2014, Dubuquoy et al., 2003). Further studies support an important role for PPARγ in protecting against intestinal inflammation since treatment with a PPARγ ligand, rosiglitazone, has been shown to be effective in ameliorating intestinal inflammation in experimental mouse colitis models and in clinical trials involving UC patients (Dubuquoy et al., 2006, Dubuquoy et al., 2003).

1.1.2.2 Immune response and IBD

The mucus layer which overlies the colonic epithelium provides an environment for the development of a symbiotic relationship between commensal bacteria and the
host. These interactions are characterised by down-regulation of inflammatory genes and inhibition of the NF-κB signalling pathway, thus, attenuating immunological responses to microbes and food antigens which are continuously present in the GIT (Johansson et al., 2011). In IBD, loss of this tolerance is evident, leading to prolonged and destructive immune responses towards commensal microflora. Indeed, the microflora itself has been shown to be a key factor in the development of spontaneous colitis in the interleukin (IL)-10−/− mouse model (Sellon et al., 1998), with further studies showing that IL-10−/− mice maintained in a germ-free environment do not develop colitis (Kitajima et al., 2001, Kobayashi et al., 2014).

Several studies have shown that IBD is associated with alterations in the colonic microbiome. For example, mucosal inflammation in IBD is associated with a loss of normal anaerobic bacteria, introducing an imbalance in the normal microbiome population (Ott et al., 2004). Dysregulated microbiome affects the mucosal innate immune response and also alters the synthesis of colonic bile acids. Furthermore, colonic biopsies from CD patients contain decreased numbers of Lactobacillus and Bifidobacteria species, whereas the mucosa and intraepithelial layer contain increased populations of commensal but not harmful opportunistic pathogens (Wills et al., 2014, Hansen et al., 2012, Sokol et al., 2009). In order to study the abundance of bacterial species present in the colon of IBD patients, faecal samples were collected and the numbers of bacterial phyla were identified by genomic sequence screening. Regardless of disease state, bacteria from the Firmicutes, Bacteroidetes, Proteobacteria, and Actinobacteria phyla represent the vast majority of sequences identified (Morgan et al., 2012). Sequences representative of the Bacteroidetes and Lachnospiraceae, a Firmicutes species that includes Clostridium, were greatly depleted in samples from IBD patients, whereas Actinobacteria and Proteobacteria were substantially more abundant in IBD patients than in controls. These changes did not correlate with treatments that the patients had received (Artemov et al., 1990, Greenblum et al., 2012, Duboc et al., 2013). A reduction in the diversity of mucosa-associated bacteria in patients with IBD supports the hypothesis that an imbalance between potentially beneficial and pathogenic bacteria may contribute to disease pathogenesis (Balistreri et al., 1990, Lozupone et al., 2012) (Figure 1.3).
Defective epithelial integrity and immune responses in IBD

In addition to dysbiosis of the colonic microbiome, it is also clear that dysregulated host defence, including altered epithelial barrier function and immune cell activation, has a crucial role to play in the pathogenesis of IBD. While epithelial barrier integrity and function have been well-documented to be involved in the pathogenesis of IBD (Balistreri et al., 1993, Ivanov et al., 2010, Chichlowski and Hale, 2008), the cellular and molecular pathways involved are complex and still poorly defined. It is important to enhance our understanding of these mechanisms if we are to develop new targets for IBD therapy that act by promoting epithelial barrier function.

During the early immune response, neutrophils migrate from the circulation through the vascular endothelium to penetrate the mucosa. Here, they release a variety of pro-inflammatory mediators, including antimicrobial peptides, cytokines, and reactive oxygen species which target pathogens but can also cause extensive tissue damage (Luster et al., 2005). Additionally, neutrophils activate other...
immune cells, such as macrophages which, through the production of cytokines, such as IL-12 and IL-10, can induce varying patterns of T cell activation (Hanauer, 2006). Different mediators are involved in the inductive and effector phases of mucosal immune responses, which eventually acquire distinctive T helper (Th) type 1, Th2, Th17, or alternative profiles (Wynn, 2005, Iwakura and Ishigame, 2006). Evidence indicates that CD is a Th1/Th17-like condition, whereas UC appears to represent an atypical Th2 condition (Fuss et al., 2004, Annunziato et al., 2007). These conclusions are largely based on T-cell cytokine profiles from patients with long-standing disease and numerous animal models of IBD (Strober et al., 2002).

Increasing evidence suggests that the immune system also modulates epithelial tight junctions (TJs) and intestinal permeability in IBD. Affected patients have increased mucosal concentrations of pro-inflammatory cytokines, such as tumour necrosis factor-α (TNF-α), interferon-γ (IFN-γ), IL-8, IL-6, IL-15, IL-23 and IL-16 (Seegert et al., 2001, Leon et al., 2009, Dionne et al., 1997). In vitro studies demonstrate that these cytokines attenuate the barrier function of intestinal epithelial monolayers and induce reorganisation of several TJ-associated proteins, including zonula occludens-1 (ZO-1), junctional adhesion molecule 1 (JAM-1), occludin, and claudins (Naydenov et al., 2013, Bruewer et al., 2003, Musch et al., 2006). It appears that IL-1β and TNF-α increase intestinal permeability through the induction of myosin light chain kinase (MLCK) gene transcription and consequent increases in its activity (Al-Sadi et al., 2008). Such increases in intestinal permeability lead to increased paracellular uptake of luminal antigens, bacteria and the perpetuation of the inflammatory response (Brun et al., 2007, Hardin et al., 2000). This idea is also supported by studies in mouse models where it has been shown that there is a pre-disposition to severe intestinal inflammation in regions of the intestine where permeability is compromised (Eckmann et al., 1997, Bischoff et al., 2014).

1.1.2.4 Current therapies for IBD

Existing treatments for IBD include pharmaceuticals, such as aminosalicylates, corticosteroids, immunomodulators, anti-TNF-α therapy, anti-adhesion medications, antibiotics, complementary therapies, investigational treatments, and surgery. Aminosalicylates reduce leucocyte migration, activation of NF-κB, IL-1 synthesis, degradation of prostaglandins and act as TNF-α antagonists (Nielsen
and Munck, 2007). These drugs reduce apoptosis, induce heat shock proteins, and reduce major histocompatibility complex (MHC) class II expression in the intestinal epithelium resulting in decreased immune activation. Corticosteroids activate intracellular glucocorticoid receptors resulting in upregulation of the expression of anti-inflammatory genes or repression of pro-inflammatory proteins (Triantafillidis et al., 2011). The main drawback of steroid therapy lies in the multitude of side effects, particularly associated with prolonged use (Pithadia and Jain, 2011). Another group of therapeutics include immunomodulatory agents, such as azathioprine, which are thought to inhibit immune activation through inhibition of T cell DNA synthesis (Armstrong et al., 2010). Their most serious side effects are related to the induction of agranulocytosis in immune and blood cells. Opportunistic infection, cholestatic hepatitis, pancreatitis, skin malignancy, and lymphoma are also problematic and these drugs require regular monitoring by blood testing for the duration of their use (Schwab et al., 2002).

Therapeutics classified as anti-TNF therapy includes infliximab and adalimumab. Therapeutic effects of infliximab can be impressive, with mucosal healing occurring in up to 70% of patients (Guo et al., 2013). Despite the high efficacy of these pharmaceuticals, serious infections can be associated with the use of anti-TNF antibodies, including tuberculosis (TB), Salmonella, cellulitis, and pneumonia. Furthermore, infliximab use has been linked to an increased risk of developing malignancies in young patients, particularly T cell lymphomas which are almost universally fatal (Hanauer et al., 2002).

Another therapeutic strategy is to remove circulating inflammatory cells from the peripheral blood by leukapheresis. However, this approach is associated with several adverse effects, such as sepsis (Eberhardson et al., 2013). Anti-adhesion drugs are designed to block inflammatory cells from migrating from the bloodstream to the mucosa, thereby preventing intestinal inflammation. However, it has been reported that 1:1000 patients develop progressive multifocal leukoencephalopathy (PML), a rare but often fatal brain infection (Ghosh and Panaccione, 2010). Recently, clinical studies have revealed that vedolizumab, a $\alpha4\beta7$ integrin antibody, which does not appear to cause PML due to its more gut specific actions, is effective in treating CD and UC (Löwenberg and D’Haens, 2013). Other therapeutic approaches, such as antibiotics, are effective only for short periods with the beneficial effects being lost upon discontinuation. Some
complementary therapies are also being developed around the use of probiotics to beneficially alter the balance between commensal and pathogenic bacteria in the colon (Borody et al., 2003, Jonkers and Stockbrugger, 2003). Such an approach has shown beneficial effects in humans by decreasing the severity of inflammation and relapse rates by more than 2 fold (Sartor, 2004).

Considering that UC and CD are chronic diseases, the therapeutic goals are not only to induce, but also to maintain remission. Unfortunately, current therapies often fail to fully satisfy these requirements. Furthermore, adverse effects of aminosalicylates and corticosteroids are common, occurring in 45% to 50% of patients (Carter et al., 2004, Mowat et al., 2011). Immunomodulators have also been reported to cause septicaemia, cardiac failure and malignancy. Furthermore, patients can also develop resistance to treatments, leading to relapses (Khanna et al., 2013).

Given that currently-available therapies often lack efficacy, which can have severe side effects, and can be very expensive, there is an urgent need for the development of new approaches with improved efficacy and safety. In this regard, in recent years we have seen a great deal of interest in the potential for targeting dysregulated epithelial barrier function. However, in order to realise such potential, a more in-depth understanding of the basic mechanisms by which alterations in epithelial barrier function contributes to the pathogenesis of mucosal inflammation is required.

1.2. Intestinal barrier function

The epithelial layer, which lines the intestinal lumen, is constantly exposed to a wide range of toxic substances and pathogens and also represents the first line of host defence against microbial antigens. It also plays an essential role in recruiting and regulating immune responses in the mucosa. In addition, a layer of mucus, composed of secretions from goblet cells, forms a crucial physical barrier between the intestinal mucosa and the luminal contents, further enhancing epithelial barrier function.

1.2.1. Structural organisation and function of the gastrointestinal tract wall

The intestinal wall is composed of 4 layers: the serosa, muscularis, submucosa, and mucosa (Figure 1.4). The serosa is the innermost layer and consists of loose
connective tissue coated with mucus to prevent frictional damage to the intestine due to contact against other tissues. Adjacent to the serosa is the muscularis externa. In the mouth and pharynx this layer is composed of striated muscle cells that aid in swallowing, while in the rest of the GIT it consists of circular and longitudinal muscle layers that aid in the movement of food throughout the tract (Reed and Wickham, 2009).

The submucosa overlies the muscularis externa and is a connective tissue that contains blood vessels, lymphatic vessels, submucosal glands and the Meissner’s plexus. Overlying the submucosa is the mucosa, which consists of the muscularis mucosa, lamina propria and the epithelium. The muscularis mucosa is a thin layer of smooth muscle that separates the mucosa from the submucosa and serves to keep the mucosal surface and underlying glands in a constant state of motion. This layer aids in the expulsion of substances from the crypts and in the absorption of nutrients from the lumen.

The lamina propria is composed of connective tissue and serves several key functions, one of which is to provide the epithelium with blood and lymphatic vessels, for delivering nutrients to, and transporting nutrients from, the lumen. The lamina propria also facilitates lymphocyte infiltration as it contains lymph nodules, in the Peyer’s patches, which play a crucial role in mucosal immunity.
1.2.2 Intestinal epithelium – structure and function

The epithelium is the outermost layer of the mucosa and it lines the entire lumen of the GIT. Epithelial cells are closely associated with each other, forming a continuous physical barrier between the lumen of the gut and the body. The colonic epithelium includes absorptive surface cells, goblet cells, undifferentiated columnar crypt cells, caveolated cells, tuft cells, Paneth cells, and M-cells (Figure 1.5a). Due to the unique organisation of these cells, the colonic epithelium has the ability to create a resistant barrier. The plasma membrane is anchored to an extracellular matrix as well as to neighbouring cells and is divided into 2 functionally distinct domains, termed the apical (luminal) and basolateral (mucosal) domains. Segregation of apical and basolateral membrane proteins into the different membrane domains is facilitated by TJ (Figure 1.5b), which prevent lateral diffusion of integral membrane proteins. This allows the distinct transport
functions of the apical and basolateral sides to be maintained and is essential for transepithelial ion and nutrient transport to occur (Niessen, 2007).

**Figure 1.5 The cells of intestinal epithelium and the apical junctional complex.**

**a)** A schematic representation of the crypt-villus axis. **b) Left panel:** transmission electron micrograph of apical junctional complexes between two villous enterocytes. The tight junction (TJ) is just below the microvilli (Mv), with the adherens junction (AJ) located below this, and the desmosomes (D) further below. **Right panel:** Freeze-fracture electron micrograph of a TJ. **Right panel:** Schematic representation of the interactions between the cytoskeleton, zonula occludens-1 (ZO-1), claudins, tight junction-associated marvel proteins (TAMPs), junctional adhesion molecule (JAM), and the coxsackie adenovirus receptor (CAR). Adapted from (Anderson and Van Itallie, 2009) and (Niessen, 2007).
The integrity of colonic epithelial barrier function is critical for the separation between body luminal contents. The quality of the separation between the two is determined by the epithelial cells themselves together with the TJs that connect them. TJs are one of the mammalian junctional complexes, which also include adherent junctions and desmosomes (Chantret et al., 1988). Epithelial TJs are dynamic structures and are subject to modulation during epithelial remodelling, wound repair and inflammation (Soler et al., 1999). Structurally, TJs are composed of 4 different types of transmembrane proteins; occludin, claudins, tricellulin, and junctional adhesion molecule, with claudins playing the most critical role in regulating barrier permeability (Anderson and Van Itallie, 2009). Other proteins that stabilise and regulate TJ permeability include ZO-1, tight junction–associated marvel proteins (TAMPs), and the coxsackie adenovirus receptor (CAR). The composition, structure, and permeability of TJs are tissue-specific and subject to close regulation by physiological and pathophysiological stimuli (Hering et al., 2012).

Gastrointestinal epithelia form a barrier between the body and the luminal environment, which not only contains nutrients but is also laden with potentially harmful micro-organisms and toxins. Thus, a crucial function of the epithelium is to allow the efficient uptake of nutrients, while excluding the passage of harmful substances and organisms into the body. The gastrointestinal barrier is often considered to have 2 components; intrinsic and extrinsic. The intrinsic barrier is composed of the epithelial cells themselves and the tight junctions that hold them together to form a physical barrier. The extrinsic barrier is comprised of a number of factors, including secreted proteins, chemokines, cytokines and mucus. These factors are not physically a part of the epithelium itself but they regulate epithelial and neighbouring cells to maintain barrier function (Verstege, 2010, Camilleri et al., 2012).
1.2.3 Mucus and the microbiome

Mucus is produced by goblet cells, and is primarily composed of the gel-forming large glycoprotein mucins, Muc2 and Muc3. The thickness of the mucus layer is thought to be determined by the secretion rate and the rate of erosion through mechanical shear and enzymatic degradation. Mucus is organised into inner and outer layers. The inner layer is dense, adherent and does not allow bacteria to penetrate, thus protecting the epithelium from pathogens. However, another important function is that it constitutes an environment that houses commensal bacteria present in the gut. This is important in maintaining a balanced microflora, which facilitates digestion. The outer layer is a dynamic compartment that is continuously degraded by luminal flora and replaced by the underlying cells (Johansson et al., 2011).

The most abundant population of commensal bacteria is found in the colon (Canny and McCormick, 2008) where the thickest layers of mucus are found (Johansson et al., 2011). Mucus not only provides an environmental niche for bacteria but also creates a micro-environment for epithelial cells and their products, such as antimicrobial peptides and growth factors. When secreted, these products are captured in the mucus and exert their effects on the epithelium or surrounding bacteria, providing protection against pathogen entry and infection. The protective function of the epithelial barrier is enhanced by intestinal trefoil factors that increase mucus viscosity and also serve to promote restitution, a process by which epithelial cells migrate to cover an injured luminal surface, thereby maintaining epithelial integrity (Buda et al., 2012).

As mucus layers are strategically positioned between the mucosa and the bacterial contents of the colon, changes in mucin structure and/or quantity could contribute to the pathogenesis of inflammatory disorders (McGuckin et al., 2009). In support of this, recent data from animal models have shown that intestinal inflammation can be initiated by molecular defects restricted to the epithelium only, where epithelial cells fail to produce adequate amounts of mucus. This occurs in patients that display normal microbial flora and normal underlying innate and adaptive immunity (Heazlewood et al., 2008).

The GIT of a normal foetus is sterile. However, during birth and rapidly thereafter, bacteria from the mother and the surrounding environment colonise the gut. In the intestine, it is estimated that there are up to 100 trillion bacterial cells, which is
tenfold the number of human cells (Ley et al., 2006). With up to $10^{12}$ bacterial cells per gram of luminal content, the colon is one of the most densely populated microbial ecosystems (Garrett et al., 2010), and bacteria make up to 60% of the dry mass of faeces (Stephen and Cummings, 1980). The intestinal microbiome appears to be necessary for healthy gut development as shown in the poor development of the colon in germ-free mice and immune deficiency states in humans. Remarkably, throughout the length of the intestinal tract there are more than 1000 different microbial species, the majority of which are found in the colon (Maynard et al., 2012). The metabolic capacity of intestinal bacteria is extremely diverse and enzymes produced by the microbiome play a central role in the enterohepatic circulation by de-conjugating compounds, such as bilirubin, bile acids, cholesterol, oestrogens, metabolites of vitamin D and multiple drugs (Bäckhed et al., 2005). The intestinal microflora also synthesises vitamin K, biotin, vitamin B12, folic acid and thiamine (LeBlanc et al., 2013). The microbiome is essential for several aspects of host biology. For example, they facilitate the metabolism of otherwise indigestible polysaccharides; they are required for the development and differentiation of the host intestinal epithelium and immune system; they confer protection against invasion by opportunistic pathogens; and they have a key role in maintaining immune tissue homeostasis (Bäckhed et al., 2005).

The intestinal microbiota has also been shown to be important in extra-intestinal roles, in particular by influencing fat and glucose metabolism (Kau et al., 2011). Furthermore, imbalances in the microbiota have been shown to have an ability to alter mood and behaviour (Nicholson et al., 2012, Cryan and O'Mahony, 2011). Mice colonised with intestinal bacteria had reduced anxiety-like behaviour when compared to germ-free mice, supporting the hypothesis of the brain-gut axis relationship (Nishino et al., 2013).

1.2.3.1 The mucus in IBD

Differences in mucus producing colonic cells, goblet cell numbers and secreted mucus represent important variations between the 2 IBD subtypes. In CD, there is typically an increase in goblet cell numbers and a thicker mucus layer, whereas in UC there is a reduction in goblet cells, decreases in MUC2 production and sulphation and a reduction in secreted mucus (Dvorak et al., 1980, Hanski et al., 1999). Previous studies have shown that genetic mutations in the mucin gene can
lead to increased accumulation of abnormal protein in the endoplasmic reticulum (ER), which, in turn, leads to ER stress. ER stress-related mucin depletion could be a fundamental component in the pathogenesis of intestinal inflammation (Heazlewood et al., 2008). While mucin gene expression in UC does not appear to be significantly altered compared to controls, major changes in protein production, involving accumulation of the MUC2 precursor in the ER of both inflamed and non-inflamed tissue from UC patients, have been described (Heazlewood et al., 2008). In CD, ileal mucin gene expression abnormalities have also been reported (Shirazi et al., 2000). Atypical structures of colonic mucins have been discovered in UC patients, which may affect their function as a protective barrier, for example, decreased sulphation of mucins, most likely due to altered faecal bacteria populations, leads to increased susceptibility of the mucus layer to degradation by faecal glycosidases (Corfield et al., 1996). Increased sulphatase activity has been reported in UC, particularly in the active state of the disease (Bensch et al., 1995).

Moreover, differences in the thickness of mucus layers and the degree of mucin sulphation have been reported in CD and UC patients (Barnich et al., 2005, Bodewes et al., 2012). Numerous genetic mutations associated with these changes have also been identified and described in animal models of intestinal inflammation, highlighting the importance of the epithelial barrier in protection from pathogens and other luminal contents (Boltin et al., 2013). Moreover, in Muc2−/− transgenic mice, depletion of goblet cells triggers spontaneous inflammation (van der Sluis et al., 2006). However, there is still much to be learnt about the role that mucus plays in development of IBD.

1.2.3.2 The microbiome in IBD

Recent studies have highlighted that alterations in the composition of commensal bacterial populations are linked to multiple metabolic and inflammatory diseases in humans, including: IBD (Kau et al., 2011, Hill and Artis, 2010), obesity (Musso et al., 2011), type 2 diabetes (Tremaroli and Backhed, 2012), allergy (Flint et al., 2012) and colon cancer (Sears and Garrett, 2014). For example, the composition of the microbial flora is altered in IBD, as it contains fewer commensals and a greater number of opportunistic bacteria, which is reflected in the reduced numbers of protective *Bacteroides fragilis* and increased numbers of *Enterobacteriaceae*, especially virulent *Escherichia coli* (Chassaing and Darfeuille-Michaud, 2011, Morgan et al., 2012). However, when considering the diversity of
IBD pathogenesis and disease progression, and the fact that no single pathogenic agent can routinely be isolated from diseased tissue, there is no conclusive evidence that a single pathogen is the cause of the disease, though there is mounting evidence that reduced diversity of the colonic microbiota population can contribute to IBD development (Ott et al., 2004, Tlaskalová-Hogenová et al., 2011, Duboc et al., 2013).

1.2.4 Defensins

Defensins are among the most important antimicrobial peptides of the innate immune system in humans and other mammals. They have molecular masses of 3.5 – 6 kDa and encode a family of small cationic AMPs characterised by 6 conserved cysteine residues cross-linked through disulphide bridges. Based on the arrangement of the cysteines and the disulphide-bonding pattern, these peptides are divided into 2 major subfamilies: α-defensins, β-defensins (O'Neil et al., 1999, Yang et al., 2000). In humans, most of the genes encoding α- and β-defensins are located in clusters on chromosome 8p23.1 but they also appear in other clusters on chromosome 6 and 20 (Jia et al., 2001). The disulphide linkages of cysteine residues in α-defensins are between the first and the sixth cysteine residues, i.e., Cys1-Cys6, Cys2-Cys4, and Cys3-Cys5. In contrast, in β-defensins, the linkages are Cys1-Cys5, Cys2-Cys4, Cys3-Cys6, as shown in Figure 1.6 (Klotman and Chang, 2006). Defensins are present at high levels in cells and tissues involved in host defence against microbial infections. They are an important part of innate immune responses and assist in maintaining the balance between protection from pathogens and tolerance to normal flora (Schneider et al., 2005).
Figure 1.6 Structural diversity of natural antimicrobial peptides. a) Human alpha defensin (HNP-1) with indicated bridge-forming cysteine (Cys) residues (Birrenbach and Bocker, 2004). b) Human beta defensin (HβD-1) with indicated bridge forming Cys residues (Bloomgren et al., 2012). c) The amino acid sequence alignment was performed in ClustalW. In α-defensins, sequence conservation is limited to the 6 Cys residues (disulphide bonds form between Cys 1 and 6, 2 and 4 and 3 and 5), an arginine residue and a glutamic acid residue that are known to form a salt bridge in HNP-3, and 2 glycine residues. In β-defensins, there are disulphide bonds between Cys 1 and 5, 2 and 4, and 3 and 6. The residues shown in green are more towards the amino terminal with respect to those aligned in the diagram and may or may not appear in the fully processed peptide (Klotman and Chang, 2006).
1.2.4.1 α-defensins

α-defensins, also referred to as human neutrophil peptides (HNPs), are small peptides that are found in the skin, lung epithelia, phagocytes and intestinal mucosa of all mammals. They are particularly abundant in neutrophils, certain macrophage populations, and Paneth cells of the small intestine (Ganz, 2003). HNPs1-4 are constitutively produced by neutrophils, whereas HNPs 5 and 6 are produced in Paneth cells (Klotman and Chang, 2006).

HNPs are found in azurophil granules and endoplasmic vesicles of neutrophils which upon full maturity are stored in endoplasmic vesicles, ready to be released upon stimulation. HNPs are also released from Paneth cells of the small intestine and by natural killer cells upon activation by bacterial specific-proteins, such as flagellin and lipopolysaccharide (LPS), and are likely to play a role in phagocyte-mediated host defence (de Leeuw and Lu, 2007, Lehrer and Lu, 2012). During phagocytosis, the defensin-containing granules fuse to phagocytic vacuoles where defensins exert their bactericidal activity (Ganz, 2003). α-defensins have been shown to have broad and very powerful anti-microbial, fungal and even anti-viral activity, such as the anti-HIV activity recently shown for HNP-1 (Estrada-Aguirre et al., 2014, Wang, 2013). There are indications that HNP deficiency may predispose to the development of chronic intestinal inflammation, particularly CD (Wehkamp et al., 2005).

1.2.4.2 β-defensins

To date, 31 individual β-defensin peptides have been identified across the species, of which, 4 (HβD1-4) are found in the human colon and are expressed from the DEFB coding genes (Pazgier et al., 2006). The process of HβD protein production includes post-translational modifications, in which proteolytic cleavage of the signal sequence and subsequently of the N-terminal pro-piece yields a mature and functional peptide. Before release, the mature defensin peptide is further truncated at the N-terminus, to produce multiple small peptides ready to be realised from the cell (Bals et al., 1998, Cobo and Chadee, 2013, Garcia et al., 2001a).

The first identified β-defensin was extracted from bovine tracheal mucosa and showed high antimicrobial activity. In humans, β-defensins were discovered in 1995, when extracted from blood plasma (Bensch et al., 1995). Human β-defensin-1 (HβD-1) is primarily expressed in the epithelial lining of the urinary and
respiratory tracts and the colon, and its level of expression has been shown to be moderately regulated during inflammatory responses, but only in vivo. Known stimulants of HβD-1 include LPS, heat-inactivated *Pseudomonas aeruginosa*, and interferon gamma (IFN-γ) in vitro (O'Neil et al., 1999, McCray and Bentley, 1997, Valore et al., 1998, Duits et al., 2002, Sorensen et al., 2005, Fang et al., 2003). While levels of HβD-1 in human blood can fluctuate, they normally remain within the nanomolar range (Cobo and Chadee, 2013). However, alterations in levels of HβD-1 have been shown to occur in HIV infection (Zapata et al., 2008) and in Alzheimer’s disease (Williams et al., 2013). Increased release of HβD-1 has also been associated with decreased tumour cell migration in oral carcinoma (Abiko et al., 2007). Interestingly, Wilson et al. have recently reported the possible involvement of polymorphisms in the DEFB1 gene in CD, particularly in cases where the colon is affected by inflammation and ulcers (Wilson et al., 2014).

In contrast to HβD-1, which is constitutively expressed, HβD-2, 3 and 4 mRNA levels are induced in response to a broad range of microbial agents (Garcia et al., 2001b, Ganz, 2003, García et al., 2001). HβD-2, in particular, is found in respiratory, skin and intestinal epithelial cells where it is expressed in response to bacterial pathogen-associated molecular patterns (PAMPs) and pro-inflammatory mediators, such as LPS, IL-1β, and TNF-α, indicating a function for this peptide in epithelial host defence (Diamond et al., 2001, Jang et al., 2004, Liu et al., 1998). Interestingly, in the colon, LPS does not induce an expression of HβD-2, which may reflect an adaptation of intestinal epithelial cells to the naturally high levels of LPS present in the lumen (Vora et al., 2004).

Biophysical properties of defensin molecules allow them to form dimers or octamers in aqueous solutions and to create pores in target membranes, thereby causing lysis of bacterial cells (Ganz, 2003). The activities of HβDs have been reported to be predominantly effective against gram negative bacteria, such as *Escherichia coli* and *Pseudomonas* (Dhople et al., 2006, Abiko et al., 2007), with weak or no activity against gram positive bacteria, such as *Staphylococcus aureus* and *Streptococcus pyogenes* (Dinulos et al., 2003). HβD-2 has been found to be 10-fold more potent than HβD-1 and exhibits lytic activity against *P. aeruginosa* at concentrations of 100 ng/ml (Vylkova et al., 2007, Huang et al., 2007a). HβD-4 appears to be less effective than HβD-2 against selected gram positive and gram negative bacteria and yeasts, with the exception of *P. aeruginosa*, for which it
displays greater antimicrobial activity than the other defensins. In contrast, HβD-3 has broad-spectrum activity against both gram negative and gram positive bacteria at concentrations much lower than those for other members of the defensin family (Korneva and Kokryakov, 2003).

Although they are clearly very important in mucosal protection against infection, there is still little known about the factors that regulate the expression of antimicrobial peptides in the human GIT.

1.2.4.3 Immune regulatory roles of β-defensins

In addition to their anti-microbial functions, defensins have been implicated in regulating the release of cytokines and chemokines from cells involved in innate and adaptive immune responses and can therefore participate in the pathogenesis of inflammation (Wehkamp et al., 2007a). In fact, human defensins have multiple activities relevant to inflammatory responses, including regulation of complement activation, degranulation of mast cells, induction of cell proliferation, inhibition of glucocorticoid production, blockade of LPS binding to lipopolysaccharide binding protein (LBP), chemoattraction of naïve resting T cells, CD8 T cells and immature dendritic cells, and enhancement of antigen-induced cellular and humoral immune responses (Verbanac et al., 1993, Territo et al., 1989, Zapata et al., 2008, Chen et al., 2012). HβDs can also induce prostaglandin production, activate monocytes, and induce chemokine and cytokine release by activation of TLR4 receptors (Ryan et al., 2013, Niyonsaba et al., 2006, Biragyn et al., 2002). HβDs can also have anti-apoptotic effects on polymorphonuclear leukocytes by activation of the chemokine receptor (CCR) 6 (Suarez-Carmona et al., 2015). Taken together, there is strong evidence to support the role of HβDs in driving pro-inflammatory immune responses.

1.2.4.4 Molecular pathways involved in defensin regulation

One of the primary factors regulating β-defensin production from epithelial cells, including those of the colon, is NF-κB (Tsutsumi-Ishii and Nagaoka, 2002, O'Neil et al., 1999, Tsutsumi-Ishii and Nagaoka, 2003). The complex molecular mechanisms involved in regulating the activation of NF-κB have been extensively reviewed elsewhere, and are summarised in Figure 1.7 (Wang et al., 2009). NF-κB-mediated expression of HβDs can be regulated by several pro-inflammatory
cytokines, including IL-1β, TNF-α and EGF-receptor ligands (Wehkamp et al., 2007c, Wehkamp et al., 2006, Hasegawa et al., 2011).

