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MODEL SYSTEMS OF INTESTINAL INFLAMMATION: A STEP TOWARDS PERSONALIZED MEDICINE

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Abstract

Inflammatory bowel disease (IBD) is a lifelong chronic inflammatory condition of the gastrointestinal tract (GIT), with incidence and prevalence increasing worldwide. It is considered a complex, multifactorial disease with no cure. Even though large progress has been made in recent years, current therapies are far from satisfactory, and show extreme variability of outcomes due to patient heterogeneity. The traditional therapy consists of anti-inflammatories, corticosteroids, antibiotics, and immunomodulatory drugs. This non-specific immunosuppression guarantees disease-control in some patients although the long-term use of these drugs is correlated with a significant number of therapy-associated complications and side-effects. A dramatic improvement in disease management was achieved by the introduction of biological agents targeting pro-inflammatory cytokines such as anti-TNF-α. Despite the revolutionary impact of these agents in IBD disease management, treatments such as anti-TNF-α do show several drawbacks – for example, up to 50% of patients do not respond at all or eventually lose response. This variability in clinical outcome is reflecting the variability of individuals due to different genetics, life style and inflammatory state. Therefore, there is a need to define the specific inflammatory state of a given patient, considering individual complications and develop new in-vitro systems and biomarkers that predict drug responsiveness and allow developing patient-specific treatment

In this thesis different in-vitro models were developed addressing different aspects and compartments of IBD pathology including the enteric nervous system, the ECM component fibrillin-1, as well as patient-derived, three dimensional short-term and long-term cultures that will bring us a step closer towards personalized medicine.

Aim of the study and study design

The overall aim of this project was to develop new culture systems of intestinal inflammation, that will help us to better understand complex pathological processes underlying IBD and allow for in vitro drug screening of novel or alternative therapeutics and to potentially serve as a platform for individual personalized medicine in the future.

The thesis was divided in four parts:

Study I. Establishment of a novel in-vitro ENS model

Study II. Unravelling the molecular mechanism of intestinal inflammation and the anti-inflammatory effects of phytotherapeutic agents using the zebrafish model

Study III. The role of extracellular matrix component Fibrillin in intestinal inflammation

Study IV. Establishment of long-term and short-term cultures of IBD Patient derived mucosal biopsies

CONTENTS

LIST OF ABBREVIATIONS

2-AG	2-arachidonoylglycerol
5-HT	5-hydroxytryptamine
ACh	Acetylcholine
AM	Acellular matrix
AMPA	a-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid
AMPs	Antimicrobial peptides
a-SMA	Alpha-smooth muscle actin
ATG16L	Autophagy-related protein 16-1
AVPR	Arginine vasopressin receptor
BCL-2	B-cell lymphoma 2, apoptosis regulator
BMP	Bone morphogenetic proteins
BSA	Bovine serum albumin
CD	Crohn's Disease
CD(number)	Cluster of differentiation
CGRP	Corticotropin-releasing hormone
cKit	Mast/stem cell growth factor receptor
CNR	Cannabinoid Receptor
CO_2	Carbon Dioxide
CRC	Colorectal Cancer
DAMPs	Damage-associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DCS	Deep crypt secretory
DLL	Delta like canonical Notch ligand
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DSS	Dextran sulfate sodium
ECL	Enhanced chemiluminescence
ECM	Extracellular matrix
EGF	Epidermal growth factor
ENS	Enteric nervous system

FADD	Fas-associated protein with death domain
FBN1	Fibrillin-1
FBS	Fetal bovine serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
FOX	Forkhead box protein
FZD	Frizzled receptor
GABA	Gamma-aminobutyric acid
GALT	Gut-associated lymphoid tissue
GDNF	Glial cell-derived neurotrophic factor
GIT	Gastrointestinal Tract
gp	Glycoprotein
GSNO	S-nitrosoglutathione
GWAS	Genome-Wide Association Studies
HA	Hyaluronan
HBD	Human beta-defensin
HBSS	Hank's Balanced Salt Solution
HD5	Human alpha-defensin
HE	Hematoxylin/eosin
HIV	Human immunodeficiency virus
IBD	Inflammatory Bowel Disease
IBS	Inflammatory bowel syndrome
IECs	Intestinal epithelial cells
IF	Immunofluorescence
IFN	Interferon
Ig	Immunoglobulin
iGluR	Ionotropic glutamate receptor
IL	Interleukin
IPANs	Intrinsic primary afferent neurons
IRGM	Immunity-related GTPase family M protein
ISCs	Intestinal stem cells
JAK	Janus kinase
LGR	Leucine-rich repeat-containing G-protein coupled receptor
LPS	Lipopolysaccharide
LRP	Low-density lipoprotein receptor-related protein

mAchR	Muscarinic acetylcholine receptors
MAMPs	Microbe-associated molecule patterns
MIF	Geometric mean fluorescence
MMP	Matrix metalloproteinase
mRNA	Messenger ribonucleic acid
MUC	Mucin
MYD88	Myeloid differentiation primary response 88
nAChR	Nicotinic acetylcholine receptors
NaCl	Sodium Chloride
NCC	Neural crest cell
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NGF	Nerve growth factor
NMDA	N-methyl-D-aspartate
NOD	Nucleotide-binding oligomerization domain
NOTCH	Notch homolog 1, translocation-associated
OCT	Optimal cutting temperature compound
P4H	Prolyl-4-hydroxylase
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PE	Phycoerythrin
Pen-Strep	Penicillin-Streptomicin
PNS	Peripheral nervous systems
PRR	Pattern recognition receptors
PVDF	Polyvinylidene fluoride
qPCR	Quantitative polymerase chain reaction
REG	Regenerating islet-derived protein
RNA	Ribonucleic acid
RNase	Ribonuclease
RNF	Ring finger protein
RT	Room temperature
RT-PCR	Reverse transcription-polymerase chain reaction
SCFAs	Short-chain fatty acids
SD	Standard deviation
SEM	Scanning Electron Microscopy
SMAD	Mothers against decapentaplegic homolog

SOX	SRY (Sex Determining Region Y)-Box
SP	Substance P
SSc	Systemic sclerosis
TA	Transit amplifying
TBS	Tris-buffered saline
TGFβ	Transforming growth factor beta
T_H	T helper cell
THC	Delta-9-tetra-hydrocannabinol
TIMPs	Tissue inhibitors of metalloproteinases
TLR	Toll-like receptors
TNBS	Trinitrobenzenesulfonic acid
TNF	Tumor Necrosis Factor
TNFR1	Tumor necrosis factor receptor 1
TNFR2	Tumor necrosis factor receptor 2
TP53	Tumor protein p53
TRADD	Tumor necrosis factor receptor 1-associated death domain protein
TRAF	Tumor necrosis factor receptor-associated factor
T_{REG}	T regulatory cell
TSK	Tight skin
UC	Ulcerative Colitis
VIP	Vasoactive intestinal peptide
WNT	Wingless-related integration site
WT	Wild type
ZNRF	Zinc and ring finger
ZO	Zonula occludens

1 INTRODUCTION

1.1 Inflammatory Bowel disease

Inflammatory bowel disease (IBD) is a lifelong chronic inflammatory condition of the gastrointestinal tract (GIT). IBD prevalence is increasing globally, and about 2-3 million people in Europe and over 1 million in the US estimated to be effected by IBD (Kaplan 2015). The two main forms of IBD are represented by Crohn´s Disease (CD) and Ulcerative Colitis (UC). UC is characterized by superficial inflammation limited to the mucosa of the colon. In contrast, CD is presented by discontinuous and patchy inflammation that can occur in any part of the GIT with inflammation deeper into the gut wall (transmurally). The symptoms, characterizing both, UC and CD, include abdominal pain, diarrhea (often with blood or mucus), malabsorption, weight loss and fatigue (Fakhoury et al. 2014). About one third of IBD patients develop additional extra intestinal symptoms including cutaneous manifestations, ocular inflammations, peripheral arthritis, spondylarthritis, and primary sclerosing cholangitis (Veloso 2011). Quality of life is decreasing as disease progresses due to individual complications regarding the natural evolution of the disease (Cosnes et al. 2011; Cosnes et al. 2002; Langholz et al. 1996; Moum et al. 1999). Due to its transmural nature, CD patients

frequently develop fistulae (Schwartz et al. 2002), abscesses (Keighley et al. 1982), and the formation of inflammatory or fibrotic strictures (C.-W. Chang et al. 2015; Gordon et al. 2014) which in most cases require surgical interventions. Another severe, life-threatening complication especially in UC patients, is the development of toxic megacolon coming along with acute colitis and systemic toxicity (Gan and Beck 2003; Greenstein et al. 1985). Furthermore, IBD patients show a high risk in the development of dysplasia (precancerous lesions) and colorectal cancer (CRC) with increase in disease duration (M. Chang et al. 2017). To date, no efficacious cure for IBD patients is reported and the patients require lifelong medical care for appropriate management of the disease. The traditional therapy consists of anti-inflammatories, corticosteroids, antibiotics, and immunomodulators (Kozuch and Hanauer 2008). This non-specific immunosuppressive regimen leads in some cases to disease-control but not complete remission. Moreover, severe side effects and therapy-associated complications have been associated with long term use. A dramatic improvement in disease management was achieved by the introduction of biological agents (such as cytokine-targeted drugs) in the late nineties.

1.2 Biologics in IBD

The first biologics approved in IBD were targeting tumor necrosis factor α (TNFα), one of the key cytokines mediating inflammatory processes (Bazzoni and Beutler 1996). Anti-TNF-α agents such as infliximab (IFX), adalimumab (ADA), and golimumab (GOL) are reported to neutralize the activity of TNFα, and to have a significant efficacy in promoting and maintaining IBD clinical remission and therefore reducing the need for surgery when compared to conventional treatments (Berns and Hommes 2016;

Dryden 2009). Despite the revolutionary impact of these agents in IBD treatment, anti-TNF-α agents do show several drawbacks. Firstly, severe side-effects are observed in some patients, and there is an increased risk of infections (Crawford and Curtis 2008) besides the development of neoplasia (Siegel et al. 2009; Mackey et al. 2007). Secondly, the efficacy of anti-TNF-α is limited, with a total of 20-30% of CD patients and 40% of UC patients not responding at all to treatment (Yanai and Hanauer 2011). In addition, a proportion of initial responders treated with anti-TNF-α eventually lose response (Yanai and Hanauer 2011). To overcome this issue, several new biological drugs targeting different cytokines and cellular molecules are being developed as alternative for anti- TNF-α resistant patients. Vedolizumab is targeting the cell adhesion molecule, in a gut-specific manner, through the subunit of integrin α4β7. Vedolizumab has been shown to be effective in one-third anti- TNF-α non responding patients (Amiot et al. 2016). Inhibiting immune-protective functions of TGF-β, SMAD7 is present in elevated levels within the inflamed gut mucosa of CD patients. Mongersen, an oral SMAD7 antisense oligonucleotide, re-establishes TGF-β activity. It has been shown to induce remission and clinical responses in a subset of patients with active CD in a phase II trial (Ardizzone, Bevivino, and Monteleone 2016). The JAK1,2 and 3 inhibitors are inhibiting a number of pro-inflammatory cytokine signalling pathways (IL-2, -4.-7, -9,-15 and -21). A subset of UC, but not CD patients respond with clinical remission towards Tofacitinib (Sandborn et al. 2012). Ustekinumab, a novel bispecific antibody that targets both, IL-12 and IL-23.

However, these biologics only seem to have beneficial clinical effects in certain subgroups of IBD patients and come along with adverse effect including high risk of infections (bacterial, fungal, and viral), lymphoma and malignancies.

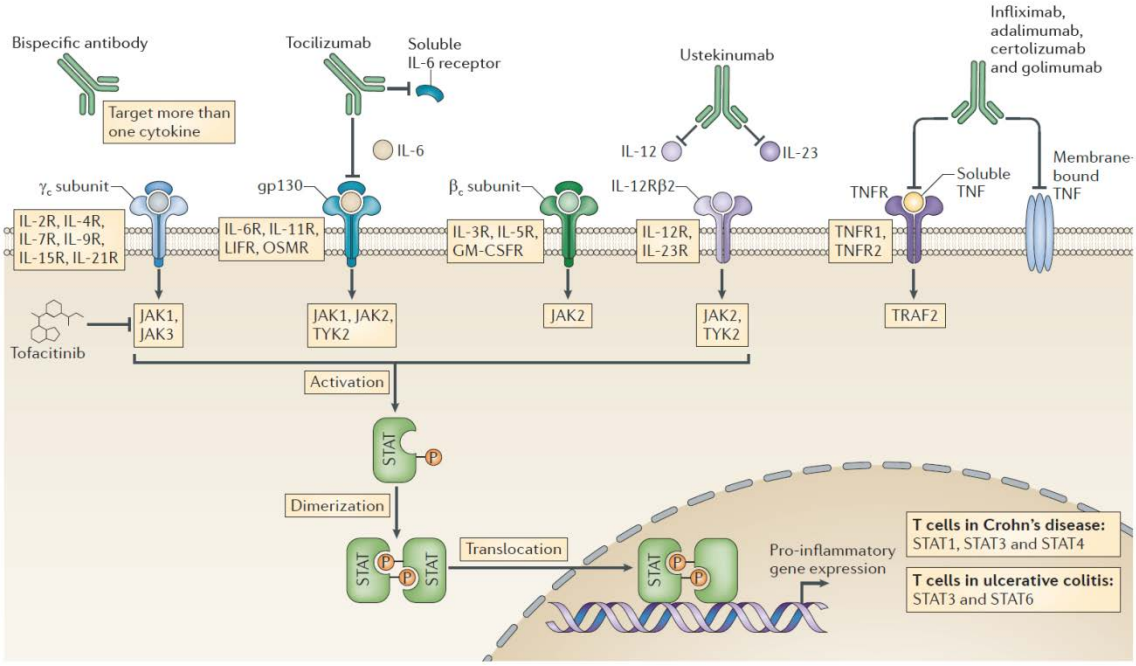


Figure 1. Biological agents in IBD treatment. Antibodies targeting soluble tumor necrosis factor (TNF) and membrane-bound TNF (such as infliximab, adalimumab, certolizumab pegol and golimumab) are standard therapies in IBD management. Recently, novel biologic agents blocking other pro-inflammatory cytokines such as interleukin-6 (IL-6) (tocilizumab), or subunits of IL-12 and IL-23 (ustekinumab) are being under investigation. Similarly, agents inhibiting the JAK /STAT pathway (tofacitinib), involved in pro-inflammatory cytokine signaling are being tested in clinical trials (Neurath 2014).

The variability in clinical outcome reflects the variability of patients, with respect to their individual genetic background, microbiota, location and lifestyle. Furthermore, patients with longer disease history may show symptoms not directly related to inflammatory symptoms but to fibrosis, bacterial overgrowth and motility dysfunction, and therefore may be non-responding to anti-inflammatory agents. As the current medical strategies have proven to be not efficacious to adequately manage and rescue the symptomatology of IBD patients, intense research involving pharmaceutical companies, gastroenterologists, biologists and pharmacologists has been recently set in motion. This research aims to identify new effective biomarkers and preclinical screening tests that may be useful to better characterize the inflammatory state of patients and to predict the efficacy of biological agents in each individual subject.

1.3 Etiology of IBD

IBD is considered as a multifactorial disease and is thought to arise from an uncontrolled inflammation caused by the unbalanced immune system of genetically susceptible individuals, in the context of environmental triggers. Genetic involvement in IBD pathogenesis was initially suspected due to differences in prevalence among different ethnic groups and aggregation of IBD in families, with 5 - 23% of patients having an affected first-degree relative (Ek, D'Amato, and Halfvarson 2014; Jess et al. 2005). Subsequently, genome-wide association studies (GWAS) have become an important tool with which to research the genetic background of IBD. To date, more than 200 genetic risk regions have been associated with IBD (McGovern et al. 2010; Duerr et al. 2006; Parkes et al. 2007; Rivas et al. 2011; J. Z. Liu et al. 2015). The first IBD-based GWAS revealed that IL23R, a gene encoding the receptor of the proinflammatory cytokine IL-23, was associated with both CD and UC (Duerr et al. 2006). While many of the CD- and UC- associated variants are shared, some were exclusive for one IBD form (Rivas et al. 2011), indicating that targeted treatment of each specific IBD type is possible. CD risk candidate genes are encoding proteins involved in immunological pathways such as autophagy (ATG16L1 and IRGM) (Hampe et al. 2007; Parkes et al. 2007) and microbial sensing (NOD2) (Homer et al. 2010; Parkes et al. 2007). UC specific risk loci include IL10 and ECM1 (Franke et al. 2008; Fisher et al. 2008). Genetic predisposition, however, cannot be solely responsible for IBD development. The increase in IBD prevalence over the last few decades, especially in developed countries, has linked to environmental factors such as increased hygiene and a lack of contact with pathogens during childhood to increased susceptibility of developing IBD during adulthood (Gent et al. 1994). Other environmental risk factors for the development of CD include smoking (Calkins 1989)

and appendectomy (Frisch and Gridley 2002). Paradoxically, both of these factors are correlated with a decrease in UC development (Koutroubakis and Vlachonikolis 2000; Calkins 1989). Furthermore, the use of contraceptive pills (Godet, May, and Sutherland 1995), antibiotics (Card et al. 2004; Hildebrand et al. 2008), as well as nonsteroidal anti-inflammatory drugs (Felder et al. 2000) are also associated with IBD. Undoubtedly, diet may influence the development of IBD and has been especially linked to alterations in the intestinal microflora (Chapman-Kiddell et al. 2010; De Filippo et al. 2010; Guzman, Conlin, and Jobin 2013; Lewis and Abreu 2017). An imbalance in microbiota has been associated with IBD, including alteration of commensal bacteria, the overgrowth and invasion of pathogens and abnormal cellular responses. This may be partly explained by alterations of the intestinal epithelium of IBD patients, who often present with increased permeability and reduced barrier function (McGuckin et al. 2009)

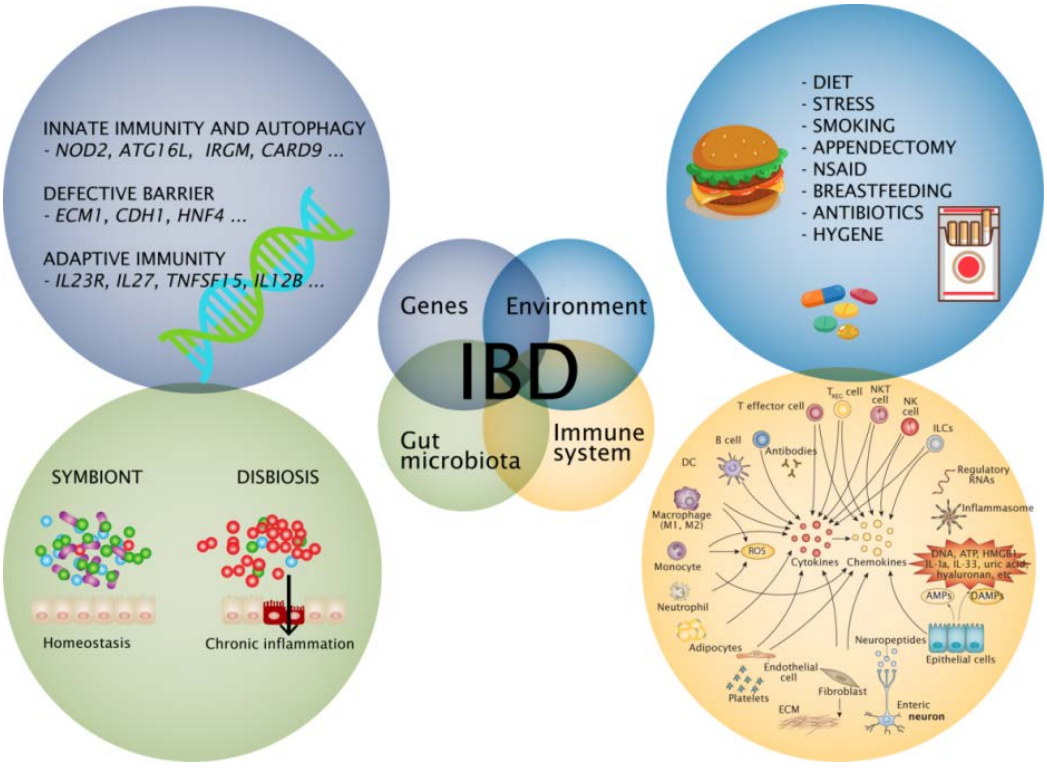


Figure 2. Factors that influence the development and progression of IBD can be grouped into four subtypes: genetic, environmental, gut microbiota, and immune factors. Key genes, pathways, and associated elements of each group are indicated. Modified from (De Souza and Fiocchi 2015)

1.4 Abnormal immune regulation and epithelial barrier

The gastrointestinal epithelium is the largest interface between the body that, as it is continuously exposed to antigens, serves as a point of communication with the immune system (Brandtzaeg 2011). Presenting a surface area of about $32m^2$, the adult intestinal epithelium is specialized in the digestion, absorption and processing of nutrients (Helander and Fändriks 2014). Furthermore, it provides a physical, biochemical and immunological barrier to prevent invasion by pathogens present in the intestinal lumen. The adult gut homes about 80% of the body's immune, over 500 million neurons and even more glial cells (John B. Furness 2012), as well as up to 100,000 extrinsic nerve endings (Grundy and Brookes 2011). The microbiome consists of as many as 40 trillion cells and hundreds of different species (Lozupone et al. 2012; Sender, Fuchs, and Milo 2016). Constantly exposed to bacteria, dietary antigens, and other environmental inputs, an important function of the GIT is to sense and appropriately respond to external stimuli. The balance of the innate and adaptive immunity is critical for intestinal homeostasis, by promoting immune tolerance against harmless commensal bacteria and antigens while also protecting against pathogens. Along with cellular, environmental, and genetic factors, the deregulation of immune responses in the intestinal mucosa has been associated with the etiology of IBD. Furthermore, the proper maintenance and function of these systems is also dependent on the proper structure of the intestinal epithelium.

1.4.1 Intestinal epithelium

Intestinal epithelial cells (IECs) form a protective fence between the intestinal lumen and the underlying lamina propria. Structurally the small intestine is organized into crypt-villus units (Figure 3). Villi are projections into the gut lumen which act to

maximize available surface area. While villi are not present in the caecum and colon, the crypts of Lieberkühn (Lieberkühn 1745) are present in all parts of the gut. Crypts are in direct contact with the basement membrane and houses multipotent intestinal stem cells (ISCs) that self-renew and give rise to all mature epithelial cell types necessary for maintaining gut homeostasis. ISCs cycle slowly (24-30h) and give rise to fast dividing transit amplifying (TA) cells which move apically towards the villus zone, which contains only differentiated, post-mitotic cells (Potten 1998). While migrating they gradually lose their stem cell features and switch on differentiation programs, to replace the epithelial cells being lost at the villus tip.

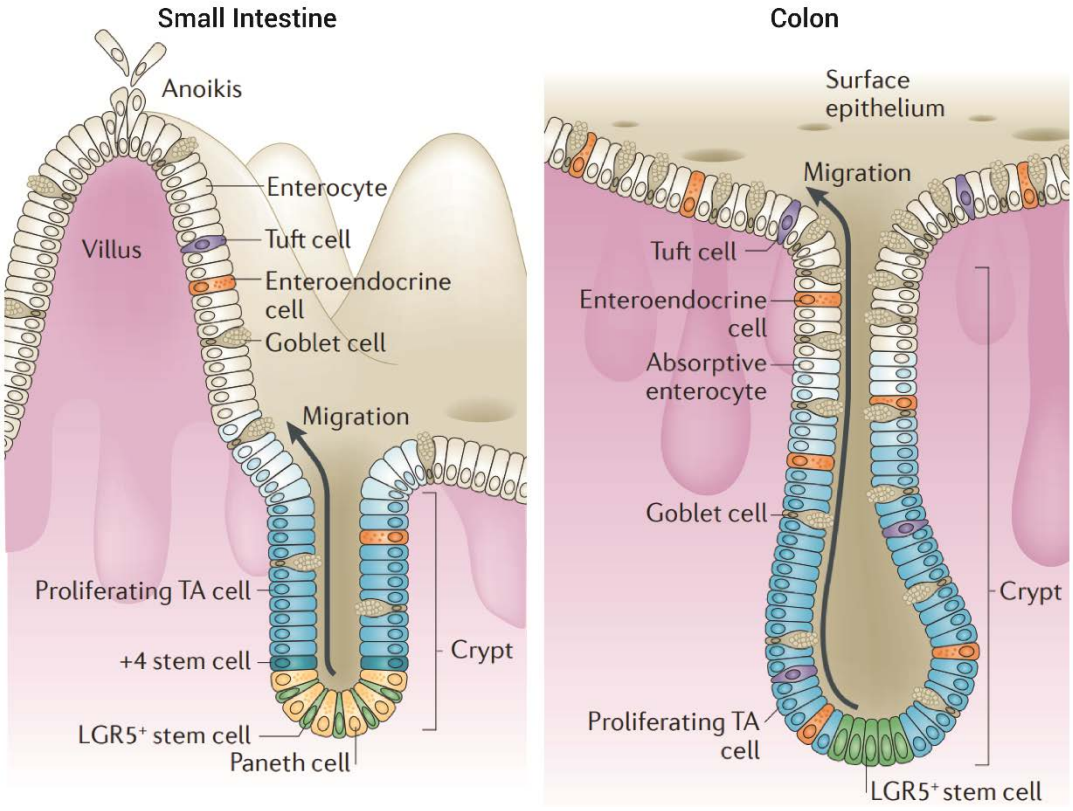


Figure 3. Schematic diagram of the intestinal epithelial cell organization. (Left) Intestinal stem cells within the small intestine are located along with Paneth cells at the base of the crypt. These multipotent stem cells generate transit-amplifying (TA) cells and are continuously differentiating into various adult epithelial cells such as Paneth cells, Enterocytes, Goblet cells, Enteroendocrine cells or Tuft cells while migrating upwards into the villus region. (Right). In the colon, intestinal stem cells are located in the base of the crypt and proliferating TA cells migrate upwards towards the surface epithelium while differentiating into adult epithelial cells. Paneth cells are not present within the colon. Figure adapted from (Barker 2013).

