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**DEVELOPMENT OF HIGH THROUGHPUT MICROFLUIDIC GUT-ON-A-CHIP
MODELS FOR DRUG DISCOVERY AND TARGET VALIDATION IN
INFLAMMATORY BOWEL DISEASE**

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SUMMARY

Inflammatory bowel disease (IBD) is a group of chronic relapsing inflammatory diseases of the gastrointestinal tract. Diseased patients have currently limited options in terms of treatment caused by the lack of physiologically relevant models to study IBD. Recently, the organ-on-a-chip technology has offered new insights into IBD modelling that could alleviate some of the limitations of the models currently used. This study reports the development of two novel gut-on-a-chip microfluidic platforms that can uniquely contribute to the advancement of research in the IBD field.

The first model, robust and simple, is composed of the human cell line Caco-2 submitted to a clean inflammatory cocktail. The epithelium displayed a disrupted organization and an increased cell activation denoted by elevated cytokine release. The second model, more complex and physiologically relevant, integrates patient-derived intestinal organoids together with human macrophages, triggered by a mixture of cytokines and bacterial components.

We show that these gut-on-a-chip models can be used to support daily drug discovery activities in IBD, both at the target validation and compound testing levels. Our results set the ground to improved, more relevant models of the human intestine, in order to better understand this complex pathology, which could in turn lead to the discovery of more efficient and accurate treatments for IBD patients.

DECLARATION

I, Claudia Beaurivage, confirm that the Thesis is my own work. I am aware of the University's Guidance on the Use of Unfair Means (www.sheffield.ac.uk/ssid/unfair-means). This work has not been previously been presented for an award at this, or any other, university.

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LIST OF ABBREVIATIONS

AdV	Adenovirus
AJ	<i>Adherens</i> junction
ALI	Air-liquid interface
AMP	Antimicrobial peptides
ANOVA	Analysis of variance
A.U.	Arbitrary units
CBC	Crypt base columnar cell
CD	Crohn's disease
CMV	Cytomegalovirus
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
DC	Dendritic cells
DNA	Deoxyribonucleic acid
DSS	Dextran sodium sulfate
ECM	Extracellular matrix
EEC	Enteroendocrine cell
EMA	European Medicines Agency
EMT	Epithelial-mesenchyme transition
ER	Endoplasmic reticulum
FDR	False discovery rate
GALT	Gut-associated lymphoid tissue
GAS	Gamma-Activated Sequence
GFP	Green fluorescent protein
GI	Gastrointestinal
GWAS	Genome wide association studies
HCC	Hepatocellular carcinoma
HIO	Human intestinal organoids
HISC	Human intestinal stem cell
HLA	Human leukocyte antigen
HS	Healthy subjects
IBD	Inflammatory bowel disease
IESC	Intestinal epithelial stem cells
IEC	Intestinal epithelial cells
iPSC	Induced pluripotent stem cells
IEL	Intra-epithelial lymphocytes
IU	Infective units
IgA/sIgA	Immunoglobulin A / Secreted Immunoglobulin A
LPS	Lipopolysaccharide
M cells	Microfold cells
MF	Macrophage
MFI	Median fluorescence intensity
MOI	Multiplicity of infection
NLS	Nuclear localization signal
NO	Nitric oxide
OTU	Operational taxonomic unit
PAMPs	Pathogen-associated molecular patterns
PBMC	Peripheral blood mononuclear cell
PDMS	Poly-dimethylsiloxane
PIN	Percentage of inhibition
PX	Phox homology

RCC	Renal cell cancer
RQ	Relative quantification
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SCFA	Short chain fatty acid
shRNA	Short hairpin ribonucleic acid
SLC	Solute carrier
SNP	Single nucleotide polymorphism
TCA	Tricarboxylic acid
TEER	Transepithelial electrical resistance
TFF	Trefoil factors
Th	T helper lymphocyte
TJ	Tight junction
TLR	Toll-like receptor
TNBS	Trinitrobenzene sulfonic acid
UC	Ulcerative colitis
2D	Two-dimensional
3D	Three-dimensional

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INTRODUCTION

The gastrointestinal tract

1.1. Generalities

The digestive system, composed of the digestive tract and various accessory organs, is the set of organs necessary for the breakdown and assimilation of nutrients. Its role is to receive food, break it down into nutrients and absorb it into the bloodstream before promoting the elimination of residues (Martini, 2012).

The digestive tract, also named gastrointestinal (GI) tract, starts at the mouth, goes through the pharynx, the oesophagus, the stomach, the small intestine, the colon and ends in an orifice, the anus. The small intestine and colon are separated by the appendix in humans and the cecum in mice. The organs attached to the digestive system, i.e. the teeth, tongue, gallbladder, salivary glands, liver and pancreas, are also important to the digestion process. The intestine, made up of the small intestine and the colon, makes up the majority of the digestive tract and is the site of importance for digestion and absorption.

1.2. The small intestine

The small intestine is the first segment of the intestine, directly following the stomach. It is the main place for digestion and selective absorption of nutrients. It also produces some hormones, enzymes and mucus. The small intestine is separated in three main regions; the duodenum, jejunum and ileum. First, the stomach chyme mixes with the pancreatic juices and bile from the liver in the duodenum and thus becomes less acidic. Then, the nutrients produced are captured mainly in the jejunum. Finally, bile salts and vitamins are absorbed into the ileum. The small intestine also absorbs more than 95% of the water entering the digestive tract. In a normal individual, digestion is significantly complete when the chyme enters into the colon (Smith and Morton, 2010).

1.3. The colon

The colon is the second segment of the intestine, which connects the ileum to the anus. It is divided into four main regions; the ascending, transverse, descending and sigmoid colon. The colon ends in the rectum, before connecting with the anus. The colon is responsible for the absorption of residual water, vitamins and electrolytes from the chyme as well as the digestion of certain fibers with the help of intestinal bacteria. The intestinal microflora is present

throughout the digestive tract, but is particularly concentrated and diversified in the colon, where the concentration can reach up to 10^{11} bacteria per gram of faeces (Gorbach, 1996).

1.4. General structure of the intestine

Although the intestine has different segments, each with their own function, the general structure of the intestinal wall is preserved between these segments (**Fig. 1**). The intestinal wall is divided into four different layers; the mucosa, submucosa, muscularis externa and serosa (Smith and Morton, 2010).

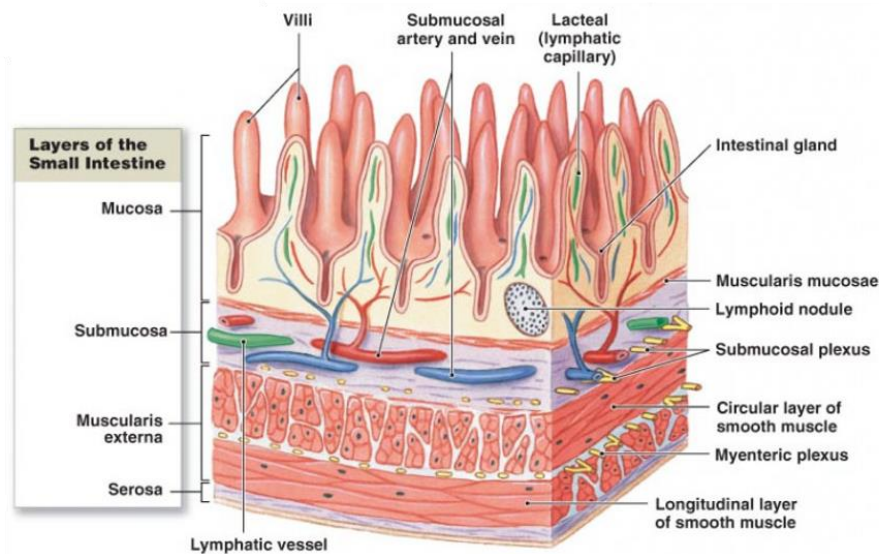


Figure 1. Architecture of the intestinal wall

The intestinal wall is divided into 4 layers: the mucosa, submucosa, muscularis externa and serosa. A network of muscular fibers, called *muscularis mucosae*, is separating the mucosa and the submucosa. The intestinal wall is highly innervated and vascularized. Authorized reproduction of (Martini, 2012).