In addition to NF-κB, other signalling pathways, such as the mitogen-activated protein kinases (MAPK), are also involved in regulating defensin production. In particular, HβD expression has been shown to be regulated by p38 and c-Jun N-terminal kinase (JNK) MAPK pathways (Jang et al., 2004, Semlali et al., 2012, Tan et al., 2007). Both of these MAPKs can be activated in response to various stimuli, including cytokines, growth factors, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in regulating cell differentiation, apoptosis, autophagy, proliferation and inflammatory responses (Grossi et al., 2014, Schmid et al., 1987, Oltmanns et al., 2003). ERK1/2 MAPKs also participate in regulating a large variety of physiological processes, including cell adhesion, cell cycle progression, cell migration, cell survival, differentiation, metabolism and proliferation. ERK1/2 has also been found to be involved in the prevention of intestinal inflammation and its activation is necessary to induce HβD expression (Maeng et al., 2006, Moon et al., 2002). Erk1/2, p38 and JNK MAPK signalling pathways are summarised in Figure 1.8.
Figure 1.7 NF-κB signaling pathway. The canonical NF-κB activation pathway occurs in response to TNF-α, IL-1α, viral stimulation or activation of a number of receptors, such as TLRs. Intermediate kinases convey signals to the IκB complex formed by IκBα, IκBβ, IκBγ, and proteasomal degradation. p65 is processed to p50/p65, which translocates to the nucleus and binds the promoter regions of genes that regulate innate immunity and cell survival.
Figure 1.8 MAPK signalling pathways. The ERK, JNK and p38 MAPK cascades all contain a similar series of 3 kinases. A MEK kinase (MEKK) phosphorylates and activates a MAP kinase kinase (MEK), which then phosphorylates and activates the MAP kinases (MAPKs). The MAPK networks mediate cellular responses to various extracellular inputs, including growth factors, GPCRs and stress, resulting in various biological responses depending which pathways are activated. Blockade of specific MAPK pathways with pharmacological inhibitors can be useful in treating disease conditions associated with aberrant kinase signalling.
The expression and release of fully-functional defensins is a highly complex mechanism that is closely regulated during normal and inflammatory states. Dysregulation can lead to either insufficient or prolonged immune responses. Studies have shown that altered antimicrobial defence, due to inappropriate release of defensins by Paneth cells, epithelial cells, or neutrophils appears to be a critical factor in the pathogenesis of inflammatory diseases (Wehkamp et al., 2007c, Xavier and Podolsky, 2007). For example, abnormal expression of HβD-1 is associated with dysregulated immune responses in diseases such as atopic dermatitis (Mohamed et al., 2009), pulmonary tuberculosis (Wu et al., 2012), and periodontitis (Loo et al., 2012). There is also strong evidence suggesting that HβD-1 mRNA expression is decreased in patients suffering from IBD (Kocsis et al., 2008). Moreover, in about 70% of CD patients, where the disorder involves the small intestine (ileal CD), production of both HNP-5 and HNP-6 by Paneth cells is greatly decreased (Kim, 2014). There is a relative lack of Paneth cell defensins in ileal CD. This deficit is not dependent on the presence of active inflammation and results in diminished antibacterial activity (Wehkamp et al., 2007b). Such a loss of antimicrobial protection in the small intestine is linked to bacterial overgrowth and prolonged inflammation.

In contrast to HβD-1, HβD-2 is found only in trace amounts in the healthy colon but its expression is induced by inflammation and other pathogenic stimuli (Watanabe, 2013, Cobo and Chadee, 2013). Recent genome-wide DNA copy number profiling of the HβD-2 gene showed that healthy individuals had a median of 4 HβD-2 gene copies per diploid gene, whereas Crohn’s disease patients had lower gene copy numbers (≤ 3) resulting in decreased defensin expression (Fellermann et al., 2006). It is well-established that pro-inflammatory cytokines, such as TNF-α and IL-1, induce the expression of HβD-2 (Peyret-Lacombe et al., 2007). In addition to directly regulating barrier function, epithelial cell defensins also regulate the composition of the bacterial stool flora, which in return can alter innate immune responses, increase the production of pro-inflammatory cytokines, decrease epithelial permeability and aggravate inflammation in the lungs, kidneys and intestine (Nygaard et al., 1993, Bdeir et al., 2010, Vora et al., 2004, Wehkamp et al., 2009). There has been increased interest in the pattern of HβD-2 expression in healthy and inflamed colon. In normal colon, trace levels of HβD-2 expression are present in the epithelium. Inflammation increases defensin secretion, with UC
patients presenting extremely high levels of HβD-2 release (Aldhous et al., 2009, Wehkamp et al., 2002). Furthermore, there are significant differences in HβD-2 expression patterns between CD and UC patients, where HβD-2 release appears to be attenuated in inflamed tissue from CD patients but is upregulated in UC (Fahlgren et al., 2004).

In summary, defensins are not only antimicrobial peptides but they are also important regulators of mucosal barrier function and, as such, they are essential contributors to innate and adaptive immune responses. In the gut, dysregulated expression and/or alterations in defensin functions can play an important role in the pathogenesis of uncontrolled inflammation.

1.2.5 Epithelial restitution

Epithelial cells are constantly migrating from the base of the crypts, where stem cells reside, to the surface of the colon at a speed of 5 – 10 μm/h, with crypts being renewed every 2 – 5 days (Wong et al., 2010, Mizoguchi, 2012). Luminal contents, inflammatory responses, microbial interactions, and pharmaceuticals can all bring about injury to the intestinal epithelium. In turn, this may result in the increased penetration of toxic and immunogenic factors into the body leading to inflammation and uncontrolled immune responses. Thus, rapid regeneration of the epithelial barrier following damage is an essential process in preserving intestinal homeostasis (Palileo and Kaunitz, 2011).

After injury, the continuity of the epithelial surface is re-established by a very distinct mechanism. Cells alongside the injured area migrate into the wound to cover the exposed lamina propria. These migratory cells undergo dramatic molecular and cellular changes such as, remodelling of cell-cell adhesion, mediated via loss of E-cadherin and cell-matrix adhesion, together with remodelling of the actin cytoskeleton (Yilmaz and Christofori, 2010). Following migration into the damaged area, epithelial cell proliferation is necessary to replenish the decreased cell numbers. Proliferation rates vary and depend on the size of the wound inflicted. The mitotic activity of epithelial cells has been measured in in vitro rat studies and was shown to be highly efficient; with complete re-establishment of the epithelial barrier occurring within days post injury (Sorbye et al., 1988). Appropriate re-differentiation of immature epithelial cells is necessary to maintain the numerous functional activities of the epithelium. A schematic representation of the epithelial restitution process is shown in the Figure 1.9.
Some injuries penetrate deeper layers of the colonic wall and require additional repair mechanisms that involve inflammatory processes and non-epithelial cell populations. However, inflammation may interfere with cell migration and proliferation and thus modulate intestinal epithelial healing (Iizuka and Konno, 2011).

1.2.5.1 Modulators of epithelial restitution

There are a variety of extracellular regulators of epithelial restitution, including neurotransmitters, commensal and pathogenic microflora, regulatory peptides, and a broad spectrum of non-peptide factors. These factors have been recognised to modulate multiple intestinal epithelial functions that affect restitution, including cell migration, proliferation, and differentiation (Karrasch and Jobin, 2009). Growth factors such as epidermal growth factor (EGF), fibroblast growth factor (FGF), keratinocyte growth factor and insulin-like growth factors can also increase cell migration, thereby promoting epithelial restitution (Jones et al., 1999, Rowland et al., 2013). For example, epidermal growth factor receptor (EGFR) activation initiates multiple intracellular signalling cascades, such as through the release of intracellular calcium, leading to activation of phospholipase C, PKC, phosphatidylinositol-3 kinase, and mitogen-activated protein kinases, all of which

Figure 1.9 A schematic representation of epithelial restitution. Following injury, epithelial cells adjacent to the wound migrate to cover the denuded area. Epithelial cells that migrate into the wound undergo dedifferentiation, form pseudopodia-like structures, reorganize their cytoskeleton, and then re-differentiate after closure of the wound. Subsequently, epithelial cell proliferation is necessary to replenish the decreased cell pool. Finally, maturation and differentiation of undifferentiated epithelial cells is required to maintain the numerous functional activities of the epithelium.
are critical effectors involved in cell migration (Polk, 1998, Katz et al., 2007). Other modulators of epithelial restitution include adenine nucleotides and phospholipids, such as lysophosphatidic acid (LPA), which influence epithelial cell differentiation. It has been shown that epithelial wounding activates NF-κB at wound edges in intestinal epithelial cells and that blocking the activation of this pathway inhibits restitution (Egan et al., 2003b). However, activation of NF-κB does not always stimulate restitution. For example, while TNF-α activates NF-κB in intestinal epithelial cells, it does not enhance epithelial migration after injury. In contrast, IL-1β, which also activates NF-κB, does promote intestinal epithelial cell migration after injury (Dignass and Podolsky, 1993, Sturm and Dignass, 2008). Therefore, while NF-κB is important in the regulation of intestinal epithelial migration, it is not likely to be the only signalling pathway involved. MAPKs have been implicated in corneal wound healing (Terai et al., 2011), protein kinase A (PKA) and protein kinase C (PKC) signalling are also involved at different stages of endothelial wound healing processes (Shen et al., 2010).

1.2.5.2. Epithelial restitution and IBD

Epithelial and mucosal lesions associated with recurrent damage of the intestinal surface are fundamental characteristics of a number of intestinal disorders, including IBD. The importance of mucosal healing has been highlighted in a cohort study which showed that this process serves as the most significant prognostic factor for maintaining remission in IBD patients (Froslie et al., 2007). It has been demonstrated that increasing the epithelial restitution rate has a protective effect in the DSS-induced model of mucosal injury (Zeeh et al., 1996). Further, in a double-blind clinical trial, patients receiving EGF enemas showed significant decreases in disease index activity when compared to control patients (Sinha et al., 2003). This finding is substantiated by an in vivo study which showed that rectal administration of growth factors, stimulated mucosal healing and ameliorated inflammation by suppressing TNF-α gene expression in the DSS-induced murine model of colitis (Matsuura et al., 2005, Sturm and Dignass, 2008). However, it should also be considered that cytokines which promote restitution can have pleiotropic activities. For example, FGF also induces fistula formation in Crohn’s disease, which might limit their therapeutic use (Di Sabatino et al., 2004).
Taken together, these studies support the hypothesis that the development of therapeutics that modulate epithelial restitution and promote tissue regeneration holds promise for the development of new approaches to treat IBD.

1.3. Bile acids

1.3.1. Synthesis and biochemistry of bile acids

Bile acids are steroid molecules released into the GI tract which aid in food processing and digestion. The main, naturally-occurring bile acids in humans are cholic acid (CA), chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), lithocholic acid (LCA), and ursodeoxycholic acid (UDCA). The primary bile acids, CA and CDCA, are formed in the liver from cholesterol and before excretion from hepatocytes they are conjugated to the amino acids, taurine or glycine, through the carboxyl group of their side chain. Bile acids are stored in the gallbladder and upon ingestion of a meal, they are discharged into the duodenum where they are important in facilitating the digestion and absorption of lipids (Amaral et al., 2009).

Bile acids are primarily released into the small intestine in their conjugated form. Since conjugated bile acids possess both polar and non-polar regions, they act as a detergent to emulsify dietary fat droplets through the formation of mixed micelles. This significantly increases the surface area of the fat droplets, making them available for digestion by lipase, which otherwise would be unable to access the interior of the droplets. Bile acids can solubilise many lipids by forming mixed micelles with fatty acids and cholesterol for the solubilisation and absorption of fat-soluble vitamins such as, vitamin E (Werner et al., 2004). The ability of bile acids to solubilise biliary cholesterol is the most prominent mechanism of cholesterol elimination from the body and prevents cholesterol accumulation and consequently, the risk of atherosclerosis (Pullan et al., 1994, Roberts et al., 2002, Clerici et al., 1992).

The secondary bile acids, DCA and LCA, are formed by bacterial 7α-dehydroxylation of the primary bile acids in the colon. Furthermore, deconjugation of bile acids also takes place in the colon resulting in major changes in water solubility, as shown in Figure 1.10a. In all bile acids, the core ring structure is the same, however, the number and position of hydroxyl groups and the presence or absence of conjugated amino acids bring about important differences in the structure and physical properties (Bukiya et al., 2008). Subtle changes, such as
the addition of one hydroxyl group at positions 3, 7 or 12 or changes of the hydroxyl group from the α- to β- configuration may give rise to very different crystalline packing, solubility and behaviour in aqueous solutions. For example, α-hydroxy groups all lie on one side of the ring, giving bile acids their amphipathic character with the polar and non-polar faces being responsible for their fat solubilising properties (Chiang, 2004). These characteristics are highlighted in Figure 1.10b.
Figure 1.10 Bile acid biosynthesis and metabolism in humans. a) In human hepatocytes, the 2 primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), are synthesized as result of cholesterol metabolism. CA and CDCA differ in their hydroxylation at C-12. These primary bile acids are usually conjugated to glycine or taurine before they enter biliary flow. Once they reach the colon, microbes in the gut can remove the conjugated glycine or taurine and they can transform the primary bile acids into secondary bile acids via dehydroxylation at C-7. CA is transformed into deoxycholic acid (DCA) and CDCA is transformed into ursodeoxycholic acid (UDCA) and lithocholic acid (LCA). b) Primary and secondary human bile acids showing the position of their hydroxy groups, their α- to β-configuration, and their water solubility (Holm et al., 2009).
1.3.2. Enterohepatic circulation

Humans discharge between 400 – 800 ml of bile from the gallbladder per day, 67% of which is bile acids (Legido-Quigley et al., 2011). Typically, after secretion into the intestine, bile acids are efficiently reabsorbed via the apical sodium-dependent bile acid transporter (ASBT) in the terminal ileum. Reabsorbed bile acids are recycled back to the liver by the enterohepatic circulation (EHC). Normally, this process is highly efficient, with only 2 – 5% of bile acids entering the colon with each cycle of the EHC. Our knowledge of faecal bile acids is based mainly on qualitative and quantitative analysis using gas-liquid chromatography-mass spectrometry (Sjovall et al., 2010). Quantitative determination of faecal bile acid excretion provides important information about bile acid kinetics, whereas qualitative analysis provides an insight into intraluminal events involving bacteria/bile acid interactions (Samuel et al., 1968).

In general, the total faecal bile acids contained in healthy adults has been shown to be in the range of 200 – 300 mg/day, mainly in their unconjugated forms due to bacterial metabolism. Bile acids are bound to dietary residue and intestinal microorganisms, however, in the colon; passive absorption has been demonstrated, contributing significantly to the conservation of the bile acid pool. This is also demonstrated by the presence of numerous unconjugated and secondary bile acids in peripheral blood (Wan Norhana et al., 2009). Our knowledge of faecal bile acid composition in humans is mainly based on faecal samples that have passed through the whole length of the colon. However, Hamilton et al. examined the concentrations and spectrum of bile acids in the human caecum. They found that 90% of bile acids were unconjugated. The total 3-hydroxy bile acid concentration was 0.6 ± 0.3 mM, of which DCA comprised 34 ± 16%, LCA comprised 26 ± 6%, CA was 6 ± 9% and CDCA made up 7 ± 8% (Hamilton et al., 2007). The most common of the colonic bile acids is DCA, which is formed by dehydroxylation of CA, and which is normally present in the range of 100 – 200 µM (de Kok et al., 1999, Garcia et al., 2001b). Various factors can influence colonic bile acid levels, such as circadian clock, food intake, diet, and active reabsorption in the terminal ileum which is the most crucial factor in the conservation of the bile acid pool (Fiorucci et al., 2010, Hamilton et al., 2007, Westergaard, 2007).
At normal intraluminal pH levels, conjugated bile acids are present principally in their ionized form and have high water solubility. Active transport processes favour ionized conjugated bile acids and decreases in intraluminal pH can influence bile acid uptake. The intestinal microflora metabolizes the bile acids through a number of reactions, mainly by hydrolysis of the amide bond to cause deconjugation and 7α-dehydroxylation (Figure 1.11). Thus, changes in the microflora of the gut can alter the patterns of faecal bile acids, both quantitatively and qualitatively (Walters et al., 2015). Furthermore, pathological conditions that increase intestinal motility and decrease transit time can lead to the increased entry of primary bile acids into the colon, leading to rapid changes in epithelial function (Setchell, 1988).

Figure 1.11 Schematic representation of the enterohepatic circulation in humans. Bile acids are formed in the liver via cytochrome P450-mediated oxidation of cholesterol. After conjugation with glycine or taurine, bile acids are stored in the gallbladder and released into the small intestine upon food ingestion. Although, the majority of bile acids are reabsorbed in the ileum and returned to the liver via the portal vein, a small percentage of the bile acid pool leaks into the colon. Here, bacterial metabolism yields secondary bile acids via processes of deconjugation, dehydroxylation, and epimerisation. Image adapted from (Hamer et al., 2012).
In the last decade we have learnt a lot about the role of bile acids as important modulators of intestinal physiology. They can regulate many aspects of epithelial physiology such as, migration, ion transport, mucus production and cytokine secretion. The primary focus of this thesis is on the physiology and pathophysiology of bile acids the colon, particularly, DCA and UDCA, both of which are secondary bile acids which are known to have distinct actions on the epithelium and intestinal barrier function.

1.3.3. Deoxycholic acid

DCA is a secondary bile acid, produced by the bacterial dehydroxylation of CA in the intestinal lumen. This lipophilic bile acid is primarily a fat emulsifier and can be released, in its conjugated form, to glycine or taurine and at high concentrations into the intestinal lumen upon food ingestion (Martínez-Augustin and de Medina, 2008). Under pathological conditions, when increased amounts of bile acids enter the colon, they can have a profound influence on epithelial physiology. For example, studies have shown a role for DCA as a stimulant of the innate immune system, inducing IL-8 release from epithelial cells via activation of p38 MAPK and protein kinase A (Shan et al., 2013). The physiological processes regulated by bile acids include colonic epithelial secretion (Mroz and Keely, 2014), programmed cell death (Ignacio Barrasa et al., 2011) and inflammatory responses such as cytokine release (Kakiyama et al., 2014). Previous studies have also shown DCA to influence epithelial permeability, thereby increasing the passage of intestinal bacteria from the lumen to the submucosa (Stenman et al., 2013). DCA is a known modulator of epithelial death and survival pathways. DCA increases rates of autophagy, which can lead to cellular transformation and cancer development (Swidsinski et al., 2002a). At high concentrations, DCA also causes DNA damage and increases the levels of apoptosis leading to disruption of the epithelial colonic barrier (Alkarakooly et al., 2014)

1.3.4. Ursodeoxycholic acid

UDCA is a relatively hydrophilic bile acid, which is derived from CDCA in the colon by the bacterial metabolism. It can be further metabolised to LCA by dihydroxylation (Amaral et al., 2009). UDCA has many unique properties among the family of bile acids. It is been shown to be chemopreventative, acting by inducing colonic epithelial differentiation and inhibiting proliferation (Rodrigues et al., 1998, Shah et al., 2005b). UDCA also inhibits the translocation of PKC, a
pathway involved in the promotion of tumour growth and malignancy (Shah et al., 2005a). Chemopreventive actions of UDCA have also been shown in UC patients, where the bile acid decreased the risk for developing colorectal dysplasia or cancer (Kim, 2009). In addition, UDCA, and its taurine-conjugated form (TUDCA), also exert cytoprotective effects on colonic epithelial cells. The protective actions of UDCA on DCA-induced apoptosis are mediated via the modulation of EGFR/Rapidly accelerated fibrosarcoma (Raf-1)/ERK signalling pathways (Im and Martinez, 2004). In addition to its effects on cell cycle and survival, UDCA also regulates other aspects of epithelial physiology. For example, it has recently been reported that, unlike other dihydroxy bile acids, UDCA exerts anti-secretory actions on colonic epithelial cells in vitro, an effect that appears to be mediated by attenuation of sodium-potassium adenosine triphosphatase (Na⁺/K⁺ ATPase) and basolateral K⁺ channel activities. However, interestingly, when UDCA was administered to mice in vivo, enhanced colonic secretory responses occurred (Kelly, 2014). This effect was attributed to the bacterial conversion of UDCA to LCA, or some other metabolite. These data indicates that the metabolism of UDCA in the colon modulates its actions and suggests an important role for the microbiome in regulating its therapeutic effects. UDCA has also been used for centuries in traditional Chinese medicine to successfully treat a range of conditions, from cholelithiasis to epilepsy (Boatright et al., 2009, Sabariah, 2014). Furthermore, UDCA has been used in western medicine to treat primary biliary cirrhosis (PBC) (Lindor et al., 2009a).

In other studies, UDCA has also been shown to exert anti-inflammatory actions (Goto et al., 2001, Pardi et al., 2003). For example, TUDCA has been shown to inhibit TNF-α-induced IL-8 release from cultured colonic epithelial cells and to ameliorate inflammation in rat and mouse models of IBD (Martínez-Moya et al., 2013). The protective effects of TUDCA in these models are also likely to involve many effects on barrier functions as it has been found to induce the production of colonic mucins, decrease epithelial apoptosis and to prevent the bacterial translocation into the mucosa.

1.3.5. Bile acid receptors

In the past decade, there has been growing interest in the potential for targeting bile acids for the treatment of inflammatory disorders. This interest largely has been driven by the discovery of bile acid receptors. These receptors include
nuclear receptors, namely, farnesoid X receptor (FXR) and pregnane X receptor (PXR), and more recently a membrane bile acid receptor, TGR5 (Maruyama et al., 2006, Kawamata et al., 2003a, Makishima et al., 1999). Subsequent to the discovery of these proteins as bile acid receptors, a great deal has been learnt of the many roles that bile acids play as hormones which exert important paracrine and endocrine functions (Tiwari and Maiti, 2009). Bile acids are also known to activate other receptors present in the colonic epithelium. A derivative of LCA, LCA acetate, has been shown to potently bind to the Vitamin D receptor (VDR) and subsequently induce the expression of VDR target genes in intestinal cells (Adachi et al., 2005). Moreover, bile acids have been found to activate muscarinic receptors, an action which may contribute to cellular proliferation (Raufman et al., 2003a). In addition, previously described cytoprotective actions of UDCA have been shown to be mediated via the glucocorticoid receptor (GR) (Solá et al., 2005). Furthermore, UDCA have been found to activate constitutive androstane receptor (CAR) which appears to have a role in sulphonation and detoxification of dihydroxy bile acids (Halilbasic et al., 2013).

1.3.5.1. TGR5

TGR5 was identified in 2003 as a G-protein coupled receptor for bile acids (Kawamata et al., 2003a). The highest expression of TGR5 is found in the hepatocytes (Yang et al., 2007), bile duct and gallbladder epithelial cells (Keitel et al., 2009), although brown adipose tissue (Pols et al., 2011) and some regions of central nervous system and intestine also express the protein (Duboc et al., 2014). Bile acid-mediated activation of TGR5 has been reported to prevent diet-induced obesity and insulin resistance by increasing the energy expenditure in brown adipose tissue (BAT). This effect is mediated via cAMP-dependent induction of iodothyronine deiodinase 2 (D2), an enzyme that is involved in thyronine transformation (Watanabe et al., 2006). In macrophages, TGR5 has been shown to have anti-inflammatory properties by decreasing levels of pro-inflammatory cytokine release (Keitel et al., 2008). TGR5 has also been shown to be expressed in colonic epithelium, where its activation decreases basal fluid secretion, implicating TGR5 as a potential therapeutic target for intestinal transport disorders (Ward et al., 2013a). Furthermore, studies on DSS- and Trinitrobenzene sulfonate (TNBS)-induced colitis in TGR5 knockout mice studies reveal the protective effects of TGR5 (Cipriani et al., 2011). As depicted in Figure 1.12, primary and secondary bile acids are ligands for TGR5, with LCA being the most potent, followed by DCA,
CDCA, and CA. While conjugation with glycine has little influence on their TGR5 agonistic activity, the conjugation of bile acids with taurine increases their efficacy as TGR5 agonists (Kawamata et al., 2003a).

![Figure 1.12 Efficacy of various bile acids at membrane bound and nuclear receptors. Bile acids bind to and activate various membrane bound G protein-coupled receptors, including TGR5 and muscarinic receptors, and nuclear receptors: FXR, VDR and PXR, at varying efficacy.](image)

1.3.5.2. **FXR**

FXR was the first nuclear receptor for which bile acids have been identified as endogenous ligands. Its cDNA was first cloned in 1995, from mouse and rat livers (Seol et al., 1995, Forman et al., 1995). FXR belongs to the class 2 nuclear receptors which, for their activation, require heterodimer formation with the retinoid X receptor (RXR) which binds the DNA with an inverted element separated by 1 nucleotide, inverse repeat 1 (Laffitte et al., 2000, Modica and Moschetta, 2006). FXR is highly expressed in the liver, small intestine, kidney, adrenals and colon (Forman et al., 1995). Two isotypes of FXR have been identified: FXRα and FXRβ. While both FXRα and FXRβ are functionally active in rodents, only FXRα is functional in humans and FXRβ is present as a pseudogene. The most potent FXRα activator is the primary bile acid CDCA and its conjugates (Makishima et al., 1999), whereas UDCA is not efficient for FXR activation.
Bile acids can exert a negative feedback regulation on their own synthesis by inhibiting cholesterol 7α-hydroxylase activity and expression (Gupta et al., 2001). FXR exerts its actions by 2 major regulatory pathways, including the small heterodimer partner (SHP)/liver receptor homolog-1 (LRH-1) pathway in the liver and the fibroblast growth factor 19 (FGF19) pathway in both, the gallbladder and the intestine (Walters, 2014, Zweers et al., 2012).

FXR activation has been demonstrated to interfere with the NF-κB inflammatory signalling pathway, thus inhibiting the production of pro-inflammatory mediators in hepatocytes (Stojancevic et al., 2012). Moreover, FXR activation was shown to reduce intestinal inflammation in a mouse model of IBD (Gadaleta et al., 2011a), whereas pro-inflammatory cytokines repress FXR activation (Gadaleta et al., 2011b). The activation of FXR has also been linked to the modulation of inflammation and preventing gut barrier dysfunction and beneficial effects in experimental models and patients with bile acid diarrhoea (Tsuei et al., 2014, Mroz and Keely, 2014, Walters et al., 2015). In addition, FXR ligands can inhibit bacterial overgrowth and regulate the expression of antimicrobial genes in the intestine, without exerting bacteriostatic effects (Inagaki et al., 2006b).

1.3.6. Bile acids in IBD

As discussed above, IBD patients are well-established to have imbalances in gut microbiota composition. Since colonic bacteria are responsible for the formation of secondary bile acids, dysbiosis of the microbiota is closely linked to imbalances in the levels, sulphation and conjugation of colonic bile acid (Duboc et al., 2013). Additionally, imbalances in colonic bile acids can be attributed to altered reabsorption in ileum (Lenicek et al., 2011). Previous studies have shown that bile acids can have opposing actions on colonic epithelial physiology depending on their concentrations (Keating et al., 2009). Furthermore, other secondary bile acids can also have differential effects on colonic epithelium. For example, DCA induces IL-8 and activates NF-κB, while these actions are inhibited by TUDCA (Lee et al., 2004, Pardi et al., 2003, Zhang et al., 2014).

Interestingly, UDCA has been used as an anti-inflammatory agent in traditional Chinese medicine and more recently for treatment of PSC and PBC in Western medicine (Dathe et al., 2001, Hood et al., 1988, Tsuei et al., 2014). The unique properties of UDCA make it desirable for its development as a therapeutic target.
and studies are ongoing into its potential for colon cancer prevention, treatment of Alzheimer's disease (Ramalho et al., 2008), and retinitis pigmentosa (Drack et al., 2012). Numerous clinical trials are ongoing currently to assess the benefits of UDCA on glycaemic control (Clinical trial identifier: NCT01337440), Huntington's disease (Clinical trial identifier: NCT00514774), Barrett's esophagus (Clinical trial identifier: NCT00858858), and treatment of Hepatitis C (Clinical trial identifier: NCT00200343). Furthermore, UDCA is also under investigation in ongoing clinical trial for treatment of IBD in Europe (EU Clinical Trials Register Number: 2014-003141-10). UDCA has been proven to be a very safe drug, with very few side effects or adverse drug interactions.

On the basis of its previously reported cytoprotective and anti-inflammatory effects, the current studies were based on the hypothesis that UDCA is a good target for therapeutic development in patients with IBD. However, there is still little known about the effects of UDCA on various important aspects of epithelial barrier function and these gaps in our knowledge must be addressed before its therapeutic potential can be realised.
1.4. Aims of the thesis

Given that alterations in innate intestinal barrier function are important in the pathogenesis of IBD and that increased levels of bile acids are also associated with disease progression, the overall goal of this thesis is to investigate the role of bile acids in regulating the intestinal barrier function and the potential for targeting bile acids in the treatment of IBD.

More precisely, the specific aims of this thesis were to investigate:

i. the effects of DCA and UDCA on colonic epithelial HβD secretion

ii. the effects of DCA and UDCA on colonic epithelial restitution

iii. molecular mechanisms by which DCA and UDCA exert their effects on colonic barrier function.
CHAPTER 2

Materials and Methods
2.1 Cell Culture

2.1.1 Cell lines

The T84 cell line was originally isolated from a lung metastasis of a colon carcinoma in a 72 year old male patient and was injected subcutaneously into BALB/c nude mice. The subsequently developed tumour was isolated, homogenised, and cultured in growth medium (Murakami and Masui, 1980). When allowed to polarise on semi-permeable supports, T84 cells create a columnar epithelium which possesses the appearance of native crypt epithelial cells and retains many of their characteristics, including formation of an electrically-resistant monolayer and apical Cl⁻ secretion (Dharmsathaphorn et al., 1984, Madara and Dharmsathaphorn, 1985). For the current studies, T84 colonic epithelial cells were obtained from laboratory of Kim Barrett (University of California, San Diego) and cultured in DMEM Ham’s F-12 media, supplemented with 5% (w/v) bovine calf serum (BCS), 2 mM L-glutamine, 50 U/ml penicillin, and 55 µg/ml streptomycin. The conditions of cell culture are shown in Table 2.1. Cells were maintained in a humidified atmosphere, containing 5% CO₂ at 37 °C. While in culture, cells were fed every 2-3 days, and passaged every 7 days. Cell passages between 17 and 33 were used for experimentation.

The HT29Cl.19A cell line, which has been used in this thesis, shows characteristics of well-differentiated epithelial cells, and thus provides a good model for studying colonic epithelial function, such as proliferation and migration. The parental cell line HT29 is derived from a colorectal adenocarcinoma which was isolated in 1964 from a Caucasian 44 year old female. HT29Cl.19A was selected for its ability to develop transepithelial resistance (TEER) when cultured on semi-permeable supports. The HT29Cl.19A cell line was a gift kindly donated by Dr. Christian Laboisse (Augeron and Laboisse, 1984). The cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% (w/v) fetal bovine serum (FBS which was inactivated by heating for 30 mins at 56 °C), penicillin (100 U.ml⁻¹) streptomycin (100 µg.ml⁻¹) and glutamine (2 mM)).

Cells were grown on inserts (Millipore) in a humidified atmosphere containing 5% CO₂ at 37 °C until TEER reached approximately 1 KΩ/cm² (Figure 2.1). Cells were cultured in 75 cm² polystyrene culture flasks and culture media was changed every 2-3 days. Experiments were carried out using cells between passage 20 and 40.
2.1.1.1 Subculture of adherent cell lines

For subculturing T₈₄ cells were passaged by enzymatic detachment when they reached approximately 90% confluence. Following aspiration of waste medium, cells were rinsed with pre-warmed PBS. The purpose of this was to remove any naturally occurring trypsin inhibitors, which would be present in residual serum. Fresh trypsin was pipetted onto the cells (3 ml/75 cm²) and the flasks were incubated at 37 °C until the cells were observed to have detached (10 – 15 mins). The trypsin was deactivated by addition of a quadruple amount of growth medium (i.e., containing serum). The entire cell suspension was transferred to a 15 ml tube (Fisher Scientific CFT-420-075L) and centrifuged at 600 x g for 3 mins. The resulting pellet was re-suspended in pre-warmed (37 °C) fresh growth medium, cells were counted, and used at the required density to seed semi-permeable supports or stock flasks (Table 2.1).

Table 2.1 T₈₄ and HT29Cl.19A cell culture-seeding conditions. Cells were seeded at varying densities depending on the size of insert area and material; Polyester (PET), Mixed Cellulose Ester (MCE).