During the process of cell shedding at the villus tip, IECs undergo a complex, cytoskeletal remodeling process that allows to maintain the barrier function without the formation of gaps in the monolayer (Watson and Hughes 2012). This renewal process happens continuously; thus, more than 300 million cells are generated daily to compensate, and the single layer IEC is completely replenished within 5 days (L. G. van der Flier and Clevers 2009).

The diversity of epithelial functions is demonstrated by the numerous specialized cell types present in the intestinal epithelium (Figure 3). Most cells bordering the intestinal lumen are absorptive enterocytes, which are characterized by apical microvilli structures, adapted for metabolic and digestive function. The luminal secretion of mucins is shaped by Goblet cells that are scattered throughout the epithelium of the small and large intestine. Mucins are heavily glycosylated proteins which form a protective barrier and medium for molecular exchange between environment and epithelium (Johansson and Hansson 2016). Paneth cells, which are present throughout the bottom of the crypt in the small intestine produce antimicrobial peptides (AMPs) and enzymes (defensins and lysozymes) and establish a barrier against microbial contact (Bevins and Salzman 2011). Additionally, Paneth cells provide essential niche factors to maintain the intestinal stem cell niche (T. Sato et al. 2009). Enteroendocrine cells represent only 1% of all epithelial cells and represent a link between the central and enteric neuroendocrine system through the secretion of hormones regulating appetite and digestive function (Sternini, Anselmi, and Rozengurt 2008). Important for mucosal immunological response to luminal antigens, Microfold cells (M-cells) overlie gut-associated lymphoid tissue (GALT), and function as interface between the luminal content and the underlying immune cells (Neutra 1998). On the luminal side of GALT such as Peyer's patches and lymphoid follicles, M cells are often in close contact with

dendritic cells (DCs). Overall, it's clear that the myriad of cell types present in the intestinal epithelium have a variety of critical functions necessary for the maintenance of gut health, and it is likely that we will discover even more functions of these cells in the coming years. One such role involves the regulation of various functions of an organism's innate immune response, an area in which the gut has become a major player.

1.4.2 Abnormalities of innate immunity

The innate immune response is the first line of defense against pathogens and involves cells of the innate immunity, including neutrophils, monocytes, macrophages, and dendritic cells (DCs), but also IECs, mesenchymal and ENS cells. Those cells can sense intestinal microbiota and are specialized in identifying pathogen-associated molecular patterns (PAMPs) by using the pattern recognition receptors (PRR), such as toll-like receptors (TLR) and nucleotide-binding oligomerization domains (NOD). This allows the initiation of rapid and effective inflammatory responses against microbial invasion. The subsequent activation of downstream signaling pathways results in nuclear factor (NF)-κB activation, thus inducing the expression of pro-inflammatory cytokines. In the healthy gut mucosa, human macrophages do not produce pro-inflammatory cytokines, but retain their phagocytic and bactericidal activity in vitro (Smythies et al. 2005). In CD patients however, innate immune cells produce abundant IL-6, IL-23 and TNF, and IFN-γ (Kamada et al. 2008). The key function of DCs is to monitor the surrounding microenvironment, sample antigens and regulate the immune response towards tolerance or inflammation (Rescigno and Di Sabatino 2009) thus interacting between innate and adaptive immunity (Rossi and Young 2005). In IBD patients, the expression of TLR2 and TLR4 and CD40 on DCs is markedly increased. Additionally, they show a

higher production in pro-inflammatory cytokines such as IL-12 and IL-6 (Hart et al. 2005).

It is now clear that the cells of the gut are key players in the innate immune response. Additionally, since organismic immunity not only involves the recruitment and activation of classical immune cells and pathways, but also the physical barriers that protect the body, it is important to note that the intestinal epithelium also plays a role in this physical aspect of immunity.

1.4.3 Compromised epithelial barrier and secretory defense function

The essential epithelial barrier function is primarily determined by the integrity of the epithelium through adherent and tight junctions (TJ). TJs are composed of transmembrane proteins (occludins, claudins and junctional adherent molecules) connected to the cytoskeleton via a complex of multiple proteins (zonula occludens; ZO-1, ZO-2). Intestinal TJs are selectively regulating the transport of soluble molecules through the paracellular space between epithelial cells. A number of factors, such as nutrients, pathogens, immune cells, cytokines as well as the enteric nervous system are able to control and alter the intestinal permeability (Berkes et al. 2003; Awad, Hess, and Hess 2017). In fact, altered TJ structure and epithelial permeability have been observed in IBD patients (Clayburgh, Shen, and Turner 2004; Secondulfo et al. 2001). Furthermore, the abundance of the pore-forming protein claudin-2 increases in UC (Heller et al. 2005) and CD (Zeissig et al. 2007) which functionally leads to a flux in cations and water, that, in turn results in diarrhea (Sandle 2005). Moreover, the expression of occluding and barrier-forming claudins such as claudin-4 and -7 (Oshima, Miwa, and Joh 2008) in UC and claudin-5 and -8 (Zeissig et al. 2007) in CD are

downregulated. In this context, in vitro and in vivo studies have shown that disease-related cytokines such as TNF, interferon-γ (IFN-γ) or interleukin-13 (IL-13) are able to modify TJ expression and organization (Clayburgh et al. 2005; Weber et al. 2010; Amasheh et al. 2009). Consequently, the epithelium shows an increased permeability to luminal pathogens such as food antigens and bacterial lipopolysaccharides (LPS). Notably, there is evidence, that those changes already occur before the clinical disease-onset (Miele et al. 2007), and is also present in asymptomatic relatives of patients (Söderholm et al. 1999). Overall, several studies in humans and animals demonstrate intriguing links between IBD and intestinal barrier permeability.

The intestinal epithelium is not only a physical barrier but has innate immune cell functions to actively fight off pathogens through the secretion of protective mucus and antimicrobial peptides (AMPs) from secretory Goblet and Paneth cells.

While within the small intestine, a single mucus layer is charged with AMPs and immunoglobulin A (IgA), in the colon, the mucus consists of two layers. While the outer layer entraps bacteria, the inner layer is relatively sterile (Johansson et al. 2008). The most abundant of secreted mucins is the gel-forming glycosylated mucin 2 (MUC2) which has a key protective role. Mucus, together with AMPs acts as the first defense line against microbiota, therefore defects in these systems allowing bacterial penetration of this normally sterile zone triggers intestinal inflammation in several mouse colitis models and IBD patients (Johansson et al. 2014; Dorofeyev et al. 2013; Van der Sluis et al. 2006; Velcich et al. 2002; Gersemann, Wehkamp, and Stange 2012; Alipour et al. 2016; Zheng et al. 2011).

Numerous clinical studies have linked IBD to the altered expression and secretion of AMPs produced by different IECs and innate immune cells (J. Wehkamp et al. 2005; Elphick, Liddell, and Mahida 2008; Simms et al. 2008; Gulati et al. 2012). The

expression profile of AMPs, including defensins, cathlecidins and lysozymes, varies along the intestinal tract. In the small intestine, Paneth cells secrete AMP containing granules into the crypt lumen via exocytosis in response to acetylcholinergic agonists (Satoh 1988; X. D. Qu et al. 1996; J. Liu et al. 2012; Satoh et al. 1995) or bacterial lipopolysaccharide (LPS) (Ayabe et al. 2000; Tanabe et al. 2005; Rumio et al. 2004; Foureau et al. 2010; Rumio et al. 2012). Human α-defensins (HD5 and HD6) are specifically expressed by both Paneth cells and neutrophils in the small intestine, and act to actively protect against bacteria (Ganz 2003). The location of Paneth cells within the crypts suggests an essential role of these cells in protecting the ISC (Ouellette 2010). While normal colonic mucosa does not express HD5, it can be detected in colon of IBD patients due to a phenomena called Paneth cell metaplasia and can be explained by the attempt to counteract bacterial invasion in colonic mucosa (Simmonds et al. 2014; Tanaka et al. 2001). Furthermore, in the colon, epithelial cells and plasma cells in the lamina propria express human b-defensins (HBD), which are anti-microbial peptides important in the prevention of colonization by pathogenic bacteria. Epithelial cells constitutively express HBD-1, while the expression of HBD-2, 3, and 4 is induced by various inflammatory and bacterial stimuli. A defective expression of antibacterial peptides, with reduced levels of β-defensins HBD2, HBD3 and HBD4, has been reported in colonic Crohn's disease compared with ulcerative colitis, that causes an altered colonic microflora, microbial invasion, and an inflammatory state (Jan Wehkamp et al. 2003; Zilbauer et al. 2010).

Overall it is clear that the intestinal epithelium is a key player in the establishment and maintenance of innate immunity defenses against invading pathogens, and that these factors are crucial in the development of IBD.

1.4.4 Adaptive Immunity in IBD

Effector T cells (T_H cell) are key players in the adaptive immune system and their specificity for antigens is a result of a complex maturation due to external stimuli (Murphy and Stockinger 2010), inducing their differentiation in T_H1, T_H2, T_H17 and T_{REG} cells (Weaver and Hatton 2009). Dysregulated T-cell activation provokes the onset of inflammation by several pathological mechanisms through an excessive release of cytokines and chemokines. Several studies have associated CD and UC to different subtypes of the pro-inflammatory response. CD is usually described as a Th1 and Th17 condition with prominent production of IL-12, IL-23, IFN-γ, and IL-17., whereas UC is usually characterized as a Th2 and Th9 condition with high abundance of IL-13, IL-5, and IL-9 (Fuss et al. 1996; Bamias et al. 2005). Of particular importance are regulatory T cells (T_{REG}) which are essential to develop tolerance to self and non-self-antigens (Harrison and Powrie 2013). In particular, T_{REG} produce anti-inflammatory cytokines IL-10 and TGF-β and prevent over-activation of effector T cells (Valencia et al. 2006). Given their importance in both innate and adaptive immunity, defects in T_{REG} cell function are often associated with autoimmune and chronic inflammatory conditions, including IBD (Mayne and Williams 2013). T_{REG} cells are sensitive to changes in the gut microbiota in regard to number and activation state (Mayne and Williams 2013). Protective TGF-β signaling is important for function and is impaired in IBD mucosa, due to an overexpression of its inhibitor molecule Smad7 (Monteleone et al. 2001). Consequently T cells have been shown to be unresponsive to T_{REG} actions, a process that can be reversed by treatment with Smad7 antisense treatment (Fantini et al. 2009). In order to avoid a prolonged and destructive immune response, antigen-activated T cells in the lamina propria usually undergo enhanced apoptosis (Boirivant et al. 1996; Strasser and Pellegrini 2004). However, in CD patients mucosal T cells lost this

mechanism due to the abnormal ratios of Bcl-2 family proteins (Ina et al. 1999) and the upregulation of inhibitors of apoptosis (and associated gene families) such as survivin (De Souza et al. 2012). Additional mechanisms contributing to T-cell resistance to apoptosis in IBD are mediated by IL-6 signaling (Atreya et al. 2000). CD patients have been reported to show abnormal T cell cycling due to dysfunctions in the tumor suppressor gene TP53 (Andreas Sturm et al. 2002), that causes, in turn, an increased number of mucosal T cells (A Sturm et al. 2004).

1.4.5 Microbiota in IBD

Microbiota are thus clearly associated with several arms of both the adaptive and the innate immune responses. It has also been reported that IECs have the ability to sense microbiota through microbe-associated molecule patterns (MAMPs) by PRRs and dynamically activate appropriate immune response (Medzhitov 2007). A defective inflammatory cascade could affect the clearance of antigens and initiate a compensatory adaptive immune response, and eventually trigger a chronic inflammatory response (Sewell, Marks, and Segal 2009). The GIT harbors the largest microbial community of the body, with more than 40 trillion microorganisms colonizing this $32m^2$ of surface area (Helander and Fändriks 2014). Commensal bacteria consist of gram-negative strains, such as *Bacteroidetes*, and gram-positive bacteria, such as *Firmicutes* as well as some minor divisions of *Proteobacteria*, *Actinobacteria*, *Fusobacteria*, and *Verrucomicrobia* (Eckburg et al. 2005). Those mucosa-associated bacteria are reduced in diversity and quantity in IBD patients (Matsuoka and Kanai 2015). In homeostatic conditions, host and microorganism live in a mutualistic relationship in which the microbiota contributes to many physiological processes and, in turn, the host provides niches and nutrients for microbial survival (Hooper and Macpherson 2010). Early environmental exposures, including neonatal delivery mode, source of milk, food,

hygiene and several other factors exert a fundamental effect on shaping the intestinal microbiota in childhood, whilst in adulthood the gut microbiota is relatively stable (Dominguez-Bello et al. 2011). The intestinal microbiota metabolizes carbohydrates, is involved in the production of vitamins and counteracts the colonization by pathogenic bacterial strains (Renz, Brandtzaeg, and Hornef 2011; Stecher and Hardt 2011). Furthermore microbiota is important during development and maturation of the gut specific immune system with the goal of establishing a symbiotic relationship of tolerance and protective immunity (Lathrop et al. 2011). Given the abundance of commensal, non-pathogenic microorganisms within the gut, the response is tightly controlled in order to develop immune tolerance towards harmless symbiotic organisms (Franchi et al. 2012). This relationship is mutually beneficial, as the microbiota is controlled by epithelial and immune cell products such as mucus and AMPs, while in-turn, microbiota shapes mucosal immune cell composition. Beneficial subsets of commensal bacteria tend to have anti-inflammatory activities and can fight off pathogens, partly through the induction of regulatory immune responses, involving regulatory T (T_{Reg}) cells. Commensal bacteria also promote mucosal barrier function, through the production of metabolites such as short-chain fatty acids (SCFAs), which for example, can be produced by *Bifidobacterium spp.*, and can act to inhibit the translocation of Shiga toxin produced by enterohaemorrhagic *Escherichia coli* (Fukuda et al. 2011).

The protective effect of commensal bacteria and their products is evident from studies using germ-free mice, which are more susceptible to DSS-induced colitis (Kitajima et al. 2001). Deficiency in TLR2, TLR4, TLR9 or MYD88 is associated with increased susceptibility to DSS-induced colitis in these mice (Rakoff-Nahoum et al. 2004; Lee et al. 2006b; Kirkland et al. 2012). Furthermore, protection of these mice from colitis can be achieved by the administration of bacterial lipopolysaccharide (LPS) (a TLR4

ligand) or CpG (a TLR9 ligand), which act by promoting epithelial barrier function following the upregulation of beneficial heat shock proteins or by the induction of type I (Interferon) IFN production, respectively (Rakoff-Nahoum et al. 2004; Katakura et al. 2005; Lee et al. 2006a). The administration of human commensal bacterium *B. fragilis* protects mice from colitis by promoting the accumulation of IL-10-producing T_{Reg} cells in the colon (Mazmanian, Round, and Kasper 2008; Round and Mazmanian 2010). When this mutualistic relationship between the host and microbiota is disrupted, the normally beneficial gut microbiota can instead cause or contribute to disease (Honda and Littman 2012; Littman and Pamer 2011). Certain conditions, for instance, immune defects, acute inflammation, or dietary changes can result in the disruption of the normal balanced state of the gut. Quantitative and qualitative abnormalities of the gut microbiota (dysbiosis) are present in both forms of IBD (Chassaing and Darfeuille-Michaud 2011). A combination of genetic factors (*Nod2, Atg16l, Il23r*) combined with environmental factors such as infection, stress, diet or antibiotics, result in disruption of the microbial community structure. In CD patients, there is an increased abundance in *Bacteroidetes* and *Proteobacteria* and a decrease in abundance of *Firmicutes* (Man, Kaakoush, and Mitchell 2011), as well as a decreased bacterial diversity (Hansen et al. 2012). Furthermore, CD patients develop serum antibodies against microbial antigens such as *Escherichia coli* outer membrane protein C, anti-Cbir1 flagellin, anti-Pseudomonas fluorescens, glycans and saccharomyces cerevisiae (Quinton et al. 1998; Mow et al. 2004; Lodes et al. 2004; Murdoch et al. 2012; Dotan et al. 2006), which results in the targeted destruction of these bacterial species. Similarly, a decrease in numbers of protective microorganisms might be present in IBD. For instance, polysaccharide A, a product of the human symbiont *Bacteroides fragilis*, can suppress IL-17 production and improve experimental colitis (Mazmanian, Round, and Kasper 2008), and a reduced number of *Faecalibacterium prausnitzii*, which have anti-

inflammatory properties, is found in CD patients (Sokol et al. 2008). An increase in the abundance of *Enterobacteriaceae, Pasteurellaceae, Veillonellaceae* and *Fusobacteriaceae* and a decrease in the abundance of *Erysipelotrichales, Bacteroidales* and *Clostridiales* has also been reported in pediatric CD patients, which strongly correlated with levels of inflammation (Gevers et al. 2014).

Overall these studies have shown that the dynamic and comprehensive composition of the microbiota can both influence, and be influenced by, IBD and other immune and inflammatory alterations. These and other previously described aspects of the gut microenvironment can also be affected by the enteric nervous system, which will be discussed forthwith.

1.5 The enteric nervous system and its involvement in IBD

The ENS is the most complex division of the peripheral nervous systems (PNS) in vertebrates. The human ENS is comprised of 500 million enteric neurons organized into interconnected ganglia, embedded in the wall of the GIT within the *myenteric plexus* (Auerbach's) and of the *submucosal plexus* (Meissner's and Henle's) (John Barton Furness 2006) (Figure 4). So far about 20 different neuronal cell types were identified by their neurochemical coding that provides a local neuronal circuitry consisting mainly of intrinsic primary afferent neurons (IPANs) ascending/descending interneurons, and inhibitory/excitatory motor neurons. In addition, enteric glia (which largely exceed the number of neurons) also play an important role in different functions of the ENS (Gershon and Rothman 1991; Rühl, Nasser, and Sharkey 2004). They have been proven to be active in enteric neurotransmission, maintaining the integrity of the mucosal

barrier and acting as a link between the ENS and immune system (Rühl 2005). Enteric glial cells are also located in close proximity to mucosal immune cells and the intestinal epithelium, allowing direct communication between compartment.

Mechanical, chemical, nutritional or bacterial stimuli are able to activate the ENS, which then controls all key aspects of gut functionality including bowel motility, gastric acid secretion, transport of water and electrolytes, the regulation of blood flow, secretion of mucins and cytokines, interaction with the immune and endocrine system, and the regulation of epithelial barrier function (John Barton Furness 2006). The control of these functions is mediated via the release of neuronal and glial factors targeting different components of the gut, such as muscle, epithelium, endothelial and immune cells. Each of these physiological aspects are compromised in IBD and it is therefore not surprising that there is an increasing amount of research interest in elucidating the role of the ENS in the pathogenesis of IBD.

Experimental data obtained from human IBD biopsies and animal models has found considerate structural and functional changes and ENS abnormalities due to inflammation (Vasina et al. 2006; Mawe, Strong, and Sharkey 2009; Lakhan et al. 2010; Villanacci et al. 2008; Geboes and Collins 1998). These abnormalities consequently are associated with dysmotility, hypersensitivity, and other ENS dysfunctions, even after the inflammatory actions are resolved (Isgar et al. 1983; Gracie and Ford 2017; Beyak and Vanner 2005). Some studies have shown that the activation of enteric neurons can influence intestinal permeability, through vasoactive intestinal peptide (VIP) release causing an increase in ZO-1 expression in IECs (Michel Neunlist et al. 2003) and regulating epithelial cell proliferation (Toumi et al. 2003). Other data indicates that acetylcholine, another enteric neuromodulator, can increase permeability (Cameron and Perdue 2007; Greenwood and Mantle 1992).

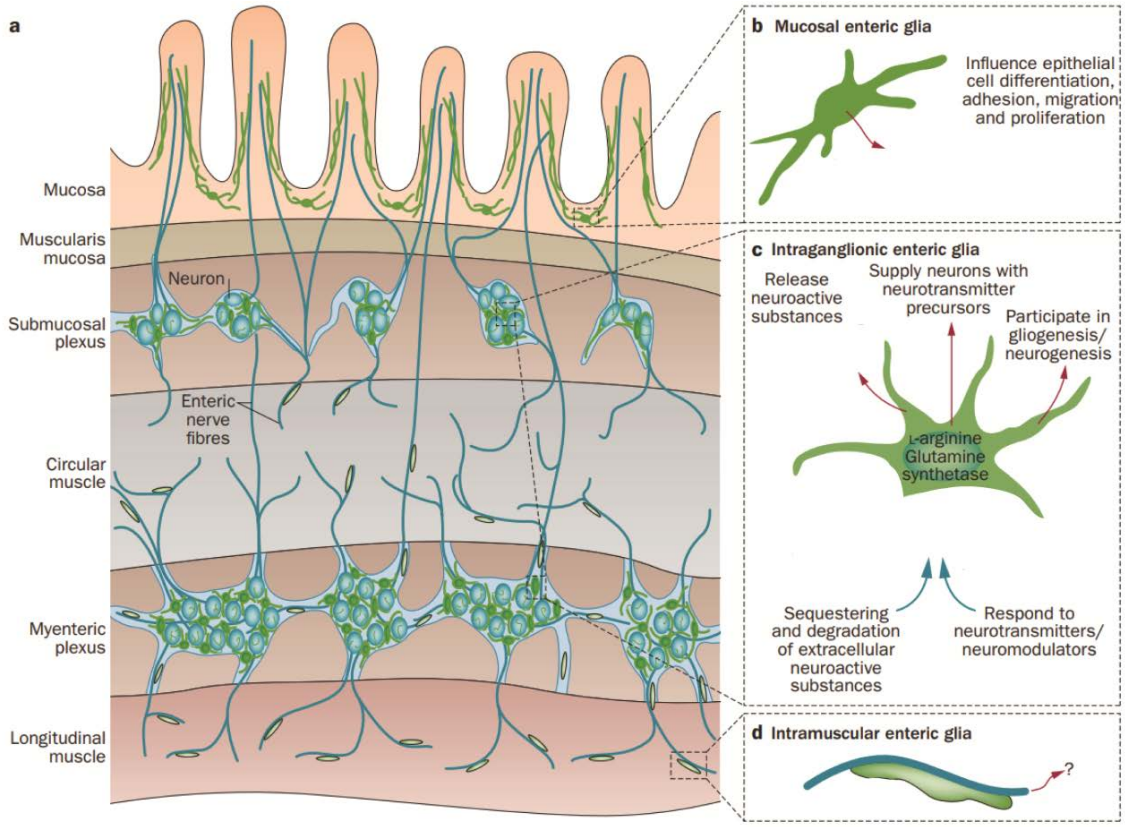


Figure 4. Organization of the enteric nervous system. (a) The ENS comprises two ganglionated plexuses, the submucosal and myenteric, which interconnect with each other and have far reaching projections also within the mucosa. (b-d) Different glial cells can be distinguished by their location and function. (b) Mucosal enteric glia cells are located underneath the intestinal epithelium and directly and indirectly influence epithelial cell behavior. (c) Intraganglionic enteric glia cells interact with enteric neurons and have neuroprotective actions. (d) Intramuscular enteric glia Adapted from (Gulbransen and Sharkey 2012)

A major role in intestinal barrier function has also been designated to enteric glia (Savidge, Sofroniew, and Neunlist 2007). In particular, enteric glial cells increase barrier resistance (Cornet et al. 2001; Bush et al. 1998), inhibit IEC proliferation (M Neunlist et al. 2007) and increase IEC adhesion (Van Landeghem et al. 2011). These studies have also identified various glial mediators involved such as TGFβ1 and S-nitrosoglutathione (GSNO) (M Neunlist et al. 2007; Savidge et al. 2007), which could allow for targeted activation or inhibition of these cells in future experiments.

It's clear from these and other studies that the enteric nervous system is closely associated with several processes implicated in the development of IBD. Furthering the

investigation of intestinal structural elements as contributing factors to IBD and related disorders, the role of the extracellular matrix has also been extensively researched.

1.6 The role of the extracellular matrix in IBD

The ECM network provides structural and signaling cues to tissues interacting with the cellular compartment, via the action of integrins and mechanosensors, as well as via the regulation of growth factor availability (Hynes 2009; Lu et al. 2011). Constantly adapting to changes in the microenvironment, the ECM acts as a dynamic structure that continually undergoes several remodeling processes to preserve tissue homeostasis.

In the presence of mild intestinal damage, mesenchymal cells such as fibroblasts, myofibroblasts and smooth muscle cells accumulate in the insulted area and secrete ECM components, such as collagens and fibronectins to resolve tissue damage (Pucilowska, Williams, and Lund 2000). Chronic and severe inflammation, as present in IBD, leads to ongoing tissue damage which can result in abnormal ECM remodeling. Subsequently many of the patients develop penetrating or fibrotic tissue damage, altered architecture of the intestinal wall, thickening of the muscularis propria, and excessive matrix deposition that reciprocally contributes to inflammatory process as IBD progresses. Intestinal fibrosis is a common complication of IBD and occurs in more than 30% of the patients with CD and 5% patients with UC (Latella et al. 2015). Since anti-inflammatory therapies are not always resolving CD-associated strictures, invasive procedures and bowel resection are the only possible interventions (Rieder, Fiocchi, and Rogler 2017).