The mucosa is the layer directly in contact with the intestinal lumen and is made up of the epithelium, the lamina propria and the *muscularis mucosae*, a smooth muscle layer. The epithelium is a single layer of epithelial cells lining the entire intestinal tissue and has multiple functions which will be discussed in the next section. The lamina propria contains the blood and lymphatic vessels necessary for the absorption of nutrients. It mainly contains connective tissue as well as fibroblasts, smooth muscle cells and immune cells. Finally, the *muscularis mucosae* contains the muscle cells allowing movement of the mucosa and the shape of the lumen (Martini, 2012).

The submucosa is a flexible connective tissue containing blood vessels and nervous plexuses composed of ganglionic neurons that allow the smooth muscle of the mucosa to contract (Martini, 2012).

The third layer of the intestinal wall is an external muscle layer, composed of a succession of internal circular smooth muscles and external longitudinal smooth muscles. Between these two layers are nerve plexuses which, in combination with the muscle layer, allow peristalsis to propel food chyme along the digestive tract (Martini, 2012).

Finally, the entire wall is surrounded by the serosa. The serosa is made up of a layer of connective tissue that surrounds the wall and which, in turn, is surrounded by a simple squamous epithelium. The serosa thus protects the intestinal wall by preventing possible friction or displacement.

The intestinal mucosa

The intestinal mucosa exists in a functional balance with the complex luminal milieu that is dominated by a variety of microbial species and their products. Maintaining this functional equilibrium is essential to preserving normal intestinal physiology and its perturbation contributes to the pathophysiology of many gastrointestinal conditions, such as IBD. Along with nutrient absorption, IEC accomplish both barrier and signal-transduction functions, with the ability to sense luminal contents through surface receptors and, in response, secrete regulatory products that can coordinate a suitable reaction in the underlying lamina propria. The mucosa holds a large array of cell types in close proximity to the epithelium. Most, if not all of these cells, produce molecules that have the potential to affect the epithelium.

2.1. The intestinal epithelium

The intestinal epithelial cells (IEC) organize into a tight sheet that forms a single layer of cells lining the entire intestinal wall. The primary function of the epithelium, in addition to digestion or absorption of nutrients, is to physically segregate commensal bacteria in the lumen of the intestine from the intestinal mucosa. The intestinal epithelium consists of four principal differentiated cell types as well as stem and progenitor cells (**Fig. 2**) (Crosnier et al., 2006). However, the composition of the intestinal epithelium is not exactly the same in the small intestine and the colon, which gives the two segments different functions.

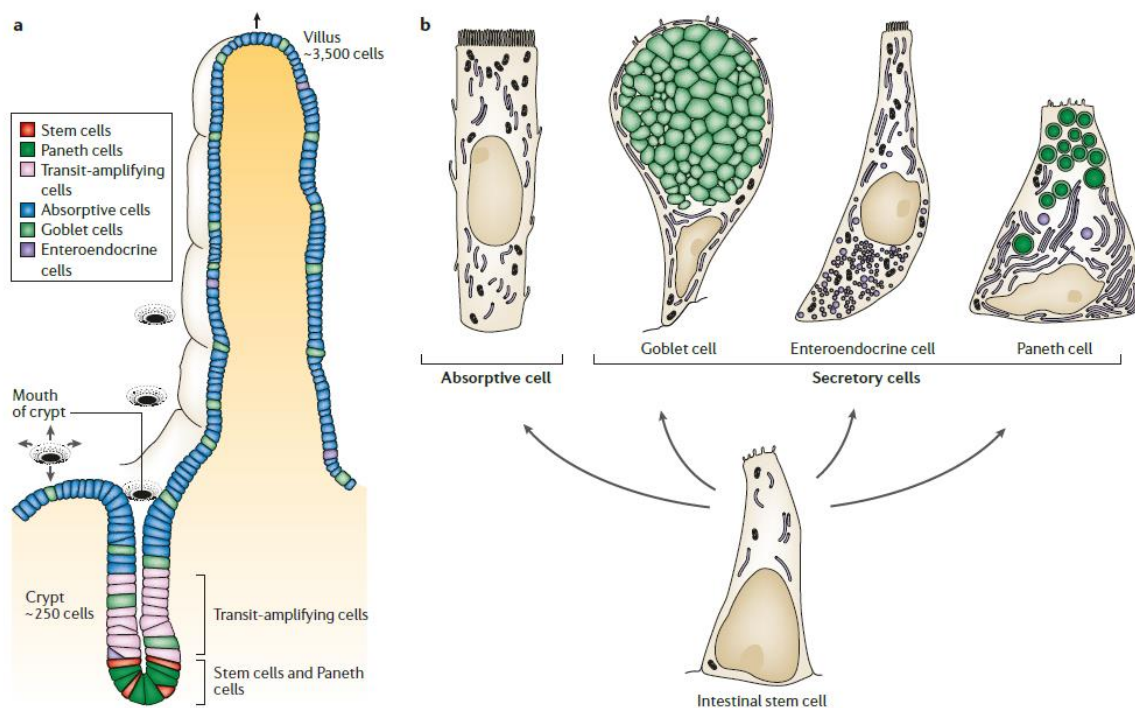


Figure 2. Composition of the intestinal epithelium

A. Schematic representation of the small intestinal crypt-villus axis. Stem cells are located at the base of the crypts where they divide to give rise to progenitor cells that will enter a cellular differentiation program. During differentiation, all the different cell types will migrate towards the top of the crypt. Once their differentiation is terminated, Paneth cells will return to the base of the crypts. Once the cells reach the apex of the villi, they are exfoliated through anoikis. The human intestinal epithelium renews itself every 3 to 5 days. **B.** The intestinal epithelium is composed of four main differentiated cell types: absorptive cells, goblet cells, enteroendocrine cells and Paneth cells. Authorized reproduction of (Crosnier et al., 2006)

2.1.1. Composition of the intestinal epithelium

In the small intestine, the epithelium is organized into crypt-villus axes. The crypt is made up of stem cells, progenitor cells, differentiating cells and Paneth cells. The villus is composed of differentiated post-mitotic cells; goblet cells, enteroendocrine cells (EEC) and absorptive cells, also called enterocytes or colonocytes depending whether they are located in the small intestine or the colon (van der Flier and Clevers, 2009). There are also Microfold cells (M cells) that cover the surface of the gut-associated lymphoid follicles which capture and transfer antigens to *bona fide* dendritic cells (DC) for antigen presentation (Jepson et al., 1993; Dillon and Lo, 2019) as well as cup cells (Madara, 1982) and tuft cells (Hammond and Ladeur, 1968) which are in minority and less well characterized. Tuft cells, however, are thought to regulate Th2 immune response in answer to parasitic infection (Gerbe et al., 2012).