<table>
<thead>
<tr>
<th>Application</th>
<th>Porosity</th>
<th>Material</th>
<th>Supplier</th>
<th>Cat no</th>
<th>Area</th>
<th>Number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth and maintenance</td>
<td>T75</td>
<td>Plastic</td>
<td>Sarstedt</td>
<td>83.1813.500</td>
<td>75cm²</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>Confocal Microscopy</td>
<td>Hanging insert</td>
<td>1 µm</td>
<td>PET</td>
<td>PIRP12R48</td>
<td>0.33cm²</td>
<td>1 x 10⁵</td>
</tr>
<tr>
<td>Protein/</td>
<td>Standing insert</td>
<td>0.45 µm</td>
<td>MCE</td>
<td>PIRP3050</td>
<td>4.2cm²</td>
<td>2 x 10⁶</td>
</tr>
<tr>
<td>RNA isolation</td>
<td>Hanging insert</td>
<td>1 µm</td>
<td>PET</td>
<td>PIRP30R48</td>
<td>4.5cm²</td>
<td>2.1 x 10⁶</td>
</tr>
<tr>
<td>Wound assay</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1.1.2 Cell counting

Samples were trypsinised, transferred to a 15 ml tube, and then centrifuged at 600 x g for 3 mins. The resulting pellet was re-suspended in 1 ml of pre-warmed fresh growth media, from which 10 µl was taken and loaded onto a haemocytometer (Neubauer). Cells in the 16 squares of the 4 outer corner grids of the chamber were counted microscopically. An average number per corner was calculated with the dilution factor being taken into account and final cell number was multiplied by 10⁴ to determine the number of cells per ml. The volume of the cell suspension in the chamber was 0.1 cm x 0.1 cm x 0.1 cm, i.e., 0.0001 cm³. Therefore, the number of counted cells x 10⁴ represents the number of cells per ml.
2.1.1.3 Cell treatments

As soon as TEER had stabilised, T84 and HT29Cl.19A cell monolayers were washed bilaterally with serum-free medium and cultured in fresh serum-free medium for 12 hrs, unless stated differently. Incubation of monolayers in serum-free medium synchronises the cells in the quiescent G0 growth phase, and precludes serum constituents from binding or enzymatically cleaving the compounds used for treatments (Pirkmajer and Chibalin, 2011). The 12 hrs serum starvation period elicited an increase in basal TEER from 1014 ± 137.5 to 1426 ± 98 Ω*cm² (n = 15; p ≤ 0.05). Subsequently, cells were treated for 1 – 48 hrs in serum-free medium with compounds diluted in serum-free medium. The drugs used in the current studies, and their mechanisms of action, are listed below:

BMS-345541 is a highly selective inhibitor of IkB Kinase (IKK) and thus is an inhibitor of NF-κB activity. BMS-345541 binds to either the peptide binding site on IKK, or an allosteric site that changes the binding site, thus preventing peptide-binding (McIntyre et al., 2003).

INT-777 is a derivative of the primary human bile acid CA and is a potent and selective agonist of the TGR5 receptor (Table 2.2). Cells were treated with INT-777 at a concentration of 10 μM (Pelliacciari et al., 2009).

GW4064 is a synthetic FXR agonist, known to activate the nuclear bile acid receptor, FXR (Bass et al., 2009). Treatments were carried out at a concentration of 5 μM.

PD98059 is a potent and selective inhibitor of the ERK-specific MAP kinase MEK, and therefore prevents phosphorylation of ERK MAPKs 1/2 (p44/p42 MAPK). PD98059 was used at a concentration of 20 μM (Dejaco et al., 2001).

SB203580 (10 μM) is a specific inhibitor of p38α and p38β MAPK which suppresses downstream activation of MAPKAP kinase-2 and heat shock proteins. At relevant concentrations, it does not inhibit JNK MAPK or ERK MAPK activity (Lali et al., 2000).

CFTRinh-172 (10 μM) blocks channel opening with a Ki of 0.3 to 5 μM from an intracellular site. Its mode of action involves interaction with Arg347, which is located near the CFTR cytosolic surface. At relevant concentrations, CFTRinh-172 is specific and without effect on several other ion channels and transporters found
in epithelial tissues, including Ca\(^{2+}\)-activated and volume-regulated Cl\(^{-}\) channels and the ATP-binding cassette transporter, P-glycoprotein (Diamond et al., 2001, Dong et al., 2015b).

GlyH-101 (25 μM) is a specific and potent CFTR channel blocker. It acts in a voltage-dependent manner by occluding the channel pore from its extracellular side. GlyH-101 (10 – 50 μM) completely abolishes cAMP-stimulated short-circuit current, a measure of CFTR-mediated transepithelial electrolyte transport, in respiratory and intestinal epithelia from humans and mice.

Cell treatments were carried out for a number of different time points, depending on the processes under investigation. Assessments of MAPK activation were performed at 1hr and NF-κB activation at 1 – 6 hrs. The time dependency of bile acid effects on HβD mRNA and protein expression was examined over 1 – 48 hrs and 6 – 48 hrs, with the time points at which maximal responses were seen being chosen for subsequent studies.

Table 2.2 TGR5 and FXR potency of key bile acids, bile acids derivatives and pharmaceuticals. The potency of bile acids and pharmaceuticals activating bile acid receptors, TGR5 and FXR. Effective concentration (EC\(_{50}\)) refers to the concentration of drug which induces 50% of the maximan response (Colliva, 2013).

<table>
<thead>
<tr>
<th>Agonists</th>
<th>EC(_{50}) (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDCA</td>
<td>6.71</td>
</tr>
<tr>
<td>CA</td>
<td>13.6</td>
</tr>
<tr>
<td>DCA</td>
<td>1.25</td>
</tr>
<tr>
<td>LCA</td>
<td>0.57</td>
</tr>
<tr>
<td>OCA</td>
<td>No activity</td>
</tr>
<tr>
<td>UDCA</td>
<td>No activity</td>
</tr>
<tr>
<td>INT777</td>
<td>0.82</td>
</tr>
<tr>
<td>GW4064</td>
<td>0.065</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Naturally-occurring</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA</td>
</tr>
<tr>
<td>DCA</td>
</tr>
<tr>
<td>LCA</td>
</tr>
<tr>
<td>OCA</td>
</tr>
<tr>
<td>UDCA</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Pharmaceuticals</th>
</tr>
</thead>
<tbody>
<tr>
<td>INT777</td>
</tr>
<tr>
<td>GW4064</td>
</tr>
</tbody>
</table>
Figure 2.1 Time course of the TEER development across monolayers of T\textsubscript{84} and HT-29Cl.19A cells seeded on permeable supports. a) T\textsubscript{84} cells were cultured on 4.2 cm\textsuperscript{2} permeable standing supports and TEER was recorded until it stabilised (n = 10 – 12). b) T\textsubscript{84} cells were cultured on 4.5 cm\textsuperscript{2} permeable hanging supports and the TEER was recorded each day until it reached plateau. c) HT-29Cl.19A cells were cultured on 4.2 cm\textsuperscript{2} permeable standing supports and TEER was recorded until it stabilised (n = 8). d) HT-29Cl.19A cells were cultured on 4.5 cm\textsuperscript{2} permeable hanging supports and the TEER was recorded each day until it reached a plateau (n = 10). In each case, data are expressed as Ohms (\(\Omega\)).cm\textsuperscript{2}.
2.2 Animal models

2.2.1 TGR5 knockout mice

To investigate the role of TGR5 in vivo, targeted disruption of the receptor was performed in C57BL/6 mice kindly provided by Professor Kristina Schoonjans from Ecole Polytechnique Fédérale in Lausanne (EPFL) in Switzerland (Vassileva et al., 2006). TGR5$^{-/-}$ mice display a healthy and fertile phenotype with normal development. Light microscopic examination of tissue sections from organs, such as the heart, brain, liver, and intestine, did not reveal developmental abnormalities in TGR5$^{-/-}$ compared to wild type (WT) and TGR5 knock-in (TGR5$^{+/+}$) mice. Other cellular parameters, such as haematological indices and serum chemistries (bilirubin, low and high-density lipoproteins, triglycerides, and bile acids), were similar in knockout and WT mice, as were measurements of glucose and other metabolites in the urine (Maruyama et al., 2006). However, the liver mRNA expression of various cytochromes and proteins involved in the hepatic synthesis and transport of bile acids was higher in TGR5$^{-/-}$ than in WT mice, especially Cyp7a1, the rate-limiting enzyme in bile acid synthesis. One would expect that this would lead to an increase in bile acid synthesis. However, the bile acid pool was not increased, suggesting that other homeostatic mechanisms may be involved in the knockout animals (Vassileva et al., 2006, Maruyama et al., 2006).

2.2.2 DSS-induced Colitis mouse model

Male, 10-week-old C57BL/6 mice weighing 20 – 22 g were housed in rooms at a controlled temperature and a 12 hr day/night cycle. Animals were fed standard mice chow pellets, had access to tap water supplied in bottles ad libitum, and were acclimatised to environmental conditions for at least 5 days before they were studied in experiments. Mice were killed by cervical dislocation under isoflurane anaesthesia. Daily disease activity index (DAI) was calculated. All experiments were approved by the RCSI Ethics Committee and are in agreement with the guidelines for the proper use of animals in biomedical research. Clinical and histological scoring of colitis were performed in a blinded fashion. Animal licence numbers: B100/4453, B100/4159, Research Ethics Committee number: Ae19127/i076.
2.2.2.1 DAI score

Body weight, stool consistency, and anal bleeding were monitored daily. The DAI was assessed essentially as described by Hartmann et al. (Hartmann et al., 2000) with minor modifications by Cummins et al. (Cummins et al., 2008). Each score was determined as shown in Table 2.3 below. The presence of blood in the stool was assessed by HemDetect® (Dipromed, GmbH, Vösendor, Austria). In addition to DAI, spasmodic colon shortening, another marker of colonic inflammation, was recorded. Colons were carefully dissected and their length was measured from the ileocecal junction to the anal verge (Okayasu et al., 1990).

Table 2.3 Scoring of disease activity index. Mice were monitored daily and DAI was scored on the basis of weight loss, stool consistency and anal bleeding.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Animal ID:</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight (g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Weight loss %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No weight loss</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>1 – 3 %</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>3 – 6 %</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>6 – 9 %</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>&gt; 9%</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Stool</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Loose</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
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<td></td>
</tr>
<tr>
<td>No blood</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Blood</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Gross Bleeding</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>0 – 12</td>
<td></td>
</tr>
</tbody>
</table>
2.2.2.2 Histological scoring

For histological scoring, approximately 1 cm length of the distal colon was removed and fixed in 10% formaldehyde-saline. The immunocytochemistry was performed by H and E staining of colonic sections. All histology sections were kindly stained and examined by Dr. Murtaza Tambuwala from Saad Centre for Pharmacy and Diabetes, Ulster University, Coleraine, UK. All scoring was performed in a blinded fashion independently by 2 observers. Arbitrary histologic scoring was used to quantify colon damage. Histological damage was quantified by the histological scoring system described by Williams et al. In brief, the sections were graded to access inflammation severity, inflammation extent, and crypt damage (Williams et al., 2001). The grading index for inflammation severity was as shown in the Table 2.4.

Table 2.4 Scoring of severity of histological damage. Histological sections were scored according to the guidelines described before (Williams et al., 2001).

<table>
<thead>
<tr>
<th>Feature</th>
<th>score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Severity of inflammation</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mild</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Nmoderate</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Severe</td>
</tr>
<tr>
<td>Extent of inflammation</td>
<td>0</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Mucosa</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Mucosa and Submucosa</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Transmural</td>
</tr>
<tr>
<td>Crypt damage</td>
<td>1</td>
<td>1/3 damaged</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>2/3 damaged</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Crypts lost, surface epithelium present</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>Crypts lost and surface epithelium lost</td>
</tr>
<tr>
<td>Percentage Involvement</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1-25%</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>26-50%</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>51-75%</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>76-100%</td>
</tr>
</tbody>
</table>

Scores were calculated by multiplying the score for the first three parameters by their percentage involvement, giving a maximum score of 40.
2.3. Analytical techniques

2.3.1 Protein extraction and quantification

T84 cells were cultured for protein extraction, as described in Section 2.2.1.4. Following treatment, monolayers were washed twice in ice-cold 1 x phosphate buffered saline (PBS). Cells were then lysed in situ using Nonidet P40 (NP40) lysis buffer, containing protease and phosphatase inhibitors (Table 2.5) for 45 mins on ice. Lysed cells were then scraped from inserts. Cell lysates were centrifuged at 15,294 x g for 10 mins at 4 °C. The supernatant was retained and analysed for protein content. Quantification of protein was performed using the Lowry method (Lowry et al., 1951).

For each experiment, a standard concentration curve was prepared from bovine serum albumin (BSA) and a coefficient of determination (R²) was created. R² is a computed statistical measure, which determines the degree of linear-correlation of variables in regression analysis. An R² close to 1.0 indicates that a regression line fits the data well, while an R² closer to 0 indicates a regression line that does not fit the data. A coefficient of determination ≥ 0.97 was determined as the cut-off point. The amount of protein in the sample was calculated from the standard curve equation of the line: y = ax + b. An example of standard curve is shown in Figure 2.2.

The Lowry protein assay relies on the interaction of proteins with copper tartrate and Folin reagent. In the first step of the assay, the reaction of copper ions with peptide bonds under alkaline conditions and oxidation of aromatic protein residues occurs. The next step involves reduction of Folin reagent by copper-treated proteins. This process of reduction releases free oxygen species, which leads to colour development with optimal absorbance at 750 nm wavelength (Everette et al., 2010).
Table 2.5 Composition of NP40 lysis buffer. NP40 is a non-ionic, non-denaturing detergent used to solubilise membrane proteins. Additional inhibitors include Ethylenediaminetetraacetic acid (EDTA) phenylmethanesulfonyl fluoride (PMSF), a protease inhibitor that reacts with serine residues to inhibit trypsin, chymotrypsin, thrombin and papain, Sodium orthovanadate is an inhibitor of protein tyrosine phosphatases, alkaline phosphatases and ATPases.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonident P40</td>
<td>1%</td>
</tr>
<tr>
<td>NaCl</td>
<td>150 mM</td>
</tr>
<tr>
<td>Tris base</td>
<td>50 mM</td>
</tr>
<tr>
<td>PMSF</td>
<td>0.1 mg/ml</td>
</tr>
<tr>
<td>Complete mini EDTA free</td>
<td>1 tablet/ 10 ml</td>
</tr>
<tr>
<td>protease inhibitor tablet</td>
<td></td>
</tr>
<tr>
<td>Na₃VO₄</td>
<td>1 mM</td>
</tr>
</tbody>
</table>

Figure 2.2 An example of a standard curve for protein quantification. A standard curve was prepared using a range of concentrations of bovine serum albumin (BSA) (0 mg/ml – 4 mg/ml).
When performing the assay 5 µl of each sample (standard and protein lysate) was placed into a 96-well plate in triplicate followed by addition of 25 µl of reagent A’. A’ reagent consists of alkaline copper tartrate solution and surfactant solution in a v/v ratio of 50:1. Subsequently, 200 µl of Folin reagent was added to each well and samples were incubated for 20 mins. Absorbance was measured at 750 nm using a multiscan EX multiplate reader (Thermo Scientific) 5 µl of lysis buffer was used as a blank.

2.3.2 Electrophoresis and Western Blot

Samples, normalised for protein content were mixed with an equal volume of 2 x loading buffer (1/1, v/v) and then heated to 95 °C to aid in the protein-denaturing process. Samples were then electrophoretically resolved by SDS-PAGE using the appropriate percentage of 30 % acrylamide solution (Mini-Protean system, Bio-Rad Laboratories, Hemel Hempstead, UK) to separate proteins based on their size as indicated in Table 2.5

Separated proteins were transferred onto methanol pre-permeabilised polyvinylidene difluoride (PVDF) membranes using a semi-dry electrophoretic transfer apparatus (Trans-Blot semidry, Bio-Rad Laboratories, Hemel Hempstead, UK). The PVDF membrane was stained with Ponceau S red following transfer in order to insure proteins had transferred equally. Membranes were then blocked with appropriate blocking reagents for 1 hr with horizontal rotation prior to addition of selected primary antibodies diluted in the blocking reagents as shown in Table 2.6.

Following 2 washes in Tris buffered saline with 1% (w/v) tween (TBST), secondary HRP-linked antibodies were diluted in blocking reagent and were added for 1 hr with rotation at room temperature. Secondary antibody solutions were then removed and the membranes were washed a further 3 times. Immunoreactive proteins were detected by enhanced chemiluminescence (ECL). Table 2.7 summarises sample proteins concentrations, transfer times, blocking reagents, primary and secondary antibody concentrations used, and the optimal exposure time in ECL prime reagent.
Table 2.6 Composition of Polyacrylamide gels. Lower percentage gels were used to analyse larger proteins (180 kDa - 30 kDa) of interest and high SDS percentage gels were used for small proteins (8 kDa – 40 kDa).

<table>
<thead>
<tr>
<th>Reagent</th>
<th>10% resolving</th>
<th>20% resolving</th>
<th>5% stacking</th>
</tr>
</thead>
<tbody>
<tr>
<td>30% (v/v) acrylamide/bis-acrylamide mix</td>
<td>2</td>
<td>2</td>
<td>2.66</td>
</tr>
<tr>
<td>1.5 M Tris (pH = 8.8)</td>
<td>1.3</td>
<td>1.3</td>
<td>-</td>
</tr>
<tr>
<td>0.5 M Tris (pH = 6.8)</td>
<td>-</td>
<td>-</td>
<td>1.332</td>
</tr>
<tr>
<td>10% (w/v) SDS</td>
<td>0.05</td>
<td>0.05</td>
<td>0.052</td>
</tr>
<tr>
<td>TEMED</td>
<td>0.002</td>
<td>0.002</td>
<td>0.001</td>
</tr>
<tr>
<td>10% (w/v) APS</td>
<td>0.05</td>
<td>0.05</td>
<td>0.052</td>
</tr>
<tr>
<td>H$_2$O</td>
<td>1.6</td>
<td>7.9</td>
<td>12.26</td>
</tr>
</tbody>
</table>

Table 2.7. Conditions for western blot analysis of proteins extracted from $T_{84}$ cells. Parameters listed above were optimised for analysing proteins of interest in $T_{84}$ cell lysates.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein size (kDa)</th>
<th>Lysate quantity (µg)</th>
<th>Transfer time/Current (min/A)</th>
<th>Blocking reagent</th>
<th>Primary antibody dilution</th>
<th>Source/Cat number</th>
<th>Developing reagent</th>
<th>Exposure time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NFkB Phospho-p65</td>
<td>65</td>
<td>40</td>
<td>120/15</td>
<td>Milk</td>
<td>1/4000</td>
<td>Cell Signaling #3033L</td>
<td>ECLprime</td>
<td>5</td>
</tr>
<tr>
<td>NFkB p65 (Total)</td>
<td>65</td>
<td>40</td>
<td>120/15</td>
<td>Milk</td>
<td>1/2000</td>
<td>Cell Signaling #69565</td>
<td>ECLprime</td>
<td>10</td>
</tr>
<tr>
<td>Phospho-p38 MAPK (Thr180/Tyr182)</td>
<td>38</td>
<td>30</td>
<td>120/30</td>
<td>BSA</td>
<td>1/3000</td>
<td>Cell Signaling #9211</td>
<td>ECLprime</td>
<td>1</td>
</tr>
<tr>
<td>p38 MAPK</td>
<td>38</td>
<td>30</td>
<td>120/30</td>
<td>BSA</td>
<td>1/3000</td>
<td>Cell Signaling #92165</td>
<td>ECLprime</td>
<td>15</td>
</tr>
<tr>
<td>CFTR (A-3)</td>
<td>165</td>
<td>30</td>
<td>90/15</td>
<td>BSA</td>
<td>1/3000</td>
<td>Santa Cruz Biotech sc-376683</td>
<td>ECLprime</td>
<td>3</td>
</tr>
<tr>
<td>B-Actin</td>
<td>42</td>
<td>30</td>
<td>30/15</td>
<td>Milk</td>
<td>1/4000</td>
<td>Sigma #A5316</td>
<td>ECLprime</td>
<td>1.5</td>
</tr>
</tbody>
</table>
2.3.3 Molecular Biology

2.3.3.1 Total RNA extraction

Following treatment, monolayers of T84 or HT29Cl.19A cells were washed twice in ice-cold PBS. The cells were scraped from the inserts, re-suspended in 1 ml of ice-cold PBS and then centrifuged (600 x g; 10 mins; 4 °C). The supernatant was discarded and the cell pellet was snap frozen in liquid nitrogen and stored at -80 °C. Total RNA was isolated using a Qiagen RNeasy® Mini Kit (Qiagen, UK), according to the manufacturer's protocol. The RNA was then treated with DNase-I to digest and remove any genomic DNA contamination. RNA quality was determined by spectrophotometric methods. RNA has an absorption maximum at 260 nm and the ratio of absorbance at 260 and 280 nm was used to assess the purity of RNA preparations, with proteins absorbing strongly at 280 nm. Pure RNA measured in water has an A260/A280 of 1.8–2.0. A secondary measure of RNA purity is the A260/230 ratio. Carbohydrates and phenols absorb light optimally at 230 nm. This ratio is used as a secondary measure of nucleic acid purity. Expected 260/230 values are commonly in the range of 2.0 – 2.2. If the ratio is appreciably lower than expected, it may indicate the presence of contaminants, which absorb at 230 nm.

2.3.3.2 cDNA preparation

To obtain single stranded cDNA, reverse transcription was performed on purified RNA (0.5 µg), using the Improm-II Reverse Transcription kit (Promega, Germany). Oligo (dT)15 primers (Promega, Germany) were used in the reaction to ensure reverse transcription of mRNA only. cDNA was then used to perform PCR reactions using appropriate, self-designed primers, specificity of each primer set was confirmed by nucleotide-The Basic Local Alignment Search Tool (BLAST) analysis. GoTaq® green master mix was used in all PCR reactions. GoTaq is a pre-mixed, ready to use solution, containing a non-recombinant form of Taq DNA polymerase that lacks 5’-3’ exonuclease activity. The mix contains 400 µM dATP, 400 µM dGTP, 400 µM dCTP, 400 µM dTTP, and 3 mM MgCl₂. Each reaction contained the following: 12.5 µl of GoTaq master mix, 1 µl of forward primer (10 µM), 1 µl of reverse primer (10 µM), 0.5 µg – 1 µg of cDNA template and nuclease-free water to bring the final volume to 25 µl.
All reactions were carried out using the Touch-down PCR procedure (Don et al., 1991). Touch-down PCR is a method that avoids nonspecific binding of primers to cDNA. The PCR programme is set to decrease annealing temperature by 1 °C every cycle, over a range of 10 °C. The upper limit of the temperature range is determined by the highest melting temperature of the primer set. As the temperature drops, the reaction primarily contains the desired amplicon, and therefore decreases nonspecific binding at lower temperatures. The RT-PCR reaction physical conditions are presented in Table 2.8 below.

Table 2.8. The RT-PCR reaction performed to obtain single stranded cDNA.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>50</td>
<td>2 min</td>
<td>1</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>95/60/72</td>
<td>15 s/30 s/30 s</td>
<td>40</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>10</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>4</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

2.3.3.3 Primer design

A number of the primer sets used in these studies were obtained from previously published literature (Table 2.9). Primer design software was also employed for genes for which no primer sequences had been previously published. The sequences obtained were cross-referenced with the NCBI primer design BLAST utility to ensure that there were no off-target amplicons. Primers were ordered from Invitrogen (Carlsbad, CA, USA) through Biosciences.

Control reactions were performed to determine the quality and purity of RNA samples. The first control reaction was performed to ensure that there was no genomic DNA contamination of RNA samples. In situations where DNAase treatment does not completely remove contamination with genomic DNA, the PCR reaction will amplify not only target cDNA but also genomic DNA. The control reactions were performed by carrying out RT-PCR on RNA samples used as a binding template. To insure there was no contamination of solutions and equipment with genomic DNA the appropriate control reactions were performed and proved no contamination of the samples tested.
Table 2.9 Sequences of primer sets used to amplify cDNA and quantitatively compare mRNA expression between samples. Primers were designed with approximately 50% – 60% GC content and were examined for the presence of hairpins, the ability to form self-dimers, and runs of G or C. All primers used were designed for human transcripts. GAPDH was used as a ‘housekeeping’ control gene to normalise gene expression data for differences in sample concentration and loading. GAPDH is constitutively expressed in many tissues and is therefore useful as an internal control. Specificity of each primer set was confirmed by nucleotide-BLAST analysis (http://blast.ncbi.nlm.nih.gov/).

<table>
<thead>
<tr>
<th>Protein target name</th>
<th>Gene name</th>
<th>Species</th>
<th>Gene ID OMMM</th>
<th>Genomic location (cM)</th>
<th>Primer Sequence (Forward / Reverse)</th>
<th>Annealing Temp. Range</th>
<th>Product Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human β Defensin 1</td>
<td>BEFB1</td>
<td>Human</td>
<td>602215</td>
<td>Ch: 8p23.1</td>
<td>5’TGTTCAGATGCGCTCAGGCTGGTAAC-3’ 5’TTCAGCTCCTGCTCAATTCTCTCCTG-3’</td>
<td>58 - 62</td>
<td>202</td>
</tr>
<tr>
<td>Human β D-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>5’-GAGGACACAGGTTGCAAATTT-3’</td>
<td>58 - 60</td>
<td>172</td>
</tr>
<tr>
<td>Human β Defensin 2</td>
<td>DEFB4A</td>
<td>Human</td>
<td>602215</td>
<td>Ch: 8p23.1</td>
<td>5’-AGCTAGCTGACATGAGATGATC-3’ 5’CTTCGCGCAGATTCTCCTGCA-3’</td>
<td>58 - 64</td>
<td>208</td>
</tr>
<tr>
<td>Human β Defensin 3</td>
<td>DEFB103B</td>
<td>Human</td>
<td>55894</td>
<td>Ch: 8p23</td>
<td>5’-AAGAGGTCAACAGGAAATGGA-3’ 5’TCCAGATGATGGTGTCATA-5’</td>
<td>58 - 64</td>
<td>208</td>
</tr>
<tr>
<td>Mouse β Defensin 1</td>
<td>DEFB1</td>
<td>Mouse</td>
<td></td>
<td>Ch: 8 A4; 8 10.35</td>
<td>5’-CTCCACATCGAGCCCTTTAC-3’ 3’GTGACATCCCCCTGAACTGGA-5’</td>
<td>68</td>
<td>250</td>
</tr>
<tr>
<td>Mouse β Defensin4 mβ D-4</td>
<td>DEFB4</td>
<td>Mouse</td>
<td></td>
<td></td>
<td>5’-CTCCACATCGAGCCCTTTAC-3’ 3’GTGACATCCCCCTGAACTGGA-5’</td>
<td>70</td>
<td>134</td>
</tr>
<tr>
<td>G protein-coupled bile acid receptor TGR5</td>
<td>GPBAR1</td>
<td>Human</td>
<td>610147</td>
<td>Ch: 2q35</td>
<td>5’-ATCGATCAGCAGCTGCTGATC-3’ 5’-TGTCGCTGCTCAGGATA-CG-3’</td>
<td>68 - 70</td>
<td>990</td>
</tr>
<tr>
<td>G protein-coupled bile acid receptor TGR5</td>
<td>GPBAR1</td>
<td>Mouse</td>
<td>610147</td>
<td>Ch: 1 C3; 1 38.53</td>
<td>5’-CTCCACATCGAGCCCTTTAC-3’ 5’-TGTCGCTGCTCAGGATA-CG-3’</td>
<td>59 - 60</td>
<td>990</td>
</tr>
<tr>
<td>Farnesoid X receptor</td>
<td>NR1H4</td>
<td>Human</td>
<td>603826</td>
<td>Ch: 12q23.1</td>
<td>5’-ATCCAAAGGGGATGACCTGATC-3’ 5’-TGTCGCTGCTCAGGATA-CG-3’</td>
<td>68 - 88</td>
<td>591</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase GAPDH</td>
<td>GAPDH</td>
<td>Mouse</td>
<td>2597</td>
<td>Ch: 6 F2; 6 59.32</td>
<td>5’-ATCGATCAGCAGCTGCTGATC-3’ 5’-TGTCGCTGCTCAGGATA-CG-3’</td>
<td>59</td>
<td>444</td>
</tr>
<tr>
<td>Glyceraldehyde 3-phosphate dehydrogenase GAPDH</td>
<td>GAPDH</td>
<td>Human</td>
<td>2597</td>
<td>Ch: 12p13</td>
<td>5’-ATCGATCAGCAGCTGCTGATC-3’ 5’-TGTCGCTGCTCAGGATA-CG-3’</td>
<td>59</td>
<td>444</td>
</tr>
</tbody>
</table>
Quantitative Polymerase Chain Reaction

Real-time, quantitative reverse transcription polymerase chain reaction (qRT-PCR) is a technique that was developed in 1992 to overcome the limitations of end-point detection in standard RT-PCR. qRT-PCR uses fluorescent reporter molecules to monitor the production of amplification products during each cycle of the PCR reaction. This combines the nucleic acid amplification and detection steps into one homogeneous assay and obviates the need for gel electrophoresis to detect amplification products. cDNA (0.75 µg) was loaded into the wells of a 96-well plate with 0.5 µM forward and reverse primers (the same primer sets described in Table 2.7). SYBR Green I Master mix (Roche) in a total volume of 20 µl was used in each reaction. Amplification was carried out in a LightCycler 480 (Roche). Variables that were changed in order to optimise conditions were cycle number, annealing temperature, and elongation time. PCR was then performed using the optimised conditions (Table 2.10), and samples were separated by 2% agarose gel electrophoresis to visualise the amplified target and ensure its size is compatible with the expected product size.
### Table 2.10
The q-PCR reaction performed on single stranded cDNA with desired sets of primers to obtain target gene amplification. Melting curves were analysed for each primer set to confirm specificity of the primer sets.

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pre-incubation</td>
<td>95</td>
<td>10 min</td>
<td>1</td>
</tr>
<tr>
<td>Denaturation/Annealing</td>
<td>95 / 58</td>
<td>10 sec / 10 sec</td>
<td>40</td>
</tr>
<tr>
<td>elongation</td>
<td>72</td>
<td>10 sec</td>
<td>1</td>
</tr>
</tbody>
</table>

### 2.3.4 Electrophysiological Measurements

#### 2.3.4.1 Human colonic tissue preparation

Resected colonic tissue was obtained from adult patients undergoing colorectal surgery in Beaumont Hospital between November 2012 and May 2013. Patients agreed to participate by written informed consent and this study was approved by Beaumont Hospital Medical Ethics Committee (approval ID: 12/33). Patients who had received neoadjuvant chemo/radiotherapy, with a background of IBD, or with active inflammation at time of resection were excluded for the purposes of this study. Patient demographics are summarised in the table below (Table 2.11). All surgical specimens were collected from the pathology department, fresh, on ice, bathed in Ringers solution (Table 2.11). Normal colonic mucosa was identified macroscopically and microscopically by 2 independent pathologists. All specimens were taken at least 3 cm clear of tumour margins and at least 3 cm clear of resection margins to avoid injured or cancerous tissue. Sheets of isolated colonic mucosa were obtained by blunt dissection of the overlying muscle layers. Table 2.11
Muscle-stripped sections of colonic mucosa were cut laterally into 4 – 6 sections, which were then mounted on inserts, and bathed in Ringer’s solution (Table 2.9) in Ussing chambers (aperture 0.5 cm²), at 37 ± 1 °C and gassed with O₂/CO₂. Tissues were equilibrated for 30 mins during which time the basal electrical parameters, potential difference (PD) and conductance (G) were recorded. Following equilibration, various concentrations of bile acids were added bilaterally. Transepithelial conductance was monitored (G) throughout the experiment to determine viability and integrity of the tissues. Apical and basolateral solutions were collected and stored at – 80 °C until required for further experimentation.