Such mesenchymal cells (like fibroblasts and myofibroblasts as mentioned previously) are considered central ECM producing cells, and are activated and expanded during inflammatory processes. Besides resident fibroblasts, myofibroblasts and smooth

muscle cells, other cell types may also become activated via transition from epithelial or endothelial cells, such as stellate cells, pericytes, and bone marrow derived fibrocytes (Rieder and Fiocchi 2009a). Myofibroblast activation is the outcome of paracrine signals from immune cells (Curciarello, Docena, and MacDonald 2017), autocrine factors, and damage-associated molecular patterns (DAMPs) (Fiocchi and Lund 2011) derived from injured cells. In particular, growth factors such as TGF-β1 (Babyatsky, Rossiter, and Podolsky 1996), insulin-like growth factor (Pucilowska et al. 2000), platelet-derived growth factor (Kumagai et al. 2001), or cytokines such as TNFα, IL6 or IL13 (Manetti et al. 2007; Bailey et al. 2012; Fichtner–Feigl et al. 2008; Curciarello, Docena, and MacDonald 2017), are all known to drive the fibrotic processes. Furthermore, pathogen-associated molecular patterns (PAMPs) (Rieder et al. 2011; Otte, Rosenberg, and Podolsky 2003) and dietary components (Tao et al. 2015; Yang et al. 2016) have been correlated with fibrotic processes.

The major fibrillar collagen types in normal intestinal tissue are type I (70%), Type III (20%) and type V (12%) (M. F. Graham et al. 1988). In the fibrotic intestine, both, total and relative content are altered. In particular, levels of Collagen III (M Stumpf et al. 2001; Stallmach et al. 1992), Collagen V (M. F. Graham et al. 1988), Laminin (Koutroubakis et al. 2003; Spenlé et al. 2014), Hyaluronan (HA)(de la Motte 2011), Collagen I (Michael Stumpf et al. 2016), Collagen XVI (Ratzinger et al. 2010b) have been reported to be modified in IBD. Once matrix accumulates in the bowel wall it enhances tissue stiffness, which in itself acts as a mesenchymal cell activator via integrin-mediated mechanisms (Wells 2008; Johnson et al. 2013). Additionally, matrix remodeling enzymes such as matrix metalloproteinases (MMPs), and their inhibitors; tissue inhibitors of metalloproteinases (TIMPs) have been reported to be modulated in IBD (O'Sullivan, Gilmer, and Medina 2015). Furthermore, matrix degradation products have been shown to amplify inflammatory processes. MMP9 for example induces the

formation of collagen-derived fragments, which is a chemoattractant for neutrophils. And conversely, neutrophils are the main producers of MMP9 (Xu et al. 2011), thus setting in motion a self-perpetuating cycle of inflammatory activation.

There is evidence that the ECM component Fibrillin-1 might be involved in IBD pathology and associated fibrotic processes (Olivieri et al. 2010). Fibrillin-1 is a large extracellular matrix glycoprotein and major constituent of fibrillin-rich microfibrils, which are key components of elastic fibers (Ramirez et al. 2004). Furthermore fibrillin interacts with a number of other ECM components, forming a network that controls the bioavailability and activity of growth factors of the TGF-β superfamily (Kaartinen and Warburton 2003). Within the intestinal mucosa TGF-β plays an essential role in the maintenance of immune homeostasis by preventing abnormal inflammatory responses (A. B. Kulkarni 1993; Gorelik and Flavell 2002). Furthermore, RGD binding sites within the fibrillin-1 proteins allow direct cell-matrix interactions directly modifying cell attachment, gene expression, migration and proliferation (Bax et al. 2007; Bax et al. 2003). A study by Ratzinger and colleagues demonstrated a downregulation of Fibrillin-1 expression in the lamina propria of patients suffering Crohn's disease (Ratzinger et al. 2010b). Consequently, disturbances in the fibrillin network structure and stability may result in impaired biomechanical properties and cell-matrix interactions, pathological immune response and TGF-β induced fibrosis. Considering the changes in ECM components and high incidence of fibrosis in IBD (Latella et al. 2015), a major role of Fibrillin-1 interactions in disease development and progression is very likely.

2 MATERIALS AND METHODS

2.1 Three-dimensional in-vitro model of primary ENS cells

2.1.1 Animals

All animal procedures were performed under the guidelines of the local ethic committee and according to Italian laws for animal protection.

2.1.2 Preparation of gut acellular matrix (AM)

The decellularization protocol of small intestinal and colon tissue from rats was adapted from previous decellularization protocols of tracheas (Maria Teresa Conconi et al. 2005). Briefly, adult rats (Sprague Dawley rats, 3-month-old) were sacrificed by cervical dislocation, the intestine was removed and rinsed several times with PBS to remove any faecal contaminants following 2 washes with an antifungal and antibiotic agent (Amuchina, sodium hypochlorite, Angelini ACRAF S.p.A., Ancona, Italy). Subsequently, the luminal surface was treated with 4U/mL Dispase II (Roche

Diagnostics, Monza, Italy) and the epithelial layer was removed. Remaining tissue underwent six decellularization cycles each consisting of treatment with sterile Milli-Q water supplemented with 1% of antibiotic solution (Sigma-Aldrich, Milan, Italy) for 72h, 4% sodium deoxycholate (Sigma-Aldrich) for 4h, and 2000kU DNase-I (Sigma-Aldrich) in 1M NaCl (Sigma-Aldrich) for 3h. RNase A (Sigma-Aldrich) was added to the decellularization solution prior to performing the VI cycle. AMs were stored in PBS supplemented with 1% antibiotic solution at 4°C until use. The absence of cellular elements was verified by hematoxylin/eosin (HE) (Sigma-Aldrich) and DAPI staining (Invitrogen, Thermo Fisher Scientific, Monza, Italy) of cryosections (Cryostat DM2000, Leica Microsystems, Buccinasco (MI), Italy). After III, IV, V, and VI cycles, AMs were processed for DNA and RNA extraction using TRIzol® Reagent (Invitrogen) following the manufacturer's instructions. Untreated samples were considered as positive control. Quantification was performed using a Nano Drop 2000 spectrophotometer (Thermo Fisher Scientific).

2.1.3 Morphological and protein characterization of AM

2.1.3.1 Hematoxylin/Eosin overview staining

AMs were opened and pieces of approximately 5mm^2 were embedded in cryostat embedding medium (Killik, Bio-Optica, Milano, Italy). Then, samples were cut (7µm) using a Cryostat DM2000 (Leica Microsystems), put onto microscope slides and stained with hematoxylin/eosin (H&E, Sigma-Aldrich) according to manufacturer's instructions.

2.1.3.2 Scanning electron microscopy

Surface morphology was evaluated by SEM. After fixing, the samples were dehydrated, subjected to Critical Point Drying and metalized with gold. The images were acquired using the scanning electron microscope (JSM 6490) (JEOL, Peabody, USA).

2.1.3.3 Immunofluorescence

The AM was characterized for the presence of laminin. Cryosections (7μM) were permeabilized with Triton X-100 (Sigma-Aldrich) for 30 min and nonspecific sites were blocked with 1% bovine serum albumin (BSA, Sigma-Aldrich) in PBS. AMs were stained with rabbit anti- laminin (1:50, CosmoBio, Tokyo, Japan) antibody, incubated overnight at room temperature (RT) and then treated for 1h at RT with secondary antibody goat anti-rabbit-FITC (1:100, Santa Cruz Biotechnologies, Inc., Heidelberg, Germany). AM sections were only treated with secondary antibody and were considered as a negative control. Samples were mounted with Anti-Fade Mounting Medium (Immunological Sciences, Rome, Italy) and examined using a fluorescence microscope (Leica SP2, Leica Microsystems).

2.1.3.4 Quantification of DNA and RNA from AM

AM samples (0.020g), were processed for DNA and RNA extraction after III, IV and V cycles using TRIzol® Reagent (Invitrogen) following manufacturer's protocol. Untreated samples were considered as positive controls. Quantification was performed using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific).

2.1.3.5 Western blot

Collagen I and IV expression were evaluated by western blotting. Briefly, total proteins extracted from AMs (20μg) were separated by SDS/PAGE and electrophoretically transferred onto PVDF membranes (0.45μm, Bio-Rad Laboratories). Rabbit anti–

collagen I and collagen IV (CosmoBio) antibodies were used for immunoblotting at a concentration of 1:1000 in TBS-0.5% non-fat dry milk. The detection of target proteins was performed using peroxidase-conjugated anti-rabbit secondary antibodies (1:2000 in TBS-0.5% non-fat dry milk, Bio-Rad Laboratories). The development of immunoreactivity was enhanced by chemiluminescence substrate (ECL) (Sigma-Aldrich) and then visualized by VersaDoc Imaging System (Bio-Rad Laboratories).

2.1.4 Isolation and culture of ENS cells (ENSc)

Sprague Dawley rats (3 days old, P3), were sacrificed by decapitation. Briefly, the smooth muscle layer was stripped from the mucous layer and incubated in a digestion medium at 37°C, according to previously described protocols (K.-H. Schäfer et al. 1997). The digestion medium was composed of Hank`s balanced salt solution (PAN, Aidenbach, Germany), 50ng/ml trypsin-chymotrypsin inhibitor (Sigma-Aldrich), 1mg/mL collagenase type 2 (Worthington, Lakewood, USA) and 200µg/mL deoxyribonuclease (DNAse) (Roche). Myenteric nets were collected and treated with Accutase (PAA, Pasching, Austria) for 10min. After the digestion, cells were dissociated by aspiration through a 27G needle. The preparation yield per animal was approximately $1x10^6$ cells. The cells were seeded at a density of $2x10^5$ cells/cm^2. The culture medium was composed of Neuronal Base P (PAA) supplemented with 2% neuronal stem cell supplement (PAA), 1% bovine serum albumin (Sigma-Aldrich), 0.1% β-mercaptoethanol (Invitrogen), 1% penicillin/streptomycin (Invitrogen) and 1% L-glutamine (Sigma-Aldrich), 10 ng/mL EGF (ImmunoTools, Friesoythe, Germany), 20ng/mL b-FGF (ImmunoTools) and 10ng/mL GDNF (ImmunoTools).

2.1.5 In vitro cultures of ENS cells on AM

After seven days in culture, primary ENSc were seeded at a density of $2x10^4$ cells/cm^2 on the top of the outer layer of AM. Before seeding, the AM was opened and cut into pieces of approximately $5mm^2$. The matrices were stretched on a glass coverslip, transferred into 24-well plates (Falcon, BD Biosciences, Milan, Italy) and anchored using stainless steel rings. Cultures were fixed at two different time points (7 and 14 days) for SEM analysis, wholemount immunofluorescent staining and gene expression analysis. These procedures are described below.

2.1.5.1 Wholemount immunofluorescence staining

For wholemount staining, the specimens were fixed using BD Cytofix (BD Biosciences, Milan, Italy) for 2h and permeabilized with 0.5% triton X-100 for 30min following blocking with 1% BSA for 2h at 4°C. Primary antibodies against ßIII-tubulin (1:50, Merck Millipore, Vimodrone (MI), Italy) and α-sma-FITC (1:50, Abcam, Cambridge, UK) were incubated overnight at RT. Visualization resulted from a secondary antibody anti-mouse PE (1:100, Santa Cruz Biotechnology) which was incubated for 3h at RT. Samples were mounted with Anti-Fade Mounting Medium (Immunological Sciences) and examined using a fluorescence microscope DMI4000B (Leica Microsystems).

2.1.5.2 Gene expression: RT^2 profiler assay

Repopulated AMs (treated) and ENS cells on tissue culture plates (control) grown for 14 days were treated with RNeasy Mini Kit (Qiagen, Milan, Italy) for extraction of total RNA that was quantified using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). The gene expression evaluation of rat neurotrophic and neuronal receptors was performed using RT^2 Profiler™ PCR Array Rat Neurotransmitter Receptors (Qiagen) following manufacturer's protocol. Data were acquired using DNA Engine

Opticon® Real Time Thermal Cycler (MJ Research, St. Bruno, QC, Canada) and analyzed using the ΔΔCt Livak method (Livak and Schmittgen 2001) normalizing to housekeeping gene expression (hypoxanthine-guanine phosphoribosyltransferase, HPRT). Data were presented as mean fold change ($2^{-\Delta\Delta Ct}$) ± SD of n=3 replicates of three independent experiments.

2.2 Zebrafish model of intestinal inflammation

2.2.1 Animals

All animal procedures are performed under the guidelines of the local ethic committee and according to Italian laws for animal protection. Zebrafish (*Danio rerio)* were kept in a 12 h light/ 12 h dark light cycle with light turning on at 8.00 am and off at 8.00 pm. Adult Zebrafish (Danio rerio, older than 3 months) were anesthetized by immersion in 1.3mM tricaine mesylate solution (Sigma-Aldrich). After decapitation, whole gut was extracted under the dissecting microscope and surrounding tissues including mesenteries and fat were removed. Gut samples were further processed for immunofluorescence analysis and primary cell culture establishment.

2.2.2 Morphological analysis of Zebrafish gut

A preliminary morphological analysis, wholemount and immunofluorescence (IF) were performed in adult Zebrafish in order to explore ENS organization.

2.2.2.1 Histology

After dissection from adult Zebrafish (3 months old), the whole intestine was fixed in BD Cytofix solution (BD Biosciences) overnight at 4C° and then fixed in paraffin. Gut

sections (5µm) were prepared for histological analysis by standard hematoxylin and eosin (H&E) staining. The samples were analyzed using a DMI4000B microscope.

2.2.2.2 Wholemount immunofluorescent staining

After gut digestion with Dispase II solution (Roche), muscle layer containing ENS was collected and processed for wholemount immunofluorescent staining. Briefly, following fixation with BD Cytofix solution (BD Biosciences) overnight at 4C°, the samples were treated with 10% bovine serum albumin (BSA, Sigma-Aldrich) for 1h at RT to block the aspecific sites and afterward were submitted to permeabilization with 0.5% triton X-100 (Sigma-Aldrich) for 30min. Target markers were detected by incubation at room temperature (RT) for 15min with primary antibody anti-PAN neuronal marker (1:50, Millipore) and AlexaFluor®488 conjugated secondary antibody (1:100, Invitrogen). After mounting with Anti-Fade Mounting Medium (Immunological Sciences), the samples were analyzed using a Leica TCS SP5 confocal microscope (Leica).

2.2.2.3 FZD9 and Wnt3a analysis by immunofluorescence (IF)

Detection of Frizzled-9 (FZD9) and Wnt3a in the gut of zebrafish was undertaken by immunofluorescence staining (IF) using sections (5µm) of gut samples from wild-type animals. After a fixation (at 4C°, overnight) in BD Cytofix solution (BD Biosciences), all samples were embedded in paraffin (Carlo Erba Reagents, Milan, Italy) and slides were prepared using a Leica RM2125RTS microtome (Leica Microsystems). After deparaffinization in xylene and rehydration, the gut sections were immersed in citrate buffer (pH 6) and treated four times (5 min/each) by microwave irradiation. Following a blocking step with 10% BSA for 2h at RT, the membrane permeabilization was performed with 0.2% triton X-100 (Sigma-Aldrich) for 30min. The immunodetection of target markers was executed using polyclonal primary antibodies against Pan Neuronal Marker, FZD9 and Wnt3a (1:50, Immunological Sciences) and AlexaFluor®488- and

AlexaFluor®594-conjugated secondary antibodies (1:100, Invitrogen). In parallel, separate sections treated with conjugated secondary antibodies alone were used as controls. After DAPI staining (1:1000, Invitrogen) and mounting with Anti-Fade Mounting Medium (Immunological Sciences), the samples were analyzed using a Leica TCS SP5 confocal microscope (Leica Microsystems).

2.2.3 Isolation of intestinal cells and assessment of in vitro models

In order to isolate both intestinal epithelial and ENSc, the epithelial layer was separated from muscle tissue and connective tissue by enzymatic digestion with 4U Dispase type II (Roche) prepared with Hank's Balanced Salt Solution (HBSS) (PAN-Biotech), and was carried out at 28°C for 40 min.

2.2.3.1 Zebrafish epithelial cell culture

The epithelial layer was centrifuged at 500g for 5min and the pellet was resuspended in AccuMax (Sigma-Aldrich) for 30min at 28°C and processed by gentle pipetting. Cells were seeded on collagen IV (10µg/cm^2 Sigma- Aldrich) coated glass coverslips. Cells were cultured (28°C, 5% CO2 and 95% humidity) in Dulbecco′s Modified Eagle′s Medium high glucose (DMEM) (Sigma-Aldrich), 15% fetal bovine serum (FBS) (Invitrogen), 1% L-glutamine (Sigma-Aldrich), 1% Antibiotic/Antimycotic Solution (Sigma-Aldrich) and 20ng/mL epithelial growth factor (EGF) (ImmunoTools). Epithelial cells were morphologically characterized by optical microscopy (DMI4000B microscope, Leica Microsystems) and cytokeratin expression was evaluated by immunofluorescence using anti-pan Cytokeratin (1:50, Abcam, Cambridge, UK) and AlexaFluor®594 secondary antibody (1:100, Invitrogen)

2.2.3.2 Zebrafish ENS cell culture

The muscle layer containing ENSc was further incubated with 5µg/mL collagenase type II (Worthingthon, Lakewood, NJ, USA), 200µg/mL DNAse (Roche) and 50ng/mL trypsin-chymotrypsin inhibitor (TCI) (Sigma-Aldrich) in HBSS (PAN-Biotech) at 28°C for 50min. After centrifugation at 200g for 5min, the pellet was resuspended in AccuMax solution and incubated at 28°C for 20min. To dissociate cell clusters, gentle pipetting was performed before centrifugation at 500g for 5min. Cells were seeded on glass coverslips coated with 10µg/cm^2 laminin (Sigma-Aldrich) and cultured in ENS culture medium as described for primary mouse ENS cultures. At 7 days from isolation, the expression of neuronal markers was evaluated in ENS cultures by immunofluorescence using a Pan Neuronal Marker (1:50, Immunological Sciences) and AlexaFluor®488 secondary antibody (1:100, Invitrogen). After DAPI staining (1:1000, Invitrogen) and mounting with Anti-Fade Mounting Medium (Immunological Sciences), the samples were analyzed using a Leica TCS SP5 confocal microscope (Leica). The presence of neural progenitors/precursors in ENS cultures was investigated by flow cytometry (FCM) evaluating the expression of Sox[SRY (Sex Determining Region Y)-Box] 2 and Sox10. The analysis was performed at time of isolation (T0) and after 1 (T1) and 7 (T7) days of culture, incubating the samples at RT for 15min with primary antibodies against Sox2 and Sox10 (1:20, Santa Cruz Biotechnology) and goat anti-mouse- and anti-rabbit PE conjugated secondary antibodies (1:50, Santa Cruz Biotechnology). In parallel, secondary antibody-matched samples were used as controls. Data were acquired using a FACSCanto II Flow cytometer (BD Biosciences) and the analysis was performed with FACSDiva v6.1.3 software (BD Biosciences).

2.2.4 In vitro evaluation of STW5 anti-inflammatory activity

Zebrafish epithelial and ENS cell cultures were stimulated with 5µg/mL lipopolysaccharide (LPS, Sigma-Aldrich) or dextran sodium sulfate (DSS, Sigma-Aldrich) for 24h to obtain functional in vitro inflammation models. Untreated cells were used as controls. Cells under inflammatory or basal conditions were stimulated with 0.12mg/mL ethanol-free STW5 (Steigerwald Arzneimittelwerk GmbH, Darmstadt, Germany). At different time points (6h and 24h), the cells were collected and processed for qPCR analysis.

2.2.4.1 Gene expression analysis by quantitative Real-Time PCR

Total cellular RNA was extracted using TRIzol® (Invitrogen), quantified by measuring the absorbance at 260nm and then stored at −80°C until use. cDNA was retrotrascribed and amplified using the MyTaq one-step RT-PCR kit (Bioline) on an AriaMX Realtime PCR system (Agilent Technologies, Cernusco sul Naviglio (MI), Italy) according to the manufacturer's instructions. The relative mRNA expression of *il1b, il6, il8, il10, tnfa* and *wnt3a* (Table 1) was determined using the ΔΔCt Livak method, considering the expression of eukaryotic translation initiation factor 1A (*eif1a*) as reference. Data were presented as mean fold change ($2^{-\Delta\Delta Ct}$) ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's *t*-test comparing to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$); versus DSS- or LPS- treated samples (▲ $p \leq 0.05$, ▲▲ $p \leq 0.01$, ▲▲▲ $p \leq 0.001$).

2.2.5 In vivo evaluation of STW5 anti-inflammatory activity

In vivo inflammation was induced by placing adult zebrafish into individual bowls containing 0.25% DSS (Sigma-Aldrich) for 24h. After a quick water change, the stimulation with 0.12 mg/mL STW5 was performed for 48h. Untreated fish were used as controls. At the end of stimulation, the animals were sacrificed, and gut was extracted, divided into three groups: the bulb, midgut and hindgut, and thus submitted to histological analysis.

2.2.5.1 Histochemistry

Zebrafish gut tracts from animals treated with DSS and stimulated with STW5, together with controls, were processed for histological study as described previously. Alcian Blue staining was performed on slices incubating at first phase with 1% Alcian Blue (Sigma-Aldrich) in 3% acetic acid (pH 2.5) (Sigma- Aldrich) for 30 minutes and at second phase with 0.1% nuclear fast red (Sigma-Aldrich) for 5 minutes. After dehydratation in ethanol, the sections were cleared with xylene and mounted with Pertex® mounting medium (Leica). As their increased number is commonly considered as indicative of active inflammation state, goblet cells were quantified in all samples - from each sample 5 different sections from three independent experiments were analyzed. Data were expressed as number of goblet cells per villus ± standard deviation (SD). Data are presented as mean of goblet cells per villus ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's *t*-test comparing to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$); versus DSS-treated samples (▲ $p \leq 0.05$, ▲▲$p \leq 0.01$).

Table 1. Primer pairs used for gene expression analysis (F = forward; R = reverse).

Danio rerio

Gene (*Danio rerio*)	Abbreviation	Sequence 5'-3'	Amplicon length
Eukaryotic translation initiation factor 1a	*eif1a*	F: GACAAGAGAACCATCGAG R: CCTCAAACTCACCGACAC	271 bp
Interleukin 1 beta	*il1b*	F: GACATGCTCATGGCGAACG R: GCAAATCGTGCATTGCAAGACG	102 bp
Interleukin 6	*il6*	F: GTGAAGACACTCAGAGACG R: GTTAGACATCTTTCCGTGCTG	149 bp
Interleukin 8	*il8*	F: TGTTTTCCTGGCATTTCTGACC R: TTTACAGTGTGGGCTTGGAGGGG	151 bp
Interleukin 10	*il10*	F: CTGACTGTTGCTCATTTGTGG R: CTCGTTCATGACGTGACATC	170 bp
Tumor necrosis factor alpha	*tnfa*	F: CCGTCTGCTTCACGCTCC R: GTCTTTGATTCAGAGTTGTATCC	148 bp
Wnt family member 3a	*wnt3a*	F: CCCGCTCTGCTATGAATCG R: CATTTCCGATGCGCTGTCG	186 bp

2.3 Role of fibrillin-1 in intestinal inflammation

2.3.1 Animals

All animal procedures were performed under the guidelines of the local ethic committee and according to Italian laws for animal protection.

Fbn1Tsk/J (FBN Tsk/+) mice were purchased from Jackson laboratory (Bar Harbor, ME). The Tsk mutation is a genomic duplication within the fibrillin1 (Fbn1) resulting in a larger than normal in-frame Fbn1 transcript. FBN1 Tsk/+ mice thus synthesize and secrete both normal fibrillin-1 (~330 kDa) and the mutant oversized Tsk fibrillin-1

(~450 kDa). This model allows us to investigate the consequences of fibrillin-1 perturbations on different levels within the gut. For genotypization, genomic DNA was isolated from the tail of the mice using the MyTaq™ DNA Polymerase (Bioline, London, UK) as described by manufacturer's protocol. Wild type C57BL/6J and $Fbn1^{Tsk}$/J mice (3 months old) were sacrificed by cervical dislocation and the gut was dissected and processed for further histological, immunofluorescence or gene expression studies. In parallel, primary cells from the myenteric plexus were isolated as described previously and then processed via expression studies, western blot, and flow cytometric analyses.

2.3.1.1 Histology

After dissection from adult mice (C57BL/6J and $Fbn1^{Tsk}$/J), colon sections were fixed in BD Cytofix (BD Biosciences) solution overnight at 4°C and then mounted in paraffin. Gut sections (5 µm) were prepared for histological analysis by standard hematoxylin and eosin (H&E, Sigma-Aldrich) and Masson's trichrome staining (Sigma-Aldrich). The samples were analyzed using a DMI4000B microscope (Leica Microsystems).

2.3.1.2 Gene expression analysis by quantitative real-time PCR

For gene expression analysis, whole tissue homogenates were prepared form all parts of the intestine (stomach, duodenum, jejunum, ileum, caecum, proximal colon and distal colon). The relative mRNA expression of target and housekeeping genes (Table 2) was determined as described previously. Data are presented as mean ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's *t*-test comparing FBN1 Tsk/+ to wild-type samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Table 2. Primer pairs used for gene expression analysis (F = forward; R = reverse).