On the other hand, the colonic epithelium has deeper crypts and lacks villi. Pluripotent stem cells are found at the base of the crypts and begin a differentiation program that will lead to

goblet cells, enteroendocrine cells, and absorptive cells, also called colonocytes. Paneth cells are normally absent from the colon (van der Flier and Clevers, 2009). M cells and tuft cells are also found in the colon, while cup cells are mostly restricted to the ileum (Fujimura and Iida, 2001).

2.1.1.1. Stem cells and progenitor cells

The intestinal epithelium is a rapidly dividing organ that renews itself every three to five days on account of the constant division of stem cells, or columnar base cells (CBC) located at the base of the crypts (Barker, 2014). Following an asymmetric division, a stem cell gives birth to another stem cell and a progenitor cell. The progenitor cell begins to migrate upwards the crypt, during which it divides four or five times (van der Flier and Clevers, 2009). When it receives differentiation signals, it stops dividing and then enters a cellular differentiation program to generate one of the differentiated cell types listed above, which each have their specialized function (Bloemendaal et al., 2016).

2.1.1.2. Cellular differentiation signals of the intestinal epithelium

Progenitor cells, as they migrate along the crypt, are subject to many differentiation signals originating from the epithelium and the mesenchyme. These signals will determine whether they belong to the secretory or the absorptive lineage (**Fig. 3**). It is in fact a lateral inhibition system combined with the expression of transcription factors that allows the differentiation and maturation of IEC (Crosnier et al., 2006). Particularly, WNT and NOTCH signalling pathways are the main drivers of IEC differentiation. It all starts in the stem cell niche where the combination of WNT and NOTCH signals is required for maintaining the stem cell pool (Korinek et al., 1998; Van Es et al., 2005). Paneth cells are providing NOTCH signalling input to the stem cell compartment through delta-like ligands DLL1 and DLL4 (Pellegrinet et al., 2011; Sato et al., 2011a). When comes the time of progenitor differentiation, high WNT and low NOTCH will favour secretory lineages while low WNT and high NOTCH will favour the absorptive lineage.

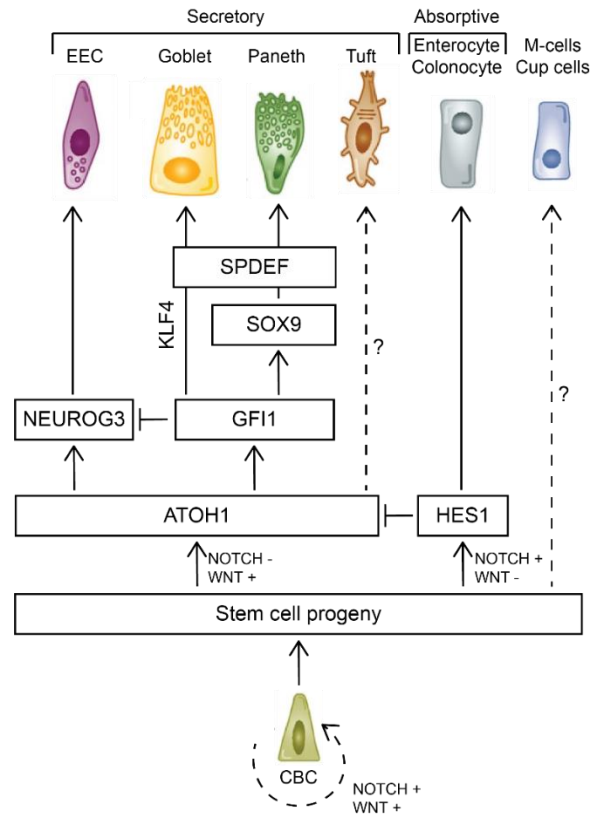


Figure 3. Differentiation of intestinal epithelial cells

Under active NOTCH and WNT signalling, columnar base cells (CBC) will retain their proliferative state. Transcription factor HES1 is a target gene from the NOTCH pathway, which inhibits ATOH1 expression. HES1 is essential for the differentiation of the absorptive lineage while ATOH1 is essential to the secretory lineage. Other transcription factors control the further differentiation and maturation of IEC. NEUROG3 controls the production of enteroendocrine cells (EEC). GFI1, SPDEF and KLF4 regulate the production of goblet cells while GFI1, SPDEF and SOX9 control Paneth cell maturation. Downstream of ATOH1, the events leading to the production of tuft cells are uncharacterized. M cells and cup cells have yet to be classified into the secretory or absorptive lineage. Authorized modification from (Sancho et al., 2015).

In the absorptive lineage, an active NOTCH signalling will release NICD, the active fragment of NOTCH (Sancho et al., 2015). This results in the elevated expression of HES1 transcription factor. The HES1 protein is an inhibitory transcription factor for genes associated with the secretory line, including ATOH1. Therefore, in the presence of an active NOTCH pathway, the expression of ATOH1 is inhibited and the progenitor cells are directed away from the secretory and towards the absorptive lineage (Fre et al., 2005). Lateral inhibition warrants that a cell expressing high levels of NOTCH (which is skewed towards the absorptive lineage) limits the activation of NOTCH in neighbouring cells, which promotes secretory differentiation. Committing to the absorptive lineage does not instantly stops proliferation, while cells committed to the secretory lineage no longer divide, leading to fewer secretory cells overall (Stamatakis et al., 2011).

When the progenitor cells are not exposed to a stimulus of NOTCH signalling, ATOH1 is then expressed and the formation of secretory precursors is favoured. By mechanisms still poorly characterized, tuft cells differentiate from the secretory precursor. For the other secretory subtypes, they result from a combination of the expression of several transcription factors (Shroyer et al., 2005). Initially, the expression of NEUROG3 induced by ATOH1 leads to differentiation of EEC (Jenny et al., 2002). Paneth cells and goblet cells come from a common precursor, which expresses the factor GFI1 (Noah et al., 2011). Expression of GFI1 inhibits the expression of NEUROG3 to direct progenitors to differentiate into one of these two cell types. Subsequent specific differentiation into Paneth cells is done through SOX9 expression, while differentiation into goblet cells is supported by the factor KLF4 (Bastide et al., 2007; Katz et al., 2002). Supporting this model that offers a common precursor to Paneth and goblet cells, it has been shown that the factor SPDEF was necessary for the maturation of these two cell types (Gregorieff et al., 2009).

2.1.1.3. Absorptive cells

Enterocytes and colonocytes are the most abundant cell type in the intestine, accounting for 80% of IEC (van der Flier and Clevers, 2009). They are found in the villi of the small intestine and in the surface epithelium of the colon. They are polarized, cylindrical-shaped cells and present a brush border composed of microvilli on their apical surface (Noah et al., 2011). This structure increases the surface of contact with nutrients to promote their capture and absorption. Nutrients are then released at the basal surface so that they can be absorbed and transported into lymphatic and blood vessels.

In order to properly perform their role in digestion, absorptive cells express many enzymes, including sucrase-isomaltase and alkaline phosphatase, which cleave disaccharides and control lipid absorption, respectively (Ferraris et al., 1992). It is generally admitted that colonocytes do not express digestive hydrolases (Zweibaum et al., 1984; Foltzer-Jourdainne et al., 1989), they are rather involved in the absorption of water and salts, but also in the secretion of β -defensins and type-C lectins like RegIII β and RegIII γ that act as antimicrobial peptides (AMP) (Cobo and Chadee, 2013; van Ampting et al., 2012). Enterocytes and colonocytes also produce proteins such as FABP2 that binds and promotes the absorption of fatty acids (Gajda and Storch, 2015) and GLUT2 which promotes the transcellular transport of glucose and fructose (Kellett et al., 2008).