<table>
<thead>
<tr>
<th>Patients (n)</th>
<th>Male:Female ratio</th>
<th>Median Age</th>
<th>Region of colon</th>
<th>Indication for surgery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>06:04</td>
<td>57</td>
<td>50% left</td>
<td>60% tumour resection</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>50% right</td>
<td>40% high grade dysplastic polyp resection</td>
</tr>
</tbody>
</table>
Table 2.12 Composition of Ringers solution. Ringer’s solution is a physiological salt solution that is isotonic at physiological pH. It is named after Sydney Ringer, who determined that the liquid around a frog’s heart must contain a set proportion of salts if the heart is to remain beating. Once prepared, Ringer’s solution was aerated with a 95 % O₂, 5 % CO₂ mix to achieve a pH of 7.5 and an osmolarity of 283 ± 7 mOsms.

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na⁺</td>
<td>140</td>
</tr>
<tr>
<td>K⁺</td>
<td>5.2</td>
</tr>
<tr>
<td>Ca²⁺</td>
<td>1.2</td>
</tr>
<tr>
<td>Mg²⁺</td>
<td>0.8</td>
</tr>
<tr>
<td>Cl⁻</td>
<td>119.8</td>
</tr>
<tr>
<td>HCO₃⁻</td>
<td>25</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>2.4</td>
</tr>
<tr>
<td>D-glucose</td>
<td>10</td>
</tr>
</tbody>
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2.3.5 Wound healing assay

T₈₄ cells were grown on hanging 4.5 cm² transparent semi-permeable supports, as described in Section 2.1. Fully polarised cells were washed twice with pre-warmed PBS and placed in new empty wells. Wounds were created by scraping the monolayers with a 10 μm pipette tip. Media containing drugs treatments was then applied bilaterally (unless stated differently). Five random wound points were chosen and marked for further assessment. Images were taken using a Samsung I310W camera and wounds were measured using Image J® software, developed at the National Institutes of Health. The area between the 2 edges of the wound was measured at time zero (T = 0), 24 and 48 hrs. Evaluation of epithelial restitution was based on the rate at which the distance between the wound edges decreased.

2.3.5.1 Boyden chamber

Boyden chambers were used to measure cell migration. Cells were seeded at a concentration of 5 x 10⁵ on 0.33 cm² PET membrane filters with 8 μm pores (Millipore). The upper side of the insert was filled with serum free media. The lower compartment was filled with media containing 20% BCS, which acts as a chemoattractant. Cells were seeded apically and all treatments were added bilaterally. Cells were then incubated for 24 – 48 hrs at 37 °C and 5% CO₂ to
allow cell migration. After treatment, cells were washed in PBS 3 times and
fixed in 100% methanol for 10 mins before staining in 0.05% (w/v) crystal violet
for 5 mins. Cells remaining on the upper layer of the support were removed with
cotton swabs. The support was washed carefully and cells that had migrated
into the lower compartment and attached to the lower surface of the support
were dissolved in 0.1 M acetic acid for 30 mins. The optical density was read at
560 nm using a Multiskan EX multiplate reader (Thermo Scientific) to quantify
the number of cells migrated.

2.3.6. Enzyme-Linked Immunosorbent Assay

HβD levels in T84 monolayers were measured using a commercially-available
kit, as per the manufacturers’ instructions. The kit contains an enzyme-linked
immunosorbent assay (ELISA) that uses anti-HβD antibodies to bind HβD
peptide, either in experimental samples or in standard solutions. Ninety-six well
plates were incubated with 0.5 µg/ml of HβD capture antibody overnight. All
incubations were performed at room temperature unless stated otherwise. Four
washes with wash buffer were then followed by incubation with blocking buffer
for 1.5 hrs. Samples and standards were pipetted into appropriate wells and
were simultaneously incubated at room temperature for 3 hrs. Excess reagents
were then washed away. Avidin-HRP conjugate was then added and incubated
for 1 hr, after which 2,2'-azino-bis solution (ABTS) liquid substrate was added.
The resulting colour generated was spectrophotometrically analysed at 405 nm
(Figure 2.4), with colour intensity being representative of the amount of HβD
peptide present. For each experiment, a standard concentration curve was
prepared from HβD standard solutions and a coefficient of determination (R²)
was created. A coefficient of determination ≥ 0.97 was determined to be the cut-
off point. The amount of peptide in each sample was calculated from the
standard curve equation of the line: y= ax + b. An example of a standard curve
is shown in Figure 2.5.
Figure 2.4 Schematic representation of the HβD sandwich ELISA. Capture antibody was incubated in the well overnight, washes were performed and HβD peptide was introduced. The detection antibody recognising HβD and avidin-HRP substrate was added. Wells were washed, ATBS substrate was added and the absorbance of the solution was read at 405 nm.

Figure 2.5 Examples of a standard curves for HβD quantification. Standard curves were prepared using a range of a) HβD-1 concentrations (0 pg/ml – 1000 pg/ml) or b) HβD-2 concentrations (0 pg/ml – 2000 pg/ml). Both standard curves were read at 405 nm wavelength.
2.3.7. Lactate Dehydrogenase Assay

The lactate dehydrogenase (LDH) assay was performed using a commercially-available kit sourced from Sigma-Aldrich Inc. LDH is a relatively stable enzyme in the cytosol and is rapidly released from the cell upon damage to the ER. LDH is an enzyme that interconverts pyruvate and lactate with the coincidental conversion of nicotinamide adenine dinucleotide (NAD⁺) and (NADH). In the assay, NAD is reduced to NADH by LDH. NADH is then used to reduce a tetrazolium dye, thereby forming a coloured compound. Tetrazolium redox dyes scavenge electrons from oxidation/reduction reactions and are intracellularly reduced to brightly coloured formazan precipitates.

T84 cells were cultured as described in Section 2.2.1.4. Following DCA (200 µM; 9, 12 and 24 h) treatment, culture medium from control and DCA-treated cells was analysed for LDH release. A separate set of cells was incubated with lysis buffer at 37 °C for 45 mins and the culture medium from these cells served as the positive control. Sterile serum-free culture medium was used as a negative control, the value of which was subtracted from sample readings. An equal volume (40 µl) of apical and basolateral media were combined and placed in the wells of a 96-well plate. Assay reagent was added to each well at a volume equal to twice that of the sample, and the reaction was incubated in the dark at room temperature for 25 mins. The reaction was terminated by addition of a 1/10 volume of HCl (1 M) (24 µl). The absorbance of the solution was then measured spectrophotometrically at 490 nm.

2.3.8 Proliferation Assays

Proliferation was measured using the acid phosphatase assay. Cells were seeded in 96-well plates (Sarstedt, 83.1835-100CS) at a concentration of 1 x 10⁵ cells/well in 100 µl volume. Doubling time was established at 12 hrs post seeding. Seeding concentrations were determined in order to allow cells to attain 80-90% confluency over the course of the experiment. Plates were incubated for 4 hrs at 37 °C to allow cells to adhere. Treatments were added to the plates at specific concentrations. 100 µl of 2 x treatments were added to 100 µl of culture medium to obtain the desired final concentration. Plates were then incubated at 37 °C for up to 24 hrs during which time control cells attained 80-90% confluency. Following this, media was removed and the plates were
washed once with PBS (Sigma-Aldrich). 10 mM acid phosphate substrate (Sigma-Aldrich) in 0.1 M sodium acetate buffer with 0.1% Triton X (Sigma-Aldrich) was added to each well and incubated at 37 °C for 1 hr. 50 μl of 1 M NaOH was added to halt the reaction and absorbance was read at 405 nM.

2.4 Statistical Analysis

The $n$ number reported for each experiment refers to the technical replicates. All results are expressed as mean ± standard error of the mean (SEM) for a series of $n$ experiments. Statistical analyses were performed using GraphPad Prism 5 software. Paired t-tests were used for comparisons of paired treatments between 2 groups and one way Analysis of variance (ANOVA), using the Student Newman-Keuls post-test for comparisons of 3 groups or more. The p values of $\leq 0.05$ were considered to be statistically significant.
CHAPTER 3

Regulation of colonic epithelial human β-defensin secretion by secondary bile acids
3.1 Introduction

As discussed in Chapter 1, HβDs form an essential component of intestinal innate immunity. Defensins are released into the luminal space, chaperoned by mucins, and are found at their highest concentrations in the thick layer of intestinal mucus, thereby promoting sterility of the epithelial surface. In addition to their bactericidal activities, defensins have also been found to have immunomodulatory properties. HβD-1 is expressed constitutively, whereas HβD-2 has been shown to be upregulated by pro-inflammatory stimuli in oral, intestinal and colonic epithelial cell lines (O’Neil et al., 1999, Ogushi et al., 2004, Vora et al., 2004, Krisanaprakornkit et al., 2000), together with various lung epithelial cell lines (Singh et al., 1998). HβD-2 is believed to be an important component of host defence, since it has been demonstrated to be induced by both commensal and pathogenic bacteria in the oral mucosa and keratinocytes, although by different mechanisms (Krisanaprakornkit et al., 2000, Chung and Dale, 2004, Dale and Fredericks, 2005).

3.1.1 Antimicrobial functions of HβDs

Mammalian and bacterial cell membranes vary in their lipid bilayer composition. Mammalian cell membranes are rich in phosphatidylethanolamine, phosphatidylcholine and sphingomyelin, which generally confers them with a neutral net charge (Shekels et al., 1996). In contrast, microbial membranes have a strong negative charge, due to the large number of acidic phospholipids, such as phosphatidylglycerol, phophatidylserine and LPS in gram-negative, and teichoic or teichuronic acids in gram-positive bacteria (Schwab et al., 2008b, Sears, 2005, Sekirov et al., 2010). The mechanisms by which HβDs specifically target bacteria involves electrostatic attraction between the cationic AMPs and the anionic charged sites on bacterial membranes (Shekels et al., 1996). Following HβD binding to the bacteria, the amphipathic domain of the peptide is inserted into the membrane. The interaction between a charged arginine on HβD protein side chains and polar lipid head groups, in the lipid bilayer, creates transmembrane channels which results in leakage of ions and metabolites from the microbial cell. Pore-like channels created by HβDs in microbial membranes result in membrane depolarisation, loss of membrane-coupled respiration, impaired peptidoglycan synthesis and ultimately cell death (Shekels et al.,
Another mechanism by which AMPs exert their bactericidal effects involves binding to negatively charged nucleic acids in the cytoplasm resulting in direct inhibition of nucleic acid synthesis (Shibata et al., 2007).

In addition to their bactericidal activities, HβDs can also prevent inflammation by binding to LPS on the membranes of pathogens, thereby preventing it from exerting pro-inflammatory actions. HβDs can also neutralise several bacterial toxins, such as anthrax and clostridium difficile toxin B, (Fiorucci et al., 2009, Fournier and Parkos, 2012, Francois et al., 2013, Yang et al., 2004). Furthermore, binding of HβDs to bacteria and their products results in their opsonisation and internalisation into immature dendritic cells, leading to induction of antigen-induced pro-inflammatory cytokines (Yang et al., 2004, Garcia et al., 2001b).

3.1.2 Chemotactic functions of HβDs

HβDs have potent chemoattractant activities and can cause infiltration of immune cells to the mucosa, thus enhancing antigen-specific immune responses (Freel et al., 1983, Funderburg et al., 2007, Fuss et al., 2006, Rohrl et al., 2010a). HβD-1 seem to have no significant inflammatory role, however, HβD-2 specifically targets immature dendritic cells and memory T cells via chemokine receptor (CCR) 6, inducing chemotaxis (Rohrl et al., 2010a, Wehkamp et al., 2004). HβD-driven infiltration of neutrophils, monocytes and macrophages occurs through activation of CCR2, TLR1 and TLR2, leading to stimulation of NF-κB signalling and consequently production of pro-inflammatory mediators, such as IL-1α, IL-6, IL-8 and TNF-α (Smahi et al., 2002, Jin et al., 2010). Furthermore, HβDs are known suppress neutrophil apoptosis, further perpetuating inflammatory responses (Nagaoka et al., 2008).

3.1.3 HβDs in physiology and pathophysiology

In healthy colon, HβD-1, and trace levels of HβD-2, are released from the epithelium where they are thought to be beneficial, since low concentrations of HβD can promote healing processes and epithelial barrier function (Vongsa et al., 2009). HβD-2 selectively targets intestinal epithelial cells to induce mucin secretion and strengthen the mucosal barrier from bacterial penetration (Sonawane et al., 2005). However, dysregulated expression of HβDs is
associated with several inflammatory diseases, such as psoriasis, atopic dermatitis, and eosinophilia esophagitis (Tabata, 2011, Schroeder et al., 2013).

Genome-wide association studies have also shown altered expression of HβDs in IBD patients, with UC patients expressing extremely high levels of HβD-2 in the colon (Aldhous et al., 2009, Wehkamp et al., 2002, Otte and Vordenbäumen, 2011). Given that increased levels of HβDs can trigger inflammatory cascades, upregulation of defensin secretion can lead to increases in pro-inflammatory cytokine release and inappropriate activation of the immune system.

### 3.2 Specific aims

Although levels of colonic bile acids are known to be elevated in UC, where they are thought to be important in regulating several aspects of innate intestinal barrier function, there are still no studies of their potential roles in regulating colonic secretion of HβDs. Therefore, this chapter set out to address this gap in our knowledge by investigating the hypothesis that bile acids may contribute to changes in intestinal barrier function through modulation of HβD expression. In particular, using *in vitro* and *ex vivo* models of the human colon, we set out to determine:

I. The effects of the most common colonic bile acid, DCA, on the expression of HβD-1 and HβD-2

II. The effects of the “anti-inflammatory” bile acid, UDCA, on the expression of HβD-1 and HβD-2
3.3 Results

3.3.1 DCA increases secretion of HβD-1 and HβD-2 from cultured human colonic epithelial cells

3.3.1.1 TNF-α increases HβD-1 and HβD-2 gene expression in polarised T84 colonic epithelial cells.

First, in order to provide a positive control for our studies with bile acids, we needed to identify an agonist known to induce HβD expression. As one of the most important pro-inflammatory cytokines in mucosal immune responses, TNF-α has been previously shown to potently upregulate levels of secretory HβDs (García et al., 2001, Gitai, 2005, Hau et al., 2013, McDermott et al., 2003). However, since not all human epithelial cell lines respond to TNF-α stimulation with increased HβD production (Fahlgren et al., 2003b), we investigated if the cytokine has the ability to promote HβD expression in our in vitro model of colonic epithelium, T84 cells. Polarised monolayers of T84 cells were incubated with TNF-α (10 ng/ml) basolaterally for periods of 1 – 12 hrs. TNF-α concentrations used were based on previously published studies (Narayanan et al., 2003, Tsutsumi-Ishii and Nagaoka, 2003). RNA was extracted, reverse transcription performed, and changes in HβD-1 and HβD-2 gene expression were assessed by qPCR. Levels of HβD mRNAs were normalised to those of the housekeeping gene, GAPDH, in order to correct for unequal cDNA loading. We found that while HβD-1 was not significantly altered by treatment with TNF-α, HβD-2 expression was significantly upregulated after 3 hrs of treatment with the cytokine. Cells stimulated for 6 and 12 hrs showed 10.1 ± 3.1 (n = 5; p < 0.05) and 15.3 ± 2.1 (n = 5; p < 0.01) fold increases in expression of HβD-2, respectively, relative to untreated cells (Figure 3.1).
Figure 3.1 TNF-α significantly alters HβD-2, but not HβD-1, gene expression in polarised T₈₄ colonic epithelial cells. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then stimulated with TNF-α (10 ng/ml) for the time points indicated. After treatment, RNA was extracted, cDNA synthesised and semi-quantitative RT-PCR analysis was performed with primers specific for HβD-1 and HβD-2. The graph shows the relative quantity (RQ) of HβD-1 and HβD-2 mRNA after treatment with TNF-α. All values were normalised to GAPDH gene expression and expressed as fold change over untreated controls. Data represent the mean ± SEM (n = 5) and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to untreated controls; * p < 0.05, ** p < 0.01.
3.3.1.2 DCA increases expression of HβD-1 and HβD-2 mRNA in T₈₄ colonic epithelial cells.

In order to examine the effects of bile acids on colonic HβD secretion, polarised monolayers of T₈₄ cells were grown in complete media and were switched to serum-free media for 4 hr prior to treatment. Cells were then treated with DCA (150 μM) in serum-free media bilaterally for periods of 1 – 48 hrs. Expression of HβD-1 mRNA was significantly upregulated after 12 hrs with the highest expression occurring after 24 hrs of DCA treatment, reaching an 18.8 ± 3.9 fold increase of that in untreated controls (n = 4; p < 0.001) (Figure 3.2a). HβD-2 expression was upregulated more rapidly, as early as 6 hrs post-treatment with DCA, and persisted up to 48 hrs, with the highest level of expression (4182.5 ±1017.9 fold increase) occurring after 24 hrs (Figure 3.2c).

Next, a concentration-response analysis for DCA effects on HβD secretion was carried out. Cell monolayers were incubated with DCA (50 – 150 μM) for 24 hrs after which HβD-1 and HβD-2 mRNA expression was measured by q-PCR. DCA (150 μM) stimulated HβD-1 mRNA production to 18.3 ± 5.1 fold compared to untreated controls (n = 4; p < 0.01), but was without significant effect at lower concentrations (Figure 3.2b). In contrast, HβD-2 expression was significantly increased by DCA treatment at concentrations as low as 50 μM, with a maximal increase of 4003.1 ± 1140.7 fold over control observed with 150 μM treatment (Figure 3.2d). Such differences in the concentration-dependence for DCA in stimulating HβD-1 and HβD-2 expression may indicate different regulatory mechanisms underlie these responses.
Figure 3.2 DCA increases expression of HBD-1 and HBD-2 mRNA in human colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with DCA (150 μM) for 1 – 48 hrs, mRNA was extracted and levels of (a) HBD-1 and (c) HBD-2 were assessed by q-PCR (n = 4). Cells were also treated with DCA (50 – 150 μM) for 24 hrs, mRNA was extracted and levels of (b) HBD-1 and (d) HBD-2 were assessed by q-PCR (n = 4). All values were normalised to GAPDH gene expression and expressed as a fold change over untreated controls. Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to untreated controls; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.3.1.3 DCA increases secretion of HβD-1 and HβD-2 protein from T₈₄ colonic epithelial cells.

Having shown that DCA induces expression of HβD mRNA in colonic epithelial cells, we next went on to examine the effects of the bile acid on secretion of HβD protein. Monolayers of T₈₄ cells were incubated with DCA (10 - 150 µM) bilaterally for periods of 6 – 48 hrs, after which the apical media was collected and levels of HβD-1 and HβD-2 were determined by ELISA. In these experiments, we found that at a concentration of 150 µM only, DCA stimulated secretion of HβD-1 (Figure 3.3a). This response was significant after 48 hrs treatment compared to untreated controls (Untreated: 204 ± 42 pg/ml; DCA 48 hrs: 412 ± 34 pg/ml, p < 0.01; n= 4) (Figure 3.3b). HβD-2 secretion was also maximally stimulated by DCA into the apical media at a concentration of 150 µM (Figure 3.3c). This concentration of DCA increased HβD-2 release into the media at 24 hrs with a maximal effect occurring at 48 hrs (untreated: 28 ± 3 pg/ml; DCA 48 hrs: 241 ± 70 pg/ml, p < 0.01; n= 4) (Figure 3.3d).
Figure 3.3 DCA increases secretion of HβD-1 and HβD-2 from human colonic epithelial cells. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with DCA (10 – 150 μM) for 48 hrs, apical bathing media was collected and levels of (a) HβD-1 and (c) HβD-2 were assessed by ELISA (n = 4). Cells were also treated with DCA (150 μM) for 6 – 48 hrs apical bathing media was collected and levels of (b) HβD-1 and (d) HβD-2 were assessed by ELISA (n = 4). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; ** p < 0.01.
3.3.1.4 DCA does not exert toxic effects on T₈₄ cells.

In order to determine if DCA treatment was associated with cellular toxicity, TEER was measured throughout the experiments. As shown in Figure 3.4a, TEER was not altered at any of the time points tested. Another index of cellular toxicity is release of the cytoplasmic enzyme, LDH, into the culture media. Using a commercially-available kit to measure LDH, DCA (10 – 150 µM) did not appear to cause cell damage.

Figure 3.4 DCA does not exert toxic effects at concentrations which induce HBD secretion from T₈₄ cells. T₈₄ cells were cultured on permeable supports until TEER stabilised at plateau levels. a) Cells were treated bilaterally with DCA (150 µM) for the indicated times and successive TEER measurements were recorded using an epithelial voltohmeter (n = 3). b) Cells were treated bilaterally with indicated concentrations of DCA for 48 hrs. T₈₄ cells were treated with lysis buffer for a minimum of 30 mins prior to analysis. An equal aliquot of apical and basolateral culture medium was taken and LDH was measured using a commercially-available kit (n = 4). Data represent mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; *** p < 0.001).
3.3.1.5 Sidedness of DCA and TDCA actions in promoting HβD secretion from T84 colonic epithelial cells.

To further characterise the actions of DCA on HβD secretion from colonic epithelial cells, we examined the sidedness of the bile acid, and its taurine-conjugated derivative, TDCA, on HβD-1 and 2 secretion. Polarised monolayers of T84 cells were treated bilaterally, apically, or basolaterally with DCA (150 μM) or TDCA (150 μM) for 48 hrs. Apical media was then collected for measurements of HβD-1 or HβD-2 secretion by ELISA. Interestingly, we found that neither basolateral nor apical addition of DCA (150 μM) significantly increased secretion of HβD-1 into apical media (Figure 3.5a). Moreover, TDCA, which is more hydrophillic than DCA and is unable to cross cell membranes unless a transporter is present, was also ineffective when added either apically or basolaterally. However, similar to DCA, bilateral stimulation with TDCA increased HβD-1 secretion from 169.8 ± 21.1 pg/ml to 318.8 ± 42.8 pg/ml (n = 6; p < 0.01) after 48 hrs treatment (Figure 3.5b). In contrast, apical or basolateral addition of DCA (150 μM) significantly increased secretion of HβD-2 from 34.6 ±5.4 pg/ml to 251.9 ±75.5 and 192.5 ± 39.9 pg/ml, respectively (Figure 3.4c). Bilateral addition of TDCA (150 μM) also induced significant increases in HβD-2 secretion into the apical media from 34.6 ±5.4 pg/ml to 147.1 ± 33.8 pg/ml. Interestingly, basolateral treatment with TDCA (150 μM) was more effective in stimulating HβD-2 secretion (94.3 ± 14.3 pg/ml) (n = 6; p < 0.05) than was apical addition (74.6 ±8.3 pg/ml) (Figure. 3.4d).
Figure 3.5 Sidedness of DCA and TDCA effects on HβD secretion from colonic epithelium. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were stimulated with DCA (150 μM) or TDCA (150 μM) apically, basolaterally, or bilaterally for 48 hrs. Apical media was then collected and ELISA performed for HβD-1 or HβD-2. HβD-1 secretion into the apical medium after treatment with (a) DCA (n = 8) and (b) TDCA (n = 6). HβD-2 protein secretion into the apical medium after treatment with (c) DCA (n = 6) and (d) TDCA (n = 6). Data represent mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to untreated controls; * p < 0.05, ** p < 0.01, *** p < 0.001.
3.3.2 UDCA inhibits HβD-1 and HβD-2 secretion from human colonic epithelial cells

3.3.2.1 UDCA inhibits basal expression of HβD-2, but not HβD-1, in T₈₄ colonic epithelial cells.

So far this chapter has revealed that DCA, which is the most common colonic bile acid (Hofmann, 2011), can upregulate epithelial HβD production, an effect that may promote inflammatory responses in vivo. Next, we turned our attention to UDCA, often referred to as the “therapeutic” bile acid. Previously published data suggests UDCA also exerts anti-inflammatory effects in the colon in vivo (Martínez-Moya et al., 2013) and in vitro, where in cultured colonic epithelial cells, we have found it to attenuate secretion of TNF-α and IL-8 (Ward et al., 2013b). With this in mind, we were interested in determining the effects of UDCA on expression of HβDs in colonic epithelial cells. Monolayers of T₈₄ cells were incubated with UDCA (50 – 150 µM) bilaterally for periods of 6 – 48 hrs. Apical media was collected and ELISAs were performed to measure HβD-1 and HβD-2 secretion. We found that although UDCA (50 – 150 µM) tended to decrease basal secretion of HβD-1 into the apical media over the time course tested, no significant effects were observed (Figure 3.6a, b). In contrast, UDCA (150 µM) significantly inhibited HβD-2 secretion into the apical media after 6 hrs of treatment with a maximal inhibitory effect occurring after 48 hrs (Untreated: 20.3 ± 2.7 pg/ml; 6 hrs UDCA: 4.0 ± 2.9 pg/ml; 48 hrs UDCA (150 µM): 1.3 ± 0.94 pg/ml) (Figure 3.6c). Using the time point at which it exerts its maximal inhibitory effects, we also tested the concentration-dependence of UDCA in inhibition of basal HβD-2 release into the apical medium. We found that effects of UDCA were dose-dependent with maximal inhibition occurring at 150 µM (Figure 3.6d).
Figure 3.6 UDCA inhibits HB̂D-2 release from colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised. Cells were stimulated with UDCA at a range of concentrations for the indicated times. Apical media was collected and ELISA performed for HB̂D-1 or HB̂D-2. Time-dependence of UDCA (150 μM) effects on (a) HB̂D-1 and (b) HB̂D-2 secretion (n = 4). Concentration-dependence of UDCA (50 – 150 μM) effects on (b) HB̂D-1 and (d) HB̂D-2 secretion after treatment with UDCA (n = 4). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test. *denotes significant differences compared to untreated controls; * p < 0.05, ** p < 0.01, *** p < 0.001, ns – not significant.
3.3.2.2 Neither DCA nor UDCA induce basolateral secretion of HβDs in colonic epithelium.

To more fully investigate the effects of bile acids on HβD secretion, we analysed levels of HβD-1 and 2 in basolateral medium collected from treated cells. Polarised monolayers of T₈₄ cells were treated bilaterally with DCA (150 μM) or UDCA (150 μM) for 48 hrs, after which the basolateral media was collected and ELISA was performed. We found that neither DCA nor UDCA altered basolateral secretion of HβD-1 (Figure 3.7a) or HβD-2 (Figure 3.7b) in these cells (n = 5).

3.3.2.3 UDCA attenuates DCA-induced HβD-1 and HβD-2 expression in T₈₄ cells

Our data indicates that DCA and UDCA have differential effects in regulating the production and secretion of HβD. Since UDCA has been previously shown to exert opposing effects to DCA in regulating other aspects of epithelial barrier function, such as ion transport (Kelly, 2014), apoptosis (Im and Martinez, 2004) and epithelial permeability (Stenman et al., 2013), we next carried out studies to...
determine if it may also regulate DCA-induced HβD production. In order to confirm effects observed in T₈₄ cells, we also carried this experiment out on another colonic epithelial cell line, HT-29Cl.19A. Monolayers of T₈₄ or HT-29Cl.19A colonic epithelial cells were treated for 24 or 48 hrs with DCA, either alone or in the presence of UDCA (20 – 150 µM) and HβD mRNA or protein expression was measured by qPCR or ELISA, respectively. Interestingly, we found that UDCA potently inhibited DCA-induced increases in both HβD-1 and HβD-2 mRNA (Figure 3.8) and protein expression (Figure 3.9). Furthermore, these effects were not specific to T₈₄ colonic epithelial cells, since we found that in HT29Cl.19A cells UDCA also attenuated DCA (150 µM)-induced secretion of HβD-1 and HβD-2 (Figure 3.9c, d). In the majority of these studies, UDCA exerted maximal inhibitory effects on HβD secretion at the lowest concentration of 50 µM.

![Figure 3.8 UDCA inhibits DCA-stimulated HβD mRNA expression in colonic epithelial cells.](image)

T₈₄ cell monolayers were treated with DCA (150 µM) alone or in combination with UDCA (20 – 100 µM) for 24 hrs. mRNA was extracted and qPCR performed using primers for HβD-1 or HβD-2. Relative quantity (RQ) of (a) HβD-1 and (b) HβD-2 mRNA expression is expressed as fold change over untreated controls (n = 4). All values were normalised to GAPDH mRNA expression and are presented as the mean ± SEM for a series of n experiments. Data were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test. *denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-stimulated cells, * p < 0.05, ** p < 0.01; # p < 0.05.
Figure 3.9 UDCA attenuates DCA-induced secretion of HβDs from colonic epithelial cells. T84 or HT29Cl.19A cells were cultured as monolayers on permeable supports until TEER stabilised. Cells were then treated with DCA (150 μM) alone or in combination with UDCA (50 – 100 μM) for 48 hrs. Apical media was collected and sandwich ELISA performed for HβD-1 or HβD-2. (a) HβD-1 and (b) HβD-2 levels in media collected from T84 cells (n = 8). (c) HβD-1 or (d) HβD-2 levels in media collected from HT29Cl.19A cells (n = 7). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated cells; * p < 0.05, ** p < 0.01, *** p < 0.001, ## p < 0.01, ### p < 0.001.
3.3.2.4 UDCA attenuates IL-1α-induced secretion of HβD-2 from T84 cells.

We next examined if the effects of UDCA in inhibition of HβD secretion are specific for DCA or if they are broader, affecting other stimulants of epithelial defensin production. To do this, we examined the effects of UDCA on IL-1α, a cytokine previously shown to induce HβD-2 expression and release from colonic epithelial cells (O’Neil et al., 1999). Cell monolayers were treated bilaterally for 48 hrs with IL-1α, either alone or in the presence of UDCA (50 – 150 µM). Apical and basolateral media were collected and levels of HβD-1 and HβD-2 were assessed by ELISA. We found that, in accordance with previously published data, IL-1α (10 ng/ml) did not alter HβD-1 secretion (Figure 3.8a) (García et al., 2001). However, there was an induction in secretion of HβD-2 by the cytokine from 38.9 ± 3.7 pg/ml to 104.2 ± 10.3 pg/ml (n = 5). Furthermore, co-treatment with UDCA (50 μM, 100 μM or 150 μM) attenuated IL-1α-driven HβD-2 secretion to 47.5 ± 3 pg/ml, 70.9 ± 10.5 pg/ml and 48.3 ± 1.3 pg/ml, respectively (Figure 3.8b).

![Figure 3.10 UDCA inhibits IL-1α-stimulated secretion of HβD-2 from colonic epithelial cells.](image-url)

T84 cells were cultured as monolayers on permeable supports until TEER stabilised. Cells were then treated with either DCA (150 µM) or IL-1α (10 ng/ml) alone or in combination with UDCA (50 – 100 µM). Apical media were collected and ELISA performed for HβD-1 and HβD-2. (a) HβD-1 and (b) HβD-2 levels in media collected from T84 cells (n = 3). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to untreated controls; # denotes significant differences compared to IL-1α-treated controls; * p < 0.05, ** p < 0.01, *** p < 0.001, ## p < 0.01, ### p < 0.001, ns –not significant.
3.3.2.5 UDCA attenuates DCA-induced secretion of HβD-1 and HβD-2 from \textit{ex vivo} human colonic tissue.

Our \textit{in vitro} studies clearly demonstrate that DCA and UDCA exert opposing effects on secretion of HβD-1 and 2 from colonic epithelial cells. To determine if such actions also occur under more physiological conditions, we employed \textit{ex vivo} human colonic tissues. These tissues were resected from patients undergoing colectomies in Beaumont Hospital between October 2012 and May 2014 and ethical approval was obtained from the Beaumont Hospital Ethics Committee. The samples were divided, depending on their origin, into proximal and distal colon. Sheets of isolated colonic mucosa, obtained by blunt dissection of the overlying muscle layers, were mounted in Ussing chambers and bathed in physiological Ringer’s solution. After an equilibration period of 30 mins, tissues were treated for 6 hrs with either DCA (200 μM), UDCA (200 μM), or both DCA (200 μM) and UDCA (50 μM). Apical (mucosal) and basolateral (serosal) solutions were collected and HβD-1 and HβD-2 levels were measured by ELISA.