Mus musculus

Gene (*Mus musculus*)	Abbreviation	Sequence 5'-3'	Amplicon length
Calnexin	*Cnx*	F: ATGACTGGGATGAAGAGG R: GTCGTCTAGCCAGCCTTC	74 bp
Interleukin 1 beta	*Il1b*	F: TGTGGCAGCTACCTATGTCT R: GGGAACATCACACACTAGCA	97 bp
Interleukin 4	*Il4*	F: GAAGAACACCACAGAGAGTGAG R: TGCAGCTCCATGAGAACAC	120 bp
Interleukin 6	*Il6*	F: TTGCCTTCTTGGGACTGATG R: AGGTCTGTTGGGAGTGGTAT	98 bp
Interleukin 8	*Il8*	F: TGGGAGACCTGAGAACAAGA R: TCACTGGAGTCCCGTAGAAA	111 bp
Interleukin 10	*Il10*	F: CTATGCTGCCTGCTCTTACTG R: GGGAAGTGGGTGCAGTTATT	83 bp
Interleukin 17	*Il17*	F: GAGAGCTTCATCTGTGTCTCTG R: GCTGAGCTTTGAGGGATGAT	90 bp
Interleukin 18	*Il18*	F: CAGCCTGTGTTCGAGGATATG R: TCACAGCCAGTCCTCTTACT	109 bp
Interleukin 23a	*Il23a*	F: CCAGCGGGACATATGAATCTAC R: GCTGTTGTCCTTGAGTCCTT	114 bp
Transforming growth factor beta 1	*Tgfb1*	F: CTGAACCAAGGAGACGGAATAC R: GGGCTGATCCCGTTGATTT	101 bp
Mucin 2	*Muc2*	F: GGTCTGTGTGGGAACTTTGA R: GAAGCCTCCTTCCAGCTATTC	107 bp
Matrix metallopeptidase 2	*Mmp2*	F: GTTCAACGGTCGGGAATACA R: GCCATACTTGCCATCCTTCT	103 bp
Matrix metallopeptidase 9	*Mmp9*	F: TCTGTATGGTCGTGGCTCTAA R: GGAGGTATAGTGGGACACATAGT	102 bp
Matrix metallopeptidase 13	*Mmp13*	F: GTTGACAGGCTCCGAGAAAT R: CATCAGGCACTCCACATCTT	109 bp

2.4 Short-term and long-term models of human mucosal biopsies from IBD patients

2.4.1 Human intestinal biopsies and study population

Biopsy material was obtained from subjects affected by UC and CD, who were referred to the hospital for endoscopy. Inclusion criteria included age above 18 years old, absence of pregnancy, and absence of HIV or bacterial infections at time of endoscopy. Healthy controls were recruited from a colon cancer screening program and revealed no symptoms or signs of intestinal disease at endoscopy. All patients were recruited at the Hospital of Padova, and the signed informed consent was approved by the Italian National Committee for Health Research Ethics. The biopsies (n=3-5) were obtained from each patient or healthy subject from the descending colon to ensure low variability due to regional differences. Colonic biopsies were immediately collected in a physiological solution containing 2% antibiotics (Pen-Strep 10,000U/mL, Thermo Fisher Scientific), and maintained on ice and processed for in-vitro studies within 3h to ensure high quality DNA and protein samples.

2.4.1.1 Short-term biopsies explant culture

A method was developed to maintain human mucosal biopsies viable in vitro for a 24h period. Biopsies were maintained in the upper compartment of a transwell system (Falcon). The medium employed in these studies consisted of DMEM /high glucose (Sigma-Aldrich) supplemented with 1% antibiotic solution (Pen-Strep 10,000U/mL, Thermo Fisher Scientific). A series of pilot studies were performed to compare the air-liquid culture system to the submersion-based in vitro culture.

2.4.1.2 Histology of colonic biopsies

Biopsies were processed for histopathological evaluation of intestinal inflammation. Furthermore, histological analysis of biopsies prior (T0) and post-assay (T24h) were performed to evaluate the quality and cell survival under different explant culture conditions. Biopsies were thoroughly rinsed before fixing in 10% neutral-buffered formalin (Thermo Fisher Scientific) for 12h and embedded in paraffin. Paraffin sections (5µm) were cut, mounted onto Superfrost Plus slides (Fisher Scientific) and de-waxed prior to histochemical staining. Sections were stained with hematoxylin/eosin (H&E; Sigma-Aldrich) allowing the validation of the overall extent of inflammation considering inflammatory cell infiltrates, epithelial changes and overall mucosal architecture.

2.4.2 Treatment of biopsies with anti-TNF-alpha in the ait liquid interface

The short-term explant culture model is supposed to function as an in-vitro model to predict the patient-specific response to pharmaceuticals. In order to evaluate the value of this model, the response of UC donor derived biopsies to anti-TNF-alpha (Infliximab) was observed via transcriptional gene expression profiling in three groups of patients. The first group was comprised of patients who are currently and successfully being treated with anti-TNF-alpha (Responder) therapy, patients who have been treated with anti-TNF-alpha in the past without any effect (Non-Responder) and healthy donors (Healthy). Biopsies were cultured in the liquid-air interface and treated with (Infliximab; Remicade) at a concentration of 100µg/mL for 24h. Untreated biopsies from the same patient served as internal controls. After 24h biopsies were

processed for mRNA isolation and subsequent qPCR analysis, according to the methods described previously.

2.4.2.1 Gene expression analysis by quantitative real-time PCR

Colonic biopsies were stored in RNA stabilization solution RNAlater (Ambion, Thermo Fisher Scientific) according to manufacturer's protocol and subsequently lysed in 500 µl of TRIzol® (Invitrogen) for mRNA isolation. Primer pairs for target and housekeeping genes were designed as reported in table 3 and purchased from Invitrogen. The relative mRNA expression was determined by One-step RT-PCR as described previously. Data were presented as mean ± SD of n=3 replicates of three independent experiments. Differences were analyzed by Student's *t* test and *p* values of <0.05 were regarded as significant.

2.4.3 Isolation of colonic crypts of human IBD patients and colon organoid formation

For the growth and culture of colonic crypts, biopsies were carefully washed in PBS/Antibiotic solution, minced with a scalpel blade and digested for 1h at 37°C, in 10ml of digestion medium containing DMEM high glucose (Sigma-Aldrich), 1% FBS (Thermo Fisher Scientific), 75U/mL collagenase II (Worthington) and 2U/mL dispase I (Roche). After removing the digestion medium, crypts were resuspended in HBSS and transferred to a Petri dish. Crypts were collected under a dissection microscope and embedded in extracellular matrix hydrogel (Cultrex® Reduced Growth Factor Basement Membrane Matrix, Type R1; Trevigen). Organoids were cultured in IntestiCult™ Organoid Growth Medium. Organoids were cultured at long term condition and passaged every 10 to 14 days after fragmentation.

2.4.3.1 Passaging of human colon organoids

To keep the optimal nutrient supply, the organoids were cultured at low density. For passaging, the cultures were washed with cold PBS. Hydrogel was dissolved in cold BD cell recovery solution (BD Biosciences) and incubated on ice for 20min. The remaining gel was dissolved by pipetting. Organoids were fragmented using a 20G gauge needle before centrifuging at 500g for 5min at 4°C. The cell pellet was washed in PBS twice before embedding in fresh matrix hydrogel as described above.

2.4.3.2 Cryosectioning and immunofluorescence staining of human colon organoids

Cryosectioning of colon organoids allows the characterization of specific expressed markers by immunofluorescence staining. Briefly, organoids were incubated in a fixing solution containing 2% Paraformaldehyde and 0.1% Glutaraldehyde in PBS for 30min at room temperature. Structures were extensively washed in PBS before being kept in a 20% sucrose solution (Sigma-Aldrich) overnight at 4°C. Hydrogel domes were then placed in a mold containing optimal cutting temperature (OCT) compound and cut in 5-10µm sections using a cryotome. Sections were washed in PBS to remove OCT and permeabilized in 0.15% Triton in PBS for 10min. After blocking for 2h at room temperature in 3% BSA solution before incubation with DAPI counterstaining solution (1:1000, Invitrogen). Slides were washed in PBS and mounted using Anti-Fade Mounting Medium (Immunological Sciences).

2.4.4 Isolation of pericryptal mesenchymal cells

Present in the lamina propria and pericryptal region, mesenchymal cells are known regulators of the intestinal stem cell niche, the immune cells besides ECM production and destruction. Therefore, mesenchymal stem cells play an important role in IBD pathologies and IBD-correlated complications. To isolate cell populations underlying

the colonic crypts, the biopsies were initially processed as previously described for preparing organoid cultures. After tissue digestion, cells were seeded on collagen IV-coated (Sigma-Aldrich) culture plates. Culture medium was composed of DMEM high glucose (Sigma-Aldrich), 1% antibiotic solution (Thermo Fisher Scientific), 10% FBS (Thermo Fisher Scientific, 10ng/mL EGF (ImmunoTools) 20ng/mL FGF2 (ImmunoTools) and 40ug/mL insulin (Sigma-Aldrich). The obtained cell populations were characterized by immunofluorescent staining and processed for qPCR analysis.

2.4.4.1 Immunofluorescence characterization

For immunofluorescence staining, the cells were fixed using BD Cytofix (BD Biosciences, Milan, Italy) for 20min at 4°C and permeabilized with 0.2% triton X-100 (Sigma-Aldrich) for 10min following a blocking with 1% BSA (Sigma-Aldrich) for 2h at 4°C. The immunodetection of target markers was executed using primary antibodies against CD34 (1:50, Santa Cruz), LGR5 (1:50, Santa Cruz), prolyl-4-hydroxylase (P4H) (1:100, Dako), βIII-tubulin (1:100 Millipore), TNF-α (Immunological science) and AlexaFluor®488- and AlexaFluor®594-conjugated secondary antibodies (1:100, Invitrogen). After DAPI staining (1:1000, Invitrogen) and mounting with Anti-Fade Mounting Medium (Immunological Sciences), the samples were examined using a fluorescence microscope DMI4000B (Leica Microsystems).

2.4.4.2 Gene expression analysis by quantitative real-time PCR

Total cellular RNA was extracted using TRIzol® (Invitrogen) and relative mRNA expression was determined by One-step RT-PCR as described previously. Primer pairs for target and housekeeping genes were designed as reported in table 3 and purchased from Invitrogen. Data were presented as mean ± SD of n=3 replicates of three independent experiments. Differences were analyzed by Student's *t* test and *p* values of <0.05 were regarded as significant.

Table 3. Primer pairs used for gene expression analysis (F = forward; R = reverse).

Homo sapiens

Gene (*Homo Sapiens*)	Abbreviation	Sequence 5'-3'	Amplicon length
Transforming growth factor beta 1	*TGFB1*	F: CGTGGAGCTGTACCAGAAATAC R: CTAAGGCGAAAGCCCTCAAT	158 bp
Mucin 2	*MUC2*	F: TGTCCCTTCTACTGGTGTGA R: CCTGGCACTTGGAGGAATAAA	94 bp
Interleukin 1 beta	*IL1B*	F: ATGGACAAGCTGAGGAAGATG R: CCCATGTGTCGAAGAAGATAGG	114 bp
Interleukin 6	*IL6*	F: GAGCTGTGCAGATGAGTACAA R: GGACTGCAGGAACTCCTTAAA	190 bp
Interleukin 10	*IL10*	F: GCTGGAGGACTTTAAGGGTTAC R: GATGTCTGGGTCTTGGTTCTC	105 bp
Tumor necrosis factor alpha	*TNF*	F: CCAGGGACCTCTCTCTAATCA R: TCAGCTTGAGGGTTTGCTAC	106 bp
Tumor necrosis factor receptor 1	*TNFR1*	F: GGACAGGGAGAAGAGAGATAGT R: TGGACAGTCATTGTACAAGTAGG	115 bp
TNF receptor superfamily member 1B	*TNFR2*	F: TGCATCGTGAACGTCTGTAG R: GGAATCTGTGTCTCCCATTGT	84 bp
Matrix metallopeptidase 9	*MMP9*	F: GGGCTTAGATCATTCCTCAGTG R: GCCATTCACGTCGTCCTTAT	94 bp
Matrix metallopeptidase 2	*MMP2*	F: CGACCGCGACAAGAAGTATG R: TGTTGCCCAGGAAAGTGAAG	107 bp
Matrix metallopeptidase 13	*MMP13*	F: GTTTGGTCCGATGTAACTCCTC R: GAAGTCGCCATGCTCCTTAAT	96 bp
Hypoxanthine phosphoribosyltransferase 1	*HPRT1*	F: ATGGACAGGACTGAACGTCTTGCT R: TTGAGCACACAGAGGGCTACAATG	79 bp

3 RESULTS AND DISCUSSION

3.1 Study I: Establishment of a novel in-vitro ENS model

Physiological aspects of the intestine, including secretion of mucins and cytokines and the regulation of epithelial barrier function, are controlled by the intrinsic nervous system in the gut, the ENS (John Barton Furness 2006). In particular, enteric glial cells have been proven to be active in enteric neurotransmission, maintaining the integrity of the mucosal barrier and acting as a link between the ENS and the immune system (Rühl 2005). Each of these functions are compromised in IBD and experimental data obtained from human IBD biopsies and animal models has found considerate ENS abnormalities on a structural as well as a functional level, as well as changes in neurotransmitter signaling, accompanying inflammation (Vasina et al. 2006; Mawe, Strong, and Sharkey 2009; Lakhan et al. 2010; Villanacci et al. 2008; Geboes and Collins 1998). Recently we have demonstrated the involvement of Wnt signaling in enteric neuro-inflammation induced by bacterial lipopolysaccharide (LPS) (Di Liddo et al. 2015). These abnormalities consequently are associated with dysfunctional motility, hypersensitivity, and other ENS dysfunctions even after resolution of acute inflammation (Isgar et al.

1983; Gracie and Ford 2017; Beyak and Vanner 2005). Therefore, there is an increasing research interest in clarifying the role of the ENS in the pathogenesis of IBD.

ENS development is a highly dynamic process, involving events such as the migration of neural crest cells (NCCs) into and along the developing gut, and extensive proliferation and differentiation of neuronal and glial cells (Lake and Heuckeroth 2013). Not all NCCs within the ENS differentiate during development, and a small pool of enteric neural crest stem cells persist in the postnatal and adult gut [14] to serve as a backup system. In this way, the adult ENS has the ability to adapt itself to changing environmental cues under physiologically conditions; responding to diet, aging or injury throughout the entire lifespan. Among soluble factors, numerous in vitro and in vivo studies have shown the importance of growth factors such as Glial cell-derived neurotrophic factor (GDNF) (Moore et al. 1996; K. H. Schäfer and Mestres 1999), nerve growth factor (NGF) (Mulholland et al. 1994; Lin et al. 2005; Kuroda et al. 1994), basic fibroblast growth factor (bFGF) (Chadi et al. 2004; Hagl et al. 2013), epidermal growth factor (EGF) for enteric cell survival, proliferation and differentiation. Furthermore, gut ECM, mainly composed by collagen IV, fibronectin, laminin and heparinsulfate proteoglycans (Bannerman, Mirsky, and Jessen 1988), has long been known to provide necessary cues for the formation of enteric ganglia (Pomeranz et al. 1993; Chalazonitis et al. 1997; Letourneau, Condic, and Snow 1994) and for its role in neurotrophic signaling (Simon-Assmann et al. 1995; Adams and Watt 1993). In particular Collagen IV and laminin are known to favor neuronal outgrowth, differentiation and survival (Ali, Pappas, and Parnavelas 1998; Lein 1991) and heparan sulfate proteoglycan is implicated in GDNF signaling (Mammadov et al. 2012; Barnett et al. 2002).

ENS progenitor cells from rodents and humans have successfully been isolated and cultured in vitro and are usually grown as neurospheres in defined media, supporting ENS progenitor proliferation (Rauch et al. 2006; Grundmann et al. 2015; K.-H. Schäfer et al. 1997; T. a Heanue and Pachnis 2011). Subsequential differentiation can be induced by several soluble factors such as retinoic acid (Y. Sato and Heuckeroth 2008). Even though these two-dimensional cultures have led to improvement on the understanding of ENS development and pathologies, there is a need to define new ENS in-vitro model systems that authentically represent the in-vivo adult ENS. Such a model system would allow researchers to gain more translatable and clinically relevant data. Recently, Raghavan and colleagues tested various ECM components individually and in combination with each other and demonstrated a significant impact of neuronal and glial differentiation on ENS derived cells (ENSc) (Raghavan and Bitar 2014). Given these findings, it is evident that 2-dimensional ENS in vitro studies on plastic surfaces cannot properly mimic the organ specific ultrastructure and biological composition present in the native gut. The aim of the present study was the development of a 3-dimensional in vitro model using intestinal acellular matrices (AMs) as a scaffold for primary ENS cells. This model takes into account tissue architecture, matrix composition as well as the exogenous neurotrophic factors GDNF, bFGF and EGF to better mimic the enteric environment in vitro.

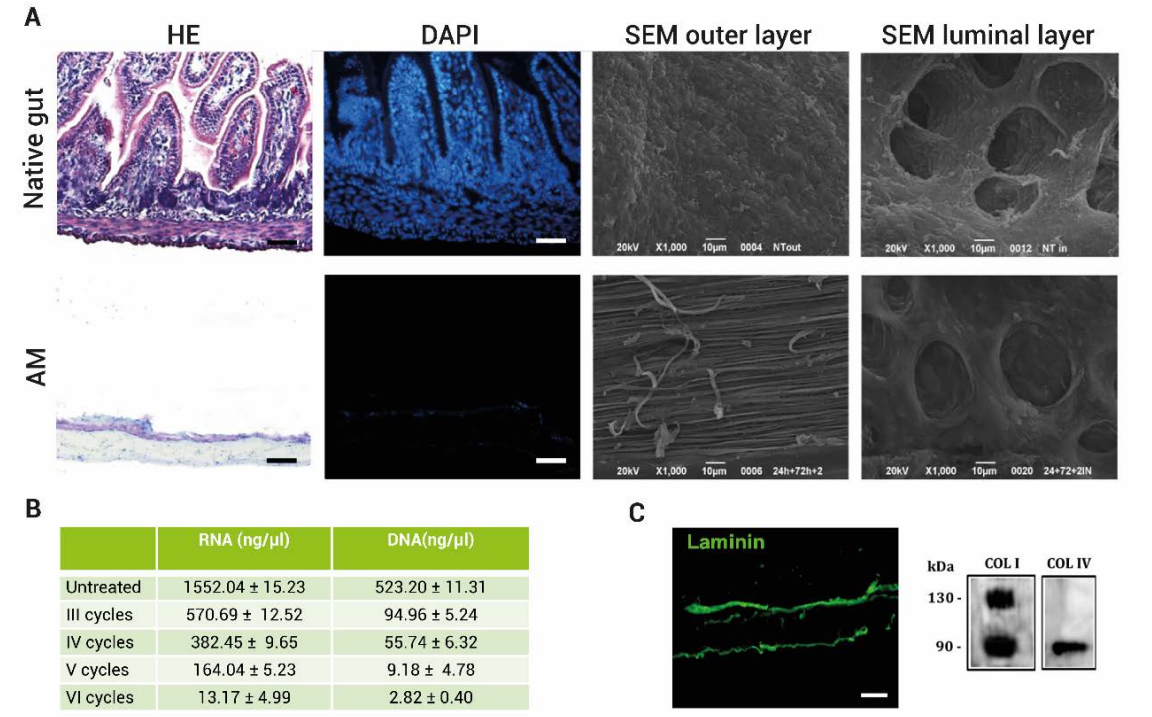
3.1.1 Establishment of intestinal acellular matrix

Biologic scaffolds derived from decellularized tissues and organs have been successfully used in both pre-clinical animal studies and in human clinical applications (Macchiarini et al. 2008). Acellular matrices (AMs) are obtained by treating tissues with various reagents that remove the cellular part while leaving almost intact the architecture of native tissue and ECM network. AMs provide an in vitro 3-dimensional

culture model supporting adhesion, growth, differentiation and function of several cell types (Maria Teresa Conconi et al. 2005; De Carlo et al. 2010), whereas in vivo they act as a template, allowing the ingrowth of the host cells.

Importantly, AMs can be remodeled in a living tissue (Macchiarini et al. 2008; Maria T Conconi et al. 2009; Dall'Olmo et al. 2014). To date, several decellularization procedures have been developed (Crapo, Gilbert, and Badylak 2011) and herein we adapted a previous protocol of trachea decellularization (Maria Teresa Conconi et al. 2005) utilized on the small intestine of adult rats. Histological analysis, DAPI staining, DNA quantification and SEM analysis verified successful decellularization (Figure 5). Hematoxylin/Eosin overview staining showed the layers of native small intestinal wall: serosa, muscularis externa, submucosa and mucosa (Figure 5). After decellularization, the thickness of the AM layer was reduced, and was free of cells, as confirmed by the absence of DAPI stained nuclei (Figure 5).

SEM analysis of the native SI demonstrated that the outer layer showed a flat and homogeneous surface, while the luminal side was characterized by the presence of villi and crypts (Figure 5). AM revealed a structure of longitudinally arranged fibers in the outer layer and a loss of villi in the luminal layer (Figure 5). Immunofluorescence and Western Blot analysis confirmed the preservation of structural proteins such as collagen IV and laminin (Figure 5) Overall, we have shown an effective procedure for decellularization of the intestinal acellular matrix.



	RNA (ng/µl)	DNA(ng/µl)
Untreated	1552.04 ± 15.23	523.20 ± 11.31
III cycles	570.69 ± 12.52	94.96 ± 5.24
IV cycles	382.45 ± 9.65	55.74 ± 6.32
V cycles	164.04 ± 5.23	9.18 ± 4.78
VI cycles	13.17 ± 4.99	2.82 ± 0.40

Figure 5. Decellularization of adult rat small intestine. (A) Hematoxylin/Eosin overview staining shows layers of native small intestinal wall: Serosa, muscularis externa, submucosa and mucosa. Successful decellularization was confirmed by histological studies and SEM studies and the absence of DAPI stained nuclei. (B) SEM analysis of the native SI outer layer showed a flat and homogeneous surface, while the luminal side was characterized by the presence of villi and crypts. (B) Quantification of nucleic acid indicated a decrease as number of decellularization cycles increased (C) Immunofluorescence and Western Blot analysis demonstrated that structural proteins such as collagen IV and laminin were preserved from enzymatic digestion. AM revealed the structure of longitudinal arranged fibers in the outer layer and a loss of villi in the luminal layer.

3.1.2 ENS cells cultured on AM form ganglion-like structures

ENSc were isolated from the myenteric plexus of postnatal rats (P3), expanded as neurospheres for 7 days in a standard cell culture system, and subsequently seeded on the outer surface of AMs. At 7 and 14 days, the morphology and the expression of specific differentiation markers were evaluated by SEM, immunofluorescence and RT-PCR. SEM analysis verified cell attachment, spreading and proliferation on the AM reflecting cell-matrix contact and interactions (Figure 6).

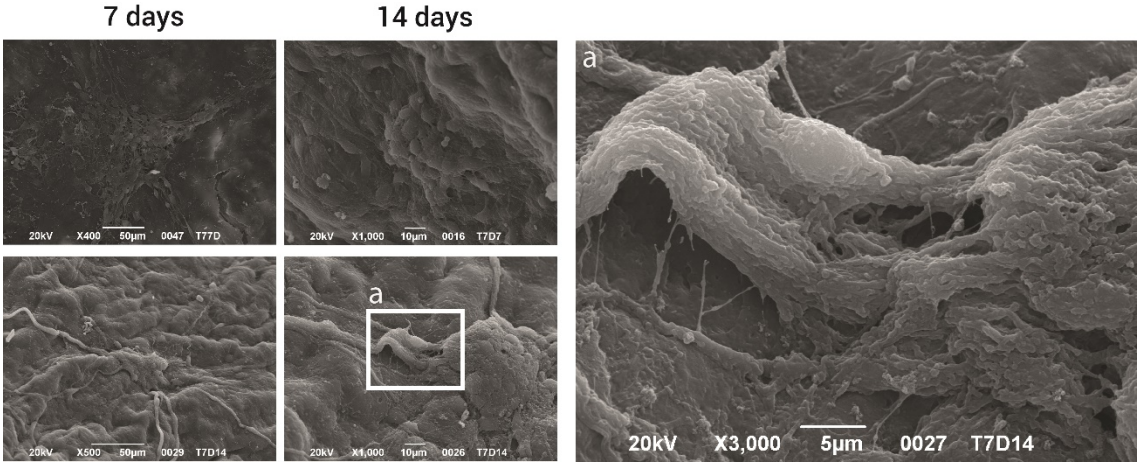


Figure 6. SEM analysis of repopulated AM. ENSc were cultured for 7 days as neurospheres and subsequently seeded on the outer surface of AMs. SEM images show cell attachment, spreading and proliferation on the AM, reflecting cell-matrix contact and interactions. At 7 days, small cell clusters were observed to form ganglion-like structures. This organizational pattern intensified during incubation time where ganglion-like structures were interconnected with each other and ENS-AM cultures reassembled networks formation similar to the in vivo situation.

This pattern intensified during incubation time and evolved in ganglion-like interconnected structures after 14 days (Figure 6). Wholemount staining confirmed βIII tubulin positive, ganglion-like formations as well as their βIII tubulin positive neuronal connections (Figure 7).