2.1.1.4. Goblet cells

Goblet cells are the most abundant secretory cell type in the intestinal epithelium (Noah et al., 2011). Their main function is to produce and secrete mucus as the first line of defence against xenobiotics, microbes or viruses contained in the intestinal lumen (Dorofeyev et al., 2013). Mucus is a gel-like structure that is composed of different mucins, large glycoproteins with many oligosaccharide chains. It is the goblet cells that secrete these mucins, either constitutively or in a regulated manner in response to factors such as pro-inflammatory cytokines or bacteria (Lindén et al., 2008). They also secrete trefoil factors (TFF) responsible for epithelial restitution, a mechanism where the damaged or inflamed surface epithelium will be re-established by the migration and division of epithelial cells (Dorofeyev et al., 2013). Mucus production is at its highest in the colon, where the concentration of bacteria is also peaking; it is indeed the presence of mucus that allows the intestine to be colonized by bacteria. The mucus layer provides a niche for bacterial colonization because it contains attachment sites and is also a carbon source (Harel et al., 1993). Effectively, the mucus is a direct source of carbohydrates that are released in the lumen. Therefore, several bacterial species of the microbiota can use mucus glycan as a carbon source (Ouwerkerk et al., 2013).

2.1.1.5. Paneth cells

Unlike other differentiated cell types of the gut, Paneth cells are found exclusively in the bottom of the crypts of the small intestine and are not normally found in the colon. Paneth cells have an apical cytoplasm filled with very dense granules (**Fig. 2**). These granules are packed with AMP such as lysozyme and α -defensins that protect the host from enteric pathogens (Wilson et al., 1999) and modulate the composition of the intestinal microflora (Biswas et al., 2010).

Besides the secretion of AMP, Paneth cells are also important for the formation and maintenance of the stem cell niche at the base of the crypts. This niche, created by the expression and secretion of growth factors such as WNT, TGF α and EGF by Paneth cells, interacts with stem cells to determine the cellular fate of IEC (Sato et al., 2011a).

2.1.1.6. Enteroendocrine cells

Enteroendocrine cells (EEC) are the least represented cell type of the gut, accounting for approximately 1% of the IEC in the small intestine and colon (Noah et al., 2011). EEC are scattered along the mucosa, often isolated from each other by non-endocrine IEC, and secrete multiple hormones responsible for regulating digestive functions (Buffa et al., 1978). There are more than 15 subtypes of enteroendocrine cells identified that secrete more than 30 different

digestive hormones (Shroyer and Kocoshis, 2011). Among these hormones are cholecystokinin, secretin, motilin and chromogranin A (Gunawardene et al., 2011).

2.2. *Immune cells*

The intestinal tract is constantly exposed to many environmental factors and the immune system must be able to activate pro-inflammatory pathways in response to pathogenic bacteria while remaining insensitive to symbiotic bacteria and food antigens. The intestine actually holds a higher number of immune cells than any other tissue in the body in order to be able to defend the host against pathogens and maintain immune tolerance in the gut (Chassaing et al., 2014).

To ensure optimal transport of nutrients while avoiding bacterial translocation, the intestinal mucosa is equipped with a well-developed immune system. As a first line of immunological defence, the epithelium is interspersed with intraepithelial T lymphocytes (IEL) that work as sentinels of the mucosal barrier. Underneath the epithelium, the lamina propria contains B cells, T cells and several innate immune cells such as DC, macrophages, eosinophils and mast cells. Most of the intestinal immune system is organized into lymphoid structures such as gut-associated lymphoid tissue (GALT) and draining lymph nodes. Those are the principal locations where adaptive immune cells are primed to generate appropriate immune responses. GALT are aggregations of subepithelial lymphocytes in the mucosa and submucosa and are covered by a follicle-associated epithelium, which comprises M cells. GALT includes Peyer's patches in the small intestine, an accumulation of B cells lymphoid follicles flanked by smaller T cells regions, and equivalent colonic structures. In addition, GALT also comprises small lymphoid regions such as isolated lymphoid follicles, which mainly contains B cells with no T cells zones.

Although T and B effector cells protect the host with powerful adaptive immune responses against the large number of antigens that are continually encountered, the intestinal immune system relies heavily on innate immune cells and their rapid effector functions to maintain intestinal homeostasis. Among these cells are monocytes, macrophages, DC and innate lymphoid cells. Macrophages and DC come from a common monocytic precursor (Tomasello and Bedoui, 2013). Macrophages are the most abundant leukocytes of the intestine (Mowat and Agace, 2014). Once differentiated, macrophages will reside in the intestinal tissue to present antigens and phagocyte dead cells and debris, thus participating in tissue remodelling (Smith et al., 2011). Dendritic cells, on the other hand, patrol the intestinal mucosa in search of

antigens. They are particularly effective in presenting these antigens to naive T lymphocytes (Tomasello and Bedoui, 2013). Finally, innate lymphoid cells do not have antigen-specific receptors and are rather involved in the production of cytokines which facilitate infection control and repair of tissue damage (Bostick and Zhou, 2016). It goes without saying that tight regulation of all these cell types is highly complex and that it is necessary in all points for the maintenance of intestinal homeostasis.

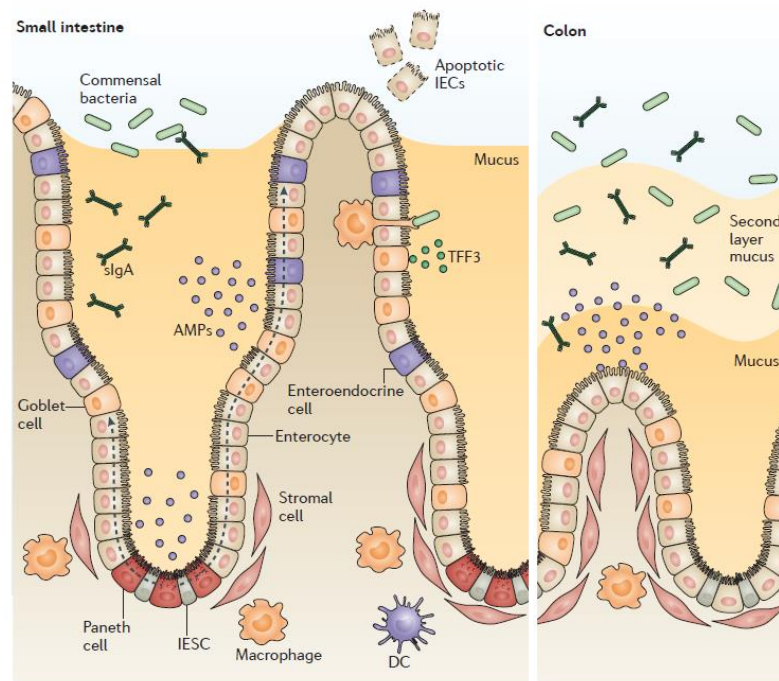


Figure 4. The intestinal epithelium

IEC form a physical and biochemical barrier that maintains segregation between commensal bacteria and the intestinal mucosa. The stem cell niche, containing epithelial, stromal and hematopoietic cells, controls the constant renewal of intestinal epithelial stem cells (IESC). IESC reside at the bottom of the crypt and upon defined signals, will differentiate into four main cellular types in the small intestine; absorptive enterocytes, goblet cells, Paneth cells and enteroendocrine cells. The colon is normally devoid of Paneth cells. Differentiated IEC migrate along the crypt-villus axis, as indicated by the dotted arrow, and will be exfoliated by anoikis at the apex of the villus. Goblet cells confer a second protection for the intestinal epithelium by secreting mucins that organize in a single layer in the small intestine and a double layer in the colon. This mucus acts as a reservoir for antimicrobial peptides (AMP), trefoil factor 3 (TFF3) and IgA, which protect the epithelium and regulate the microflora. Finally, immune cells patrol the mucosa and phagocyte bacteria that have defeated the defences of the epithelium. Authorized reproduction from (Peterson and Artis, 2014).