In the proximal colon, basal levels of HβD-1 secretion were 31 ± 6.6 pg/ml and treatment with DCA increased HβD-1 release by 1.8 ± 0.2 fold (n = 5; p < 0.01). Co-treatment with UDCA (50 μM) inhibited this response to a 1.4 ± 0.3 fold increase, compared to untreated controls (n = 5) (Figure 3.11a). Basal levels of HβD-2 in the luminal bathing solution from proximal colonic tissues were 57.7 ± 23.6 pg/ml and an increase of 11.2 ± 7.9 fold in response to DCA treatment was observed. Co-treatment with UDCA also inhibited this response to 3.6 ± 2.2 fold of controls (n = 4) (Figure 3.11b). Colonic tissues remained viable throughout the duration of the experiment, as indicated by the stability of tissue conductance during this time (Figure 3.11c).

Tissues from the distal colon showed similar responses to BA treatments. Basal levels of HβD-1 released over 6 hrs into luminal media were 222.4 ± 5.7 pg/ml, which was significantly greater than that in proximal colon (n = 5; p < 0.001). Basal HβD-1 levels were increased in response to DCA treatment by 2.4 ± 0.5 fold (p < 0.05; p < 0.001). Similar to its effects in proximal colon, UDCA also
reduced responses to DCA in distal colon to 1.3 ± 0.1 fold of that in untreated controls (n = 5) (Figure 3.12a). Basal levels of HβD-2 release from distal colon (1064 ± 48.2 pg/ml) were significantly greater than that from proximal colon (n = 4; p < 0.001) and slight increase of 1.3 ± 0.9 fold (n = 4) in response to DCA treatment was observed. Co-treatment with UDCA attenuated the effects of DCA on HβD-2 secretion from distal colon (n = 4) (Figure 3.12b). The integrity of the tissue was monitored throughout these experiments by recording tissue conductance and was found to be stable over the time course studied (Figure 3.12c).

Throughout these experiments, basolateral HβD release was also assessed and was not detectable from either control or treated tissues (n = 9).
Figure 3.11 UDCA attenuates DCA-induced HβD release from human proximal colon. Human colonic mucosal sections were obtained by blunt dissection of the overlying muscle layers. Sections were mounted in Ussing chambers and bathed in oxygenated Ringers solution. Cells were treated with DCA (200 μM) or UDCA (200 μM) alone, or were co-treated with DCA (200 μM) and UDCA (50 μM). After 6 hrs, apical solutions were collected and ELISA performed for HβD-1 and HβD-2. (a) HβD-1 (n = 5) and (b) HβD-2 (n = 4) levels released from proximal colonic tissues. Data represent the mean ± SEM for fold change over untreated controls for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test. (c) Changes in conductance across proximal colonic mucosal tissues throughout the experimental procedure. * denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated controls; ** p < 0.01.
Figure 3.12 UDCA attenuates DCA-induced HβD release from human distal colon. Human colonic mucosal sections were obtained by blunt dissection of the overlying muscle layers. Sections were mounted in Ussing chambers and bathed in oxygenated Ringers solution. Tissues were treated with DCA (200 μM) or UDCA (200 μM) alone, or were co-treated with DCA (200 μM) and UDCA (50 μM). After 6 hrs, apical solutions were collected and ELISA performed for HβD-1 and HβD-2. (a) HβD-1 (n = 5) and (b) HβD-2 (n = 4) levels released from proximal colonic tissues. Data represent the mean ± SEM for fold change over untreated controls for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test. (c) Changes in conductance across proximal colonic mucosal tissues throughout the experimental procedure. * denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated controls; *** p < 0.01, ## p < 0.01.
3.3.2.6 UDCA inhibits mβD-4 expression in the DSS model of mucosal injury.

Having shown that UDCA has the capacity to inhibit HβD secretion from epithelial cells in *in vitro* and *ex vivo* models, we next investigated if these effects can be translated into a pathophysiological model of IBD. For these experiments the widely-used DSS model of mucosal injury was employed (Wirtz et al., 2007, Chassaing et al., 2014). Male C57BL/6 mice were administered UDCA (30 mg/kg) via intraperitoneal (IP) injection. The UDCA dose employed was based on previously published studies (Y.H. Kim et al., 2013, Martínez-Moya et al., 2013). IP administration of UDCA ensures rapid delivery of UDCA to the colon by bypassing the “first pass effect”, where orally administered bile acids are absorbed from the small intestine into the enterohepatic circulation. After 24 hrs, DSS (2.5 %) was introduced in the drinking water and UDCA was administered every 24 hrs. The disease activity index (DAI), which includes weight loss measurements, stool consistency, and the presence of faecal blood, was monitored daily. On day 5, the animals were sacrificed and their colons removed and sections were taken for western blot and immunohistochemical analysis.

We found that animals treated with UDCA had a significantly reduced DAI from $10 \pm 0.3$ (DSS alone) to $7.2 \pm 0.7$ (UDCA 30 mg/kg) (Figure 3.13a).

In order to examine the effects of UDCA on colonic inflammation, histological scoring and immunocytochemical analyses were performed. UDCA (30 mg/kg) reduced inflammatory cell infiltration and prevented DSS-induced mucosal damage (Figure 3.14a). Histological scores of tissues from animals treated with UDCA (30 mg/kg) + DSS 2.5% were reduced to $29 \pm 3.5$ compared to $37.3 \pm 0.8$ in mice treated with DSS alone ($n = 6 – 12$).

Furthermore, levels of mβD-4, the mouse orthologue of HβD-2, expressed in colonic tissues were increased $1.3 \pm 0.3$ fold in DSS-treated animals compared to untreated controls ($n = 3$; $p < 0.01$), while UDCA (30 mg/kg) treatment decreased DSS-induced production of mβD-4 to $0.97 \pm 0.1$ fold of controls ($n = 3$; $p < 0.05$) (Figure 3.13b).
Figure 3.13 UDCA decreases production of mβD-4 in DSS-induced UC mice model. Male C57BL/6 mice were housed as normal and treated by IP as follows: vehicle (PBS), UDCA (30 mg/kg) or co-treated with 2.5 % DSS in drinking water *ad libitum* for 5 days. Disease Activity Index (DAI) was recorded daily. Animals were sacrificed and colons were collected. Tissues were homogenised and proteins were extracted. Western blots were prepared using mβD-4 antibodies. Densitometry was performed and all results were normalised to mouse β-actin expression in the tissues. (a) DAI score showing the disease progress in treated mice (n = 6). (b) Graph representing densitometric analysis of mβD-4 western blot (n = 3) Data represent the mean ± SEM for fold change over untreated controls and is normalised to the mouse β-actin expression for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test. * denotes significant differences compared to untreated controls; # denotes significant differences compared to DSS-treated control; * p < 0.05, ** p < 0.01, *** p < 0.001, # p < 0.05, ## p < 0.01, ### p < 0.001.
Figure 3.14 UDCA preserves colonic architecture and decreases infiltration of immune cells into mucosa in DSS-induced UC mice model. Male C57BL/6 mice were housed as normal and treated by IP as follows: vehicle (PBS), UDCA (30 mg/kg) or co-treated with 2.5 % DSS in drinking water at libidum for 5 days. Animals were sacrificed, colons were collected and fixed in 10% formaldehyde-saline for (a) Representative H and E staining of colonic sections from DSS ± UDCA treated mice. (b) Arbitrary histologic scoring in order to asses severity of inflammation (n = 6 – 12). Arrows indicate immune cells infiltrating mucosal layer. Data represents the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to vehicle-treated controls; # denotes significant differences compared to DSS-treated animals; *** p < 0.001, # p < 0.05. (Immunocytochemistry staining and histological score kindly performed by Dr. Murtaza Tambio Wala from Saad Centre for Pharmacy and Diabetes, Ulster University, Coleraine, UK).
3.4 Discussion

In this chapter we set out to investigate the effects of bile acids on secretion of HβDs from colonic epithelial cells and the current studies are the first to demonstrate that the most common colonic bile acid, DCA, induces both HβD-1 and -2 production. The HβDs gene expression is increased more potently than protein expression. This is a widely known phenomenon, usually contributed to the roles of post-transcriptional, translational and protein degradation regulation in the cell (Vogel and Marcotte, 2012). However, the precise physiological/pathophysiological roles of bile acid-induced HβD secretion in the intestine remain to be elucidated. Under normal conditions, DCA is reported to be present in the colonic faecal water at concentrations between 50 – 200 μM (Hamilton et al., 2007). However, the exact concentration of DCA to which the epithelium is exposed is difficult to estimate, as levels of bile acids constantly fluctuate and epithelial cells are also protected by a thick layer of mucus and the unstirred fluid layer. With this in mind, we propose that under normal conditions, relatively low concentrations of DCA induce HβD secretion into the lumen (Selsted and Ouellette, 2005), where they exert bactericidal actions. This would enhance protection of colonic epithelium by promoting sterility of overlying mucus. This hypothesis is supported by literature showing that HβDs are necessary for appropriate bacterial clearance in IBD patients and that a low HβD gene copy number is associated with a low rate of bacterial killing in these patients (Fellermann et al., 2006, Bentley et al., 2010). In the healthy colon, the epithelial barrier is intact, and this prevents the HβDs entering the mucosa and exerting pro-inflammatory actions. However, when the epithelial barrier is dysregulated in IBD (Maloy and Powrie, 2011), this would allow high concentrations, 50 – 200 μM normally found in the luminal water, exerts their effects on the epithelium and underlying mucosa. Disruption of the epithelial barrier also permits the passage of defensins to the underlying mucosa. Here, they would be expected to promote inflammation by increasing the release of pro-inflammatory cytokines (Boniotto et al., 2006) and stimulating the infiltration of inflammatory cells (Simrén et al., 2002, Rohrl et al., 2010a). Over the long-term, such actions would be detrimental, leading to breakdown of epithelial barrier function and increased penetration of the underlying mucosa by luminal bacteria, as is typically seen in IBD (Matricon, 2010, Vora et al., 2012).
A recently published analysis of faecal bile acids demonstrated that while deconjugated secondary bile acids are normally the most predominant in the colon, their levels are decreased in IBD patients with corresponding increases in the levels of primary and conjugated bile acids (Duboc et al., 2013). Conjugated bile acids, such as TDCA, are hydrophilic and they are unable to cross cell membranes. Their uptake into colonic epithelial cells is facilitated by bile acid transporters, which are located only on the basolateral side (Pullan et al., 1994, Rohrl et al., 2010b). In our current studies, induction of HβD-1 and HβD-2 secretion was clearly observed upon bilateral treatment with DCA or TDCA. However, while apical or basolateral challenge alone with the bile acids tended to upregulate HβD secretion, these effects were not as effective as bilateral addition. These data may suggest that separate mechanisms for bile acid-induced HβD secretion, perhaps related to differential distribution of bile acid receptors and/or transporters, are present apically and basolaterally. Further clarification of this effect is necessary by performing additional technical repeats and assessing the extent of DCA diffusion to basolateral side after apical administration.

When elucidating the effects of bile acids on colonic epithelial HβD expression, it is also important to consider such actions in the context of other aspects of barrier function known to be regulated by bile acids. HβDs are embedded in colonic mucus and studies suggest that DCA is a potent mucin secretagogue (Raufman et al., 2003b, Rieder and Fiocchi, 2009, Rodrigues et al., 1998). Thus, since mucins act as chaperones for defensins in the colonic environment, it is possible that DCA-induced increases in HβD and mucin production are coordinated at the molecular level. It has been shown recently, that mucins can ameliorate HβD-2 antimicrobial activity, further highlighting the idea of synergism between defensins and mucins in barrier dysfunction which is typical in IBD (Cobo et al., 2015).

The role of bile acids in regulating colonic fluid and electrolyte secretion may also be important when considering their actions on HβD secretion. As previously shown, DCA induces water secretion in the colon by driving Cl− secretion (Keely et al., 2007, Sun et al., 2004, Mekhjian et al., 1971, Hofmann, 1999). In the state of infection, fluid secretion and following diarrhoea are considered to be protective against pathogenic invasion (Schiller, 2000,
Phalipon and Sansonetti, 2007, Navaneethan and Giannella, 2008). Although, diarrhoea is one of the most common symptoms of patients with IBD, no specific pathogen has been confirmed as causal (Abraham and Medzhitov, 2011). Therefore, we speculate that chronic bile acid-induced ion and water secretion can result in loosening of the mucus, allowing easier access for bacteria to the epithelium. In such a scenario, increased secretion of HβDs may help to protect the epithelial barrier from bacterial invasion as the mucus layer becomes more permeable. Furthermore, during bacterial invasion, HβDs have been shown to have the capacity to increase cholinergic-induced secretory responses in the colon (Himmerkus et al., 2010), supporting the idea that HβDs themselves can regulate the microenvironment at the epithelial surface (Sartor, 2008). Further support for this idea comes from the observation that HβD-2 can increase colonic mucin production, thereby regulating the thickness of the mucosal barrier (Cobo et al., 2015). Indeed, evidence supporting the importance of HβDs in the colonic mucosa is seen in UC, where their dysregulated expression is associated with increased microbial invasion (Aldhous et al., 2009, Chamaillard and Dessein, 2011, Fahlgren et al., 2003a, Jenke et al., 2012).

Data from our current studies have shown that there are segmental differences in basal levels of HβD-1 and 2 secretion from human colonic tissues. Since the quantities of bacteria in the colon are greater in descending colon than in ascending colon (Lyra et al., 2012), we hypothesise that basal levels of epithelial HβD secretion varies accordingly along the length of the colon, with levels being higher in descending colon due to higher levels of pathogenic bacterial strains present. Furthermore, DCA more potently increased the secretion of both HβDs in the distal part of the colon. We hypothesise that such differences may be due to differential expression of receptors, transporters, or signalling molecules responsible for bile acid-induced HβD release along the length of the colon, and this is further discussed in Chapter 4 (Alemi et al., 2013, Duboc et al., 2014).

In addition to examining the effects of DCA, our studies also investigated the actions of the “therapeutic” bile acid, UDCA, on colonic epithelial HβD secretion. Previous studies have shown that UDCA can modulate AMP expression, in particular cathelicidin, in biliary epithelia, an effect associated with activation of
VDR (Schroder and Harder, 1999, D’Aldebert et al., 2009). Our results are the first to demonstrate that UDCA also regulates AMP production in the colon. Moreover, UDCA appears to have different effects on different members of the HβD family, since it does not alter basal secretion of HβD-1, but potently reduces basal levels of HβD-2. Despite these differences on basal HβD secretion, UDCA inhibits DCA-induced secretion of both HβD-1 and HβD-2. The most potent attenuation of DCA-driven HβD release can be observed at UDCA concentrations of 20 – 50 µM. In physiology, levels of UDCA in the colonic water are present at the range of 5 – 10 µM (Hamilton et al., 2007). Thus, it needs to be noted, that the levels of UDCA used in these studies could be only achieved in the colon by introduction of UDCA in the form of a therapeutic. To our knowledge, there are no reports on levels of UDCA in the faecal water in patients treated with UDCA. However, levels of UDCA can reach up to 33.9 ± 15.6 µM/L in the serum of patients treated with 12.2 ± 1.4 mg/kg UDCA/day (Invernizzi et al., 1999). These data suggest that the concentrations of UDCA used in in vitro experiments are achievable in vivo, after UDCA administration. However, studies to directly assess this in humans are required. Furthermore, the effects of UDCA are not limited to bile acid-induced defensin secretion, since we also found it to inhibit HβD-2 secretion in response to the pro-inflammatory cytokine, IL-1α. Our findings with UDCA in cultured epithelial cells were also supported by experiments in human colonic tissues, where UDCA was found to attenuate DCA-induced secretion of HβD-1 and HβD-2. Given, that the effects of UDCA on HβD protein secretion were associated with reduced mRNA levels, our data suggests that the effects of the bile acid are mediated either by inhibition of gene transcription or through a posttranscriptional mechanism, such as miRNA-mediated HβD mRNA degradation.

Given, that both colonic bile acid levels and epithelial expression of HβDs are altered in patients with IBD (Bentley et al., 2010, Rider et al., 2011, Fahlgren et al., 2003a), it is likely that our current findings are relevant to disease pathogenesis. The role of HβD-1 in IBD pathogenesis is not well-established, although studies have shown that levels of this HβD are decreased in mucosal tissues from patients with UC and CD (Wehkamp et al., 2003). However, in patients with UC it has been shown that there is increased expression of HβD-2
in colonic epithelial cells and that this correlates with elevated levels of the pro-inflammatory cytokines, IL-8 and TNF-α (Schreiber et al., 1998a, Fahlgren et al., 2003a). Indeed, other studies have shown that defensins can induce cytokine release from innate immune cells (Boniotto et al., 2006). Thus, we hypothesise that increases in chemopotent HβD secretion induced by DCA are likely to increase mucosal cytokine levels, and induce infiltration of mucosal immune cells, therefore perpetuating the inflammatory response. In support of this, it has been previously shown that high levels of defensins aggravate inflammation in chemically-induced models of mouse colitis (Hashimoto et al., 2012) and our in vivo data supports this hypothesis, since we found DSS-treated mice to express higher levels of mβD-4, the murine ortholog of HβD-2, in colonic mucosa.

Importantly, our data in the DSS model showed that, similar to its effects on HβD expression in cultured cells and human colonic tissues, mβD-4 expression in vivo was also reduced by treatment with UDCA. This effect was associated with attenuated mucosal inflammation, as measured by DAI and histological scoring of the colonic mucosa. Such protective actions of UDCA in chemically-induced intestinal inflammation have been previously reported in the literature (Laukens et al., 2014, Martinez-Moya et al., 2013, Kullmann et al., 1997), where UDCA treatment has also been shown to prevent cytokine release, epithelial cell apoptosis, and loss of barrier function. Together these preclinical in vivo data strongly support the potential for UDCA in treatment of conditions associated with intestinal inflammation.

In summary, it is clear that defensins play a crucial role in host defence and innate immunity. Our data suggests that a disturbed balance in the colonic bile acid pool could lead to altered epithelial HβD production, and that this may be a factor underlying the development of colonic inflammation in UC. Furthermore, UDCA, by virtue of its capacity to inhibit HβD production and exert anti-inflammatory actions has potential to be developed as a new therapeutic approach to dampen intestinal inflammation. However, in order to realise this therapeutic potential, a greater understanding of the molecular pathways by which bile acids regulate colonic HβD production is required.
CHAPTER 4

Molecular mechanisms underlying bile acid regulation of Human β-Defensin production
4.1 Introduction

When considering the use of bile acids or bile acid derivatives as therapeutics, it is important to understand the molecular pathways involved in their actions. Thus, we next turned our attention to studying signalling mechanisms by which bile acids regulate the production of colonic defensins.

4.1.1 Bile acid receptors and inflammation

As discussed in Chapter 1, the actions of bile acids can be mediated through both nuclear and cell surface receptors, with different bile acids having differing affinities for these receptors (Table 2.2). Furthermore, activation of nuclear and cell surface receptors by bile acids, regulate a variety of physiological processes, many of which have implications for the pathogenesis of IBD. For example, TGR5, the cell surface GPCR for bile acids, has been shown to be involved in maintaining intestinal barrier integrity (de Diego-Cabero et al., 2015), and regulation of immune responses in experimental colitis (Cipriani et al., 2011). It has also been shown that activation of TGR5 in macrophages can attenuate LPS-induced cytokine release (Cipriani et al., 2011, Francois et al., 2013). Furthermore, genome-wide association studies have identified SNPs (rs11554825) in the TGR5 gene as being associated with development of UC (Garcia et al., 2001a). The presence of TGR5 in the colon is also important for maintaining balance in the bile acid pool size, as demonstrated by a number of independent studies, which document that TGR5<sup>−/−</sup> mice had a significantly decreased total bile acid pool (Jia et al., 2001, Jonkers and Stockbrugger, 2003).

FXR, the nuclear bile acid receptor, has also been shown to have important roles in maintaining intestinal barrier function. CD patients have reduced FXR expression in the intestinal mucosa (Nijmeijer et al., 2011), while activation of the nuclear bile acid receptor has been shown to exert anti-inflammatory actions and also to protect the intestinal barrier in chemically-induced colitis in mice (Gadaleta et al., 2011b).
However, while the importance of TGR5 and FXR in regulation of innate barrier function is becoming more established, their roles in regulation of HβD secretion from epithelial cells has yet to be elucidated.

4.1.2 Molecular pathways involved in IBD pathogenesis

Many of the genetic mutations associated with IBD have been linked to the signalling pathways that regulate both innate and adaptive immune responses (Xavier and Podolsky, 2007). Particularly important aspects in this respect are the MAPK signalling pathways, which have been found to have critical roles in IBD development and progression (Caruso et al., 2007, Kwon et al., 2007). One of the MAPK pathways, namely ERK1/2 is a pathway of interest, as its involvement in IBD is evidenced by its increased expression and activation in the colonic mucosa in both mouse models of colitis and IBD patients (Waetzig et al., 2002, Ihara et al., 2009). The classical ERK1/2 activation pathway begins with activation of MAPKKKs, which activate MAPKKs, such as MEK1/2. MEK1/2 specifically binds to and phosphorylates ERK1/2 (Chen et al., 2001). ERK1/2 has been found to have a role to play in the development and progression of IBD, through inducing expression of various cytokines, including TNF-α and IL-8 in macrophages, T-cells and epithelial cells (Semlali et al., 2012, Kwon et al., 2007, Caruso et al., 2007, van Heel et al., 2002).

Another MAPK pathway strongly implicated in the pathogenesis of inflammatory responses is p38 MAPK. ERK and p38 MAPK pathways connect via the signalling node, MNK1/2, which is also closely associated with activation of immune responses (Kotlyarov et al., 1999). The p38 MAPK family proteins are known to be activated by upstream MAPKKKs, namely TAK1, ASK1, MLK3, MEKK1-4 and TAO1-3 (Schindler et al., 2007). Similar to ERK1/2, p38 MAPK regulates proteins involved in inflammatory responses, including TNF-α, IL-1α, IL-2 and IL-6 (Kim and Choi, 2010, Broom et al., 2009). Moreover, multiple studies have also demonstrated increased activation of p38 MAPK in tissues from IBD patients (Dahan et al., 2008, Waetzig et al., 2002). Kotlyralov et al., showed that disruption in the p38 signalling cascade, attenuated LPS-driven cytokine release, suggesting that p38 MAPK is directly responsible for activation
of inflammatory gene transcription (Kotlyarov et al., 1999, Houde et al., 2001). Additional in vitro studies have shown that inhibition of p38 leads to reduced IL-6 and IL-8 production in intestinal epithelial cells (Parhar et al., 2003). Collectively, these studies demonstrate that MAPKs have important roles to play in regulating various aspects of barrier function; however, their involvement in regulating HβD production in the colon is currently unknown.

One of the major regulators of inflammatory responses in mammalian cells is the NF-κB signalling pathway, which is responsible for regulating pro-inflammatory cytokine production. Levels of NF-κB activation are significantly increased in inflamed intestinal tissues from mouse models of colitis and IBD patients (Rogler et al., 1998, Neurath et al., 1996). Furthermore, mucosal macrophages from IBD patients show an increased capacity to release pro-inflammatory mediators such as TNF-α, IL-6, IL-1, IL-12 and IL-23, via increased NF-κB activation (Rogler et al., 1998, Becker et al., 2003, Becker et al., 2001). Cytokines released via NF-κB are responsible for further activation of lamina propria immune cells, e.g., T-helper (Th)1 cells, which are important in disease progression (Pallone and Monteleone, 2001, Neurath et al., 2002). Furthermore, cytokines released through NF-κB-dependent pathways, in turn, can potentiate NF-κB, activation creating a positive feedback loop (Holtmann and Neurath, 2004). In addition, stimulation of the NF-κB pathway can also prevent pathogen-induced apoptosis in macrophages (Park et al., 2005), which contributes further to prolonged inflammatory responses.

Activation of NF-κB in epithelial cells has been reported to stimulate both pro-inflammatory responses and protective effects. For example, IL-6-driven activation of NF-κB increases expression of intercellular adhesion molecule-1, which subsequently enhances recruitment of neutrophils to the site of inflammation, therefore perpetuating the inflammatory response (Wang et al., 2003a). Pharmacological inhibition of NF-κB activity has also been shown to ameliorate intestinal inflammation in chemically-induced colitis and in the IL-10–/– model (Neurath et al., 1996, Dave et al., 2007) However, other studies have shown that specific inhibition of epithelial NF-κB in vivo results in spontaneous development of severe intestinal inflammation, associated with epithelial ulceration, decreased AMP production, elevated expression of pro-inflammatory
mediators and infiltration of immune cells (Nenci et al., 2007). Moreover, activation of NF-κB in the colonic epithelium is proven to exert anti-apoptotic effects, thereby strengthening the intestinal barrier against invading bacteria (Kajino-Sakamoto et al., 2010).

Of particular interest to this thesis, previous studies suggest that NF-κB may also have a role to play in regulating epithelial defensin production (Scharf et al., 2010). The promoter region of HβD-2 contains several NF-κB binding motifs, together with truncation studies suggesting that the proximal NF-κB binding site is an important regulator of HβD-2 transcription in airway and intestinal epithelial cells (Wang et al., 2003b, Tsutsumi-Ishii and Nagaoka, 2003). Thus, when taken together, previous studies provide strong evidence for the importance of NF-κB in regulating many different aspects of epithelial barrier function (Freel et al., 1983, Funderburg et al., 2007, Lehmann et al., 2002). In the previous chapter, we demonstrated for the first time, that bile acids regulate epithelial secretion of HβDs, important components of innate intestinal barrier function. In the current chapter we set out to further elucidate the molecular mechanisms involved. In particular, since bile acids are well known to induce cellular responses through activation of MAPKs and NF-κB (Shan et al., 2013, Shah et al., 2006, Zhang et al., 2014), we sought to determine the potential role of these pathways in mediating bile acid effects on HβD expression and secretion. We hypothesise that colonic bile acids target pro-inflammatory pathways to alter levels of HβD release from the epithelium, and therefore influence the pathogenesis of inflammatory responses.
4.2 Specific aims

The primary goal of this Chapter was to investigate molecular mechanisms involved in DCA and UDCA regulation of HβD secretion from colonic epithelial cells. Specifically, we aimed to examine:

I. The roles of TGR-5 and FXR in regulating HβD secretion

II. The roles of MAPKs and NF-κB in mediating DCA-induced HβD secretion

III. Mechanisms underlying the inhibitory effects of UDCA on HβD secretion.
4.3 Results

4.3.1 TGR5, but not FXR, activation increases HβD production and release in colonic epithelium.

Expression mapping studies show wide distribution of TGR5 gene expression in tissues and organs throughout the body, such as muscle, spleen, lymph node, brain, gallbladder, small intestine and colon in both humans and mice (Kawamata et al., 2003b). The highest expression of FXR is found in liver and epithelial cells of small intestine and colon (Harrell, 2007).

As the first step in the current studies, we examined the expression of TGR5 and FXR in our T84 cell model of the colonic epithelium. Using modified sequences from previously published primers, mRNA expression of TGR5 (Figure 4.1a) and FXR (Figure 4.1b) was analysed by semi-quantitative RT-PCR. In line with previous studies in human colonic epithelium, both TGR5 and FXR receptors were detected in T84 cells (Harrison and Hanauer, 2002, Hedl and Abraham, 2012, Hempfling et al., 2003). Furthermore, previously published findings, along with studies from this laboratory, have shown that GW4064 activates FXR in T84 cells, as measured by expression of FGF19 (Harrison and Hanauer, 2002, Walters, 2014, Keely et al., 2007, Mroz et al., 2014). FGF19 is well-established as an FXR target gene and is a reliable indicator of its activation (Herwig et al., 1998, Zhang et al., 2013).
Figure 4.1 TGR5 and FXR mRNA expression in T84 colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. mRNA was isolated from the cells and RT-PCR analysis was performed with primers specific for TGR5 and FXR. Images show representative RT-PCR results and the presence of a) TGR5 and b) FXR mRNA in T84 cells (n = 3).
Next in our studies, bile acid receptor agonists were used to determine if activation of either receptor alone influences HβD production. The selective agonist of TGR5, INT777, a semi-synthetic bile acid derived from CA, has high specificity for the receptor (Table 2.2) (Pellicciari et al., 2009). Monolayers of T₈₄ cells were treated bilaterally with INT777 (50 µM) and after 24 hrs, HβD mRNA levels were measured. Upon activation of TGR5, we found that there was an increase in HβD-1 and HβD-2 mRNA production by $3.3 \pm 0.5$ fold ($n = 3$, $p < 0.05$) and $36.6 \pm 32.8$ fold ($n = 3$), respectively (Figure 4.2). The effects of TGR5 activation on HβD protein release were also investigated. Levels of HβD-1 protein secretion were increased after 48 hrs treatment with INT777 (50 µM) from $23.9 \pm 3.6$ pg/ml to $350.8 \pm 73.8$ pg/ml compared to DMSO-treated controls ($n = 5$; $p < 0.01$) (Figure 4.3a). HβD-2 protein levels were also shown to be induced by INT777 treatment from $23.6 \pm 3.6$ pg/ml to $87.7 \pm 15.4$ pg/ml ($n = 5$; $p < 0.01$) (Figure 4.3b). These data suggest that TGR5 activation can induce HβD production from colonic epithelial cells.
Figure 4.2 TGR5 activation increases HβD-1 and HβD-2 mRNA production. T\textsubscript{84} cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with the TGR5 agonist, INT777 (50 \(\mu\text{M}\)), for 24 hrs. DCA treatment was used as a positive control. After treatment, mRNA was extracted and levels of (a) HβD-1 and (b) HβD-2 were assessed by q-PCR \((n = 3)\). All values were normalised to GAPDH gene expression and expressed as a fold change over untreated controls. Data represent the mean ± SEM for \(n\) experiments and were statistically analysed by repeated measures ANOVA with the Newman-Keuls post-test; *denotes significant differences compared to untreated controls; * \(p < 0.05\), ** \(p < 0.01\).
Figure 4.3 TGR5 activation induces HβD-1 and HβD-2 release from human colonic epithelial cells. T_{84} cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with the TGR5 agonist, INT777 (50 μM; bilateral), for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = 5). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Newman-Keuls post-test; * denotes significant differences compared to DMSO-treated controls; ** p < 0.01.
Having established that activation of TGR5 induces HβD expression and secretion, we wanted to determine if the nuclear bile acid receptor, FXR, also has a role to play. Monolayers of T84 cells were treated with GW4064 (10 μM) for 24 hrs, after which levels of HβD-1 and 2 mRNA production was assessed by qPCR. Levels of HβD-1 were unchanged in cells treated with GW4064 (n = 3) (Figure 4.4a), while HβD-2 levels were increased by 3.5 ± 1.7 fold compared to DMSO-treated controls (n = 3). However, this effect was not statistically significant (Figure 4.4b). The fact that FXR does not have a role to play in HβD production was confirmed in experiments in which we found that cells treated with GW4064 (10 µM) for 48 hrs did not secrete HβD-1 or 2 into the apical bathing media (n = 5) (Figure 4.5).
Figure 4.4 FXR activation does not alter HβD expression in colonic epithelial cells. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with FXR agonist, GW4064 (10 μM) for 24 hrs. DCA treatment was used as a positive control. After treatment, mRNA was extracted and levels of (a) HβD-1 and (b) HβD-2 was assessed by q-PCR (n = 3). All values were normalised to GAPDH gene expression and expressed as a fold change over untreated controls. Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to DMSO-treated controls; ** p < 0.01.
Figure 4.5 FXR activation does not alter HβD release from colonic epithelium. 
T84 cells were cultured as monolayers on permeable supports until TEER stabilised 
at plateau levels. Cell monolayers were treated with the FXR agonist, GW4064 (10 μM) 
for 48 hrs. After treatment, apical bathing media was collected and levels of (a) 
HβD-1 and (b) HβD-2 were assessed by ELISA (n = 5). Data represent the mean ± 
SEM for a series of n experiments and were statistically analysed by repeated 
measures ANOVA with the Student Newman-Keuls post-test; *denotes significant 
differences compared to DMSO-treated controls; ** p < 0.01.
The pharmacological studies shown earlier in this chapter clearly indicate the involvement of TGR5 in stimulation of HβD release. Next, in collaboration with Prof. Kirstina Schoonjans (EFPL), we set out to further investigate this using TGR5 knockout mice (TGR5\(^{-/-}\)). The phenotype of TGR5\(^{-/-}\) mice was described in detail in Chapter 2. Animals were housed normally in a 12 hr light/dark cycle and had access to food and water *ad libitum*. Animals were euthanized, colons were removed and mucosal scrapings were taken. The cells were then treated with DMSO or INT777 (30 μM) for 6 hrs. Levels of mβD-1 and mβD-4, orthologues of HβD-1 and HβD-2 respectively, were then assessed by q-PCR. We found that in WT mice treatment with the TGR5 agonist increased expression of both mβD-1 by 52.4 ± 12.4 fold (p = 0.01) and mβD-4 by 218.6 ± 77.8 fold (p = 0.05), compared to DMSO treated controls (n = 5). In contrast, in TGR5\(^{-/-}\) animals there was no significant induction of mβD-1 or mβD-4 in response to INT777 treatment (n = 6). In fact, mβD-4 levels were significantly lowered to 0.02 ± 0.01 fold (p = 0.0004) when compared to DMSO-treated controls (n = 6) (Figure 4.6). This data confirms that TGR5 activation induces mβD release in mouse colon.
4.3.2 Effects of UDCA on TGR5 agonist-driven HβD release.