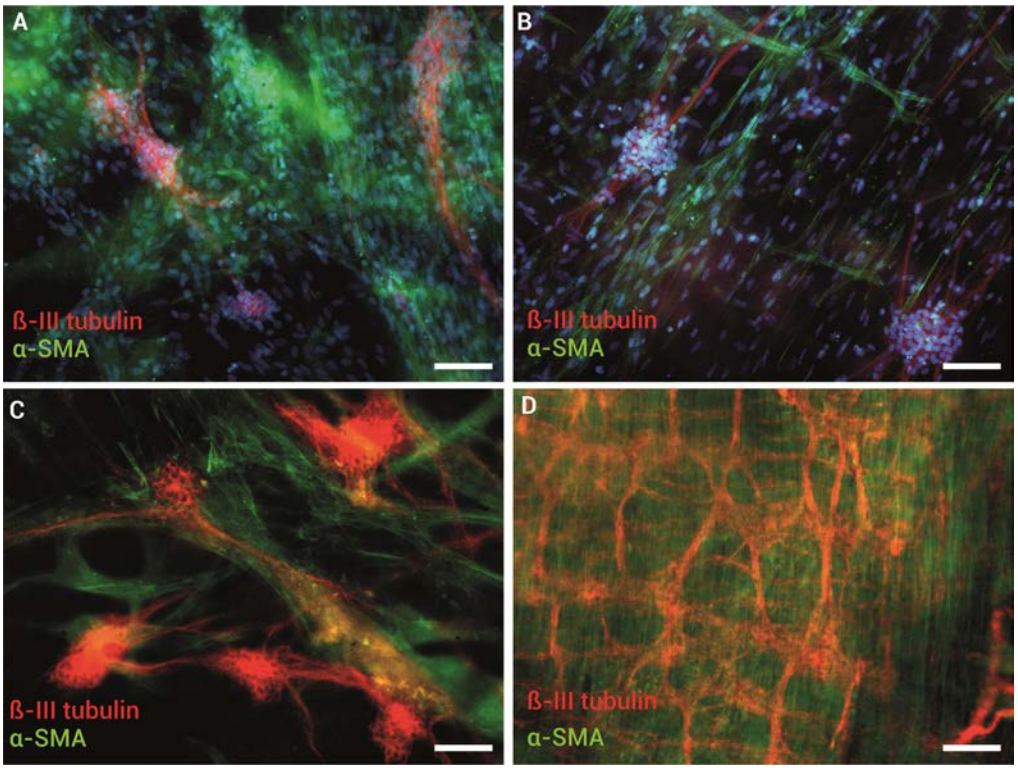


Figure 7. Wholemount staining of ENS-AM of βIII-tubulin neuronal cells and α-SMA positive cells after (A) 3 days, (B) 7days, (C) 14days, in vitro. The ENS-AM model revealed similar structural organization of neuronal networks to (D) the native myenteric plexus. Bar: 100um

In contrast, ganglion underlying supporting cells that were α-SMA-positive and did not express βIII tubulin, confirming structure and distribution of the native myenteric plexus (Figure 7). These findings in ENS-AM cultures, in which the observed structures appeared to be very similar to the myenteric plexus in vivo, prompted us to investigate whether there may also be functional improvements in terms of neurotransmitter receptors.

3.1.3 ENS-AM cultures show relevant neurotransmitter receptor expression

Morphological and electrophysiological studies revealed a huge diversity of neurons within the in vivo ENS of mammals, including enteric motor neurons, as well as sensory neurons, including intrinsic primary afferent neurons (IPANs) which contain specific types of neurotransmitters. These neurotransmitters, including acetylcholine (ACh), serotonin (5-hydroxytryptamine, 5-HT), substance P (SP), corticotropin-releasing hormone (CGRP), and vasoactive intestinal peptide (VIP), are involved in gut homeostasis and when dysregulated, in inflammation (14). Changes in neurochemical coding are of particular relevance in IBD as different studies revealed that IBD patients present with shifts of neuronal subtypes (Keränen et al. 1995; Renzi et al. 1998; Surrenti et al. 1993; M Neunlist et al. 2003; Belai et al. 1997; J. Schneider et al. 2001).

Distinctive patterns of neurotransmitter receptors allow identification of different functional neuronal subclasses. Unfortunately, knowledge of the relationship between neurotransmitter receptors and their functional role in enteric neurons, as well as their dysregulation in disease is very limited. Since altered neuronal activity within the

ENS underlies various GI disorders, including IBD, elucidating the expression and function of the neurotransmitter systems (which determine neuronal excitability within the ENS) could reveal novel therapeutic targets. To evaluate the value of the developed three-dimensional ENS in vitro models (described previously in section 3.1.2) in this context, the expression of neurotransmitter receptors was evaluated and compared to their expression in conventional two-dimensional cultures on plastic surfaces (Figure 8).

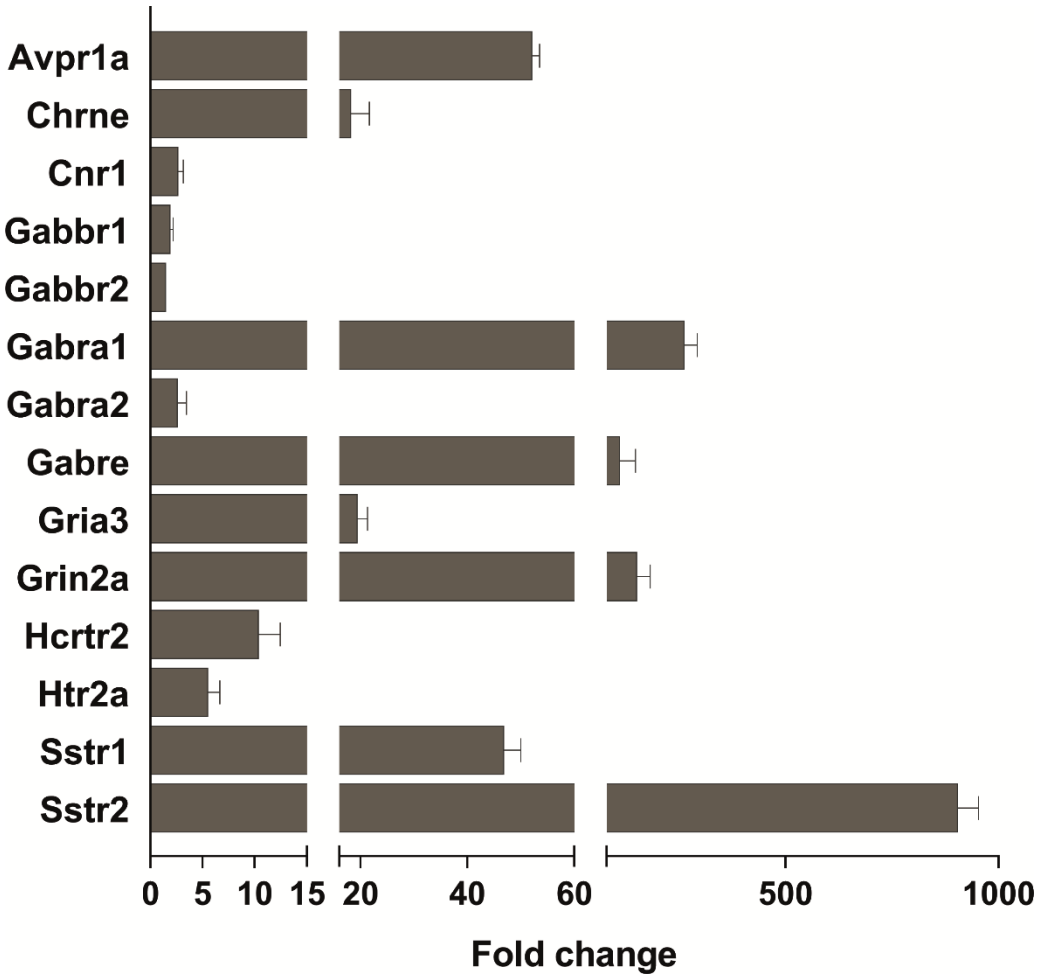


Figure 8. RT² Profiler™ PCR Array of neurotransmitter receptors. As neuronal differentiation was observed in ENSc-AM cultures in standard medium for 14 days, a quantitative gene expression of neurotrophic receptors was performed. ENSc cultured for 14 days on plastic were considered as a control. A significant increase was observed with arginine vasopressin receptor 1A (Avpr1a), gamma-aminobutyric acid (GABA) A receptor α1 (Gabra1), gamma-aminobutyric acid (GABA) A receptor ε (Gabre), cholinergic receptor, nicotinic, ε (Chrne) glutamate receptor, ionotropic, AMPA 3 (Grai3) glutamate receptor, ionotropic, N-methyl D-aspartate 2A (Grin2a), and somastostatin receptors Sstr1/Sstr2 Data are presented as mean fold change (2-ΔΔCt) ± SD of n=3 replicates of three independent experiments.

Cholinergic neurons are the most abundant neurons in the adult ENS (Z.-D. Qu et al. 2008) and therefore the main excitatory neurotransmitter in the ENS is acetylcholine (ACh), which acts through nicotinic ACh receptors (nAChRs) or muscarinic receptors (mAChR). Nicotinic receptors are ligand-dependent ion channels, and are mainly located on myenteric neurons (Kirchgessner, Liu, and Alcantara 1997), where they participate in rapid synaptic transmission and play a central role in motor function. The mRNA expression of nicotinic ACh receptor (epsilon subunit: *Chrne*) is significantly increased on AM-ENS cultures compared to control (fold increase 18.1) (Figure 8). A significantly higher mRNA expression was also observed for arginine vasopressin receptor 1A (*Avpr1a*) (fold increase: 52.03) (Figure 8), a vasopressin receptor that, within the gut, is exclusively expressed on a subset of myenteric cholinergic motor neurons. AVPR1A induces excitatory effects on the contractile fibres, and is therefore involved in motility functions (Qin et al. 2009; Mastropaolo et al. 2013). These findings are important because motility dysfunctions are commonly present in IBD patients (Bassotti et al. 2014).

Another neurotransmitter, abundant in intestinal neuroendocrine cells, is serotonin (5-hydroxytryptamine, 5-HT). About 95% of 5-HT in the human body is found in the digestive tract where it regulates a multitude of sensory, motor and secretory functions through several distinct types of receptors present within the ENS (D.-Y. Kim and Camilleri 2000; McLean, Borman, and Lee 2007). Abnormal serotonin secretion has been associated with various detrimental effects on the whole-body level. Intestinal secretion and peristaltic movements and abnormalities in 5-HT have been linked with symptoms present in patients with inflammatory bowel syndrome (IBS) (Hansen et al. 2012; Crowell 2004).

The greatest difference was present in the expression of somastostatin receptors in AM-ENS cultures compared to cultures on plastic with a fold increase of 46.79 and 903.29

of somastostatin receptor 1 (Sstr1) and somastostatin receptor 2 (Sstr2) respectively (Figure 8). Somastostatin are known for their inhibitory actions on neurotransmitter and hormone secretion and cell proliferation.

The mRNA expression of presynaptic cannabinoid receptor (*Cnr1*) is upregulated by 2.6 fold (Figure 8). Inhibiting motility, in vivo the CNR1 is present within the myenteric and submucosal plexus, mediating the inhibitory effects of several cannabinoids such as 2-arachidonoylglycerol (2-AG) or anandamide (Pertwee 2001). Cannabinoids are endogenous bioactive phospholipids (Colombo et al. 1998; Heinemann et al. 1999; Izzo et al.1999a, 1999b) which are extremely important for gut homeostasis (Pertwee 2001). Various studies have shown that modulation of the endocannabinoid system can result in the regulation of various gut functions such as motility, visceral sensation, and inflammation, and thus is likely to be involved in IBD pathogenesis (Di Sabatino et al. 2011; Alhouayek and Muccioli 2012). Cannabinoids, such as the phytocannabinoid, delta-9-tetra-hydrocannabinol (THC) have suggested to be of therapeutical use in IBD treatment (Ahmed and Katz 2016). The three-dimensional ENS model presented herein therefore allows further clarifications in this field. ENS-AM cultures present also an excellent model for studies of neurotransmitter γ-aminobutyric acid (GABA) that is located in neuronal as well as in endocrine-like cells, implicating GABA as both a neurotransmitter and secretory mediator influencing GI function (Krantis 2000). Significant mRNA expression of $GABA_A$ receptor subunits, $GABA_A$ receptor α1 (*Gabra1*) and $GABA_A$ receptor ε (*Gabre*), as well as $GABA_B$ receptor subunits (*Gabbr1*, *Gabbr2*) was detected in AM-ENS cultures. Furthermore GABAergic neuronal cells, mainly interneurons, are particularly represented in the large intestine where they account for 5–8% of the total myenteric neurons (Hyland and Cryan 2010) exerting either a stimulatory or inhibitory action on neuronal activity, via activation of GABA receptors. Given that the GABAergic signaling has been reported to be involved

not only in motor functions, but also in neuro-immune modulation, a comprehensive understanding of the GABAergic system is of particular interest in enteric IBD research of novel ENS factor-based therapeutic strategies for the treatment of inflammatory GI disorders (Auteri, Zizzo, and Serio 2015).

The ionotropic glutamate receptor (iGluRs), N-methyl-D-aspartate (NMDA) and even more so the a-amino-3-hydroxy-5-methyl-4- isoxazole propionic acid (AMPA) are present in the evaluated in vitro model as shown by the increase in their encoding genes *Grin2a* (19.31 fold increase), *Grai3* (150.54 fold increase) respectively (Figure 8). Glutaminergic neurons have been shown to modulate the release of nociceptive neurotransmitters, and have been implicated in chronic visceral pain in the inflamed colon (Q. Zhou and Nicholas Verne 2008).

3.1.4 Conclusion Study I

Intestinal AM affected the spatial organization of ENS cells and exerted a synergic effect with supplemented neurotrophic factors. The development of interconnected ganglion-like structures with a distribution similar to that of native myenteric plexus was observed. Interestingly, the gene expression of cholinergic, GABA, glutamate, serotonin and somatostatin receptors significantly increased in samples cultured on AM compared to conventional two-dimensional plastic surfaces. These results confirm that gut matrix optimizes the in vitro differentiation process of ENS cells and may be used as functional in vitro model in further clarifying the role of ENS in IBD pathology and therapeutic studies.

3.2 Study II. Unravelling the molecular mechanism of intestinal inflammation and the anti-inflammatory effects of phytotherapeutic agents using the zebrafish model

Due to its genetic and organ similarity with mammals, together with its ease of breeding, low cost of production, high fecundity and short ex vivo development time, the zebrafish (*Danio rerio*) represents an excellent model organism. Zebrafish genome sequencing revealed that 85% of disease-associated human genes have a zebrafish orthologue, making this a highly relevant model for studying and modelling human disease (Howe et al., 2013). Anatomically, the zebrafish intestine is compartmentalized in three segments: the intestinal bulb, mid-intestine and posterior intestine (Ng et al. 2005). The zebrafish intestinal wall reveals the presence of villus-like structures (epithelial folds) of a single layer epithelium and underlying lamina propria (Wallace et al. 2005), effectively mimicking the structure of the human intestinal wall as shown in (Figure 9). Besides some limitations, such as the lack of Paneth cells, as well as the absence of crypts and organized lymphoid structures (Jiminez et al. 2015; Brugman 2016), adult zebrafish intestinal epithelial cells exhibit several human-like elements such as absorptive enterocytes, endocrine and goblet cells. These cells are continuously replaced from self-renewing stem cells at the base of epithelial folds, which then migrate to the tip where they are regenerated in a manner parallel to the human intestine (Wallace et al. 2005).

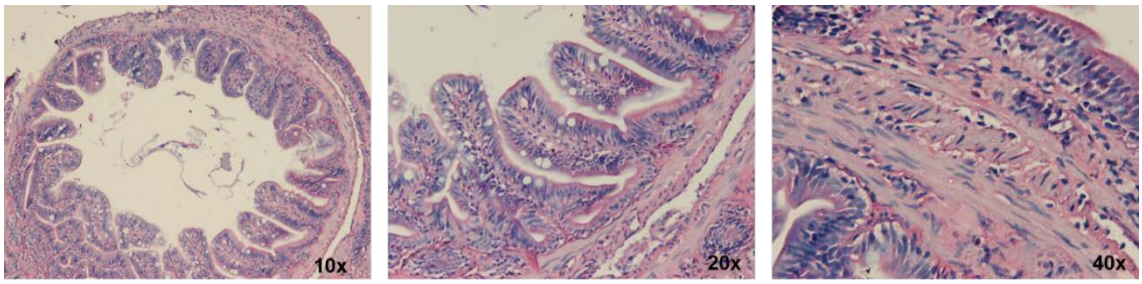


Figure 9. Hematoxylin/Eosin staining of zebrafish midgut intestinal wall showing typical structure of presence of large epithelial folds, basal lamina and underlying longitudinal and circular muscle layer.

Given the high anatomical and functional similarities of zebrafish intestine, together with the comparable innate and adaptive immune system (Fénero 2016), the zebrafish represents a powerful model organism to investigate the fundamental processes underlying intestinal inflammation present in IBD pathology, and to evaluate new therapeutics. Many aspects of the host response to microbial colonization, including intestinal alkaline phosphatase expression and NF-κB activation, have previously been investigated in this model organism (Bates et al. 2007; Kanther et al. 2011; Kanther and Rawls 2010). Furthermore the antimicrobial roles of IBD susceptibility genes encoding NOD are causing intestinal inflammation in zebrafish (Allen 2011). As discussed in the preceding chapters of this thesis, standard IBD therapies consist of anti-inflammatory and immunosuppressive drugs or biologic agents, which can interfere with pro-inflammatory cytokines. Due to severe side effects and unsatisfying clinical efficacy in some patients, there is a huge interest for alternative treatment options. In this context, interest in phytotherapy has grown and some new plant-derived products have showed promising effects (Hur et al. 2012; M. J. Schneider, Abdel-Aziz, and Efferth 2014; Ke, Yadav, and Ju 2012). Herein, we investigate the protective effect and underlying mechanisms of action of one of these compounds, STW5 (fixed combination of nine herbal extracts; Iberogast®), on the epithelial and ENS compartment from inflamed intestinal cells. STW5, used clinically in functional dyspepsia and irritable bowel

syndrome (IBS) (Madisch et al. 2004; von Arnim et al. 2007; Ottillinger et al. 2013) , has been shown to possess properties that indicate a possible supplemental therapy option in IBD disease management (Wadie et al. 2012). STW5 is a fixed combination of nine herbal extracts from *Iberis amara totalis*, peppermint leaves (*Menthae piperitae folium),* chamomile flower (*Matricariae flos*), liquorice root (*Liquiritiae radix*), angelica root (*Angelicae radix*), caraway fruit (*Carvi fructus*), milk thistle fruit (*Silybi mariani fructus*), lemon balm leaves (*Melissae folium*) and greater celandine herb (*Chelidonii herba*), all of which have anti-inflammatory, anti-ulcerogenic and protective effects (Michael et al. 2009; Khayyal et al. 2006; Awaad, El-Meligy, and Soliman 2013).

3.2.1 STW5 downregulates intestinal mucous production after DSS-induced inflammation

In the present study, the proposed protective effects of STW5 on intestinal inflammation were tested in the zebrafish model. Inflammation was induced chemically, gut sections isolated (as previously described), and analysis was conducted to clarify molecular mechanisms of action. Besides several IBD-like genetically modified zebrafish models that are used to study IBD genetics (Allen 2011), a number of chemical-based models using trinitrobenzenesulfonic acid (TNBS) or dextran sodium sulfate (DSS) to induce enterocolitis have been adapted from the murine system into both larval and adult fish (Oehlers et al. 2013; Brugman 2016). DSS is a detergent that induces inflammatory processes when applied to the zebrafish environment. These inflammatory processes resemble morphological, histopathological and symptomatic features of human IBD as described previously. In zebrafish larvae, DSS induces barrier defects and subsequent recruitment of leukocytes with elevated pro-inflammatory gene expression (Oehlers et al. 2013)

Furthermore, during inflammatory conditions an altered number of goblet cells and increased mucus secretion has been reported (Y. S. Kim and Ho 2010). Oehlers and colleagues have also hypothesized that mucin overproduction in DSS-induced enterocolitis is induced due to erosive effects, and the consequential penetration of bacterial products through defective epithelial barrier (Oehlers et al. 2013). Based on these reports, we used DSS as an inflammatory inducer in the adult zebrafish and confirmed the mucosecretory phenotype response also in adult zebrafish (Figure 10). As shown in Figure 10, DSS treatment significantly upregulated mucous production while STW5 treatment reversed this response, thus reducing the quantity of secreted mucus to the level observed in untreated samples.

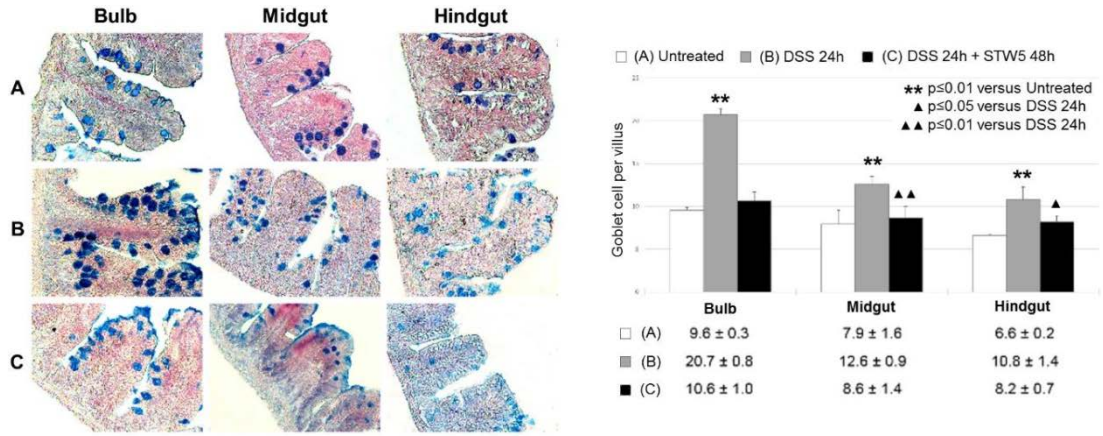


Figure 10. Alcian blue staining demonstrated that treatment of adult zebrafish with (C) DSS leads to highly upregulated mucus production in bulb, midgut, and hindgut. (B) STW5 was significantly restoring the physiological condition observed in untreated samples (A). Quantification of goblet cells was performed considering 5 different sections from three independent experiments. Data are presented as mean of goblet cells per villus ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$); versus DSS-treated samples (▲ $p \leq 0.05$, ▲▲$p \leq 0.01$).

3.2.2 Mechanism of STW5 action: Activation of anti-inflammatory WNT signaling

Several studies have demonstrated that STW5 also targets the enteric nervous system and has functional effects both motility and inflammation (K. Schäfer et al. 2015). To unravel the molecular mechanism underlying the actions of STW5, special interest was given towards the zebrafish ENS during the inflammatory processes. Since zebrafish do not have a submucosa, enteric neuronal/glial cells can only be found between the circular and longitudinal smooth muscle layer that is directly attached to the mucosa. Structurally they are organized in single neurons rather than clustered within ganglia as seen in mammals (Wallace et al. 2005).

To date there is not much knowledge about the adult zebrafish ENS, even though it has been used extensively for ENS developmental research (Shepherd and Eisen 2011a; Burzynski, Shepherd, and Enomoto 2009; T. A. Heanue, Shepherd, and Burns 2016). Like in human ENS, enteric neurons and glial cells derive from neural crest stem cells that colonize the developing gut and one of the key developmental factors are Wnt signals (Verkade and Heath 2008). Besides being the main drivers in ISC self-renewal, Wnt signalling is involved in inflammatory processes and shifts in the expression of several Wnt ligands and frizzled receptors have even been described in IBD patients (You et al. 2008). In conjunction with the experiments described in this thesis, we have recently demonstrated the anti-inflammatory activity of WNT signalling within the rat ENS, where Frizzled 9 was identified as a new receptor being expressed on ENS cells and canonical Wnt pathway was activated through Wnt3a counteracting bacterial LPS induced inflammation (Di Liddo et al. 2015). Preliminary morphological, wholemount and immunofluorescence (IF) analyses were performed in adult Zebrafish in order to explore ENS organization and expression of Frizzled (FZD) 9 and ligand Wnt3a, two

components of the Wnt canonical pathway that have been reported to exert a pivotal role in balancing the gut inflammatory response.