Maintenance of intestinal homeostasis

Since the intestinal epithelium is a rapidly renewing organ in constant contact with the microbiota and the immune system, it can be complex to maintain its integrity. It is important to continuously maintain intestinal homeostasis to prevent the onset of conditions such as IBD.

To do this, the epithelium provides different levels of defence against commensal bacteria contained in the lumen of the gut (**Fig. 4**).

3.1. *Physical barrier*

First, the epithelium acts as a physical barrier establishing segregation between the microbiota and the intestinal mucosa. A balance between the proliferation of stem cells, the production of differentiated cells, their migration into the villus and their elimination by anoikis is therefore essential to maintain intestinal homeostasis (Bjerknes and Cheng, 2005).

The epithelium also establishes a barrier with selective permeability that lets nutrients, salts and water pass, but prevents toxins and luminal bacteria from crossing the barrier. This selective permeability is possible thanks to the various protein complexes that bind adjacent cells and thus seal the intercellular space: tight junctions (TJ), *adherens* junctions (AJ) and desmosomes. These junctions are made up of transmembrane proteins which interact with neighbouring cells and intracellular adapter proteins linked to the cytoskeleton (Groschwitz and Hogan, 2009). Tight junctions, or *zonula occludens*, are localized near the apical surface of IEC and are an arrangement of CLAUDIN, OCCLUDIN, junctional adhesion molecules (JAM) and *zonula occludens* (ZO) proteins that link to the actin cytoskeleton. *Adherens* junctions are localized below tight junctions and are composed of E-CADHERIN, α -CATENIN and β -CATENIN that also connects with the actin cytoskeleton. Finally, desmosomes are localized near the basal membrane of IEC. DESMOCOLLIN, DESMOGLEIN, PLAKOGLOBIN and DESMOPLAKIN are main constituents of desmosomes and anchor keratin intermediate filaments (Citalán-Madrid et al., 2013). Being the most apically located, tight junction complexes have been shown to be the rate-limiting factor for paracellular permeability (Chelakkot et al., 2018). *Adherens* junctions mainly mediate strong cell-to-cell adhesion, however increasing evidence suggests that the assembly of tight junctions is dependent on the prior formation of AJ, and thus alteration of AJ modulates epithelial paracellular barrier function (Guo et al., 2003).

3.2. *Chemical barrier*

A thick layer of mucus covering a dense glycocalyx composed of membrane-associated mucins, which is inaccessible to most bacteria, covers the mucosal surfaces of the body, including the intestine (Maloy and Powrie, 2011). The bacteria, then trapped in the outer mucus layer, are evacuated during movements of peristalsis (Magalhaes et al., 2007).

Mucins are either secreted, like MUC2, or membrane-bound, like MUC1 and MUC3. The main intestinal mucin is MUC2 where it is organized in a single layer at the level of the small intestine and in a double layer in the colon (**Fig. 4**). The inner layer, immediately adjacent to the epithelium, is stratified into a tight filter that physically segregates the commensal microbiota from the IEC (Johansson et al., 2008). At a 50µm distance from the epithelium, the activity of endogenous proteases converts the inner layer of mucus into a second, looser layer, which allows intestinal bacteria to penetrate (Birchenough et al., 2015). Type-C lectin RegIIIγ is essential for maintaining the 50µm distance between the epithelium and the intestinal microbiota (Vaishnava et al., 2011).

The intestinal mucus also acts as a reservoir for high concentrations of antimicrobial molecules such as AMP, produced by both absorptive cells and Paneth cells, and secretory IgA that neutralize toxins and germs contained in the intestinal lumen. IEC thus regulate the colonization and penetration of bacteria into the epithelium (Maloy and Powrie, 2011).

Inflammatory bowel disease

Ulcerative colitis (UC) and Crohn's disease (CD) are relapsing chronic inflammatory conditions of the GI tract grouped under the term of inflammatory bowel disease (IBD). IBD patients suffer from intense inflammatory episodes leading to abdominal pain, weight loss, diarrhoea, anaemia and can lead to severe complications such as malnutrition, bowel obstruction or intestinal rupture. The highest reported prevalence values of IBD are in Europe and North America where 0.3 to 0.5% of the population is affected from either form of IBD (Ng et al., 2017; Xavier and Podolsky, 2007). Systemic review of IBD studies published between 1990 and 2016 suggest that while IBD prevalence is now stable in Europe and North America (Ng et al., 2017; Molodecky et al., 2012), it is rapidly increasing in newly industrialized countries, becoming globally problematic.

Although their symptoms are similar, UC and CD differ mainly by the area and the extent of inflammation they cause. The transmural inflammation caused by CD appears in skip lesions throughout the GI tract where inflammation patches are interrupted by healthy regions of tissue. However, the terminal ileum is more frequently affected. Histologically, the presence of granulomas is often noted, which corresponds to an aggregation of macrophages (Xavier and Podolsky, 2007).

On the other hand, UC mainly affects the colon or the rectum and often follows a proximal-to-distal severity gradient, leaving the terminal colon more affected. The inflammation observed is rarely transmural but is rather concentrated in the mucosa and submucosa. Histologically, we frequently observe loss of goblet cells in addition to a strong recruitment of neutrophils within cryptic abscesses (Xavier and Podolsky, 2007).

4.1. Aetiology

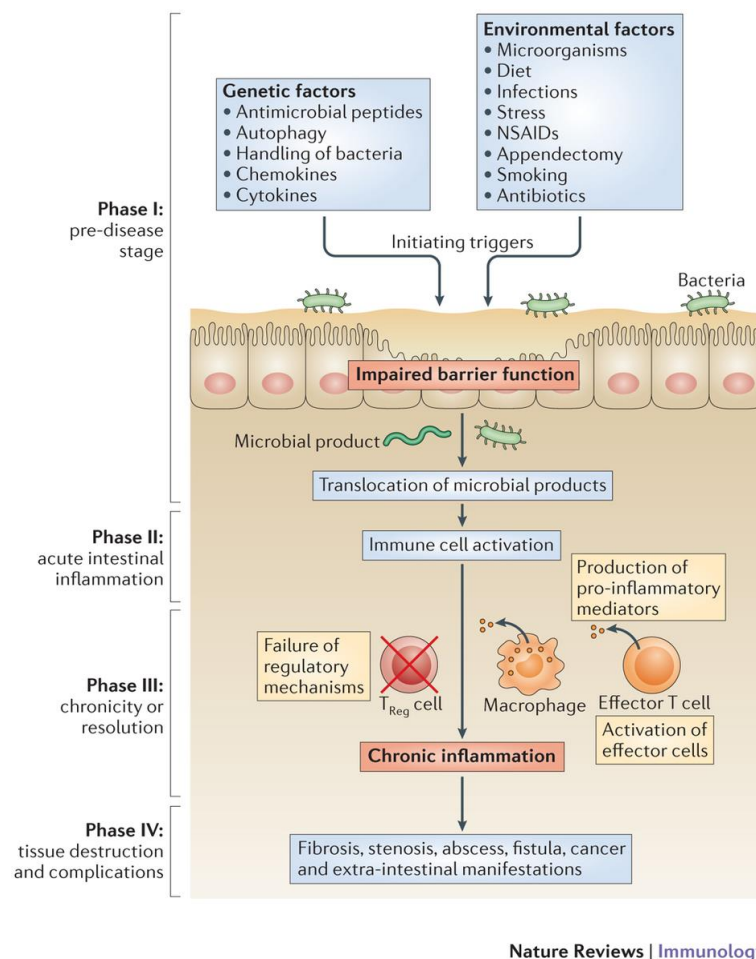


Figure 5. Pathogenesis of inflammatory bowel disease

Genetic and environmental factors induce impaired barrier function in the intestine. Altered barrier function consequently induces the translocation of microbial products from the lumen into the mucosa, which leads to immune cell activation and cytokine production. If inflammation cannot be resolved by regulatory mechanisms and the suppression of pro-inflammatory mechanisms, chronic intestinal inflammation develops. Authorized reproduction from (Neurath, 2014).