In light of our findings in Chapter 3 that UDCA attenuates HβD secretion in response to DCA, and since DCA is known to be an agonist of TGR5 (Hisamatsu et al., 2003), we next set out to examine the effects of UDCA on TGR5-induced responses. Monolayers of T84 cells were treated with INT777 alone, or were co-treated with UDCA (50 μM), levels of HβD-1 and 2 secretion were also assessed. We found that UDCA co-treatment reduced INT777 induction of HβD-1 release from $350.5 \pm 73.8$ pg/ml to $194.3 \pm 37.3$ pg/ml ($n = 5$; $p < 0.05$) (Figure 4.7a) and of HβD-2 from $87.7 \pm 15.4$ pg/ml to $40.4 \pm 3.4$ pg/ml ($n = 5$; $p < 0.001$) (Figure 4.7b).

Figure 4.6 Activation of TGR5 increases mβD-1 and 4 production in WT, but not in TGR5−/−, mice. Colonic mucosal scrapings from wild type (WT) and TGR5−/− knockout mice were incubated ex vivo with vehicle (DMSO) or INT777 (30 μM) for 6 hrs. After treatment, mRNA was isolated from tissues and semi-quantitative RT-PCR analysis was performed with primers specific for mβD-1 or mβD-4 (WT; $n = 5$; TGR5−/−: $n = 6$). All values were normalised to GAPDH gene expression and expressed as a fold change over DMSO-treated controls. Data represent the mean ± SEM for $n$ experiments and were statistically analysed by Students t-tests, $p$ values over 0.05 were considered to be statistically significant.
Figure 4.7 UDCA attenuates TGR5-induced HβD-1 and HβD-2 release from T84 colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cell monolayers were treated with the TGR5 agonist, INT777 (50 μM) alone, or in combination UDCA (50 μM) for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = 5). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to DMSO-treated cells; # denotes significant differences compared to DCA-treated cells; ** p < 0.01, # p < 0.05, ## p < 0.01.
4.3.3 Involvement of MAPKs in bile acid-induced HβD-1 and HβD-2 secretion.

Next, we focussed on investigating the p38 MAPK pathway, we wanted to determine if this may have a role to play in mediating their actions of HβD release. Polarised monolayers of T₈₄ cells were treated with SB023580 alone or were co-stimulated with DCA (150 μM) and SB203580 (10 μM) bilaterally for 48 hrs. The apical media was then collected and ELISA performed for measurements of HβD-1 and HβD-2. As shown in Figure 4.8, inhibition of p38 MAPK did not alter basal HβD-1 secretion (160.3 ± 20.9 pg/ml) when compared to vehicle-treated controls (191.9 ± 35.9 pg/ml), but significantly decreased DCA (150 μM)-stimulated HβD-1 secretion from 315.7± 22.6 pg/ml to 252.1 ± 43.2 pg/ml (n = 6; p < 0.05) (Figure 4.8a). Basal levels of HβD-2 were also unaffected by SB203580 treatment (58.7 ± 4.8 pg/ml), when compared to vehicle-treated controls (61.9 ± 3.3 pg/ml). However, inhibition of the p38 pathway significantly decreased DCA-induced HβD-2 secretion from 151.9 ± 14.2 pg/ml to 79.7 ± 5.7 pg/ml (n = 5; p < 0.001) (Figure 4.8b). These data suggest that p38 MAPK is an important step in the signalling mechanism underlying both DCA-induced HβD-1 and HβD-2 production.
Figure 4.8 Inhibition of p38 MAPK attenuates DCA-induced HβD-1 and HβD-2 secretion from colonic epithelial cells. T_84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle or with the p38 MAPK inhibitor, SB203580 (10 μM) alone, or in combination with DCA (150 μM) for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = 6). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to DMSO-treated cells; # denotes significant differences compared to DCA-treated cells; δ denotes significant differences compared to SB203580-treated cells; ** p < 0.01; *** p < 0.001, # p < 0.05, ### p < 0.001, δ p < 0.05; ns denotes not significant differences.
Next, we focussed on investigating the p38 MAPK pathway in respect of bile acid activation and to explore whether UDCA is involved in p38 mediated effects. Polarised T_{84} cells were treated bilaterally with either DCA (150 μM) or INT777 (10 μM) for 1 hr, after which levels of phospho-p38, a reliable marker of p38 MAPK activation, were investigated by western blotting (Figure 4.9a). We found that both DCA and INT777 increased p38 MAPK phosphorylation by 2.9 ± 0.43 and 2.9 ± 0.84 fold, respectively (n = 6; p < 0.01) (Figure 4.9b) when compared to controls.

Taking into consideration our previous findings regarding the inhibitory effects of UDCA on HβD secretion, we went on to investigate if UDCA also altered DCA-induced p38 MAPK activation in our model. As shown below, UDCA alone slightly induced phosphorylation of p38 by 1.9 ± 0.4 fold (n = 6). Cells co-treated with DCA and UDCA increased phosphorylation of p38 by 2.8 ± 0.71 fold (n = 6, p < 0.001) of controls, indicating that UDCA does not prevent DCA-induced p38 MAPK activation (Figure 4.9c).

Next we examined if the p38 inhibitor, SB203580, alters DCA-induced p38 phosphorylation. SB203580 decreased DCA-induced phosphorylation of p38 to 0.69 ± 0.21 fold when compared to vehicle treated controls (n = 6; p < 0.05) (Figure 4.9d).

Together, these studies indicate that although DCA and TGR5 activation stimulate p38 MAPK activation in colonic epithelial cells, this is not the target of inhibition by UDCA.
Figure 4.9 DCA and INT777 activate p38 MAPK in colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then stimulated with DCA (150 μM) or INT777 (10 μM) for 1 hr, after which lysates were prepared and changes in phosphorylation of p38 MAPK were assessed by western blotting. All samples were normalised to β-actin.


b) Densitometric analysis of western blot from cells treated with DCA and INT777 (n = 6).

c) Densitometric analysis of western blot from cells treated with DCA and UDCA alone, or co-treated with both bile acids (n = 6).

d) Densitometric analysis of western blot from cells co-treated with the p38 MAPK inhibitor, SB203580 (10 μM), and DCA (150 μM) (n = 6)

Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to vehicle-treated cells * p < 0.05, ** p < 0.01.
In further experiments, proceeded to investigate the potential involvement of ERK1/2 MAPK in DCA-induced HβD release. Polarised monolayers of T84 cells were treated with the ERK1/2 inhibitor, PD98059 (20 μM), alone or with DCA (150 M) for 48 hrs. The apical media was then collected and ELISAs for HβD-1 and HβD-2 were performed. We found that PD98059 had no effect on basal HβD-1 secretion (n = 6). Similarly, inhibition of ERK MAPK did not alter DCA-induced HβD-1 expression (n = 6) (Figure. 4.10a). Basal levels of HβD-2 were also unaffected by PD98059 (20 μM) treatment (70.6 ± 4.0 pg/ml) when compared to vehicle-treated controls (61.9 ± 3.3 pg/ml) (n = 5). However, DCA-induced levels of HβD-2 secretion were attenuated by PD98059 to 115.9 ± 13.4 pg/ml compared to 151 ± 14 pg/ml in DCA-treated cells (n = 5; p < 0.05) (Figure 4.10b). These data indicate that ERK1/2 MAPK is not involved in DCA-stimulated HβD-1 release but may have a role to play in that of HβD-2.
Figure 4.10 ERK MAPK inhibition attenuates DCA-induced HβD-2, but not HβD-1, secretion. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle or with the ERK1/2 MAPK inhibitor, PD98059 (20 μM) alone, or in combination with DCA (150 μM) for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to DMSO-treated cells; # denotes significant differences compared to DCA-treated cells; * p < 0.05, ** p < 0.01, *** p < 0.001, # p < 0.05; ns denotes not significant.
4.3.4 DCA stimulates NF-κB activation to induce HβD release

4.3.4.1 DCA but not UDCA induces NF-κB activity in colonic epithelial cells

Having demonstrated the involvement of MAPKs in driving HβD release, we next sought to investigate the role of another import pro-inflammatory transcription factor, NF-κB. To do this, we examined the phosphorylation of p65 protein, a key signalling protein downstream of the IKK complex, and a reliable index of NF-κB activation. DCA increased phosphorylation of p65 after 3 hrs of treatment by 2.3 ± 0.5 fold (n = 3; p < 0.05), and further increased it to 5.0 ± 0.9 fold (n = 3; 0.001) after 6 hrs of treatment compared to untreated controls (Figure 4.11 a). In contrast, we found that UDCA did not alter NF-κB activation (Figure 4.11b). These results suggest that DCA may exert its effects on HβD secretion via activation of NF-κB.
Figure 4.11 DCA, but not UDCA, activates NF-κB in T84 cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were left untreated or were treated with either a) DCA (150 μM) or b) UDCA (150 μM) for the indicated times. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by western blotting for phospho- and total p65. All values were normalised to β actin protein expression and expressed as fold change over untreated controls. Data represent the mean ± SEM (n = 3) for n experiments and were statistically analysed by Students t-tests, p values over 0.05 were considered statistically significant; *denotes significant differences compared to untreated controls; * p < 0.05; *** p < 0.001.
Given that UDCA can inhibit DCA-induced HβD secretion, we wanted to examine whether it might do so by altering DCA-induced NF-κB activation. Monolayers of colonic epithelial cells were co-treated bilaterally with DCA (150 μM) in the absence or presence of UDCA (10 – 100 μM) for 6 hrs. Phosphorylation of p65 was then assessed by western blotting. As previously shown, DCA increased phosphorylation of p65 (n = 4; p < 0.001), and we found this effect to be inhibited by UDCA treatment (Figure 4.12).

p38 MAPK has previously been shown to regulate NF-κB activation in other cell systems (Hoover et al., 2000), and in the current studies we found the p38 inhibitor, SB203580, decrease basal NF-κB activation in T₈⁴ cells, suggesting it is also likely to function upstream of NF-κB in colonic epithelial cells (Figure 4.13).
Figure 4.12 UDCA inhibits DCA-induced phosphorylation of p65 in colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were treated with DCA (150 μM), UDCA (200 μM), or a combination of both DCA (150 μM) and UDCA (10 – 100 μM) for 6 hrs. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by western blotting for phospho- and total p65 (n = 4). All values were normalised to β actin protein expression and expressed as fold change over untreated controls. Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated cells; *** p < 0.001, ### p < 0.001.
Figure 4.13 p38 inhibition decreases NF-κB activity in colonic epithelial cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle, DCA and p38 phosphorylation inhibitor, SB203580 (10 μM) for 48 hrs. After treatment cells were then lysed and proteins were separated by SDS-PAGE and analysed by western blotting for phospho- and total p65. All values were normalised to β actin protein expression and expressed as fold change over vehicle-treated cells. Data represent the mean ± SEM (n = 5) and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to vehicle (DMSO)-treated cells; * p < 0.05, ** p < 0.01.
4.3.4.2 NF-κB mediates basal and DCA-induced HβD secretion in colonic epithelial cells.

Since NF-κB has previously been shown to regulate HβD production in the intestine (Mondel et al., 2009), we next set out to examine its potential involvement in DCA-induced HβD secretion. Polarised monolayers of colonic epithelial cells were treated with an NF-κB inhibitor, BMS-345541 (25 μM), alone or in combination with DCA (150 μM) for 48 hrs. The apical media were collected and levels of HβD-1 and 2 were assessed by ELISA. In the presence of BMS-345541, we observed a decrease in basal secretion of HβD-1 from 153.4 ± 12.6 pg/ml in vehicle (DMSO)-treated controls to 63.4 ± 12.1 pg/ml (n = 6; p < 0.05) (Figure 4.14a). HβD-2 secretion was almost completely abolished from 40.1 ± 10.6 pg/ml in controls to 1.7 ± 1.5 pg/ml in BMS-345541 treated cells (n = 6; p < 0.05) (Figure 4.14b). These data suggest that basal NF-κB activity is necessary for both HβD-1 and 2 production.

Next, we investigated the potential involvement of NF-κB in DCA-induced production of HβDs. While we found that treatment carried out with BMS-345541 had no effect on DCA-stimulated production of HβD-1 (Figure 4.14a), HβD-2 secretion was significantly reduced to 27.1 ± 7.8 pg/ml compared to 115.1 ± 7.7 pg/ml in DCA-treated control (n = 6; p < 0.001) (Figure 4.14b). These data indicate that DCA induces HβD-2, but not HβD-1, via NF-κB activation.
Figure 4.14 NF-κB signalling is involved in DCA-stimulated HβD-2, but not HβD-1, release. T₈4 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle (DMSO) or with the NF-κB phosphorylation inhibitor, BMS-345541 (25 μM) alone, or in combination with DCA (150 μM) for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = 6). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to DMSO-treated cells; # denotes significant differences compared to DCA-treated cells; * p < 0.05; *** < 0.001, ### p < 0.001.
4.3.4.3 TGR5 activation increases NF-κB in colonic epithelial cells.

Finally, we set out to investigate the effects of bile acid receptor activation on the NF-κB pathway in colonic epithelial cells. In these experiments, cells were treated with either the TGR5 or FXR agonists, INT777 (10 μM) and GW4064 (5 μM), respectively, for 6 hrs. Activation of NF-κB was evaluated by western blotting with a phospho-p65 specific antibody. We found that INT777 significantly increased phosphorylation of NF-κB by $2.1 \pm 0.1$ fold ($n = 5; p < 0.01$) compared to vehicle-treated controls. In contrast, GW4064 was without effect on p65 phosphorylation (Figure 4.15).

To gain further insights into the involvement of NF-κB in bile acid-driven HβD release, cells were co-treated with INT777 in the presence or absence of the NF-κB inhibitor, BMS345541. Here, we found that inhibition of NF-κB did not significantly alter TGR5-induced HβD-1 release (Figure 4.16a). In contrast, INT777-induced HβD-2 levels were decreased from $97.9 \pm 5.5$ pg/ml in controls to $28.8 \pm 4.3$ pg/ml in BMS-345541-treated cells ($n = 6; p < 0.001$) (Figure 4.16b). Collectively, these data indicate that TGR5 activation induces secretion of both HβD-1 and 2, but that the pathways underlying these responses are differentially regulated by NF-κB.
Figure 4.15 TGR5, but not FXR, activation stimulates NF-κB in T84 cells. T84 cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were treated with INT777, GW4064 and DCA for 6 hrs. Cells were then lysed and proteins were separated by SDS-PAGE and analysed by western blotting for phospho- and total p65. All values were normalised to β actin protein expression and expressed as fold change over vehicle-treated cells. Data represent the mean ± SEM (n = 3) for n experiments and were statistically analysed by Students t-tests, p values over 0.05 were considered statistically significant; *denotes significant differences compared to vehicle-treated cells; ** p < 0.01.
Figure 4.16 NF-κB mediates TGR5-induced HβD-2, but not HβD-1, secretion. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle (DMSO), DCA (150 µM), TGR5 agonist, INT777 (10 µM), NF-κB phosphorylation inhibitor, BMS345541 (25 µM) alone, or co-treated with combination of INT777 and BMS345541 for 48 hrs. After treatment, apical bathing media was collected and levels of (a) HβD-1 and (b) HβD-2 were assessed by ELISA (n = 6). Data represent the mean ± SEM for a series of n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to DMSO-treated cells; # denotes significant differences compared to INT777-treated cells; * - compared to DCA-treated cells; * p < 0.05, *** p < 0.001, ### p < 0.001.
4.4 Discussion

Having shown in Chapter 3 that bile acids can differentially regulate the release of HβDs from colonic epithelial cells, in the current chapter we set out to investigate the molecular pathways involved in these effects. First, we used specific agonists to determine the potential involvement of the bile acid receptors, TGR5 and FXR in bile acids-induced modulation of HβDs release. Previous studies have shown that T84 cells express both TGR5 and FXR (Hempfling et al., 2003, Huang et al., 2007b) and this was confirmed, at the mRNA level, in the current studies. Furthermore, both receptors have repeatedly been shown to participate in regulation of innate immunity (Iguchi et al., 2011, Inagaki et al., 2006a). In the current studies, we found that stimulation with the TGR5 agonist, INT777, upregulated HβD gene expression and protein secretion in the T84 cell model. Furthermore, in collaboration with Prof. Kristina Schoonjans (EFPL), who supplied us with INT-777-treated colonic mucosal scrapings from wild type and TGR5-/- mice, we confirmed that TGR5 receptor activation upregulates secretion of mβDs in the colon. Thus, together our data suggests a new role for TGR5 in regulating HβD secretion from colonic epithelial cells.

Since TGR5 is expressed on colonic epithelial cells, which are exposed to high levels of bacteria and their metabolites, our data suggests that TGR5 may have an important role in mediating epithelial responses to changes in the microbiome. Furthermore, it is possible that dramatic changes in colonic bacterial populations, such as those that occur in UC, could lead to alterations in the colonic bile acid pool that favour TGR5 activation, in turn leading to increased levels of mucosal HβD-2 that have been reported in these patients (Wehkamp et al., 2003). In support of this idea, it has been shown that microbial dysbiosis in UC leads to increased levels of conjugated colonic bile acids (Duboc et al., 2013), that preferentially activate TGR5 (Kawamata et al., 2003a). Ultimately, whether the TGR5 induction of HβDs is beneficial or detrimental in the setting of UC remains to be determined. For example, it may serve as a barrier promoting effect which prevents bacteria entering the mucosa under
conditions of increased epithelial permeability, or alternatively TGR-5-induced HβD release may contribute to the development of inflammation through induction of cytokine release and recruitment of mucosal immune cells. The precise role of TGR5 in regulating mucosal barrier function is also made more complicated by its apparent anti-inflammatory effects on innate immune cells, such as macrophages (Pols et al., 2011) and monocytes (Yoshimura et al., 1997). However, the pro-inflammatory effects of TGR5 activation on monocytes has also been demonstrated (Mobraten et al., 2014). Furthermore, in support of an important role for TGR5 in IBD, studies have shown that TGR5−/− mice have increased intestinal permeability and susceptibility to developing colitis in response to DSS treatment (Cipriani et al., 2011), while TGR5 expression is increased in mucosal tissues from CD patients (Yoneno et al., 2013).

Having established TGR5 as an important regulator of colonic epithelial HβD production, our studies next focussed on further elucidating the molecular pathways involved in mediating bile acid effects on HβDs. Bile acids are well known to activate many intracellular signalling pathways, including MAPKs (Anwer, 2012). In the current studies, we found that DCA and the TGR5 agonist, INT777, both induce p38 MAPK activation in colonic epithelial cells. Such effects are in accordance with the literature, which have previously shown that DCA and TGR5 activation can promote p38 activation in different cell types (Qiao et al., 2001, Dent et al., 2005). To further investigate the involvement of p38 MAPK in HβD secretion, we employed a selective p38 MAPK inhibitor, SB203580 and found that in the presence of the inhibitor, DCA does not induce HβD-2 release, while HβD-1 production is significantly attenuated. In contrast, we found that ERK MAPKs appear to be less important in mediating bile acid-induced HβD secretion, since an ERK MAPK inhibitor, PD98059, was without effect on DCA-stimulated HβD-1 release and only slightly altered HβD-2 production in response to the bile acid.

The expression of HβD-2 in intestinal and colonic epithelial cell lines is increased upon stimulation with IL-1α, flagellin or bacteria, in an NF-κB -dependent manner (O’Neil et al., 1999, Ogushi et al., 2004). The evidence emerging from this chapter implies that HβD-1 and HβD-2 are regulated by
different molecular mechanisms. In the current studies, we found that p38 MAPK inhibition decreases NF-κB activation, indicating that p38 is likely to be involved in signalling upstream of NF-κB in the T84 cell model. Thus, we focussed our attention on NF-κB being a potential mediator of bile acid-induced HβD release. Interestingly, we found that both DCA and INT-777-induced phosphorylation of the p65 subunit of NF-κB, implying that bile acids acting through TGR5 can bring about activation of the transcription factor in colonic epithelial cells. Furthermore, we found that in the presence of an NF-κB inhibitor, BMS345541, bile acid and TGR5-induced HβD-2, but not HβD-1, secretion was inhibited. These results suggest that NF-κB is of most importance in bile acid and TGR5-induced HβD-2 release. This result was not surprising, as it has already been shown that HβD-2 is inducible during the course of inflammation and infection in the intestine, a process in which NF-κB plays a central role (Cobo and Chadee, 2013).

Since in the previous chapter, we found that UDCA can inhibit DCA-induced HβD secretion; consequently we proceeded to investigate the effects of this bile acid on TGR5-induced HβD production. We found that UDCA also inhibited TGR5-stimulated releases of both HβD-1 and HβD-2, indicating that the effects of UDCA are not only specific for DCA actions. While UDCA is a poor agonist of TGR5 (Pols et al., 2011), there is no data in literature which describes the antagonistic effects of the bile acid on TGR5 signalling. Thus, the mechanisms via which UDCA triggers cellular responses which lead to inhibition of TGR-5-induced HβD release are not clear. One possibility could be that it exerts its effects via activation of other bile acid receptors, such as PXR. PXR has been shown to be activated by UDCA in human hepatocytes (Schuetz et al., 2001), while its activation in the intestinal epithelium has been shown to exert protective effects in the DSS-induced mice model of colitis (Terc et al., 2014). Furthermore, activation of PXR has been shown to inhibit NF-κB in the intestinal epithelial cells (Mencarelli et al., 2011).

Exploring the molecular mechanisms by which UDCA can attenuate DCA-driven secretion of HβDs, we investigated its effect on DCA-induced levels of p38. We found that UDCA does not inhibit DCA-induced p38 activation, indicating the UDCA is likely to exert its inhibitory effects by interacting with the DCA/TGR5-
dependent signalling pathway downstream of p38 MAPK. With this in mind, we investigated the effects of UDCA on the NF-κB pathway. We found that UDCA attenuates DCA-induced NF-κB activation. This result was confirmation of an already known phenomenon previously described in human colonic epithelium (Shah et al., 2006). The mechanisms by which UDCA can exert its inhibitory effects on NF-κB are yet not fully understood. Previous reports reveal involvement of glucocorticoid receptor (GR) in UDCA-mediated NF-κB inhibition in hepatocytes and esophageal epithelial cells (Kharwanlang and Sharma, 2011, Miura et al., 2001). However, the T₈₄ cells used here lack GR expression (Tsugita et al., 2009). Thus, this is an unlikely mechanism of UDCA action in our studies. However, UDCA has also been shown to activate VDR and inhibit NF-κB activation (Halilbasic et al., 2013). Therefore, experiments investigating the effects of VDR agonist, atorvastatin or pregnenolone-16-carbonitrile, on DCA-induced HβD-2 release may yield very interesting results.

NF-κB signalling displays opposing roles in intestinal inflammation: on the one hand, inhibition of NF-κB signalling can disturb immune homeostasis triggering inflammation and disease, but on the other hand increased and sustained NF-κB activation induces inflammation and tissue damage. Previously published research shows that excessive NF-κB activation contributes to intestinal inflammation and that NF-κB inhibition could have therapeutic effects in IBD (Wullaert et al., 2011, He et al., 2015, Jian et al., 2005). This is concluded from studies showing that administration of drugs that bind and abolish activators of NF-κB, such as p65, NEMO of IKK, greatly reduces inflammation in experimental colitis mice models (Li and Chiang, 2013, Lieu et al., 2014, Liu et al., 1996). Furthermore, numerous studies have demonstrated increased activation of NF-κB in the intestinal mucosa of IBD (Longman et al., 2006, Schreiber et al., 1998b).

The results in this chapter suggest DCA increases secretion of constitutively expressed HβD-1 and infection inducible HβD-2 through activation of the TGR5 receptor and subsequent NF-κB activation. While the increased expression of HβD-1 shows no effect on inflammatory responses, HβD-2 is known as a potent inducer of chemotaxis and its elevated secretion in the absence of bacteria can
cause prolonged and uncontrolled inflammation (Schröder and Harder, 1999, Strober et al., 2007). Although we cannot completely exclude the possibility that the inhibitors used block pathways other than those mentioned, our results are in accordance with the previously published data obtained by gene reporter assays, indicating that p38 MAPK and NF-κB are required for HβD-2 induction in colonic epithelium (Wehkamp et al., 2004). However, one cannot exclude that there is a possibility that other transcription factors may also participate in HβD-2 induction by DCA in colonic epithelial cells. For instance activator protein (AP)-1, AP-2, and NF-IL-6 had been shown to be involved in modulation of HβD secretion in the inflammatory state (Harder et al., 2000).

Collectively, on the basis of previously published results and data obtained in our lab, we hypothesise that physiological concentrations of DCA confers protective actions on colonic epithelium. Such beneficial effects are likely mediated via activation of TGR5, p38 MAPK and NF-κB, resulting in DCA-induced release of HβDs. We hypothesise that under such conditions this release of HβDs maintains a germ-free environment overlying the epithelium, thereby preventing bacterial invasion and upregulation of inflammation (Figure 4.17). As the concentration of DCA rises in the pathological scenario, the inappropriate and sustained activation of the NF-κB pathway occurs and high concentrations of HβDs are released from epithelial cells. Due to its chemmoattractive properties, HβD-2, then causes an infiltration of neutrophils and monocytes to the surrounding mucosa, increasing inflammatory necrosis and scarring, which, in turn, causes further inflammation (Mahadevan, 2006). Data presented in this chapter highlights the importance of colonic bile acids balance. Interestingly, UDCA has been shown to reverse DCA-induced activation of NF-κB pathway and as shown in Chapter 1, subsequent attenuation of DCA-induced HβDs release. Under normal circumstances, levels of UDCA in the colonic water are found at the range of 5 – 10 µM (Hamilton et al., 2007). Thus, it needs to be noted, that levels of UDCA used in these studies could be only achieved in the colon by introduction of UDCA in the form of therapeutics to attain the desirable effects. Thus, on the basis of this evidence, it is tempting to speculate a new role for bile acids as mediators of epithelial
barrier function, modulators of intestinal inflammation and regulators of colonic microbiome via alternation levels of AMPs.

Figure 4.17. Proposed mechanisms of DCA and UDCA regulation of defensin secretion in colonic epithelial cells. DCA exerts its effects in colonic epithelium acting via the TGR5 receptor, increasing activation of NF-κB downstream of p38 MAPK. NF-κB translocates to nucleus where it binds to an NF-κB binding site upstream of the HβD-2 promotor. In contrast, HβD-1 is regulated by a pathway independent of NF-κB activation. UDCA attenuates DCA-induced NF-κB activation and inhibits secretion of HβD-2. The mechanisms involved in UDCA-mediated decrease of HβD-1 release remain unknown.
CHAPTER 5

Regulation of colonic epithelial restitution by secondary bile acids
5.1 Introduction

Harmful substances present in the lumen of the gut continually challenge the integrity of the intestinal epithelium, often resulting in colonic barrier damage. Epithelial damage and loss of intestinal barrier function are hallmark pathologies of mucosal inflammation (Turner, 2009). Even shallow injuries to the epithelium result in damage to the barrier and require healing (Podolsky, 1999). Following wounding, the migration of healthy epithelial cells adjacent to the wound edge occurs, a process referred to as restitution. To date, most data regarding epithelial restitution has been derived from studies involving 2-dimensional planar models. There is less known about the mechanisms involved in cell migration in the 3-dimentional matrix (Bindschadler and McGrath, 2007). In vivo studies of stratified epithelia revealed that cell-cell contacts are maintained and cells migrate as a collective sheet to close wounds (Peglion et al., 2014). Time lapse 3-dimensional imaging has further demonstrated that the epithelium surrounding the wound migrates in a smooth sliding fashion, and cells taking part in migration processes extend well beyond the wound margin (Farooqui and Fenteany, 2005). Restitution of the intestinal epithelium is modulated by a range of highly divergent factors, among which are a broad spectrum of regulatory peptides including, growth factors, cytokines or antimicrobial agents (Sheetz et al., 1999, Taupin and Podolsky, 2003, Mazuy et al., 2015).

5.1.1 The role of epithelial wound repair in IBD

The importance of wound healing in IBD is apparent. In vivo administration of DSS in animal models causes induction of mucosal inflammation resembling UC in humans, as described in Chapter 3. DSS induces inflammation in the colon by disrupting the epithelial barrier, allowing passage of luminal contents and bacteria into the mucosa, thereby triggering high levels of inflammation (Wirtz et al., 2007, Alex et al., 2009). Moreover, NF-κB transcriptional activity is necessary for maintenance of epithelial barrier function and a proper response to wounding (Fox et al., 2004, Zaph et al., 2007, Lebeis et al., 2007, Reuther-Madrid et al., 2002). In IBD, there is a considerably slower mucosal wound healing in the colon, despite high levels of NF-κB activation (Atreya et al., 2008, Sturm and Dignass, 2008). Furthermore, enhancing epithelial restitution by
various factors, such as TGF-β (Grazul-Bilska et al., 2003), prostaglandin E2 (PGE2) (Rieder et al., 2007) and glucagon-like-peptide-2 (GLP-2), *in vivo* has been shown to ameliorate inflammation in the DSS-induced mouse model of colitis (Matsuura et al., 2005, L'Heureux and Brubaker, 2003) and in UC patients (Sinha et al., 2003). However, mechanisms responsible for decreased colonic epithelial wound healing in IBD still remain unclear.