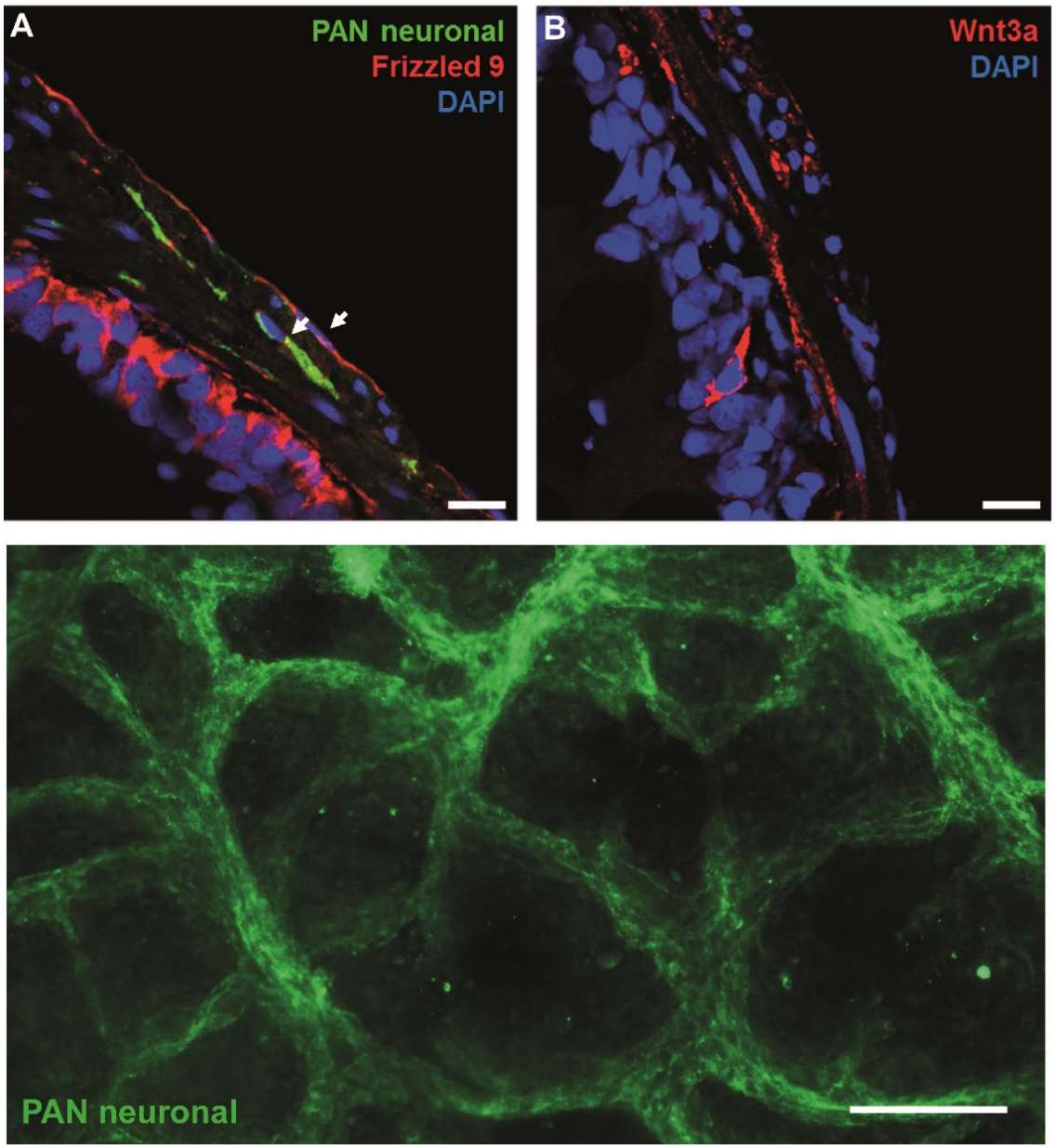


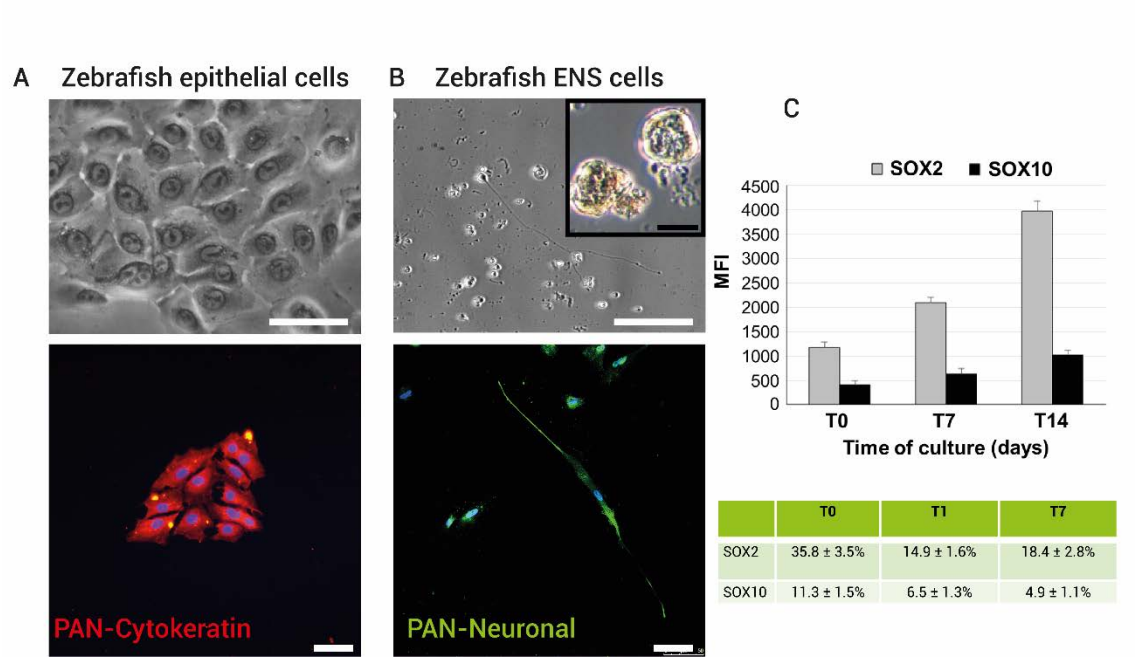
Figure 11. Immunofluorescent staining shows expression of (A) Frizzled-9 to be co-localized with neuronal cells (PAN neuronal marker) and in the basal epithelial layer. (B) Wnt3a expression was found within the muscle layer and isolated epithelial cells. (C) Wholemount staining of adult zebrafish ENS

Herein, enteric neuronal cells of adult zebrafish were visualized within the zebrafish smooth muscle layer by immunofluorescent staining using a Pan Neuronal Marker

(Figure 11). The expression of the Wnt signaling receptor, Frizzled 9, was located in the basal epithelial layer and within ENS ganglia, where it co-localized with neuronal cells (Figure 11). Moreover, Wnt3a expression was distributed in the muscle-ENS layer and in isolated epithelial cells localized in the basal layer (Figure 11). Wholemount staining of adult zebrafish intestinal tissue revealed ENS organization similar to that found in mammals.

3.2.3 Zebrafish epithelial and ENS cell characterization

The developed protocol allows the parallel isolation of cells of both epithelial and ENS compartments. Intestinal epithelial cells were adherent to collagen-coated glass coverslips, forming typical colonies composed of cells with epithelial morphology (Figure 12A). Immunofluorescent staining confirmed the expression of epithelial cytokeratin, as shown in the figure below (Figure 12A). Zebrafish ENS cells formed typical neurospheres within 7 days in culture similar to those of mammalian ENS (Figure 12B). By FCM, Sox2, a known marker of neuronal progenitors, and Sox10, a marker of neural crest cells (T. a Heanue and Pachnis 2006; V. Graham et al. 2003; Laranjeira et al. 2011) were detected in freshly isolated samples (Figure 12C). After culturing for 1 (T1) and 7 days (T7), the expression of both markers showed to be maintained on a reduced number of ENS cells, however SOX2 protein content was determined to be increased, as demonstrated by the higher intensity of geometric mean fluorescence (MIF) (Figure 12C). On the other hand, Sox10 MIF remained relatively stable, exhibiting a very slight increase over the time course. Furthermore, ENSc adhered to the laminin coating and partly differentiated into cells with a neuro/glial morphology (Figure 12B). The immunofluorescent detection of Pan Neuronal Marker confirmed that in vitro culture conditions were appropriate for neuronal survival and differentiation.



	T0	T1	T7
SOX2	35.8 ± 3.5%	14.9 ± 1.6%	18.4 ± 2.8%
SOX10	11.3 ± 1.5%	6.5 ± 1.3%	4.9 ± 1.1%

Figure 12. Primary (A) epithelial and (B) ENS cells isolated from the zebrafish gut characterized by Pan-cytokeratin and Pan neuronal marker respectively. Nuclear counterstaining was performed using DAPI (C) FCM analysis of Sox2 and Sox10 in ENS cells at time of isolation (T0) and after culturing for 1 (T1) and 7 (T7) days. Data were expressed as geometric mean fluorescence intensity (MFI)± SD and percentage (%) of positive cells ± standard deviation (SD). Bar:50 µm.

3.2.4 STW5 activates anti-inflammatory response in primary adult epithelial and ENS cells

Both LPS- and DSS-treatment for 6 and 24h induced the inflammatory response of isolated primary cells of both compartments as demonstrated by the significant upregulated gene expression of pro-inflammatory cytokines *il1b, il6, il8, il10* (Figures 13-14).

In epithelial cells, STW5 showed a protective effect as demonstrated by a reduced expression of pro-inflammatory cytokines and an increased transcription of anti-inflammatory genes *wnt3a* (6h) and *il10* (24h). Interestingly, under LPS-induced in vitro inflammation, an early and significant increase of *il10* and *wnt3a* gene expression was observed together with a reduced mRNA synthesis of pro-inflammatory cytokines,

suggesting that STW5 could exert a pivotal regulatory activity during bacterial inflammation (Figure 13).

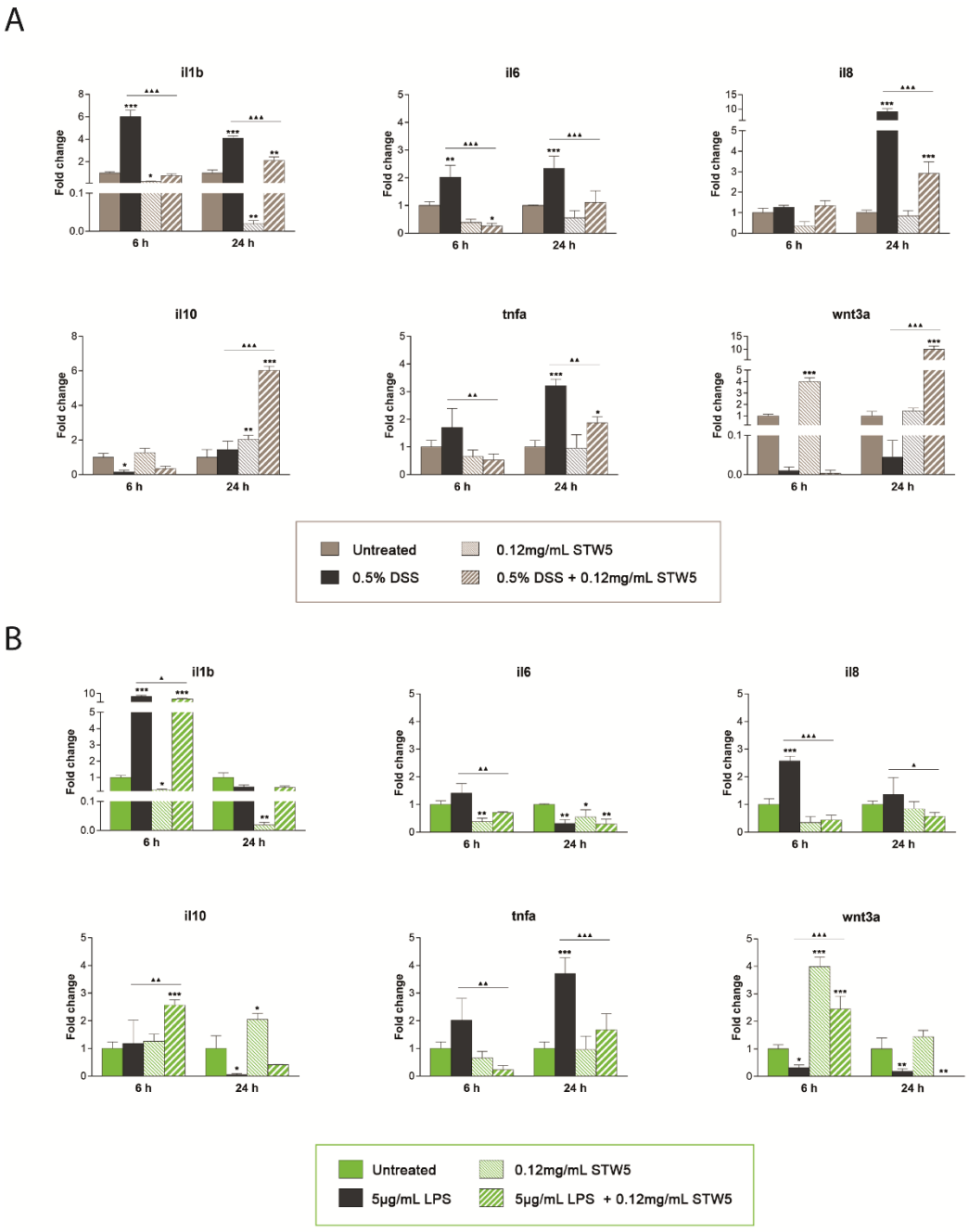


Figure 13. Quantitative RT-PCR analysis in zebrafish epithelial cells treated with STW5 for 6h and 24h after (A) DSS or (B) LPS inflammatory induction. The herbal preparation STW5 showed a protective effect as demonstrated by a reduced expression of pro-inflammatory cytokines and an increased transcription of anti-inflammatory genes in both inflammatory conditions. Data are presented as mean fold change ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$); versus DSS-treated samples (▲ $p \leq 0.05$, ▲▲ $p \leq 0.01$, ▲▲▲ $p \leq 0.001$).

As expected, in the DSS-induced inflammatory model, a significant upregulation in mRNA expression of *il10* and *wnt3a* was induced by STW5 at 24h in concomitance with a stronger inflammatory state (Figure 13).

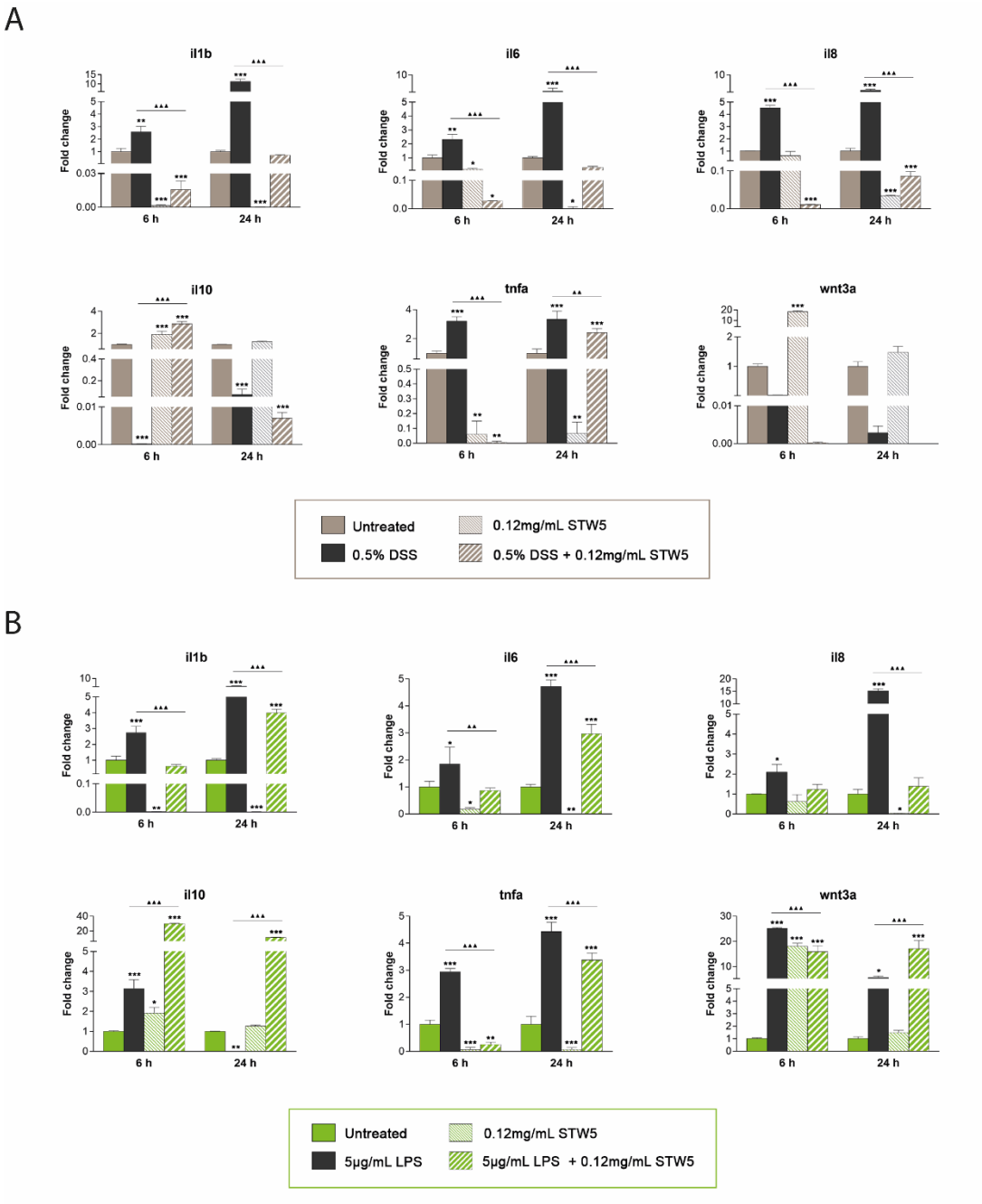


Figure 14. Quantitative RT-PCR analysis in zebrafish ENS cells treated with STW5 for 6h and 24h (A) DSS or (B) LPS inflammatory induction. STW5 demonstrated functional efficacy, inducing a downregulation of pro-inflammatory cytokines and significantly stimulating the transcription of *il10* and *wnt3a*. Furthermore, both inflammatory conditions were negatively modulated by STW5, as demonstrated by a decreased mRNA expression of pro-inflammatory cytokines. Data are presented as mean fold change ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$); versus DSS-treated samples (▲ $p \leq 0.05$, ▲▲ $p \leq 0.01$, ▲▲▲ $p \leq 0.001$).

In ENS cells, STW5 demonstrated functional efficacy, inducing a downregulation of pro-inflammatory cytokines from 6h to 24h, and significantly stimulating the transcription of *il10* and *wnt3a* (Figure 14). The inflammatory state induced by DSS was negatively modulated by STW5 since it downregulates pro-inflammatory cytokine expression (Figure 14).

3.2.5 Conclusions Study II

The adult zebrafish has proven to be an excellent model system for in vitro and in vivo experiments, and allows the investigation of the efficacy of novel and alternative therapeutics in intestinal inflammatory disorders such as IBD. Herein, we have described the development of a DSS-induced adult zebrafish inflammation model, and verified several anti-inflammatory effects of STW5. Primary cell cultures of zebrafish ENS and intestinal epithelium were developed, allowing in vitro drug screening, which is of importance for the investigation of molecular mechanisms within specialized gut compartments. In this context, the anti-inflammatory effects of STW5 within the ENS as well as the epithelial compartment were verified to be regulated through Wnt3a and Il-10. The effects of Wnt3a are hypothesized to act, at least partly, through the Wnt receptor Frizzled-9, which was demonstrated for the first time to be expressed within the adult zebrafish ENS and the basal epithelial layer.

3.3 Study III. The role of Fibrillin in intestinal inflammation

Extracellular matrix (ECM) components play an important immunoregulatory role and their composition as well as ultrastructure is involved in intestinal immune responses, pathological signaling and chronic inflammation (Shimshoni et al. 2015). Fibrillin microfibrils are a key component of elastic fibers and are responsible for many of the structural and biomechanical properties of the intestine. For example, fibrillin microfibrils are essential for peristaltic movement and their structural perturbations could underlie several intestinal pathologies. Furthermore, the fibrillin network controls the bioavailability and activity of growth factors of the TGF-β superfamily, which are essential for the activation of cellular signaling pathways. Within the intestinal mucosa TGF-β plays an essential role in the maintenance of immune homeostasis by preventing abnormal inflammatory responses (A. B. Kulkarni 1993; Gorelik and Flavell 2002). Additionally, TGF-β is one of the main pro-fibrotic cytokines driving myofibroblast activation, proliferation, and ECM synthesis. Consequently, disturbances in the fibrillin network structure and stability may result in impaired biomechanical properties, cell-matrix interactions, pathological immune responses, and TGF-β induced fibrosis (Lemaire, Bayle, and Lafyatis 2006). In this context, there is evidence suggesting fibrillin involvement in inflammatory and fibrotic processes, as they are present in chronic intestinal inflammatory disorders (Rieder and Fiocchi 2009b) .

A widely used mouse model to study consequences of fibrillin network perturbations is the tight skin mouse (Tsk), which possesses a tandem in-frame duplication of the fibrillin-1 gene (Fbn1) (Siracusa et al. 1996). In heterozygous Tsk mice, mutant fibrillin-1 copolymerizes with wild-type fibrillin-1, thus giving rise to altered morphology and unstable structure (Lemaire, Bayle, and Lafyatis 2006; Wallis et al.

2001; Filemon K. Tan et al. 1998; F K Tan et al. 2001; X. Zhou et al. 2003). This ultimately renders the mutant microfibrils more sensitive to proteolysis (Gayraud et al. 2000). This instability leads to a disorganization and fragmentation of elastic fibers due to structural abnormal microfibrils (Saito et al. 1999; Gardi et al. 1989) as well as an alteration in TGF-β signaling (Kissin et al. 2002). Phenotypically these mice manifest pathological abnormalities similar to those present in patients suffering systemic sclerosis (SSc), a connective tissue disorder that has been linked to fibrillin network disruption with excessive fibrosis, inflammation and vascular dysfunction (Pablos, Everett, and Norris 2004). Interestingly gastrointestinal manifestations appear in up to 90% of patients suffering SSc (Sallam, McNearney, and Chen 2006), indicating an important role of fibrillin within the intestine. Another mechanism supporting the involvement of fibrillin in intestinal inflammation is its regulation of MMPs, which has previously been described to be induced by fibrillin and elastin fragments due to unstable networks (Patrick Booms et al. 2005; P Booms et al. 2006). MMPs are known to be upregulated in IBD and other inflammatory conditions and disturbed ECM turnover is involved in pathological disease progression (De Bruyn et al. 2016). Reinforcing an involvement of the fibrillin network in intestinal homeostasis, a recent study confirmed a downregulation of fibrillin in the lamina propria of human IBD patients compared to control tissue (Ratzinger et al. 2010a), highlighting the involvement of fibrillin network disturbance in gut inflammation.

3.3.1 Fibrillin network perturbations change TSK mouse gut macroscopically and histologically

In order to better characterize the consequences of fibrillin-network perturbations, the Tsk mouse model was used to characterize consequences of these ECM abnormalities

within the gastrointestinal tract. Macroscopically, the GIT from stomach to the distal colon is significantly reduced in length compared to WT littermates (Figure 1A). Histological analyses also verified an increase in the size of the intestinal muscle layer of TSK/+ mice, confirming the initiation of fibrotic mechanism within the colon (Figure 1B) as it has been described for other organs within the TSK/+ mice (Manne et al. 2013)

The small intestine of Tsk mice on the other hand was characterized by a large number of immune cell infiltrates indicating underlying pro-inflammatory processes within the intestinal tissue (Figure 1B) that were prompting us to furthermore characterize these hypothesized involvement in inflammatory and fibrotic processes by measuring the expression of correlated mRNA encoding inflammatory cytokines and matrix remodeling enzymes by qRT-PCR (see 4.3.2).

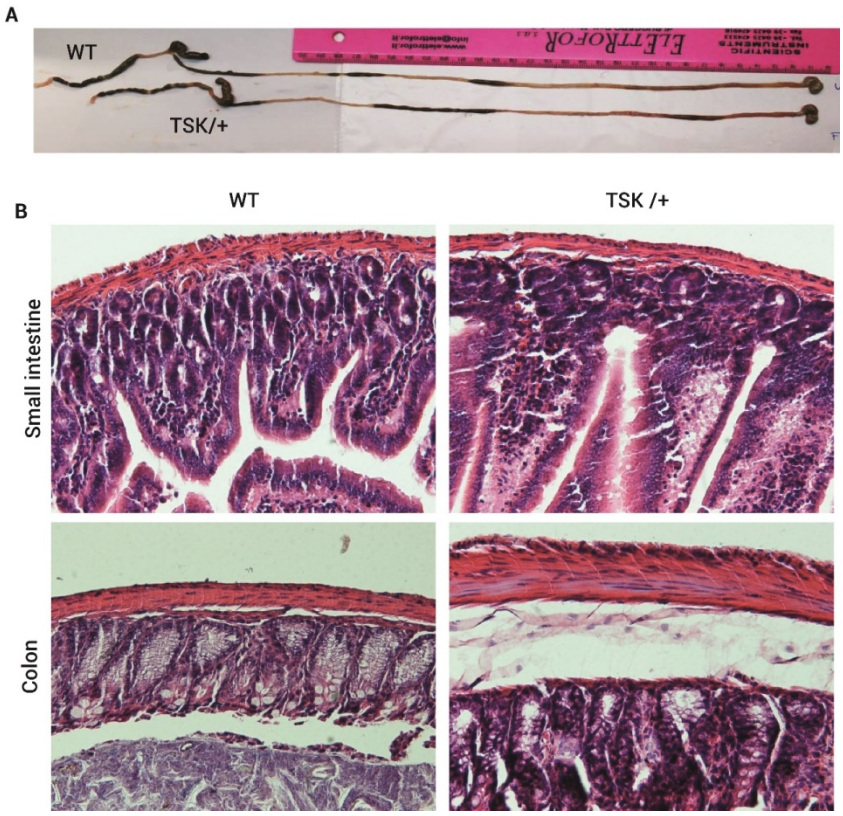


Figure 15. Macroscopical and histological changes in TSK/+ mice. (A) Macroscopically the gut of TSK/+ mice were significantly reduced in length (>10%) (B) Histological examination revealed a large number of immune cell infiltrates in the small intestinal gut wall. Within the colon gut wall an increase in muscle layer thickness was revealed. Magnification: 20x

3.3.2 Fibrillin network perturbations induce an inflammatory phenotype in several gut parts

To verify whether underlying inflammation and tissue remodeling processes are active, whole tissue lysates were analyzed for pro-inflammatory and anti-inflammatory cytokine expression and expression pro- and anti-fibrotic factors (Figure 16-17). Regional differences in gene expression profiles of target genes along the length of gastrointestinal tract were expected due to known differences in the presence, distribution, and number of immune cell subtypes in specialized gut regions ((reviewed in (Cerovic et al. 2014)). Therefore, the whole gut was divided into different parts starting from the stomach, three sub-sections of the small intestine (duodenum, jejunum and ileum), and three sub-sections of the large intestine (the caecum, proximal colon and distal colon). Consequently, mRNA expression on all sections was analyzed by qRT-PCR. Analysis revealed a strong regulation of both pro- and anti- inflammatory cytokine expression within the small intestine (Figure 16). In particular, within the jejunum and ileum, the *Il8* and *Il6* mRNA expression was strongly upregulated in Tsk/+ mice compared to WT mice (Figure 16). IL-8 is generally considered as a strong chemoattractant for neutrophils, found in increased quantities in the inflamed mucosa of human IBD patients (Daig et al. 1996). In an acute inflammation accumulated neutrophils then promote an upregulated release of pro-inflammatory cytokines such as IL-6, which also has been reported to be increased in IBD patients (Saha et al. 2010), thus implicating it as a potential player in the progression of IBD. IL-6 acts via its soluble IL-6 receptor (sIL-6R) together with the membrane bound receptor gp130 in a process defined as trans-signaling, which initiates a signaling cascade that inhibits T cell apoptosis through the upregulation of anti-apoptotic factors such as Bcl-2 (B-cell lymphoma 2) (Mitsuyama, Sata, and Rosejohn 2006; Atreya et al. 2000).