IBD is an idiopathic condition; its aetiology remains unclear to this day and we still know little about the chronology of the events leading to the establishment of inflammation. However, it is known that a strong genetic component is involved, especially in CD (Orholm et al., 1991). It is also evidenced that environmental factors such as smoking, stress and diet play an important role in the pathogenesis of IBD as western industrialized countries show high

prevalence of the disease (Ananthakrishnan, 2015). In recent years, several risk factors have been identified; genetic predisposition, intestinal barrier integrity, immune response and intestinal microflora. Based on our current level of knowledge, it is believed that IBD develops in genetically susceptible individuals who exhibit exacerbated immune responses in response to environmental or microbial stresses (**Fig. 5**). Each of the latter risk factors will be discussed separately.

4.1.1. Genetic susceptibility

Numerous genome-wide association studies (GWAS) have found loci that confer increased genetic susceptibility to IBD. A particular study by Jostins et al. identified 163 loci associated with IBD (Jostins et al., 2012). In this study combining the results of more than 15 GWAS, 110 loci were common to the two pathologies while 23 loci were UC-specific and 30 loci were CD-specific (**Fig. 6, 7**). Namely, the genetic variants identified in IBD affect genes involved in autophagy, IL-23/IL-17 axis, innate immunity, barrier function of the epithelium and failure to suppress aberrant immune responses (Liu and Anderson, 2014; Xavier and Podolsky, 2007).

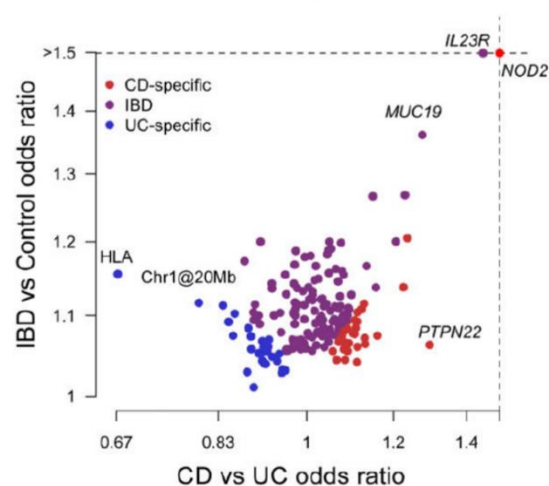


Figure 6. Identification of IBD susceptibility loci

Following the analysis of more than 15 GWAS, susceptibility loci for Crohn's disease (CD-red), ulcerative colitis (UC-blue) or inflammatory bowel disease (IBD-purple) have been identified. Among these loci, those of *NOD2* and *PTPN22* are strongly associated with CD, while that of *HLA* is associated with UC. Some loci such as those of *IL23R* and *MUC19* show different effects in CD and UC but are however strongly associated with the two pathologies. Authorized modification of (Jostins et al., 2012).

The first gene to be associated with CD was *NOD2* (Hugot et al., 2001). This gene is known to activate the nuclear factor NF- κ B, which takes part in numerous signalling cascades that stimulate the transcription of pro-inflammatory genes and protective molecules (de Souza and Fiocchi, 2016). *NOD2* has also been shown to be constitutively expressed in Paneth cells and is necessary for the production of defensins that protect the epithelium from infections

(Kobayashi et al., 2005; Ogura et al., 2003). This latter feature intrinsically links NOD2 with the autophagic pathway. Autophagy is an intracellular process that involves the lysosomal degradation of bacteria but also the self-degradation of organelles in stress situation. Patients homozygous for the risk allele *ATG16L1* (T300A) demonstrate abnormalities in the AMP granules of Paneth cells similar to those observed in mice deficient in autophagy, which therefore suggests that autophagy is necessary for the normal AMP secretion process (Cadwell et al., 2008). Interestingly, *NOD2* signalling is also able to initiate the autophagy process. For optimal intracellular digestion and elimination of bacteria, the functions of *NOD2* and *ATG16L1* must be intact (Corridoni et al., 2014).

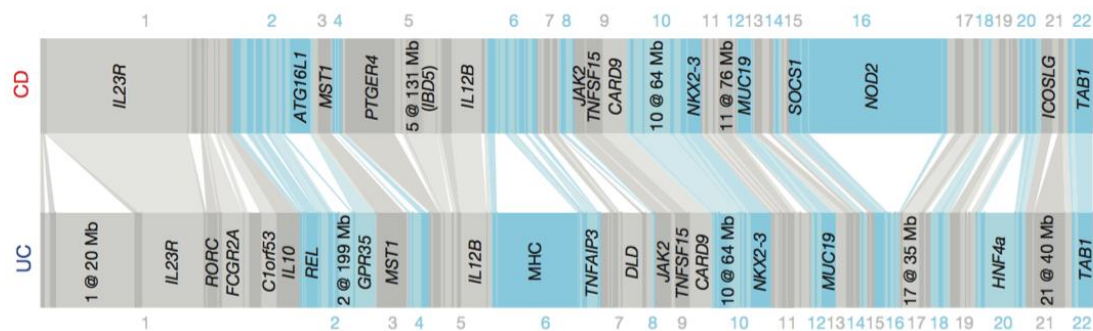


Figure 7. Genetic loci associated with increased susceptibility to IBD

Schematic representation of IBD susceptibility loci for Crohn's disease (CD) and ulcerative colitis (UC). A locus is represented if it denotes at least 1% of the total variance attributed to all susceptibility loci for a given IBD form. Authorised modification of (Jostins et al., 2012).

A critical association between IBD and the *IL23R* gene has also been described (Duerr et al., 2006). The *IL23R* gene encodes a subunit of the IL-23 receptor, a pro-inflammatory cytokine involved in the generation of Th17 cells (McGeachy et al., 2007). Several genes associated with the IL-23 / Th17 signalling cascade have also been identified as a susceptibility loci for IBD; *IL12B*, *JAK2* and *STAT3* (Barrett et al., 2008; Jostins et al., 2012).

Genes within several IBD-associated loci indicate a role for barrier integrity in disease predisposition, where polymorphisms have been reported in the *GNA12*, *HNF4A*, *CDH1* and *ERRFI1* genes (Khor et al., 2011; Jostins et al., 2012). Often, genes involved in intercellular junctions are affected. Genetic studies have shown that truncated forms of the *zonula adherens* protein E-CADHERIN, encoded by *CDH1*, are linked with CD. Intestinal biopsies from CD patients carrying these mutant alleles display abnormal protein localization and cytosolic accumulation (Muisse et al., 2009). Activated G protein $G\alpha_{12}$, encoded by *GNA12*, phosphorylates tight junction proteins ZO-1 and ZO-2, destabilizing cell junctions in epithelial cell lines (Sabath et al., 2008).