5.1.2 Epithelial wound repair and the role of ion transport

Multiple ion channels and transporters have been implicated in playing a role in various stages of the cell migration process. Ion channels are essential for regulating cell volume, intra and extracellular pH, and remodelling of the actin cytoskeleton; processes which are important for cell movement (Wang et al., 2014, Pier et al., 2014). The tight control of cell volume is critical to the process of migration, as cells undergo dramatic changes in shape, including lamellipodia lengthening and re-unification of the trailing end of the cell (Vicente-Manzanares et al., 2005, Jakab and Ritter, 2006). Amongst many ion channels and transporters involved, K⁺ channels participate in the regulation of cell volume by providing a pathway for K⁺ exit at the rear of the cell, creating a localised driving force for water efflux (Schwab et al., 2008a). Opposite effects of inhibition of K⁺ channel exists along the GIT. For example, it has been shown in a small intestinal model that K⁺ channel inhibition lowers epithelial cell migration (Rao et al., 2002), while a clear acceleration of wound healing is observed in colonic epithelial cells upon K⁺ channel blockade (Lotz et al., 2004).

The aquaporins (AQPs), channels which allow the passage of water into and out of cells, have also been shown to be important in regulating epithelial cell migration. Migration of renal and corneal epithelial cells, keratinocytes, fibroblasts, astrocytes and endothelial cells have all been shown to be inhibited upon suppression of AQP expression or function (Papadopoulos et al., 2008).

AQPs and K⁺ channels require Cl⁻ movement in order to maintain appropriate electrical and osmotic driving forces for their activity (Jacobsen et al., 2013), and the involvement of Cl⁻ channels in epithelial restitution has also been demonstrated (Marino and Kotsias, 2014, Schiller et al., 2010b, Rubenstein et al., 2011). It is thought that Cl⁻ channels may contribute to the volume
decreases at the rear end of the cell that facilitate migration. Furthermore, the impairment of epithelial restitution in CFTR−/− airway epithelial cells (Rubenstein et al., 2011), suggests an important role for this channel in regulating migration. Importantly, it should also be noted that in addition to functioning as an anion channel, CFTR is also known to be involved in regulating cell proliferation and apoptosis (Lang et al., 2005, Kirk, 2010), suggesting it is likely to play an important role in maintenance of intestinal barrier function.

5.1.3 Pathways involved in wound repair

Wound repair is a highly regulated process with multiple pathways being involved in its orchestration. For example, activation of ERK1/2 and p38 MAPKs mediate increased restitution after stimulation with TGF-α (Goke et al., 1998). Furthermore, NF-κB is also important in regulating migration since inhibition of its activity prevents intestinal epithelial restitution in vivo (Egan et al., 2003a). Interestingly, it appears that acute activation of the NF-κB pathway induces proliferation of small intestinal epithelial cells during mucosal repair, while chronic upregulation of NF-κB, in prolonged inflammatory states, results in delayed epithelial healing (Okamoto and Watanabe, 2005). These data suggest that complex signalling mechanisms are involved in the control of epithelial restitution.

5.1.4 Bile acids and wound repair

As discussed earlier, bile acids are known to modulate multiple aspects of epithelial barrier function and their levels in the colon can be altered in a number of disease states, including UC (DiBaise and Islam, 2012, Jahnel et al., 2014). When present at pathophysiological levels, bile acids increase epithelial permeability (McGovern et al., 2010, Melis et al., 2014, Ma et al., 2002), stimulate Cl− secretion via CFTR (Domingue et al., 2015), and influence cell proliferation and apoptosis. Thus, bile acids are already known to regulate many of the physiological processes that contribute to barrier function (Keating et al., 2009, Lefebvre et al., 2009, Monte et al., 2009).

Previous studies have demonstrated that bile acids may also be important in regulating epithelial restitution. Interestingly, bile acids enhance wound healing and reduces fibrosis in hibernating black bears (Iaizzo et al., 2012).
Furthermore, intestinal restitution has also been shown to be promoted by bile acids, with NF-κB playing a central role (Strauch et al., 2001, Strauch et al., 2003).

Although the etiology of IBD remains undefined, it is clear that disruptions to intestinal epithelial barrier function and repair are key features. With this in mind, understanding how epithelial wound healing is dysregulated in conditions of IBD and identifying factors that promote this process may lead to the development of new therapeutic approaches.

5.2 Specific aims

While previous chapters demonstrated how bile acids modulate different aspects of epithelial barrier function, there is still a lack of data regarding their potential roles in regulation of colonic epithelial restitution. Therefore, this chapter will aim to determine structure-activity relationships of bile acids and their effect on colonic epithelia restitution; in particular we set out to:

I. Develop a model of colonic epithelial wound healing.

II. Investigate the effects of DCA and UDCA on colonic epithelial restitution.

III. Investigate molecular mechanisms underlying DCA and UDCA actions on restitution.
5.3 Results

5.3.1 Optimisation of conditions for polarized T₈⁴ cells as a model of colonic epithelial restitution.

First, to determine if the presence of the inclusion of serum in the growth media has an influence on restitution in T₈⁴ cells, polarised monolayers were grown in inserts on transparent semi-permeable membranes. 24 hrs before wounding, cells were transferred to growth medium, either with or without BCS (5% w/v). After wounding, cells were then either maintained in either serum-containing, or serum-free medium as shown below, and the extent of wound closure was then measured after 48 hrs.

**Figure 5.1 Experimental protocol for investigating serum requirement for epithelial restitution in T₈⁴ cells.** Fully polarized cells were grown on transparent semi-permeable supports and placed in serum-containing or serum-free media 24 hrs before wound was inflicted. After the wound was performed, cells were either maintained in the same conditions or switched to the opposite serum condition for 48 hrs.
Wound sizes at time 0 were measured to ensure that any differences observed at T = 48 hrs were not due to variations in the initial wound size. These data confirmed that there were no significant differences in wound size at T = 0 hrs between the various conditions (n = 3) (Figure 5.2a).

At the end of the experiment, the wound closure in monolayers grown in serum-containing media before and after wounding (i.e., serum/wound/serum) was 65.7 ± 6% (n = 3) of that at T = 0 hrs. The wound closure in cells pre-incubated in serum-free media before being placed in serum-containing media after wounding (i.e., serum free/wound/serum) was slightly decreased to 60.9 ± 8% (n = 3) of the T = 0 value. A significant decrease in the wound closure to 41.6 ± 3% (n = 3; p < 0.05) of T = 0 values was observed in cells incubated in serum-containing media before being placed in serum-free media post-wounding. Cells grown in serum-free media before and after wounding present lowest restitution with wound closure only reaching 14.7 ± 1% of the T = 0 value (n = 3; p < 0.001) (Figure 5.2b). These results show that efficient restitution of wounded T₈ monolayer requires inclusion of serum in the culture medium after wounding. Thus, in all subsequent experiments, all treatments were carried out in serum-containing media, unless otherwise stated.
Figure 5.2 Serum is required for epithelial restitution in T\textsubscript{84} cells. T\textsubscript{84} cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Cells were treated for 24 hrs in 2 conditions: serum-containing or serum-free media. After the wound was inflicted (T = 0) the cells were either retained in the same media or switched to serum-free/serum-containing media, as indicated. After 48 hrs wound area was measured again and values were calculated as a % of the original wound area at T = 0. a) The area of wounds at T = 0 was measured. No significant differences were recorded in wound size created at T = 0 across the conditions employed. b) 48 hrs post-wounding the wounded area was measured again. The results are represented as a mean change in wound closure at 48 hrs after injury (n = 3). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences between conditions.* p < 0.05; *** p < 0.001.
5.3.2. DCA inhibits epithelial restitution in polarised monolayers of T84 colonic epithelial cells.

We began these studies by investigating the effects of the most abundant colonic bile acid, DCA, on colonic epithelial restitution Polarised T84 cells grown as monolayers in complete media were wounded, after which they were incubated with DCA (50 or 150 µM). Wound closure was monitored by taking images immediately and 48 hrs post-wounding (Figure 5.3a).

At 48 hrs post-wounding, wound closure in untreated cells was 63.3 ± 13.5% of that at T = 0, while in cells treated with DCA (50 µM) it was reduced to 47.4 ± 6.4% (n = 5; p < 0.05). Monolayers treated with DCA (150 µM) had an even greater wound area, where wound closure decreased to 24.5 ± 13.1% (n = 5; p < 0.001), compared to untreated control cells. Therefore, DCA inhibited epithelial restitution in a concentration dependant manner (Figure 5.3b).

To gain further insight into how DCA may be preventing restitution, we examined if, at the concentrations used, it may be exerting toxic effects and inducing apoptosis. Cells were grown as polarised monolayers and after wounding, were incubated with DCA (150 µM) for 48 hrs. Proteins were extracted and western blotting performed using anti-cleaved PARP specific antibodies. Cleaved PARP has been established as a reliable marker of cellular apoptosis (Moran et al., 2015, Morrison et al., 1998). As a positive control, a clear increase in cleaved PARP was observed upon exposure to DCA (500 µM) for 2 hrs. However, DCA at a range of concentrations up to 150 µM had no significant effect on levels of cleaved PARP (n = 3) (Figure 5.4). These data suggest that DCA, at concentrations which prevent restitution, do not exert toxic actions through induction of apoptosis.
Figure 5.3 DCA inhibits restitution in colonic epithelial cells. T84 cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in the serum-containing media with DCA (50 μM, 150 μM). a) Images were taken immediately and 48 hrs after the wound was inflicted. b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 5). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; * p < 0.05, *** p < 0.001.
Figure 5.4 Chronic exposure to DCA at concentrations which inhibit restitution does not induce apoptosis. T₈₄ cells were cultured as monolayers on permeable supports until TEER stabilised at plateau levels. Post-wounding, cells were incubated for 48 hrs with DCA (50 – 150 μM) or for 2 hrs with DCA (500 μM), as a positive control. Cells were then lysed, proteins were separated by SDS-PAGE, and analysed by western blotting for cleaved PARP. All values were normalised to β-actin protein expression and expressed as fold change over untreated controls. Data represent the mean ± SEM (n = 3) for n experiments and were statistically analysed by repeated measures ANOVA with the Newman-Keuls post-test; *denotes significant differences compared to untreated controls; ** p < 0.01.
Next, we wanted to examine if the effects observed with DCA are mimicked by its conjugated derivative, TDCA, which is more hydrophilic and not able to cross cell membranes unless facilitated by transporters (van der Velden et al., 2013), which are present only on the basolateral side of T84 cells (Keely et al., 2007). Thus, when applied apically, any effects of conjugated bile acids are most likely to be mediated by membrane receptors. Cells were grown on 1% (w/v) glacial acetic acid collagen-coated insert, which prevent apical media diffusing into the basolateral compartment after wounding. Polarised T84 cells grown as monolayers in complete media were wounded, after which they were incubated with DCA (150 μM) or TDCA (50, 150 μM). Wound healing was monitored by taking images immediately and 48 hrs post-wounding (Figure 5.5a). Wound closure of untreated cells was 68.1 ± 1.9% of that at T = 0, while in cells treated with DCA (150 μM) it was 41 ± 2.1% (n = 3; p < 0.001). In contrast, in cells treated with TDCA (50 and 150 μM), wound closure was 55 ± 10.8% and 56 ± 6.9% of that at T = 0 (n = 4), respectively, and was similar to that of untreated control cells (Figure 5.5b). These data suggest that membrane receptors, such as TGR5, may not be important in mediating changes in epithelial restitution in response to luminal bile acids.
**Figure 5.5 TDCA does not alter restitution in colonic epithelial cells.** T₈⁴ cells were cultured as monolayers on collagen-coated transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with either DCA (150 μM) or TDCA (50 μM, 150 μM). **a)** Images were taken immediately and 48 hrs after the wound was inflicted **b)** 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 4). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; n.s denotes not statistically significant differences, * denotes significant differences compared to untreated controls; *** p < 0.001.
5.3.3 UDCA increases epithelial restitution in polarised monolayers of T84 colonic epithelial cells.

We next went on to examine the effects of UDCA on colonic epithelial restitution. T84 cells grown as monolayers in complete media were wounded after which they were incubated with UDCA (50 μM, 150 μM). DCA (150 μM) was employed as a positive control for inhibition of restitution in these experiments. Healing of the wounds was monitored by taking images immediately and 48 hrs post-wounding (Figure 5.6a). Wound closure in untreated cells was of 68.1 ± 3.4% of that at T = 0, while in cells treated with DCA (150 μM) it was reduced to 24.5 ± 13.0% (n = 5; p < 0.001). However, there was no significant change in wound closure in monolayers treated with UDCA (50 μM), compared to untreated controls (n = 4). Interestingly, monolayers treated with UDCA (150 μM) showed a significant increase in wound closure to 88 ± 4% (n = 5; p < 0.001) of that at T = 0 (Figure 5.6b).
Figure 5.6 UDCA promotes colonic epithelial restitution. T_series cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with DCA (150 μM) or UDCA (50 μM, 150 μM). a) Images were taken immediately and 48 hrs after the wound was inflicted b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 5). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test; ns denotes not statistically significant comparison, * denotes significant differences compared to untreated controls; * p < 0.05, *** p < 0.001; ns denotes not statistically significant differences.
5.3.4 UDCA prevents DCA-induced inhibition of epithelial restitution in polarised monolayers of T_84 cells.

So far our data in this chapter has revealed that DCA inhibits, whereas UDCA promotes, colonic epithelial restitution. We next wished to investigate if UDCA has the capacity to alter responses to DCA. T_84 cells, grown as monolayers on permeable supports, were wounded, after which they were incubated with DCA (150 μM) alone or co-treated DCA (150 μM) and UDCA (50 – 150 μM). Healing of the wounds was monitored by taking images immediately and 48 hrs post-wounding (Figure 5.7a). Wound closure in untreated cells was 58.4 ± 5% of that at T = 0, while in cells treated with DCA (150 μM) it was decreased to 21 ± 15% (n = 6). Interestingly, co-treatment with UDCA prevented the inhibitory effects of DCA in a concentration dependent manner. Although, UDCA (50 μM) was without effect, in monolayers treated with DCA and UDCA (100 μM) wound closure was increased to 79.1 ± 9.8% (n = 6; p < 0.001), whereas in cells treated with UDCA (150 μM) wound closure was 55.2 ± 7.3% (n = 6; p < 0.001) (Figure 5.7b). These data suggest that UDCA has a protective effect against DCA-induced inhibition of colonic epithelial restitution.
Figure 5.7 UDCA attenuates DCA-induced inhibition of colonic epithelial restitution. T₈₄ cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with DCA (150 μM) and UDCA (150 μM) alone, or co-treated UDCA (50 – 150 μM) in the presence of DCA (150 μM). a) Images were taken immediately and 48 hrs after the wound was inflicted b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 6). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test, * denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated cells; **p < 0.01, ***p < 0.001, **** p < 0.0001.
5.3.5 Molecular mechanisms underlying bile acid regulation of restitution in colonic epithelium.

Having established the effects of DCA and UDCA on colonic epithelial restitution, we next wanted to explore the cellular and molecular mechanisms involved. Epithelial wound healing is dependent on a balance between migration, proliferation, and functional differentiation of the cells neighbouring the injured area (see Chapter 1 for more details).

5.3.5.1 DCA and UDCA regulate colonic epithelial migration.

We first tested whether the observed effects of bile acids on epithelial restitution are due to changes in cell migration or proliferation. Two colonic cell lines were used for these studies, T84 and HT-29Cl.19A, to ensure that effects observed are not cell line-specific. Boyden chamber assays were performed in order to measure cell migration through porous membranes. Cells were seeded at a density of $1 \times 10^4$ in the presence of DCA or UDCA, alone or in combination. After seeding, cells were allowed to migrate for 48 hrs and migration across the membrane was measured by staining and counting the cells that migrated to the basolateral side of the membrane. These experiments revealed that in T84 cells, DCA (150 μM) decreased cell migration to $0.7 \pm 0.1$ fold ($n = 5$, $p < 0.05$) of that in untreated controls. In contrast, UDCA increased T84 cell migration to $1.7 \pm 0.3$ fold ($n = 5$; $p < 0.05$) of untreated controls. Furthermore, in co-treated cells, UDCA prevented the inhibitory effects of DCA, where migration was $1.3 \pm 0.1$ fold compared to untreated controls ($n = 5$; $p < 0.05$) (Figure 5.8a). The pattern of bile acid effects on cell migration was very similar in HT-29Cl.10A cells, where UDCA prevented the inhibitory effects of DCA ($n = 4$) (Figure 5.8b).
Figure 5.8 UDCA prevents DCA-induced inhibition of epithelial cell migration. Boyden chamber experiments were performed with a) T84 (n = 5) and b) HT29Cl.19A (n = 4) cells in presence of DCA (150 μM), UDCA (150 μM), or DCA (150 μM) and UDCA (100 μM) in combination. After 48 hrs, cells were fixed and counted on the basolateral side of the chamber. All values were normalised and expressed as fold change over untreated controls. Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test, * denotes significant differences compared to untreated controls; # denotes significant differences compared to DCA-treated cells; * p < 0.05, ** p < 0.001; # p < 0.05, ## p < 0.001.
5.3.5.2 DCA and UDCA do not alter colonic epithelial cell growth

To gain further insights into molecular mechanisms behind bile acid-induced alterations in epithelial restitution, we examined the effects of DCA and UDCA on epithelial cell proliferation. The doubling time of T84 and HT29Cl.19A cells was established to be 8-12 hrs. Following that, cells were allowed to grow in the presence of DCA (150 μM) or UDCA (150 μM), either alone or in combination, for 48 hrs. Levels of acid phosphatase were then measured as an index of the number of viable cells. No significant differences in the number of viable cells were observed in either T84 (Figure 5.9a) or HT29Cl.19A cells (Figure 5.9b) (n = 3). These results suggest that neither DCA nor UDCA, at the concentrations used in this study, alter the growth of colonic epithelial cells.

Figure 5.9 DCA and UDCA do not alter colonic epithelial cell growth. T84 cell growth assay was performed using a commercially-available acid phosphatase assay kit. a) T84 (n = 5) and b) HT29Cl.19A (n = 3) cells were seeded on plastic and incubated with bile acids as indicated for 48 hrs. When control cells reached 80-90% confluency, media was removed from the plates, and cells were washed once with PBS. 10 mM acid phosphatase substrate in 0.1 M sodium acetate buffer with 0.1% Triton X was added to each well and incubated at 37 °C for 1 hr. 50 μl of 1 M NaOH was added to halt the reaction and absorbance was read at 405 nM. All values were normalised by expression as fold change over untreated controls. Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test.
5.3.5.3 Bile acids modulate the expression of CFTR in colonic epithelium

Next, we turned our attention to molecular mechanisms underlying regulation of cell migration by bile acids. Previously published studies indicate an important role for CFTR, since its inhibition retards wound healing in lung epithelium (Schiller et al., 2010a). It has also been demonstrated that restoration of CFTR activity promotes restitution in airway epithelial cells (Kirk, 2010, Trinh et al., 2012). Published data from our laboratory has shown that activation of the bile acid receptor, FXR, decreases Cl⁻ secretion across colonic epithelium by a mechanism involving inhibition of CFTR expression (Mroz et al., 2014). Thus, we tested whether DCA, similar to FXR agonists, also inhibits CFTR expression in colonic epithelial cells. CFTR was detected by immunoblotting using anti-CFTR carboxyl terminus antibody. The detected band (~ 168 kDa) corresponds to band C, representing the mature CFTR with complex glycosylation (Gregory et al., 1990).

We found that treatment with DCA decreased CFTR expression in polarised monolayers of T₈₄ cells to 0.2 ± 0.2 fold (n = 3; p < 0.05) compared to vehicle (DMSO)-treated controls (Figure 5.10b). Having shown this, we hypothesised that UDCA may exert its effects by preventing DCA-induced downregulation of CFTR expression. However, we found that when the cells were treated with UDCA alone, CFTR expression was decreased to 0.4 ± 0.2, while in cells treated with both DCA and UDCA, CFTR expression was only partially restored to 0.6 ± 0.1 fold (n = 3; p < 0.05) (Figure 5.10c).
Figure 5.10 Chronic exposure to DCA decreases CFTR expression. T84 cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle (DMSO), DCA (150 μM), UDCA (50 μM), DCA (150 μM) + UDCA (150 μM) for 24 hrs. Lysates were prepared, separation was performed at 8% SDS-PAGE and western blot analysis was performed in order to assess changes in CFTR expression. All values were normalised to β actin protein expression and expressed as fold change over vehicle-treated controls. a) Representative western blot. b) Densitometric analysis of western blots from cells treated with DCA (n = 3). c) Densitometric analysis of western blots from cells treated with DCA and UDCA alone, or co-treated with both bile acids (n = 3). Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test, *denotes significant differences compared to vehicle-treated cells; * p < 0.05.
Next, to determine if CFTR is an important modulator of restitution in colonic epithelial cells, we examined the effects of the CFTR inhibitors, CFTR(inh)-172 and GlyH-101, on wound healing progression. First, we assessed the effects of the CFTR inhibitors on CFTR protein expression in T84 cells. After 48 hrs of treatment with either CFTR(inh)-172 or GlyH-101 epithelial monolayers were lysed and proteins were extracted. Western blot analysis revealed that CFTR(inh)-172 decreases CFTR protein expression, while GlyH-101 has no effect (n = 3; p < 0.05) (Figure 5.11a). This effect is most probably a consequence of differential mechanisms by which these inhibitors exert their actions on CFTR activity. In this regard, it has been previously shown that CFTR(inh)-172 increases CFTR ubiquitination and proteosomal degradation (Villella et al., 2013). Thus, GlyH-101 is a more useful inhibitor when assessing purely the role of changes in CFTR activity, rather than changes in its expression, on epithelial function. With this in mind, we investigated the effects of both CFTR(inh)-172 and GlyH-101 on colonic epithelial restitution and found them both to significantly inhibit wound closure (Figure 5.11b).

Since our data suggests that CFTR activity is of great importance in regulating colonic epithelial restitution, we investigated further how it may be involved in mediating responses to bile acids. In particular, since we have previously shown that activation of the FXR decreases CFTR protein expression in colonic epithelial cells, we hypothesised that it should also prevent epithelial restitution. To explore this further, we investigated the effects of the FXR agonist, GW4064, on epithelial cell migration using Boyden chambers. Similar to CFTR(inh)-172 and DCA, which were used as positive controls for these experiments, GW4064 inhibited T84 cell migration to 0.6 ± 0.1 fold of that in DMSO-treated controls (n = 5; p < 0.05) (Figure 5.12). In the restitution model, GW4064 also inhibited wound closure compared to DMSO-treated cells (Figure 5.13). These data suggest that DCA-induced inhibition of CFTR activity and restitution is likely to be mediated via FXR activation.
Figure 5.11 Inhibition of CFTR activity decreases epithelial wound healing. a) T₈₄ cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. Cells were then treated with vehicle (DMSO), GlyH101 (25 μM) and CFTR(inh)-172 (10 μM) for 48 hrs. Lysates were prepared, separation was performed at 8% SDS-PAGE and western blot analysis was performed in order to assess changes in CFTR expression. All values were normalised to β actin protein expression and expressed as fold change over vehicle-treated controls (n = 3). To investigate epithelial restitution, T₈₄ cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media containing either DCA (150 μM), CFTR(inh)-172 (10 μM), or GlyH101 (25 μM). b) Images were taken immediately and 48 hrs after the wound was inflicted. c) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 3 - 6). Data represent the mean ± SEM for n experiments and were statistically analysed by one way ANOVA with the with the Student Newman-Keuls post-test; *denotes significant differences compared to vehicle-treated cells; * p < 0.05, ** p < 0.01, *** p < 0.001.
**Figure 5.12** CFTR inhibition decreases colonic epithelial cell migration. Boyden chamber assays were performed with T₈₄ cells in the presence of DCA (150 μM), CFTR(inh)-172 (10 μM), or GW4064 (10 μM). After 48 hrs cells were fixed and counted on the basolateral side of the chamber (n = 5). All values were normalised by expression as fold change over DMSO-treated controls. Data represent the mean ± SEM for n experiments and were statistically analysed by repeated measures ANOVA with the Student Newman-Keuls post-test, *denotes significant differences compared to DMSO-treated cells; * p < 0.05, ** p < 0.01.

**Figure 5.13** FXR agonists, GW4064 inhibit wound healing. T₈₄ cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in the serum-containing media with FXR agonists as indicated. a) Images were taken immediately and 48 hrs after wound was inflicted b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 5). Data represent the mean ± SEM for n experiments and were statistically analysed by Students t-tests, p values over 0.05 were considered to be statistically significant; *denotes significant differences compared to vehicle (DMSO)-treated controls; * p < 0.05.
We have previously shown in this Chapter that UDCA can prevent DCA-induced inhibition of epithelial restitution (Figure 5.7). Since UDCA is known to regulate ion transport across colonic epithelial cells (Ng et al., 2013, Kelly et al., 2013a) and to have the capacity to modulate CFTR activity (Neurath and Travis, 2012), we wanted to investigate if the bile acid could impact on changes in wound healing in response to CFTR inhibitors. Interestingly, we found that at 48 hrs post-wounding, UDCA (150 μM) attenuated CFTR(inh)-172 (10 μM)-mediated inhibition of wound closure from 37.8 ± 3.2% to 58.8 ± 5.1% (n = 5; p < 0.001) (Figure 5.14). These results suggest that UDCA is not only able to overcome the effects of agents that decrease CFTR expression (i.e., DCA) but also those that decrease its activity.
Figure 5.14. UDCA attenuates CFTR(inh)-172-induced inhibition of colonic epithelial restitution. T84 cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with DCA (150 μM), CFTR(inh)-172 (10 μM), or UDCA (150 μM) + CFTR(inh)-172 (10 μM). a) Images were taken immediately and 48 hrs after the wound was inflicted b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 5). Data represent the mean ± SEM for n experiments and were statistically analysed by one way ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to vehicle-treated controls; # denotes significant differences compared to CFTR(inh)-172-treated cells; ** p < 0.01; ## p < 0.01.
5.3.5.4 Involvement of NF-κB in colonic epithelial restitution.

Following on from our experiments above, we next aimed to investigate intracellular signalling mechanisms that may be involved in mediating the effects of UDCA on epithelial restitution. The first pathway that we chose to focus on was NF-κB, the master regulator of epithelial responses to inflammation and injury. It has been shown that acute activation of NF-κB increases epithelial restitution in colonic epithelium (Egan et al., 2003b). Furthermore, UDCA has been previously shown to attenuate cytokine-induced NF-κB activation (Shah et al., 2006), while in Chapter 4 we found UDCA to attenuate DCA-induced NF-κB activation in T84 cells. In order to evaluate the potential role of NF-κB in regulating wound healing, we employed an IκB phosphorylation inhibitor. We found that in BMS-345541-treated cells, wound closure was reduced to 22.2 ± 4.3% of T = 0 values, compared to 35.4 ± 3.6% in DMSO-treated cells (n = 3; p < 0.05). However, we found that treatment with UDCA reversed this effect by increasing wound closure in BMS-345541-treated cells to 37.0 ± 4.4% (n = 3; p < 0.05) (Figure 5.15). These data suggest that UDCA likely promotes epithelial restitution in an NF-κB-independent manner.
Figure 5.15 Effects of UDCA on NF-κB inhibition of epithelial restitution. T84 cells were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with treatments as indicated. a) Images were taken immediately and 48 hrs after the wound was inflicted. b) 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 3). Data represent the mean ± SEM for n experiments and were statistically analysed by one way ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to vehicle (DMSO)-treated cells; # denotes significant differences compared to BMS-345541-treated cells; * p < 0.05, ** p < 0.01, # p < 0.05.
5.3.5.5 Effects of HβDs on colonic epithelial healing.

Data from this Chapter has revealed that DCA and UDCA differentially modulate epithelial restitution, while results from Chapter 3 indicate that they also regulate HβD expression in a similar manner. Thus, we went on to consider a potential role for HβDs in regulation of restitution. Elevated levels of HβDs have been previously shown to increase pro-inflammatory cytokine release from the epithelium through mechanisms involving NF-κB and as already described, prolonged upregulation of NF-κB resulting can decrease healing processes in the colonic epithelium (Okamoto and Watanabe, 2005).

First, we assessed the effects of HβD-1 and HβD-2 on epithelial barrier function using T84 model of polarised cell monolayer in vitro. Cells were seeded as normal and treated bilaterally with HβD-1 (1 – 1000 pg/ml) or HβD-2 (1 – 1000 pg/ml) for 24 hrs. The TEER was measured at T = 0 hrs and T = 24 hrs. We observed that although HβD-1 had no effect (n = 3) (Figure 5.16a), HβD-2, at concentrations higher than 100 pg/ml, decreased TEER across the epithelial monolayer (n = 5) (Figure 5.16b). To ensure that this reduction in barrier function is not due to toxic actions, we performed an LDH assay, with no effect of HβD-2 being observed even at the highest concentration tested (n = 3) (Figure 5.16c).
Figure 5.16 HβD-2 reduces TEER but does not exert toxic effects on T₈⁴ cells. T₈⁴ cells were cultured on permeable supports until TEER stabilised at plateau levels. a) Cells were treated bilaterally with HβD-1 (1 - 1000 pg/ml) for 24 hrs and successive TEER measurements were recorded using an epithelial voltohmmeter (n = 3). b) Cells were treated bilaterally with HβD-2 (1 - 1000 pg/ml) for 24 hrs and successive TEER measurements were recorded using an epithelial voltohmmeter (n = 5). c) Cells were treated bilaterally with HβD-2 at the indicated concentrations for 48 hrs. An equal aliquot of apical and basolateral culture medium was taken and LDH levels into were measured using a commercially-available kit (n = 3). Cells treated with lysis buffer for 30 mins were used as a positive control. Data represent the mean ± SEM for n experiments and were statistically analysed by one way ANOVA with the Student Newman-Keuls post-test; * denotes significant differences compared to vehicle-treated controls; *** p < 0.001.
On the basis of these data, we hypothesised that chronic exposure to DCA might exert its negative effects on restitution by increasing levels of HβDs. Concentrations of HβDs that have been reported in literature range from pg/ml to μg/ml, depending on where it is measured. For example, HβD levels in blood are at a much lower concentration than they are in the colonic crypts (Otte et al., 2008, Ozkaya et al., 2002, Panwala et al., 1998, Park et al., 1998, Parks et al., 1999). The highest levels of HβD production induced by DCA in the current studies were found to be < 1 μg/ml for HβD-1 and 500 pg/ml for HβD-2. Thus, we used these concentrations to assess the effects of HβDs on epithelial restitution. However, we found that when treated with HβD-1 or HβD-2, wound closure in our Tβ4 cell model was not altered (n = 4) (Figure 5.17). These results suggest that DCA inhibits epithelial restitution in a HβD-independent fashion.
**Figure 5.17** HβD-1 and HβD-2 do not alter colonic epithelial restitution. 

**T84 cells** were cultured as monolayers on transparent, permeable supports until TEER stabilised at plateau levels. After wounding, cells were placed in serum-containing media with treatments as indicated. 

**a)** Images were taken immediately and 48 hrs after the wound was inflicted. 

**b)** 48 hrs post-wounding the wounded area was measured again and % closure of the original wound area was calculated (n = 4).

Data represent the mean ± SEM for n experiments and were statistically analysed by one way ANOVA with the Student Newman-Keuls post-test; *denotes significant differences compared to untreated controls; ** p < 0.01.
5.4 Discussion

The aim of this chapter was to investigate the role of the secondary bile acids, DCA and UDCA, in regulating colonic epithelial cell restitution. We have shown that, DCA decreases colonic epithelial restitution, while UDCA, not only increases it, but also prevents DCA-induced retardation of the restitution process. These opposing effects were further characterised, with both DCA and UDCA actions being found to be due to alterations in cell migration, rather than proliferation. Bile acids, such as DCA, are known to target the nuclear receptor, FXR, and we found that pharmacological activation of FXR mimicked the effects of DCA in retarding epithelial healing. Modulation of ion transport proteins has been implicated in the pathogenesis of IBD (Groschwitz and Hogan, 2009). Furthermore, these proteins have been shown to be involved in processes that regulate epithelial restitution. Previously published data from our laboratory showed that FXR activation in colonic epithelium decreases expression of the Cl- channel, CFTR (Mroz et al., 2014), and In the current studies, we found that expression of CFTR is also inhibited by DCA. Further evaluation of the role of CFTR in restitution revealed that inhibition of the channel decreases wound healing rate by inhibiting cell migration. Interestingly, UDCA was found to prevent, inhibition of wound healing by pharmacological inhibitors of either CFTR or NF-κB. Finally, since we had found DCA and UDCA to regulate HβD expression in previous chapters, we investigated the potential role of defensins in regulating epithelial wound healing, but found neither HβD-1 or 2 to be involved in this process.