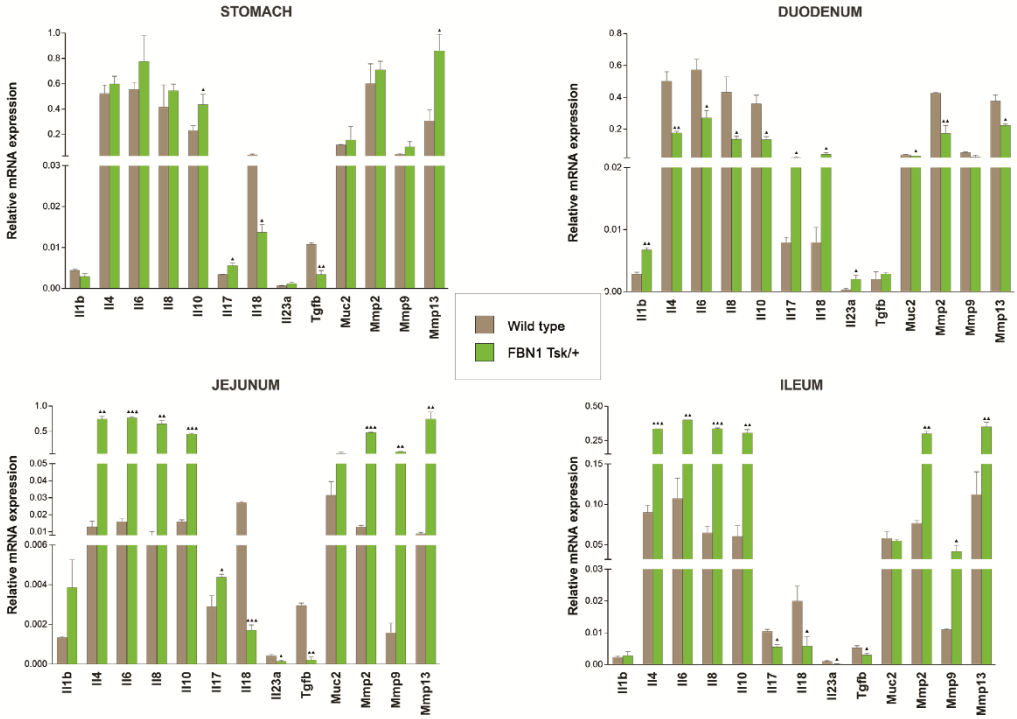


Figure 16. Quantitative RT-PCR analysis in the stomach and the three segments of small intestine (duodenum, jejunum, ileum). To verify whether fibrillin network perturbations induce an inflammatory phenotype in the GIT, whole tissue lysates were analyzed for target inflammatory and fibrotic mediators. A strong regulation of pro- and anti- inflammatory cytokine, as well as pro- and anti-fibrotic factors mRNA expression was detected within the small intestine of the Tsk/+ mice. Data are presented as mean ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing FBN1 Tsk/+ to WT samples (▲ $p \leq 0.05$, ▲▲ $p \leq 0.01$, ▲▲▲ $p \leq 0.001$).

Unexpectedly, in parallel with the upregulation of these pro-inflammatory factors, mRNA expression of the anti-inflammatory cytokines *Il4* and *Il10* was also found to be upregulated in both Ileum and Jejunum (Figure 16).

This data is in concordance with literature which reports the suppression of anti-inflammatory cytokine by IL-6 (Bettelli et al. 2006). Another pro-inflammatory cytokine, IL-17, is produced by T_H17 T-helper cells and has shown to be upregulated in acute inflammation. Its expression has been reported to be induced by the stimulation of IL-23 (Kobayashi et al. 2008), which is accordance with data reported in the present study, where mRNA expression of both *Il17* and *Il23* is upregulated within the duodenum of Tsk/+ mice (Figure 2). However, within the other two parts of the small intestine (Ileum and Jejunum) the *Il23* mRNA expression is downregulated, concurrent

with significantly higher levels of Il17 mRNA (Figure 16). These results could be explained by the fact that TH17 differentiation is not only dependent on IL-17 but also on other cytokines such as IL-6 (Bettelli et al. 2006), which has been found to be downregulated in both the ileum and jejunum (Figure 16).

Interestingly, *Il18* mRNA production is upregulated in the duodenum. Again, with apparent strong contrast to remaining small intestinal parts, jejunum and ileum, where the transcriptional levels of IL-18 are significantly downregulated compared to control. Among the cytokines implicated in IBD pathogenesis, a special role has been assigned to the cytokine IL-18 that is produced by intestinal epithelial cells and macrophages. IL-18 is mainly involved in T_H1 cell differentiation and IFN-γ production and has been especially correlated with the pathogenesis of CD (Pizarro et al. 1999; Kanai et al. 2001). High *Il18* mRNA levels were seen in duodenum (Figure 16) as well as caecum and colon (Figure 17). Using a DSS-induced mouse model of intestinal inflammation, Sivakumar and colleagues showed that colitis is diminished when IL-18 is blocked (Sivakumar et al. 2002), which is why anti-IL-18 antibodies are currently under investigation as possible therapeutics for IBD patients .

In the context of tissue damage, the expression of the gelatinases *MMP2*, *MMP9* and the collagenase *MMP13* was investigated. As mentioned earlier, MMPs are enzymes specifically implicated in dynamically shaping the ECM to retain tissues under homeostatic conditions. However, when their balance is dysregulated, MMPs are involved in chronic inflammatory disease and alterations in their expression levels have been reported to be strongly altered (Lakatos et al. 2012). Strong upregulation of all investigated MMPs has been observed within ileum and jejunum (Figure 16) as well as distal colon (Figure 17) indicating possible tissue altering processes confirming the observations of the histological study (Figure 16). The exact opposite has been observed in the duodenum where MMPs were significantly downregulated. The effect of fibrillin

network dysregulation in the TSK/+ mouse seems to only minimally affect the stomach region as the expression of most cytokines and MMPs are not significantly regulated.

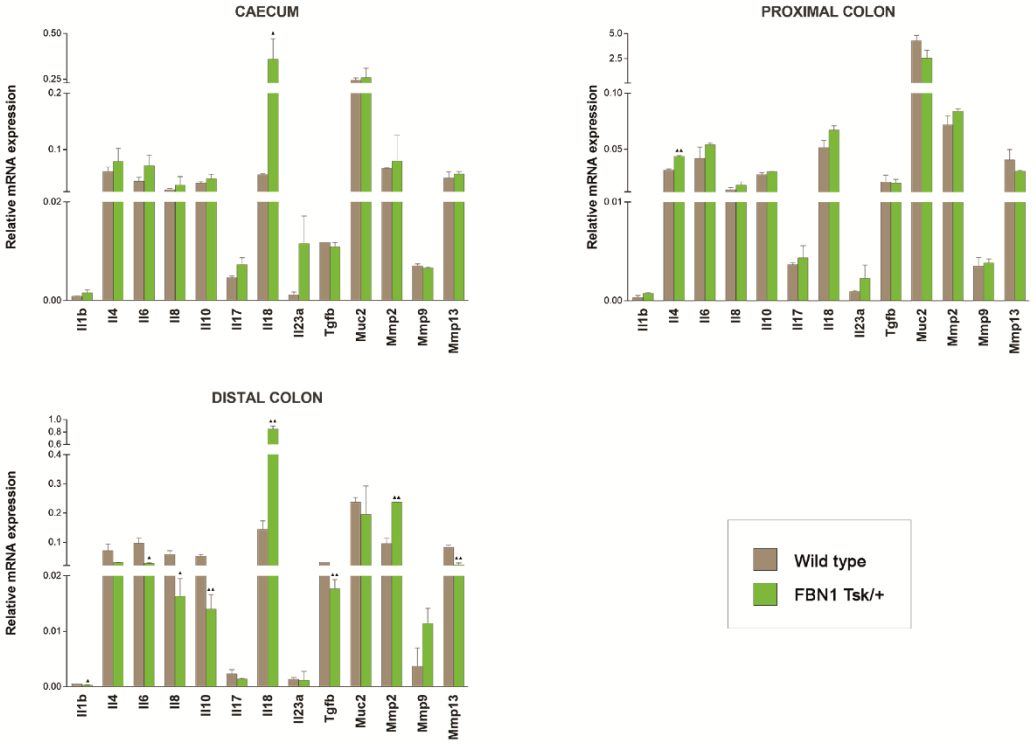


Figure 17. Quantitative RT-PCR analysis in the large intestine segments (caecum, proximal colon, distal colon). To verify whether fibrillin network perturbations induce an inflammatory phenotype in the GIT, whole tissue lysates were analysed for target inflammatory and fibrotic mediators. Structural dysregulation of fibrillin-1 seem to slightly affect the caecum and proximal colon segments. On the contrary, significant changes in mRNA expression of both inflammatory and fibrotic mediators were observed in the distal colon of the Tsk/+ mice. Data are presented as mean ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing FBN1 Tsk/+ to WT samples (▲ p ≤0.05, ▲▲p ≤0.01, ▲▲▲ p ≤0.001).

3.3.3 Conclusion Study III

Data obtained from TSK/+, fibrillin mutant mice confirmed that fibrillin-1 networks are important for maintaining intestinal homeostasis and that structurally altered fibrillin-networks are involved in inflammatory processes. Besides macroscopic changes with a reduction in intestinal length, immune infiltrates and tissue alterations, the cytokine expression was found to be extremely dysregulated. Structural changes and increased muscle layer thickness within the colon wall indicate the initiation of fibrotic processes

3.4 Part IV: Establishment of long-term and short-term cultures of IBD Patient derived mucosal biopsies

Even though huge progress in understanding the complex pathological processes has been made in the last few decades, current therapies are far from satisfactory, and show extreme variability of outcomes for different patients. Studies using genetically manipulated mouse models and cell lines, such as the CACO-2 adenocarcinoma line, have yielded significant leaps in understanding of intestinal function. This included clarifying of the role of the individual epithelial cell types, barrier function and other pathological mechanisms involved in intestinal inflammation (Sambuy et al. 2005; Ingels et al. 2004). However, these model systems do not represent the heterogeneity of IBD patients in terms of their different genetic background and immune functions. Therefore, new culture systems are needed, to improve quality and relevance of data, and to potentially serve as a platform for personalized medicine in the future.

3.4.1 Evaluation of a short-term biopsy explant culture system

The short-term explant culture model is intended to function as an in-vitro model for the evaluation of patient-specific responses to novel pharmaceuticals. For future evaluations of possible therapeutic treatments, it was necessary to develop a culture system that allowed the maintenance of the biopsy intact and viable during time of in-vitro culture. To investigate the feasibility of this system, patient-derived colonic biopsies were maintained in-vitro for 24h in static conditions either submerged in culture medium or in a liquid-air interface (as described in 3.4.1). Histological evaluation was performed before and after culturing and revealed a significant collapse and structural changes when the biopsy was maintained submerged in culture medium for 24h (Figure 18)In contrast, when the biopsies were cultured in an air-liquid-interface, structural changes

were minor. Therefore, we could conclude that the liquid-air condition could be a valuable model for short term culture (up to 24h) of human colon biopsies, as there were no significant structural changes. All further experiments described in this chapter thus use the liquid-air method of biopsy culture.

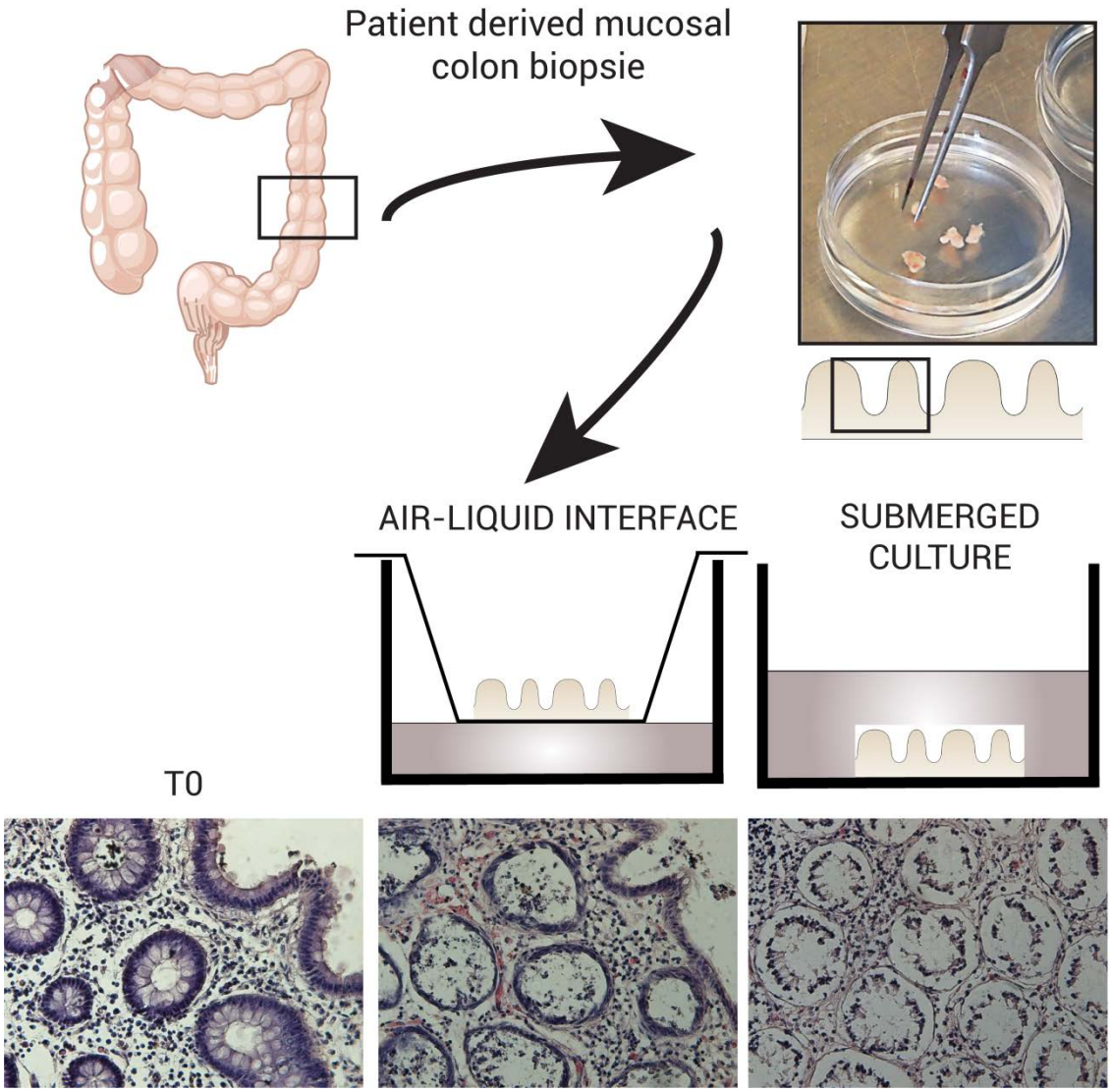


Figure 18. Evaluations of a short-term biopsy explant culture system. Patient-derived mucosal biopsies were maintained in a liquid-air interface or submerged in culture medium. Biopsies under both conditions were evaluated by histological analysis and revealed significant loss of structural features when the biopsy was maintained in the 'submerged' condition, while only minor changes were observed in the samples cultured at 'air-liquid interface'

3.4.2 Short-term culture for in-vitro screening of patient-specific therapeutic responses

Antibodies targeting TNF-alpha have been used successfully in IBD treatment for many years, highlighting the importance and clinical relevance of TNF-alpha function in intestinal inflammation. However, the treatment can cause adverse reactions and there is a large percentage of initial or secondary non-responders (which do not exhibit any effect of the treatment), thus there is a significant need for the development of new patient-specific therapies.

In a proof of principle study designed to evaluate the value of the previously described model for future pharmacological studies, biopsies were divided in three groups, all of which were treated with anti-TNF-α (Infliximab) for 24h in the short-term, air-liquid explant culture. The first group was derived from *Responders* (patients are currently and successfully in therapy with anti-TNF-α), the second one from *Non-Responders* (which have been treated with Infliximab in the past, but who have failed to improve their condition, and thus are considered a therapy failure), and the third one from healthy donors. The expression profile analyzed in this study included genes encoding pro-and anti-inflammatory cytokines as well as TNF-receptors, the tissue remodeling enzyme MMP2 and mucus forming MUC2. Data obtained from these groups are shown in the figure below. As expected, gene expression studies revealed that biopsies obtained from the Responder group strongly reacted upon treatment with Infliximab by effecting a downregulation of TNFA as well as IL1B expression (Figure 19). Interestingly, in the non-responder group the expression levels of the pro-inflammatory cytokines were significantly upregulated, thus confirming the status of these patients as Non-Responders (Figure 19).

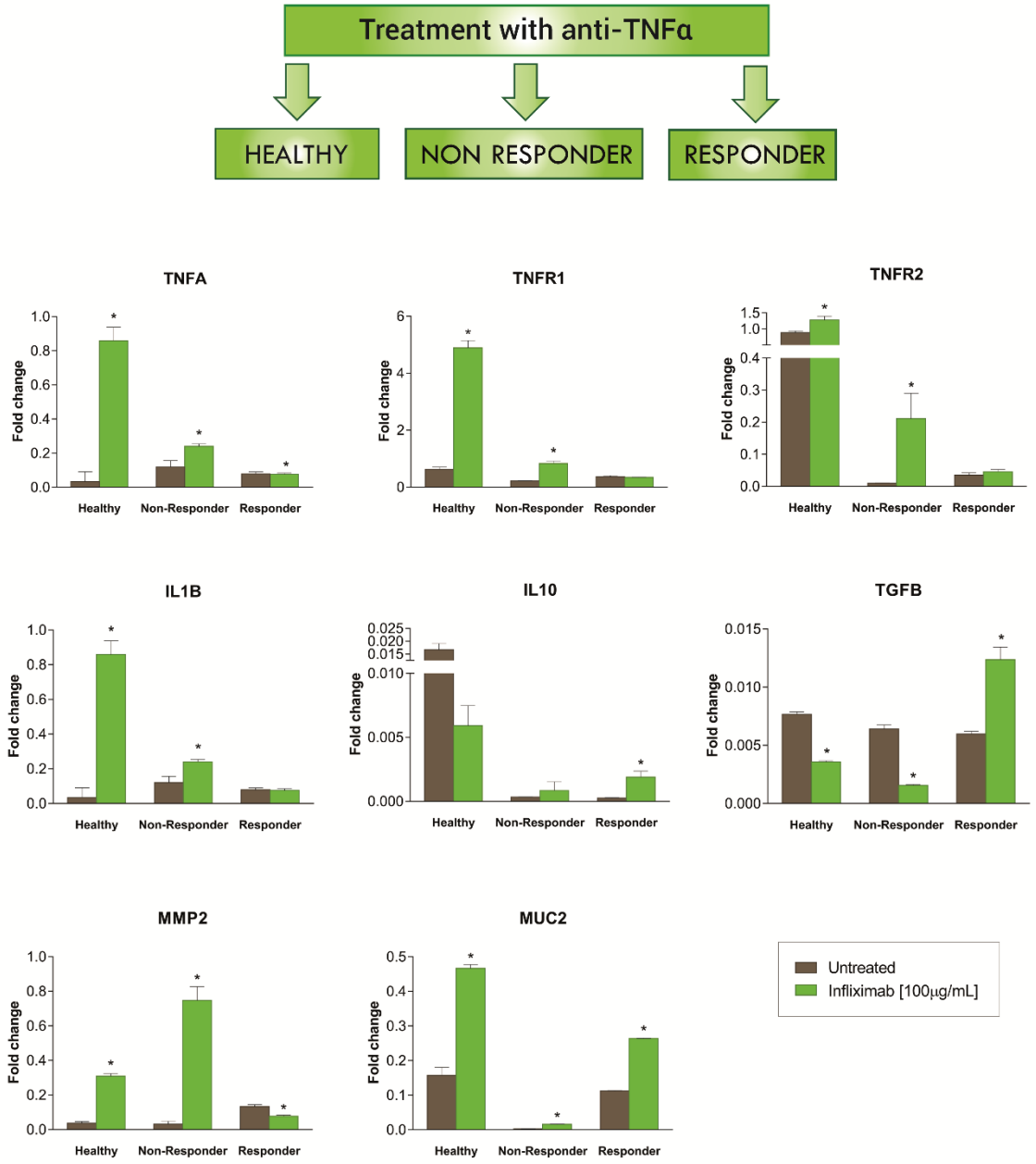


Figure 19. Proof of principle study. Biopsies obtained from three groups of patients (healthy donors, responders and non-responders to anti-TNF-alpha therapy) were treated with Infliximab for 24h in the short-term, air-liquid explant culture, and subsequently analysed by quantitative RT-PCR. Untreated biopsies were used as control. As expected, a downregulation of *TNFA* and *IL1B*, as well as an upregulation of *IL10* and *TGFB* mRNA expression were detected in the responder group. Interestingly, increase in mRNA levels of pro-inflammatory cytokines, besides the absence of significant changes in anti-inflammatory mediators, were observed in the non-responder group, thus confirming the non-responsiveness of these patients to anti-TNF-alpha treatment. Data are shown as mean ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing samples treated with Infliximab to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

TNF-alpha exerts its effects through two receptors: TNFR1 and TNFR2. While TNFR1 is ubiquitously expressed in nearly all cells of the body and is activated by soluble TNF, TNFR2 is mostly restricted to endothelial, hematopoietic, glial, and some neuronal cells, is selectively activated in response to transmembrane TNF, and promotes cell survival and proliferation, including expansion of certain populations of lymphocytes, including T-regulatory cells (Tartaglia et al. 1993). TNF binding to TNFR1 triggers apoptosis through TNFR1-associated death domain (TRADD) and Fas-associated death domain (FADD) (Hsu, Xiong, and Goeddel 1995; Chinnaiyan et al. 1995). In contrast, TNFR2 signaling relies on TRAF2 and therefore involves the nuclear pro-survival factor NFkB (Carpentier, Coornaert, and Beyaert 2004). Since TNFR1 is typically associated with cell death signaling, while TNFR2 typically promotes cell survival, the ratio of their co-expression in a given tissue or organ modulates the balance between apoptosis and survival. IL-10 is one of the main anti-inflammatory cytokines expressed by different cells of the innate and adaptive immune system and its particular importance within the gastrointestinal immune system has been demonstrated by an IL-10 knockout mouse model, which is also a well-established genetic model of IBD (Kühn et al. 1993). In the present study, both the Responder and Non-Responder groups show a significantly lower IL10 expression compared to the expression levels measured in the healthy control biopsies (Figure 19). The in-vitro explant culture model revealed significantly upregulated IL10 expression after anti-TNF treatment in the Responder group, indicating effective treatment. The Non-Responder group on the other hand did not show significant upregulation of IL10 expression (Figure 19).

In these samples MUC2 expression was also measured. This gene encodes a secretory, gel-forming component of the intestinal mucin barrier, which separates the intestinal microbiota from the intestinal epithelium. In particular, patients affected by UC have been shown to have a decrease in MUC2 gene and protein expression (Moehle et al.

2006) which can be associated with general goblet cell loss in active disease (Dorofeyev et al. 2013). A positive regulation of MUC2 expression induced by Infliximab was seen in all samples.

The pleiotropic cytokine TGF-β does not only have immunoprotective functions, as discussed earlier, but is also one of the main drivers of tissue regeneration and fibrosis. TGFB mRNA levels were upregulated in the Responder group, while the Non-Responder group showed a significant downregulation (Figure 19). Due to its importance in tissue regeneration and its involvement in disease-associated complications, the gene expression levels of the matrix degradation enzyme MMP2 were measured and showed a significant up-regulation in the Non-responder group compared to the Responder group.

Taken together, the biopsy explant culture models have a major advantage in that they offer the full spectrum of cellular diversity of a given patient. This is extremely useful as it could allow medical professionals to gain relevant data on the possible outcomes of patient-specific treatment. However, the disadvantage of this system is the limited viability of mucosal biopsies outside the body. Improvement of culture conditions, such as the establishment of new co-culture systems, 3D culture scaffolds, or dynamic rather than static culture conditions might overcome this problem in the future. Similarly, human epithelial culture must be enhanced so that biopsies from individuals can be used to rapidly expand patient derived cell lines, thus allowing functional assays to be completed in a relatively short time.