4.1.2. Intestinal barrier function

The intestinal epithelium constitutes the first barrier of protection against the microflora contained in the lumen of the intestine. A tightly regulated interaction between the epithelium and its surroundings allowing a permissive environment, while allowing the recognition of invasive bacteria, needs to be implemented. However, in IBD patients, the physical integrity of the epithelial barrier is frequently compromised. Indeed, an increase in paracellular permeability of the epithelium has been documented in both acute and chronic areas of inflammation in patients with IBD (Schmitz et al., 1999) as well as in some of their first-degree relatives (Khor et al., 2011). Animal studies support the idea that inflammation would develop in areas where permeability is affected and even that increased permeability could be a triggering event for this inflammation (Madsen et al., 1999).

The single layer of epithelial cells displays a strict balance between proliferation and apoptosis to maintain the intestinal barrier intact (Edelblum et al., 2006). If cell death is not tightly regulated, it can result in a breach in the barrier, resulting in increased permeability, microbial invasion and inflammation. In this regard, studies have shown that the proliferation and renewal of IEC is accelerated in IBD, with programmed cell death being especially high in CD and UC patients (Di Sabatino et al., 2003). Namely, IFN- γ and TNF- α are two cytokines known to regulate the proliferation and apoptosis of IEC (Kaiser and Polk, 1997; Ruemmele et al., 1998).

Although the permeability defects seen in IBD patients could possibly be caused by the marked apoptosis that occurs during the inflammation process, many studies have clearly shown that apoptosis of epithelial cells alone does not account for the permeability deficits (Laukoetter et al., 2008). The expression and localization of the proteins making up TJ have both been shown to be impaired in patients with IBD, following activation of the mucosal immune system (Landy et al., 2016). TJ are responsible for regulating cell permeability and a drop in their expression or a relocation of the proteins composing them can be directly responsible for this lack of permeability.

It is not only the integrity of the physical barrier that can be compromised in IBD, but also the integrity of the physicochemical barrier, which is provided primarily by goblet cells and Paneth cells. Paneth cells, responsible for producing AMP, may be involved in the establishment of intestinal inflammation. In CD, patients exhibit reduced ileal α -defensin expression, supporting the hypothesis that the composition of the microbiome may be different between IBD patients and healthy patients (Perminow et al., 2010; Wehkamp et al., 2005). Invalidation of the

autophagic genes *Atg16l1* and *Nod2* in mice lead to an abnormal localization and distribution of AMP similarly to what is observed in IBD patients (Cadwell et al., 2008; Vandussen et al., 2014). In UC, the presence of metaplastic Paneth cells is observed. Paneth cells, under normal conditions, are located exclusively in the small intestine. However, Paneth cells are observed in the colon of patients with UC, which may correspond to a defence mechanism against bacterial invasion and intestinal inflammation (Paterson and Watson, 1961). In addition, the expression of multiple AMP specific to Paneth cells has been detected in the colon of patients with UC (Fahlgren et al., 2003).

Goblet cells are also an important player in the protection of the intestinal epithelium and functions defects are often seen in patients with IBD. In CD, early hyper-production of mucus and abnormal glycosylation of the latter are common events. Specifically, patients with CD have moderate or increased expression of mucins in addition to having an increase in their sulfation, resulting in a thicker layer of mucus (Dorofeyev et al., 2013). On the contrary, there is rather a reduction in the number of goblet cells in UC, as well as a decrease in their glycosylation, a reduction in MUC2 and MUC3 expression and a thinner layer of mucus (Dorofeyev et al., 2013). Moreover, mice harbouring mutations in the *Muc2* gene or mice invalidated for *Muc2* expression spontaneously develop colitis, where incorrect folding of Muc2 is observed, leading to its accumulation and consequently to endoplasmic reticulum (ER) stress (Heazlewood et al., 2008; Van der Sluis et al., 2006). A phenotype similar to UC has also been observed in mice with reduced expression of *Nlrp6*, one of the key components of inflammasome signalling (Wlodarska et al., 2014). These mice show a defect in the secretion of goblet cells characterized by protuberant mucus vesicles, which do not fuse with the apical membrane, thus preventing goblet cells from secreting their contents. This secretory defect has been associated with impaired autophagy, again suggesting that autophagy is a phenomenon necessary not only for the exocytosis of PAM granules in Paneth cells but also of the mucus vesicles of the goblet cells.

In summary, the processes of apoptosis, cell permeability, autophagy and ER stress are often disturbed in patients with IBD which particularly cause defects in Paneth cells and goblet cells. Goblet cells appear to be more affected in UC while Paneth cells appear to be more affected in CD, which match the localization of inflammation observed in these pathologies.

4.1.3. Innate and adaptive immune response

In IBD patients as well as in murine models of experimental intestinal inflammation, pro- and anti-inflammatory cytokines have been shown to be produced by IEC and by a variety of mucosal immune cells. Cytokines are a large family of glycoproteins that are pleiotropic. Multiple cells types found in the *lamina propria* of the intestine are cytokine secretors; DC, neutrophils, macrophages, innate lymphoid cells, but also helper and regulatory T cells (illustrated at **Fig. 8**).

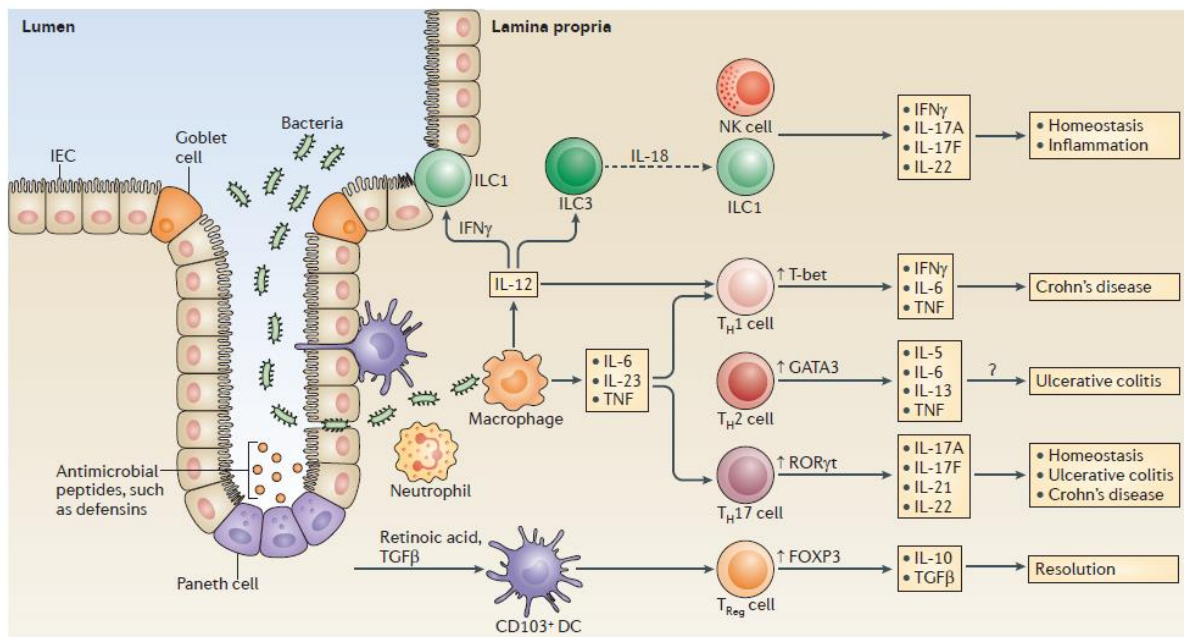


Figure 8. Influence of immune cytokines in the pathogenesis of IBD

In murine models of experimental colitis as well as in IBD patients, anti- and pro-inflammatory cytokines are produced by various cell types of the intestinal mucosa in answer to environmental trigger such as commensal bacteria. Namely, neutrophils, dendritic cells (DC), macrophages, innate lymphoid cells, mucosal effector T cells (Th1, Th2, Th17) are usually regarded as aggravating the inflammation whether regulatory T cells (Treg) help the resolution of the inflammatory onset. Cytokines and transcription factors produced by each cell type are indicated. GATA3: GATA-binding protein 3, IL: interleukin; RORγt: retinoic acid receptor-related orphan receptor-γt, TGFβ: transforming growth factor-β, TNF: tumour necrosis factor. Authorized modification from (Neurath, 2014).