Cells at the leading edge of wounds lose their cell contacts and polarity due to their transition to the migratory phenotype. Therefore, the cells at the leading edge may be undergoing apoptotic and necrotic cell death, and therefore wounds can increase in size over time (Crosby et al., 2011). The anti-apoptotic effects of UDCA have been well-established (Bellentani, 2005). Furthermore, UDCA has been shown to prevent DCA-induced apoptosis by blocking p53 apoptotic gene activation (Amaral et al., 2009). Therefore, we hypothesised, that the protective effect of UDCA on wound healing may be due to prevention of DCA-induced apoptosis at the wound edge. In order to test this hypothesis, we investigated if DCA was inducing cell death under the conditions employed in these studies. However, we found that DCA did not increase levels of cleaved PARP; suggesting
that retardation of wound healing in response to the bile acid was not as a result of increased apoptosis.

Since changes in cell proliferation rate have been linked to wound healing progression, we went on to assess the effects of DCA and UDCA on cell proliferation in our model. However, we did not observe any effects of the bile acids on epithelial cell growth. This is in contrast to previously published data, which shows that chronic exposure to DCA at low concentrations can increase cancer cell proliferation (Ha and Park, 2010, Milovic et al., 2000). We hypothesise that such differences between ours and these previous studies may be due to differences in the concentration of DCA employed, duration of exposure to the bile acid, or in the cell line under investigation. Studies of bile acid actions on proliferation/apoptosis in primary epithelial cells are required, since transformed epithelial cells, such as those used in the current and previous studies, may not accurately reflect how normal epithelial cells would respond in vivo.

The role of ion channels in IBD progression has been well established with respect to their ability to regulate fluid secretion and absorption (Yiangou et al., 2001, Hosseini-Tabatabaei and Abdollahi, 2008, Holzer, 2003). More recently, studies in other models have shown that ion channels, such as CFTR, can also regulate wound healing (Trinh et al., 2012). For example, it has been shown in bronchial epithelium that CFTR is crucial in the early phases of epithelial repair in vivo, while in CF mice, which lack functional CFTR, wound healing is delayed in the epidermis (Dong et al., 2015a). It is thought that CFTR regulates restitution by altering cell migration (Dong et al., 2015). In this regard, CFTR activity has been shown to be involved in lamellipodia protrusion at the leading edge and may also be important for retraction processes at the trailing end of the cell. Localisation of CFTR mediated Cl⁻ transport to the rear of the cell, in cooperation with K⁺ channels and AQP5s, is thought to result in a volume decrease which facilitates lifting of the cell from the extracellular matrix (Schiller et al., 2010a). However, it is clear that mechanisms underlying epithelial cell migration and restitution are very complex and the exact role that CFTR plays is still not fully understood. Furthermore, since CFTR transports not only Cl⁻, but also HCO₃⁻ (Reddy and Quinton 2003), a better understanding of how each of these ions regulates cell migration is important.
Results from our study demonstrate that pharmacological inhibition of CFTR prevents wound healing in colonic epithelium in vitro. In our laboratory, we have previously shown that activation of FXR by GW4064 inhibits CFTR protein expression (Mroz and Keely, 2014). Thus, we explored the hypothesis that activation of FXR by GW4064 will also affect colonic epithelial restitution. We discovered not only that FXR activation decreased wound healing, but also that this effect is mediated via reduction in cell migration. This is in accordance with reports that have shown silencing of CFTR expression to inhibit cell migration in airway epithelium (Peitzman et al., 2011, Peitzman, 2014).

The current studies not only reveal a novel effect of FXR activation in preventing epithelial wound healing but also point to a new role for bile acids in regulation of CFTR expression. While we have shown here that DCA can inhibit CFTR expression, recent data from our laboratory shows that CDCA and LCA are also potent inhibitors of CFTR protein expression and promoter activity. It is tempting to speculate that these effects are mediated through FXR, since DCA, CDCA and LCA are all known agonists of the receptor. Furthermore, the ability of these bile acids to inhibit CFTR promoter activity in a HEK293 cell model is dependent on expression of FXR in these cells (Mroz and Keely, 2014).

UDCA is a known regulator of CFTR activity; however, there is no data available on the effects of UDCA on CFTR expression. In biliary epithelium, UDCA promotes CFTR activity (Fiorotto et al., 2007), thereby increasing bile flow by stimulating bicarbonate secretion (Rodriguez-Ortigosa et al., 2010). This is considered to be a beneficial action of UDCA, since increased secretion of chloride and bicarbonate reduces bile acid concentrations in the canaliculus, therefore decreasing their cytotoxicity (Beuers et al., 2010). In contrast, in the colon, UDCA has been shown to have opposite effects on anion secretion. Here, acute treatment with UDCA attenuates Cl− secretory responses (Kelly et al., 2014), while preliminary data from our lab shows that UDCA inhibits CFTR expression by decreasing promoter activity (Mroz and Keely, 2014).

Interestingly, our current studies demonstrate that UDCA promotes colonic epithelial wound healing even though it also appears to inhibit CFTR expression. However, it should be noted that UDCA is less effective in this regard than is DCA or GW4064, only reducing CFTR expression by 60% of that in control cells, at the
concentrations employed. Since previous studies have shown that cellular CFTR expression must be reduced by >80% before inhibition of Cl- secretion is observed (Zhang et al., 2009), it is therefore unlikely that UDCA-induced reductions in CFTR expression are sufficient to inhibit activity of the channel and would therefore not affect migration. UDCA is also known to increase Cl- secretion independently of CFTR, where it activates calcium activate chloride channels (CaCC) by elevating cytosolic calcium levels (Shimokura et al., 1995), which could be responsible for the effects of UDCA on cell migration.

Based on recent reports, describing UDCA as an antagonist at FXR (Modica et al., 2010, Mueller et al., 2015), it is tempting to speculate that the restoration of CFTR expression by UDCA is due to antagonism of FXR. Na+-K+-ATPase pumps, located on the basolateral side of epithelial cells, are also important players in regulating epithelial ion transport and their expression has been shown to be altered in IBD patients. Thus, it is possible that they may also contribute to altered wound healing in response to bile acids in these patients (Dubé et al., 2010, Ghishan and Kiela, 2014). In support of this, previous studies from our lab have shown that FXR activation inhibits Na+-K+-ATPase activity (Mroz et al., 2014). UDCA can also acutely inhibit activity of Na+-K+-ATPase pumps (Kelly et al., 2013b), however its more long-term actions on this transporter are not yet known and whether this is important for regulation of restitution by the bile acid has yet to be determined.

Data from this chapter also revealed that the effects of UDCA in promoting wound healing appear to occur independently of NF-κB activation. NF-κB is known to be an important player in regulating intestinal restitution. It is thought that when the epithelium is wounded in vivo, this allows passage of luminal contents into the mucosa, thereby initiating a local inflammatory response. In turn this leads to rapid activation of epithelial NF-κB which then promotes cell migration into the wound, thereby facilitating wound healing (Brand et al., 2006, Nenci et al., 2007). In support of this idea, we found that inhibition of NF-κB decreases restitution in the T84 cell model.

However, even though acute activation of NF-κB appears to promote restitution and our data in Chapter 4 show that DCA induces colonic epithelial NF-κB activation, results from the current Chapter show that prolonged DCA treatment
inhibits epithelial wound healing. Moreover, results described in Chapter 4 revealed that UDCA inhibits DCA-induced NF-κB activation, while studies presented in this Chapter show that it prevents DCA-inhibition of wound healing. Thus, while NF-κB is clearly important in regulating wound healing processes, its precise role is still unclear. Interestingly, previous studies have also shown that prolonged activation of NF-κB can lead to its decreased expression, thereby resulting in a reduced restitution through decreased expression of protective peptides (Loncar et al., 2003). Whether such a mechanism may be involved in modulating the role of NF-κB in bile acid-induced alterations in restitution could be an interesting topic for future investigation.

Finally, since our findings from previous Chapters indicate important roles for bile acids in modulating HβD release from colonic epithelial cells, we set out to find out whether their effects on epithelial restitution may be mediated by defensins. HβD-2 has been previously implicated in regulation of epithelial ion transport (Himmerkus et al., 2010), curtail for migratory processes. We found that HβD-2 (300 – 1000 pg/ml) decreased TEER in our model of colonic epithelium, an effect which is indicative of dysregulation of epithelial barrier function. This effect may be mediated by altered expression of TJ proteins, such as claudins, as has been shown to occur in other cell types (Kiatsurayanon et al., 2014). However, in the current studies we found that both HβD-1 and HβD-2, at concentrations achieved in the culture medium after DCA treatment and which had the capacity to reduce TEER, did not alter colonic epithelial restitution. However, these are rather preliminary experiments and it is possible that the concentrations of HβDs used in these studies were not sufficient to affect restitution. This is an area that requires additional work in the future. For example, to more fully elucidate the potential role of HβDs in mediating the effects of bile acids on epithelial restitution, future studies could employ the use of siRNAs to knockdown HβD expression or blocking antibodies to prevent their cellular actions.

Wound healing has been shown to be of paramount importance in IBD and therefore a better understanding of intestinal damage and epithelial repair process is critical for the development of new therapeutics to prevent sustained colonic inflammation. Together, the data presented in this chapter provides a better understanding of the mechanism by which bile acids regulate colonic epithelial cell
migration as summarised in Figure 5.18. Our studies are also the first to identify a role for CFTR in colonic epithelial cell migration, a process pivotal to wound healing progression. Interestingly, results employing CFTR blockers indicated that the role of CFTR in colonic epithelial cell migration was dependent on its ion transport function, rather than its expression. Future studies to expand on these findings should focus on determining the co-ordination of other epithelial transport proteins with CFTR by investigating their expression, activity, and localisation within migrating cells, and how these are altered in conditions of inflammation. Ultimately, increased understanding of these processes may lead to the development of new therapeutics that can limit colonic epithelial damage in conditions of IBD.

![Diagram of CFTR regulation](image)

**Figure 5.18 Proposed mechanisms of DCA and UDCA regulation of restitution in colonic epithelial cells.** Prolonged exposure to DCA activates the nuclear bile acid receptor, FXR, and NF-κB. FXR translocates to the nucleus where it decreases CFTR expression through inhibition of promoter activity. This leads to decreased expression of CFTR channels in the membrane and inhibition of Cl⁻ secretion, which in turn inhibits cell migration. In addition, prolonged activation of NF-κB decreases cell migration. UDCA, through mechanisms that have yet to be defined, can reverse DCA inhibition of migration and therefore restores barrier function. The tri-directional interactions between FXR, NF-κB and CFTR activity is yet to be elucidated.
CHAPTER 6

General Discussion
With a constantly increasing prevalence in the western world, IBD represents a significant health and economic burden (Molodecky et al., 2012). Despite this, there still remains an incomplete understanding of the molecular mechanisms involved in the pathogenesis of these diseases, with the result that there is a lack of specific therapies. Current options often lack efficacy, can be expensive and can also be associated with severe adverse effects, including nausea, vomiting, and opportunistic infections (Dulai et al., 2014, van der Valk et al., 2012). Thus, there is an urgent need for new, more effective and safer therapies that can not only treat active disease but which also prevent its development. In this thesis, we aimed to address this issue by exploring the effects of secondary bile acids on colonic epithelial barrier function through studies in cells, animal models and human tissues. The data presented provides new insights into how DCA and the “therapeutic” bile acid, UDCA, influence important aspects of epithelial barrier function, namely antimicrobial peptide secretion and restitution.

While bile acids are classically known for their role in fat digestion, more recently they have become appreciated as intestinal hormones that regulate many different aspects of epithelial physiology. For example, recent studies show that physiological levels of bile acids have anti-secretory (Kelly et al., 2013a, Keating et al., 2009) and anti-inflammatory actions (Ward et al., 2013b) on the colonic epithelium. However, high levels of bile acids are known to increase colonic epithelial cell proliferation and apoptosis (Ignacio Barrasa et al., 2011), induce autophagy and stress-related pathways (Payne et al., 2009), increase fluid and electrolyte secretion (Mekjian et al., 1971), and modulate intestinal inflammatory responses (Uchida et al., 1997, Allen et al., 2011). Interestingly, considerably higher concentrations of bile acids are found in the colons of IBD patients compared to healthy subjects, suggesting that they may have a role to play in the development of these diseases (Hakala et al., 1996, Surawicz, 2010, Muller et al., 2004, Duboc et al., 2012).

Studies in this thesis were particularly focused on the secondary bile acid, UDCA, as it has been used for centuries in Traditional Chinese Medicine to treat inflammation of the liver, skin and eyes (Feng et al., 2009, Chen et al., 2002), and is also recognised in Western medicine as a safe approach to treat liver diseases, including cholestasis and PBC (Angulo, 2002). Furthermore, numerous studies have described anti-inflammatory effects of UDCA on epithelial cells, which are in
Physiologically, levels of UDCA in the colon are normally in the range of 5 – 10 μM (Hamilton et al., 2007), therefore the concentrations used in many of the current studies (50 - 200 μM) would only occur after administration of the bile acid as a drug. However, at such pharmacologically-relevant concentrations, previously published studies describe the anti-inflammatory actions of UDCA in various animal models of colitis (Goto et al., 2001, Kullmann et al., 1997). Our current data further supports the idea that UDCA exerts protective effects by showing that it attenuates production of HβDs, promotes restitution in a colonic epithelial cell model, and ameliorates intestinal inflammation in vivo. However, when considering such a use for UDCA, one needs to take into consideration multiple factors that could affect its efficacy (e.g., delivery to the colon, metabolism, and alterations to the microbiome) and also its long-term risks.

Although oral delivery is a widely used route of drug administration, the GIT presents several challenges in terms of delivering an active drug, such as UDCA, to the colon. Successful colonic delivery requires protection of the drug from degradation in the upper digestive tract and premature absorption in the stomach or small intestine (Chourasia and Jain, 2003). While rectal administration offers a feasible route for delivering drugs to the distal colon, effectively targeting the proximal colon by this route is difficult. Colon-specific drug delivery systems (CDDS), based on the pH of different regions of the GIT, could be used to overcome this problem. The pH of the stomach ranges between 1 – 2 and can increase after meals (Rubinstein, 1995). The small intestinal pH varies along its length, with a value of 6.5 in proximal sections and increasing to 7.5 in the distal small intestine (Evans et al., 1988). The pH in the caecum and ascending colon is slightly lower, in the range of 5.7 – 6.4 (Bussemer et al., 2001), and there is a gradual increase in pH along the length of the colon, reaching 6.6 in transverse colon and 7 in descending colon. Thus, pH-sensitive polymers can be used in CDDS. They are designed to take an advantage in the slight pH difference between small and large intestine, where the coated drug is released only when luminal pH reaches levels found only in the colon (Ashford et al., 1993). Another
approach for achieving colonic drug delivery is to use a time-controlled release system (TCRS). However, the large variations that occur in gastric transit time between different individuals can influence this type of approach, resulting in poor colonic availability (Gazzaniga et al., 1994). Furthermore, pro-drug (Friend and Chang, 1985, Sinha and Kumria, 2003), microbially triggered (Park et al., 2011), pressure-controlled (Jeong et al., 2001) and osmotically-controlled (Philip and Pathak, 2007) colonic delivery systems have all been under investigation for clinical use. Another approach that has been under investigation involves the conjugation of UDCA to aminosalicylates. Upon colonic bacterial degradation both drugs are released and can exert their anti-inflammatory and cytoprotective effects (Batta et al., 1998). Although each type of CDDS has its particular advantages, the pH-sensitive polymer coating could be a promising approach for development of colon–specific formulations of UDCA to treat intestinal inflammation.

Another potential limitation to the use of UDCA in treatment of UC is that of its metabolism by the colonic microbiome. When it enters the colon, UDCA undergoes metabolism by several bacteria to 7-keto-lithocholic acid, which is then further converted to LCA (Dodo et al., 1984). Thus administration of UDCA results in increased levels of both UDCA and LCA in the colon (Eaton et al., 2011, Sinakos et al., 2010). At high concentrations, LCA is considered to be toxic since it can induce colonic epithelial apoptosis (Degirolamo et al., 2011, Dulai et al., 2014). Thus, in future studies, it would be very interesting to investigate the effects of metabolically-stable analogues of UDCA, such as 6-methyl-UDCA (Roda et al., 1994), and UDCA metabolites on the development of colitis, production of defensins and epithelial healing in an animal model of disease.

As mentioned previously, UDCA is already an FDA-approved drug for treatment of PBC (Honda et al., 2013) and has been safely used for several decades. However, recent studies have shown that administration of high levels of UDCA can have adverse effects, even increasing the risk of death. Such effects are thought to be as a consequence of increased levels of LCA, due to microbial metabolism of UDCA (Lindor et al., 2009b). High levels of LCA are associated with development of colonic cancer (Kozoni et al., 2000) and also exert toxic effects in the liver (Hofmann, 2004). Therefore, further studies are necessary to more fully elucidate the impact of microbial metabolism on the balance of UDCA and LCA.
levels in the colon, and in turn, how this impacts on regulation of epithelial barrier function and liver toxicity.

Another long-term risk factor to take into account, when considering the use of UDCA as a therapeutic, is its potential to cause cancer. In particular, since UDCA prevents epithelial apoptosis, its potential for increasing the risk of development of colorectal neoplasia must be considered. In fact, a recent study demonstrated that long-term use of high-dose UDCA increased the risk of colorectal neoplasia in patients with UC and PSC (Imam et al., 2011). However, most studies to date suggest that, when used at lower doses, long-term use of UDCA is safe and relatively free of side effects (Nousia-Arvanitakis et al., 2001, Shi et al., 2006, Kuiper et al., 2011). Indeed, several studies suggest that UDCA treatment can prevent the occurrence of colon cancer in UC patients (Pardi et al., 2003, Su et al., 2013).

It is now universally accepted that the microbiome is important in the pathogenesis of IBD (Knights et al., 2013, Kostic et al., 2014). In humans, there is a considerable difference in the make-up of the colonic microbiota between patients with IBD and healthy individuals (Seksik et al., 2003, Swidsinski et al., 2002b, Schultsz et al., 1999, Greenblum et al., 2012). Sequencing of bacterial strains showed that *Bacteroidetes* and *Lachnospiraceae* were greatly reduced, whereas *Actinobacteria* and *Proteobacteria* were substantially more abundant in IBD patients than in controls. Therefore, the idea of manipulating the enteric microbiota with probiotics to restore homeostasis is currently receiving a great deal of research interest and promising outcomes have been achieved (Fedorak et al., 2015, Hansen and Sartor, 2015, Saez-Lara et al., 2015), with many clinical trials still ongoing (ClinicalTrials.gov Identifier: NCT02488954, NCT01765439, NCT02361957, NCT01887834, NCT01078935). Furthermore, the hypothesis that certain strains of bacteria may be responsible for increased conversion of CDCA to UDCA has also been explored. These studies showed that 7β-hydroxysteroid dehydrogenase (7β-HSDH), an enzyme expressed in *Ruminococcus gnavus N53* species, increased levels of UDCA in the colon (Lee et al., 2013). Zheng *et al.*, showed that UDCA conversion efficiency can also be modulated by bacteria that express 7β-HSDS (Zheng et al., 2015). Thus, using bacterial strains that strongly express 7β-HSDS could be a feasible approach to increase levels of UDCA in the colon for treatment of IBD.
In addition to their ability to modify bile acid levels, probiotics can also exert other anti-inflammatory effects in the colon. For example, they can promote epithelial protective responses by increasing production of butyrate, which prevents epithelial apoptosis (Sartor, 2008). Due to their antioxidant properties, probiotics also reduce oxidative stress, thereby promoting epithelial barrier function (Cain and Karpa, 2011). Probiotics also compete with pathogenic bacteria for adherence to the epithelium and enhance innate immune responses, thus ameliorating inflammation (Vanderpool et al., 2008). Thus, due to the close interactions between bile acids, microbiota and innate immune response, there is excellent potential for the use of probiotics to alter bile acid levels in order to dampen inflammation (Cain and Karpa, 2011, Gionchetti et al., 2003).

Another potential approach for therapeutic alteration of bile acids in treatment of intestinal inflammation is through the use of faecal microbial transfer (FMT). The idea of using faecal matter from healthy individuals to treat patients with intestinal disease originated, similar to the use of bear bile, in ancient Chinese medicine. FMT has been previously employed in the treatment of pseudomembranous enterocolitis when other treatments have failed (Kelly, 2013). More recently, a study has published the use of FMT in treating *Clostridium difficile* infection (CDI) associated diarrhoea, with cure rates ranging between 82 – 94% (Aroniadis et al., 2015). Phase II clinical studies of FMT for treatment of UC have also shown promising results, with 33% of patients achieving clinical remission in the first week, 78% having a clinical response, and 67% maintaining their response to treatment at 4 weeks post-FMT (Kunde et al., 2013). Recently published data show that FMT is safe, although efficacy appears to vary between UC and CD patients (Colman and Rubin, 2014). Furthermore, studies have shown that FMT restored normal faecal bile acid composition in patients with recurring CDI (Weingarden et al., 2014). These results suggest that FMT could be a promising approach for manipulating levels of colonic bile acids in order to restore disturbed gut microbiota in patients with intestinal inflammation.

On the basis of our results and previously published studies, it is clear that bile acids have a potential in modulating intestinal inflammation (Y.H. Kim1, 2013, Uchida et al., 1997). Moreover, it has been shown that specific agonists of the nuclear bile acid receptor, FXR, and the cell surface receptor, TGR-5, are protective in animal models of colitis (Cipriani et al., 2011, Gadaleta et al., 2011b).
It is also possible that other nuclear receptors, such as VDR and PXR, are also important, mediating the protective effects of UDCA in the colon. UDCA is known to activate PXR (Padda et al., 2011) whereas, the UDCA metabolite, LCA, is a potent VDR agonist (Adachi et al., 2005). Dysregulation of the expression or activation of these receptors could significantly compromise innate immune responses, since expression of HβDs and colonic epithelial healing has been shown to be regulated by VDR (Wang et al., 2004, Kong et al., 2008). Other studies have shown significantly reduced expressions of VDR and PXR in the colons of patients with UC (Langmann et al., 2004, Kim et al., 2013). Furthermore, activation of both, VDR and PXR, has been shown to exert the protective effects of chemically-induced models of intestinal inflammation, an effect attributed to enhanced epithelial healing and protection of barrier function (Terc et al., 2014, Liu et al., 2013). This is an area that requires more research to elucidate whether the protective effects of UDCA observed in the current studies involve PXR or/and VDR activation. Additionally, further studies are also required to elucidate how bile acid receptors couple to the downstream signal transduction pathways that regulate colonic barrier function, most notably, NF-κB, MAPKs, PKA, AKT, and PKC-dependent signalling mechanisms.

In conclusion, the studies carried out in this thesis contribute to our evolving knowledge of how bile acids and their receptors regulate intestinal physiology and pathophysiology. The work presented reveals new roles for UDCA in promoting intestinal barrier function and preventing inflammatory responses, thereby supporting its potential for development as a new therapy for IBD.
CHAPTER 7

Bibliography


COLLIVA, C. 2013. New synthetic bile acid analogue agonists of FXR and TGR5 receptors: Analytical methodologies for the study of their physico-chemical properties, pharmacokinetic activity and metabolism.


DOMINGUE, J., AO, M., SARATHY, J., ALREFAI, W. & RAO, M. 2015. Bile Acid (BA) Stimulation of Cl-
Secretion Involves Intricate Crosstalk Cascades in Human Colonic T84 Cells. The FASEB
Journal, 29, 855.1.


& WANG, Y. 2015a. Dynamically Regulated CFTR Expression and Its Functional Role in

DONG, J., JIANG, X., ZHANG, X., LIU, K. S., ZHANG, J., CHEN, J., YU, M. K., TSANG, L. L., CHUNG, Y. W.,
WANG, Y., ZHOU, W. L. & CHAN, H. C. 2015b. Dynamically regulated CFTR expression

SHEFFIELD, V. C. 2012. TUDCA slows retinal degeneration in two different mouse models
of retinitis pigmentosa and prevents obesity in Bardet-Biedl syndrome type 1 mice.

DUBÉ, J., ROCHETTE-DROUIN, O., LÉVESQUE, P., GAUVIN, R., ROBERGE, C. J., AUGER, F. A., GOULET,
potential within tissue-engineered human skin in vitro and during the wound healing

DUBOC, H., RAINTEAU, D., RAJCA, S., HUMBERT, L., FARABOS, D., MAUBERT, M., GRONDIN, V.,
Increase in fecal primary bile acids and dysbiosis in patients with diarrhea-priminated

DUBOC, H., RAJCA, S., RAINTEAU, D., BENAROUS, D., MAUBERT, M. A., QUERVAIN, E., THOMAS, G.,
BARBU, V., HUMBERT, L., DESPRAS, G., BRIDONNEAU, C., DUMETZ, F., GRILL, J. P.,
MASLIAH, J., BEAUGERIE, L., COSNES, J., CHAZOUILLERES, O., POUPON, R., WOLF, C.,
dysbiosis, bile-acid dysmetabolism and gut inflammation in inflammatory bowel

DUBOC, H., TACHE, Y. & HOFMANN, A. F. 2014. The bile acid TGR5 membrane receptor: from basic
research to clinical application. Dig Liver Dis, 46, 302-12.

DUBUQUOY, L., JANSSON, E. Å., DEEB, S., RAKOTOBE, S., KAROUJ, M., COLOMBEL, J.-F., AUWERX, J.,
PETTERRSON, S. & DESREUMAUX, P. 2003. Impaired expression of peroxisome
proliferator-activated receptor γ in ulcerative colitis. Gastroenterology, 124, 1265-1276.

DUBUQUOY, L., ROUSSEAU, C., THURU, X., PEYRIN-BIROULET, L., ROMANO, O., CHAVATTE, P.,
CHAMAILLARD, M. & DESREUMAUX, P. 2006. PPARγ as a new therapeutic target in

Expression of beta-defensin 1 and 2 mRNA by human monocytes, macrophages and
dendritic cells. Immunology, 106, 517-25.

Systematic review: monotherapy with anit tumour necrosis factor α agents versus
combination therapy with an immunosuppressive for IBD. Gut, 63, 1843-1853.

transmission electron microscopic studies. III. Target tissues. Proliferation of and injury
to smooth muscle and the autonomic nervous system. Hum Pathol, 11, 620-34.

in ulcerative colitis: a case-control study. Alimentary Pharmacology and Therapeutics,

EATON, J. E., SILVEIRA, M. G., PARDI, D. S., SINAKOS, E., KOWDLEY, K. V., LUKETIC, V. A., HARRISON,
acid is associated with the development of colorectal neoplasia in patients with


GADALETA, R. M., VAN ERPECUM, K. J., OLDBERG, B., WILLEMSEN, E. C., RENOOUJ, W., MURZILLI, S., KLOMP, L. W., SIERSEMA, P. D., SCHIPPER, M. E., DANENE, S., PENNA, G., LAVERNY, G.,


small intestine by the nuclear bile acid receptor. Proc Natl Acad Sci U S A, 103, 3920-3925.


JOHANSSON, M. E., LARSSON, J. M. & HANSSON, G. C. 2011. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. Proc Natl Acad Sci U S A, 108 Suppl 1, 4659-65.


integrity by preventing accumulation of reactive oxygen species in the intestinal epithelium. J Immunol, 185, 4729-37.


KELLY, O. B. 2014. The Actions of Ursodeoxycholic Acid and its derivatives on Colonic Epithelial Transport and Barrier Function.


MIZOGUCHI, A. 2012. Healing of intestinal inflammation by IL-22. Inflamm Bowel Dis, 18, 1777-84.


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SEMLALI, A., WITOLED, C., ALANAZI, M. & ROUABBIA, M. 2012. Whole cigarette smoke increased the expression of TLRs, HBDs, and proinflammatory cytokines by human gingival epithelial cells through different signaling pathways. *PloS One, 7*, e52614.


KHRAMTSOVA, G., TSAI, P. Y. & FU, Y. X. 2013. TNFR2 activates MLCK-dependent tight
junction dysregulation to cause apoptosis-mediated barrier loss and experimental colitis.
antimicrobial peptides or broad-spectrum molecules? Cytokine & growth factor reviews,
26, 361-370.
SWIDSINSKI, A., LADHOFF, A., PERNTHALER, A., SWIDSINSKI, S., LOENING-BAUCKE, V., ORTNER, M.,
in inflammatory bowel disease. Gastroenterology, 122, 44-54.
SWIDSINSKI, A., LADHOFF, A., PERNTHALER, A., SWIDSINSKI, S., LOENING-BAUCKE, V., ORTNER, M.,
inflammatory bowel disease. Gastroenterology, 122, 44-54.
TAN, K. S., NACKLEY, A. G., SATTERFIELD, K., MAIXNER, W., DIATCHENKO, L. & FLOOD,
P. M. 2007. Beta2 adrenergic receptor activation stimulates pro-inflammatory cytokine production
Biol, 4, 721-32.
TERAI, K., CALL, M. K., LIU, H., SAika, S., LIU, C. Y., HAYASHI, Y., CHIKAMA, T., ZHANG, J., TERAI, N.,
intestinal epithelial wound healing and repair of the intestinal barrier following the
TERRITO, M. C., GANZ, T., SELSTED, M. E. & LEHRER, R. 1989. Monocyte-chemotactic activity of
TIWARI, A. & MAITI, P. 2009. TGR5: an emerging bile acid G-protein-coupled receptor target for the
TLASKALOVÁ-HOGENOVÁ, H., ŠTĚPÁNKOVÁ, R., KOZÁKOVÁ, H., HUDCOVIC, T., VANNUCCI, L.,
TUČKOVÁ, L., ROSSMANN, P., HRNČIŘ, T., KVERKA, M. & ŽÁKOSTELŠKÁ, Z. 2011. The role
of gut microbiota (commensal bacteria) and the mucosal barrier in the pathogenesis of
inflammatory and autoimmune diseases and cancer: contribution of germ-free and
gnotobiotic animal models of human diseases. Cellular & molecular immunology, 8, 110-
120.
TONTINI, G. E., MUTTER, J., VIETH, M., ATREYA, R., GUNTER, C., ZOPF, Y., WILDNER, D., KISSLICH,
Endoscopy.
TREMAROLI, V. & BACKHED, F. 2012. Functional interactions between the gut microbiota and host
TRIANTAFILLIDIS, J. K., MERIKAS, E. & GEORGOPPOULOS, F. 2011. Current and emerging drugs for the
treatment of inflammatory bowel disease. Drug design, development and therapy, 5,
185.
TRINH, N. T. N., BARDOU, O., PRIVÉ, A., MAILLÉ, E., ADAM, D., LINGÉE, S., FERRARO, P.,
DESROSIERS, M.-Y., CORAUX, C. & BROCHIERO, E. 2012. Improvement of defective cystic
fibrosis airway epithelial wound repair after CFTR rescue. European Respiratory Journal,
40, 1390-1400.


VERSTEGE, M. I. 2010. *Epithelial barrier and dendritic cell function in the intestinal mucosa.*