3.4.3 Patient-derived long-term cultures epithelial niche cultures

In order to establish appropriate primary in-vitro cultures, it was necessary to better characterize the intestinal stem cell niche and define appropriate ISC markers. Joseph

Paneth was the first to propose that cell of the crypt villus units may share the same original cell (Paneth 1887). In 1948, Leblond and Stevens published a study on the rate and mechanism of self-renewal of the epithelium, concluding "…*the cells formed in the crypts of Lieberkühn move upward along the side of the villi to be ejected when they reach the villus tips*" (Leblond and Stevens 1948). Over decades, researchers have successfully deciphered many key aspects of intestinal stem cell biology and their native stem cell niche (Blanpain and Simons 2013). More recently it has become evident that the Wnt signaling pathway is key regulator for maintaining self-renewal and proliferation of ISCs (L. G. Van der Flier et al. 2007). Wnt signals are strongly present along the base of the crypt and fade towards the villus region (Vries, Huch, and Clevers 2010) indicating that ISCs reside within a local Wnt niche, with agonists of the canonical Wnt signaling being present only in the in the area restricted to the crypts (Gregorieff et al. 2005), which manifests in high levels of intranuclear β-catenin. The crypt epithelial cells also express frizzled 5, 6 and 7, and co-receptors LRP5 and 6, and show activation of the canonical pathway. In this context, development of clonal fate-mapping technologies has led to the discovery of robust adult ISC markers. The first marker for crypt based ISCs, Lgr5 (Leu-rich repeat-containing G protein-coupled receptor 5), was identified as being expressed at the base of mouse and human adult intestinal crypts in the small intestine and colon (Barker et al. 2007). Lgr5 together with Lgr4 and Lgr6, constitute the receptors for R-spondins, which are enhancers of Wnt signaling. The Lgr5/R-spondin complex acts by neutralizing Rnf43 and Znrf3, two transmembrane E3 ligases, which remove Wnt receptors from the stem cell membrane. Rnf43/Znrf3 are Wnt target genes and constitute a negative Wnt feedback loop. Thus, adult ISCs are controlled by a complex interplay of Wnt agonists, antagonists, and anti-antagonists.

3.4.4 Long-term culture of primary, patient derived epithelial niche cells

Within the small intestine, Paneth cells supply important niche factors like Wnt3, EGF, and Notch signals to neighboring Lgr5+ stem cells (T. Sato et al. 2011). Since the colon lacks Paneth cells, recent studies identified deep crypt secretory (DCS) cells as Paneth cell equivalents in close contact with $Lgr5^{+}$ ISCs, which reside within the colon crypt and express cKit as well as regenerating islet-derived family member 4 (*Reg4*). In a manner similar to Paneth cells, they express ISC supporting factors like EGF and Notch ligands Dll1/Dll4 (Sasaki et al. 2016). In contrast to the Paneth cells from the small intestine, however, they do not express Wnt ligands. Instead, colonic ISCs are dependent on Wnt signals, express LGR5 as well as Frizzled receptors and Lrp5 and 6 (Sasaki et al. 2016). Wnt ligands therefore must have another origin. This is also true for the small intestinal crypt stem cells, as in vivo studies have shown that epithelial cell derived Wnt is dispensable and the deletion of Paneth cells does not impair homeostasis or regeneration (Durand et al. 2012; T.-H. Kim, Escudero, and Shivdasani 2012; Metcalfe et al. 2014). Wnt and R-spondin proteins must therefore be provided by extraepithelial cells. Subepithelial myofibroblasts (SEMFs), underlying intestinal crypts were proposed to be such a source (Powell et al. 2011) since they support epithelial cell growth in vitro by Wnt2b, Wnt4, and Wnt5a expression (Gregorieff et al. 2005). However, selective inhibition of Wnt ligands from both epithelium and smooth muscle/subepithelial myofibroblasts did not cause a loss of Wnt signaling within ISCs in mice (San Roman et al. 2014). Very recently, Aoki and colleagues identified a subpopulation of pericryptal FOXL1+/CD34+ (Forkhead box l1) mesenchymal cells, describing these as an essential stem cell niche cells that expresses Wnt2b, -4, -5, R-spondin -1 and -3 together with BMP inhibitors Gremlin 1/2. Foxl1+ cells are a small

subset of mesenchymal subepithelial fibroblasts (Aoki et al. 2016). Deletion of these cells reduces intestinal epithelial cell proliferation in mice, which is associated with attenuated Wnt signaling and reduced expression of stem cell markers. Furthermore, these CD34+ mesenchymal cells reacted to DSS induced inflammation by overexpressing the stem cell niche, chemokine and inflammatory cytokines involved in innate and adaptive immunity. These findings indicate a critical role of non-epithelial, non-immune pericryptal mesenchymal cells.

Taken together, the mesenchymal compartment of the intestinal lamina propria contains multiple cell populations carrying out a multitude of functions. These include fibroblasts, myofibroblasts, pericytes, endothelial cells, enteric neuronal/glial and smooth muscle cells (Powell et al. 2011). They dynamically interact with both epithelial and immune cells, and their submucosal localization indicates their critical role in gut homeostasis as well as their clear beneficial effect in physiological processes of epithelial regenerative responses to barrier damage (Powell et al. 2011; Owens and Simmons 2013). However, when inappropriately regulated, these cells have been shown to contribute to many of the pathological and inflammatory mechanisms observed in IBD, including worsening of inflammatory processes, dysregulated mucosal healing, tissue damage and intestinal fibrosis. With the protocol described herein, it is possible to isolate and culture a patient-derived heterogeneous population of epithelial and mesenchymal cells underlying the crypt.

Patient- derived biopsies were enzymatically digested and seeded on collagen IV coated culture dishes, where they rapidly attached within the first few hours of culture. Subsequently cells migrated out of tissue fragments and started to proliferate. Even though primary cells undergo enhanced cell death within the first week of culture, which can be associated with the loss of all mature terminally differentiated cells already present within the mucosal biopsy, cultures stabilize within the first two weeks.

At this point the obtained cultures are morphologically correct, and show a mixture of fibroblast-, epithelial- and neuronal-like cells (Figure 20) which can be rapidly expanded, passaged more than 20 times until today and cryopreserved.

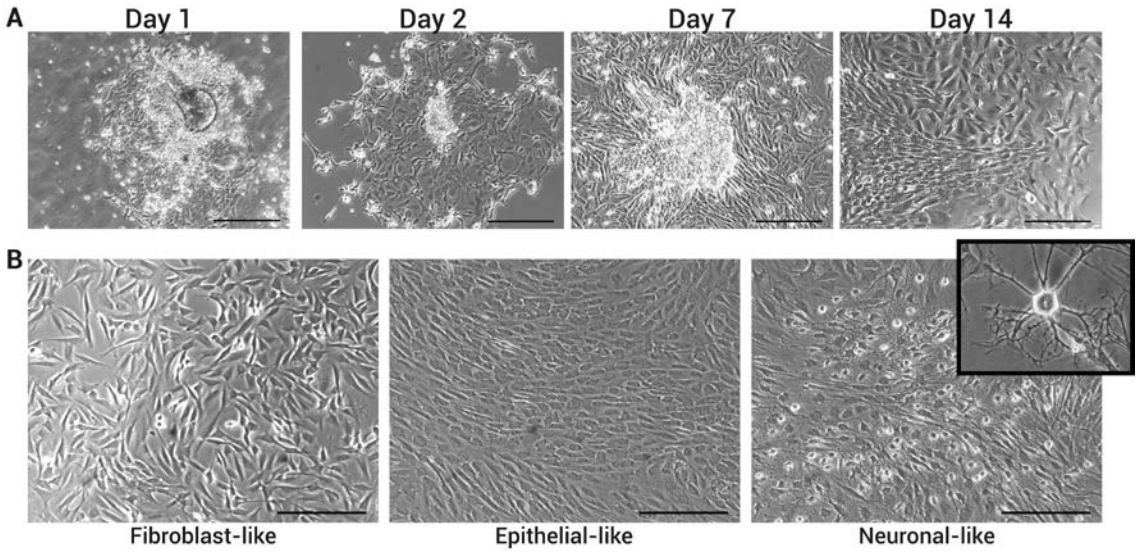


Figure 20. Primary patient-derived epithelial niche cells. (A) Patient-derived biopsies were enzymatically digested and seeded on collagen IV coated culture dishes, where they rapidly attached within the first few hours of culture. Subsequently, cells migrated out of tissue fragments and started to proliferate. Cell cultures stabilize within the first two weeks, and (B) show a mixture of fibroblast-, epithelial- and neuronal-like cells, which can be rapidly expanded, passaged, and cryopreserved. Bar 50um

These features make these patient-derived primary cells a very useful and unique in-vitro long term model to characterize possible dysfunctions in mesenchymal and stromal cell compartment in IBD pathology.

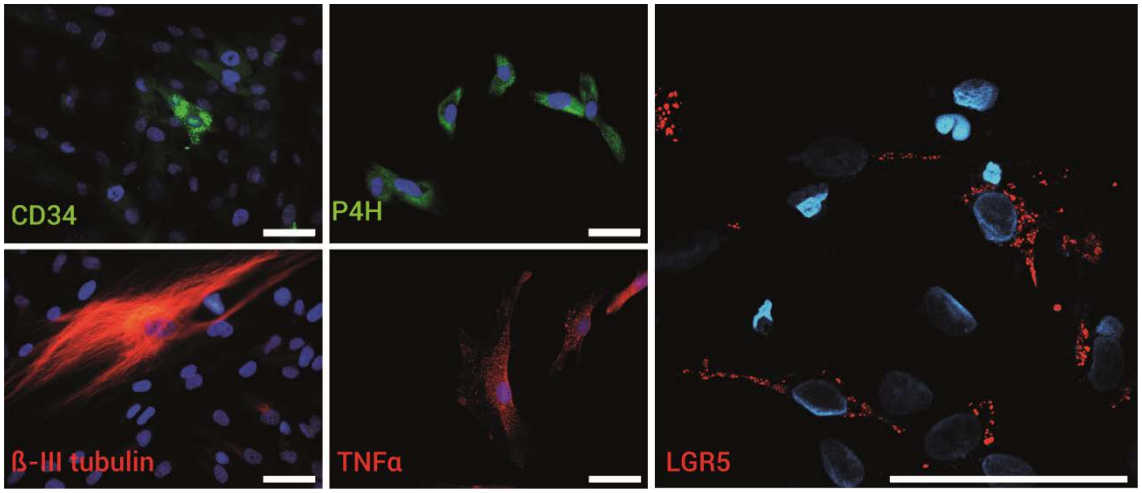


Figure 21. Immunofluorescent staining of primary intestinal epithelial niche cells isolated from human mucosal biopsies. The heterogeneous culture contains cells expressing CD34, ß-III tubulin, P4H, TNFα. A small subset of LGR5+ cells was maintained in culture. Bar 50µm.

Furthermore, this system enables the exploration of individual responses of a given patient to therapy, which would be invaluable for the personalisation of patient care in the future.

A small proportion of LGR5+ epithelial stem cells was identified by immunofluorescent staining, indicating the presence of intestinal stem cells within the culture (Figure 21). Furthermore CD34+ mesenchymal cells which recently have been identified as a major component of the intestinal stem cell niche were present and can be accounted for the survival of LGR5+ cells in-vitro (Stzepourginski et al. 2017). Immunofluorescence staining confirmed the expression of prolyl 4-hydroxylase (P4H, a key enzyme of collagen biosynthesis, which is also a marker classically associated with fibroblast and myoepithelial cells) in a large proportion of cultured cells which, at the same time, did not express α-SMA neglecting their identity as activated myofibroblasts. Furthermore, primary neuronal cells could be maintained in culture and their identity has been confirmed by β-III tubulin staining (Figure 21). The origin of these cells could be explained by the presence of the submucosal enteric plexus in mucosal biopsies. These cells are of undeniable importance for gut homeostasis and function as discussed above, and it is likely that they would not interfere negatively with analysis of future drug-based assays, however future experiments are needed to confirm this. These cells could also possibly originate from the mucosal glial cells, underlying the epithelium, which are able to transdifferentiate into neurons in vitro (Joseph et al. 2011; Laranjeira et al. 2011). Since the exact mechanisms are unclear, further experiments have to be performed for clarification of the origin and effect of these cells in primary culture.

In contrast to traditional cell lines, which often represent a homogeneous population of cells, long term primary cell cultures of IBD patient-derived biopsies represent a heterogeneous two-dimensional culture representing the epithelial niche compartment. This might be especially useful for patient-specific analysis and pharmacological

studies since they represent not only epithelial cells but also crypt surrounding cells of a given patient. Immunofluorescent analysis confirmed that a high percentage of cells within this culture express TNF-alpha (not quantified), the key pro-inflammatory cytokine involved in IBD pathogenesis, they are clearly involved in inflammatory processes. Furthermore, many patients develop specific complications such as fistulae, fibrosis or colon cancer; processes that appear when the fine balance between ECM synthesis and degradation is disturbed as discussed before. Given that mesenchymal cells are the main drivers of tissue remodelling processes, it would be extremely useful to clarify the response to treatment within the epithelial niche cell compartment before starting treatment.

In this study, we were interested in the response towards Infliximab of a patient suffering UC, before the patient was started on the therapy. In this context, we evaluated the response of a patient presenting with severe mucosal inflammation along with fibrotic stenosis. The patient has not been treated with anti-TNF-α yet, so treatment outcome is unclear. The following patient-specific experiment is stimulating an example of how the short-term biopsy-explant culture could be used in a personalized approach of in-vitro pre-treatment screenings.

Epithelial niche cells were isolated from this patient as described in (3.4.3). In short, the isolated cells were treated with Infliximab for 24h and mRNA expression was subsequently quantified by qRT-PCR. Analysis included pro-/anti-inflammatory cytokine expression (IL1B, IL6, TNFA, IL10) as well as factors involved in tissue remodelling (MMP2, MMP9, MMP13, TGFB) and mucus layer (MUC2)

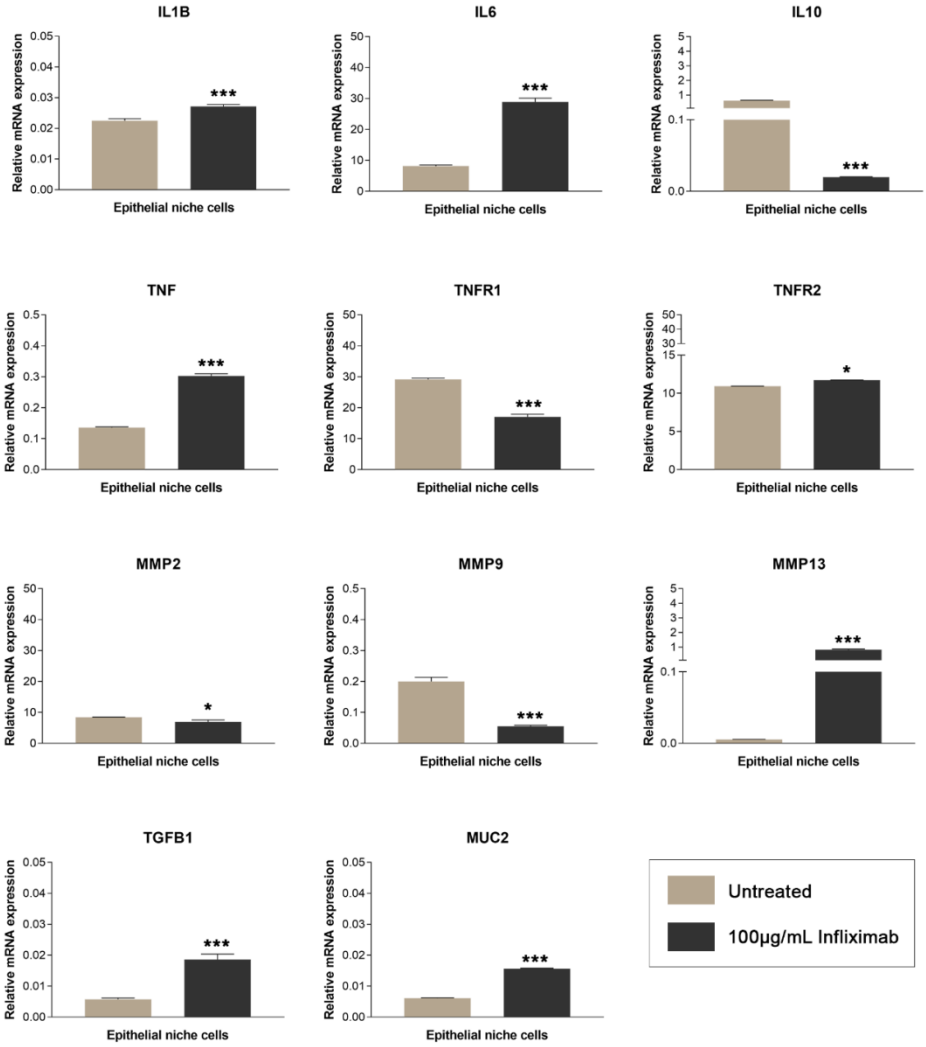


Figure 22. Example of an in-vitro pre-treatment screening via quantitative RT-PCR. Cells isolated from a patient suffering UC, who has not been in therapy with anti-TNF-alpha yet, were treated with Infliximab for 24h to predict a possible clinical outcome. A higher mRNA expression of pro-inflammatory cytokines and a downregulation of *IL10*, as well as an imbalanced mRNA expression of fibrotic mediators was observed, suggesting that anti-TNF-alpha treatment in this patient would not be beneficial. Data are shown as mean ± SD of n=3 replicates of three independent experiments. Statistical significance was calculated by Student's t-test comparing samples treated with Infliximab to untreated samples (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$).

Isolated cells of the described patient reacted on the treatment with Infliximab with a high upregulation of pro-inflammatory cytokines Il-1β, Il-6 and TNF-α. As discussed above (see 3.4.3) the ratio of TNFR1 and TNFR2 expression is important for the balance of pro-apoptotic and pro-survival signaling, respectively. Moreover, the expression of IL10 was negatively regulated due to treatment. These data indicate that the anti-inflammatory effects of anti-TNFα might not be effective in this specific

patient. Furthermore, significantly upregulated MMP13 and TGFB together with a downregulation of MMP2 and MMP9 indicates perturbations in tissue turnover and involved fibrotic processes as discussed in previous section. Taken together this data directs that anti- TNF-α treatment in the described patient would not be beneficial.

3.4.5 Establishment of colon organoid cultures

As discussed above, more physiologically relevant model systems serve to replace the cell line-based assays, in order to better clarify the pathophysiological processes associated with IBD development and progress (Ranga, Gjorevski, and Lutolf 2014). The first published report of a culture system that of intestinal stem cells was describing the culture of intestinal fragments containing both epithelial and mesenchymal cells from neonatal mice. These cells were noteworthy as they were the first example of an in vitro culture that was able to form the cyst-like structures of adult in vivo epithelium (Ootani et al. 2009). A key advance in the field was achieved in 2009 by the introduction of intestinal organoid cultures generated from adult LGR5+ mouse ISCs (T. Sato et al. 2009). This system mimics the in-vivo environment of intestinal crypts, providing appropriate soluble factors, such as EGF and R-Spondin1 to activate the EGF and WNT pathways, respectively, as well as Noggin to inhibit BMP signaling (T. Sato et al. 2009). In conjunction with 3-dimensional ECM hydrogels, these culture conditions assured long-term culture of ISCs as organoids. The structure of these organoids closely mimics the structural and functional aspects of the mammalian intestinal epithelium, with a central lumen and surrounding structures representing intestinal crypts. Within these crypt–like structures, ISCs retain their self-renewing capacity, while the villus-like domain contains terminally differentiated cells such as enterocytes, enteroendocrine cells or goblet cells representing differentiation processes

that are mimicking the situation in the adult epithelium in vivo (T. Sato and Clevers 2013).

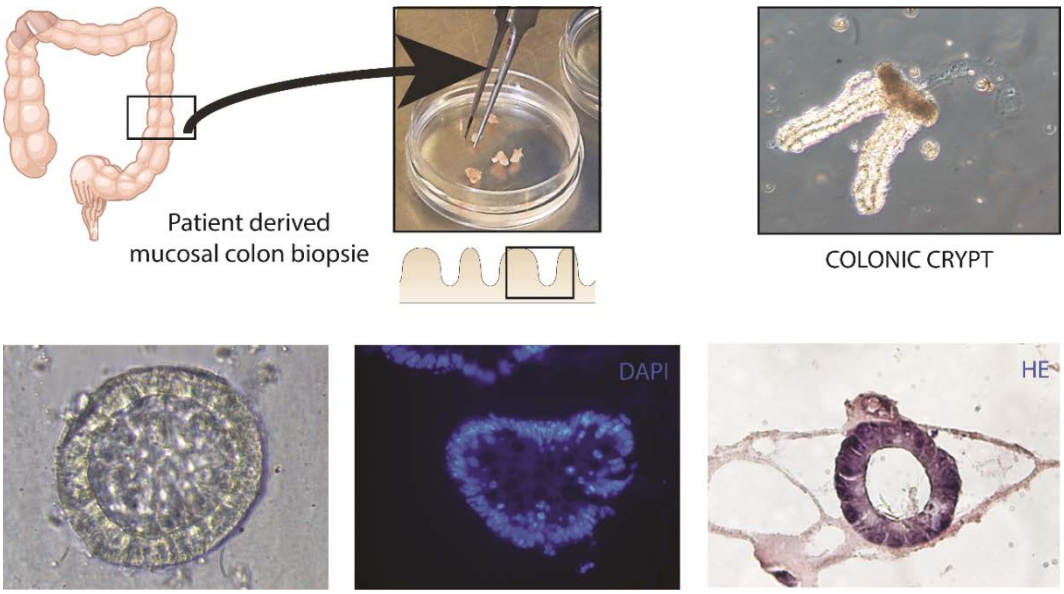


Figure 23. Establishment of organoid cultures from patient-derived mucosal biopsies. Organoids can be analyzed by immunofluorescence staining as demonstrated by DAPI staining. Also histological analyses of the organoid structures are possible as demonstrated by Hematoxylin/Eosin staining.

In the current study, colonic crypts were successfully isolated from IBD patients and were able to be maintained in long-term culture (Figure 23). The rapid expansion of cultured organoids allowed preparation of patient-derived epithelium even from a small-sized biopsy. Organoids can be maintained in long-term culture and experimental conditions can be easily before samples are processed for analysis by immunofluorescence or histology (Figure 23).

3.4.6 Conclusion Part IV

In the present study three different in-vitro models of human epithelial primary cultures were established. These models will be invaluable in the future prediction of treatment outcomes for IBD patients. By using primary derived patient tissue, accurate drug responses can be determined in vitro before patients begin therapy.

These short-term biopsy explant culture models offer the major advantage to provide a full spectrum of cellular diversity of a given patient that could be useful for help clinicians to define the correct management of gut inflammation, thus eliminating unsuccessful therapies in the very early stages of medical care. Furthermore, long term-epithelial cultures of patient-derived tissues (described herein as organoid cultures) will be invaluable as they expand rapidly and therefore allow a large number of experiments from a single mucosal biopsy. Lastly, patient-derived primary epithelial niche cells will be a great medical and therapeutical benefit as they are present not only in the epithelial compartment, but also in the crypt underlying mesenchymal cells, which are known players in IBD pathology.

These features make these patient-derived primary cells a very useful and unique in-vitro long term model to characterize possible dysfunctions in mesenchymal and stromal cell compartment in IBD pathology. Furthermore, this system enables the exploration of individual responses of a given patient to therapy, which would be invaluable for the personalisation of patient care in the future.

4 CONCLUSIONS AND PERSPECTIVES

Taken together, the models described in this thesis display powerful tools to gain a deeper understanding of processes underlying chronic intestinal inflammation. In particular, the new three-dimensional model of the ENS, closely resembling the native myenteric plexus, will be extremely useful in obtaining valuable insights into ENS dysfunction, as well as the causes and consequences of its involvement in inflammatory processes. With the prospect of demonstrating a beneficial effect of nutraceuticals as co-adjuvants in the treatment of IBD, an experimental model of intestinal inflammation was assessed using adult zebrafish to verify the anti-inflammatory activity of the herbal preparation STW5. Primary cell cultures of zebrafish ENS and intestinal epithelium were developed in order to investigate the molecular mechanisms within specialized gut compartments. Within primary ENS and epithelial cells, the protective effects of STW5 were verified to be regulated through Wnt3a and Il-10. The effects of Wnt3a are hypothesized to act, at least partly, through the Wnt receptor Frizzled-9, which was demonstrated for the first time to be expressed within the adult zebrafish ENS and the basal epithelial layer. In this context, it would be very interesting to investigate this

relatively new player, FZD9, in the human epithelial and ENS compartment and clarify its role in intestinal homeostasis and disease. Intestinal homeostasis was also found to be associated with the extracellular protein fibrillin-1. Data obtained from TSK/+, fibrillin mutant mice confirmed that Fibrillin-1 networks are important for maintaining intestinal homeostasis and that structurally altered fibrillin-networks are involved in inflammatory processes. Besides macroscopic changes with a reduction in intestinal length, immune infiltrates and tissue alterations, the cytokine expression was found to be extremely dysregulated. Structural changes and increased muscle layer thickness within the colon wall indicate the initiation of fibrotic processes. Finally, the herein described model systems of patient-derived epithelial tissue under different aspects will be particularly useful for personalized assays and individual drug screening that enable the prediction of a given patient's response to treatment, a tool that will become widespread in the future of IBD disease management. In order to achieve this, several shortcomings have to be addressed, such as the culture conditions for mucosal biopsies in order to prolong tissue survival and maintenance. In this context, a dynamic culture system, rather than static culture conditions, may be used to overcome this problem in the future. Epithelial organoids can be cultured from small-sized biopsies and present a state of the art in vitro model of the intestinal epithelium. However, while impressively reassembling the intestinal epithelium, they lack the mesenchymal cell compartments that surround the crypts in vivo. The heterogenous patient-derived epithelial niche cell-lines described in this thesis represent the natural neighbours of intestinal epithelium. Co-culturing of organoids with epithelial niche cultures derived from the same patient will further advance personalized cultures, and therefore presents exciting new possibilities.

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