Innate immunity is the first line of protection against microorganisms and other harmful agents' invasion and is quickly activated in response to invasion. It may last for several hours and has no immunological memory (Medzhitov and Janeway C., 2000). Intestinal tissue from IBD patients present increased heterogeneous populations of activated macrophages, unlike the gut of healthy subjects (Wynn et al., 2013; Silva et al., 2016). Macrophages are antigen-presenting cells that can eliminate specific pathogens such as peptides and lipopolysaccharides using proteases and free radicals. They will then present the antigens to T cells through cell membrane histocompatibility complex. The number of macrophages within the intestinal

mucosa rises significantly during an acute period of IBD. Throughout this process, macrophages release a large number of T-cells and costimulatory molecules, such as CD40, CD80 and CD86, involved in the inflammatory process (Silva et al., 2016).

The tissue microenvironment delivers a variety of signals that can direct polarization of monocytes towards a ‘classically activated’ or pro-inflammatory (M1) macrophage or towards an ‘alternatively activated’ or pro-resolving (M2) macrophage phenotype (**Table 1**). In response to Toll-like receptor (TLR) ligands such as LPS or cytokines such as IFN- γ or TNF, naive monocytes acquire an M1 phenotype driven by TLR4 activation and NF- κ B upregulation (Mosser and Edwards, 2008; Shapouri-Moghaddam et al., 2018; Martinez et al., 2008). In turn, these cells produce high levels of pro-inflammatory cytokines such as TNF, IL-1 β , IL-6, IL-12, IL-23 and CCL2 to facilitate immunity to foreign pathogens (Mosser and Edwards, 2008; Shapouri-Moghaddam et al., 2018; Martinez et al., 2008). Conversely, glucocorticoids, immune complexes and IL-4/IL-10/IL-13 cytokines can induce the polarization of alternatively activated M2 macrophages, generally considered as anti-inflammatory macrophages (Na et al., 2019).

	M1 macrophage	M2 macrophage
Polarizing stimuli	IFN- γ , LPS, IFN- γ +LPS	IL-4, IL-13, IL-10, GC, GC+TGF- β
Phenotype	Pro-inflammatory	Anti-inflammatory
Markers/products	TNF- α , IL-1 β , IL-6, IL-23, IL-12	IL-10, TGF- β
Phagocytic activity	High	Low
Antibacterial capacity	High	Low

Table 1. Polarization features of M1 and M2 macrophages

Differentiation and activation stimuli to generate *in vitro* macrophages differ. M1 and M2 macrophages also differ by their phenotype, markers, products secreted, phagocytic activity and antibacterial capacity. GC=glucocorticoids.

In IBD patients and murine models of IBD, there is a disequilibrium favouring pro-inflammatory M1 macrophages at the disadvantage of anti-inflammatory M2 macrophages (Zhu et al., 2014; Rugtveit et al., 1997; Hausmann et al., 2002) leading to incorrect responses to enteric microbiota (Xavier and Podolsky, 2007). Isolated macrophages from CD and UC patients reveal altered cytokine production in response to bacterial challenge: CD macrophages secrete more pro-inflammatory IL-23 but less of the protective cytokine IL-10, whereas UC macrophages constitutively produce high levels of pro-inflammatory cytokine IL-12 (Campos et al., 2011).

Dendritic cells are another innate immune cell type important to IBD pathogenesis. As antigen-presenting cells, DC assimilate and transport antigens to the gut-associated lymphoid tissue (GALT) where naive T cells are activated (Silva et al., 2016). Similarly to macrophages, activated DC accumulate at sites of intestinal inflammation in human IBD and in murine models of intestinal inflammation (Bell et al., 2001; Varol et al., 2010). This causes an elevated release of pro-inflammatory cytokines IL-6 and IL-12 (Hart et al., 2005).

Once the innate immune system has patrolled the *lamina propria* and eliminated the pathogens, a second line of defence is activated; the adaptive immune system. Particularly, T cells were the first subtype to be linked to IBD pathogenesis. Several subgroups of T cells can be discerned depending on the type of cytokines they secrete and their influence on the development of inflammation; Th1, Th2, Th17 and T regulatory (Treg). The subgroups Th1, Th2 and Th17 are effectors while the subgroup Treg is regulator. Traditionally, CD has been associated with a Th1 profile whereas UC is associated with a Th2 profile (Neurath, 2014). The Th17 subgroup is activated in both pathologies and has not been associated with neither of these two pathologies in particular so far (Neurath, 2014). Finally, IBD patients show decreased numbers of the Treg subgroup, which have been shown to have a protective role against experimental colitis by producing anti-inflammatory cytokines that will attenuate the Th1, Th2 and Th17 lymphocytes subgroups. They produce, among other things, IL-10 and TGF- β and express the transcription factor FOXP3, all of which participate in the attempt to resolve the inflammatory phenotype (Neurath, 2014).

4.1.4. Intestinal microflora

The GI tract is colonized by a wide range of diverse microorganisms, known as the microbiota. The intestinal microbiota has several beneficial functions for humans; it metabolizes exfoliated IEC, dietary carbohydrates and mucus to produce metabolites that affect the function of IEC, immune regulation and homeostasis. An altered balance of intestinal bacteria, called dysbiosis, is seen in patients with several diseases of the GI tract, including IBD (Sartor and Mazmanian, 2012). Despite that no persistent pathogen has been identified as being responsible for IBD, there is strong evidence indicating that the gut microbiota has a strong influence on IBD initiation and development (Yilmaz et al., 2019). Specifically, intestinal inflammation is alleviated when animal models of chronic inflammation are re-derived to germ-free status (Strober et al., 2002b), IBD patients have altered reactivity to their intestinal microbiota (Hegazy et al., 2017) and genetic loci for human susceptibility to IBD mainly encode proteins required for mucosal immune defence mechanisms and overlap with genes known to cause

microbiota-sensitive intestinal inflammation in animal models (Maloy and Powrie, 2011). It is however important to note that despite the fact that many common phyla and genera are found between the two species, the murine and human gut microbiomes differ significantly with only 4% of redundant bacterial genes between the two species (Xiao et al., 2015). A closer look reveals key divergences in microbial signatures, especially in respect to the abundance and makeup of microbes. The most apparent difference being the ratio of the two major phyla, with humans having a greater Firmicutes-to-Bacteroidetes ratio, whereas the inverse is true for mice (Park and Im, 2020). Animals might therefore not be an ideal model to study pathologies involving bacterial dysfunctions such as IBD .

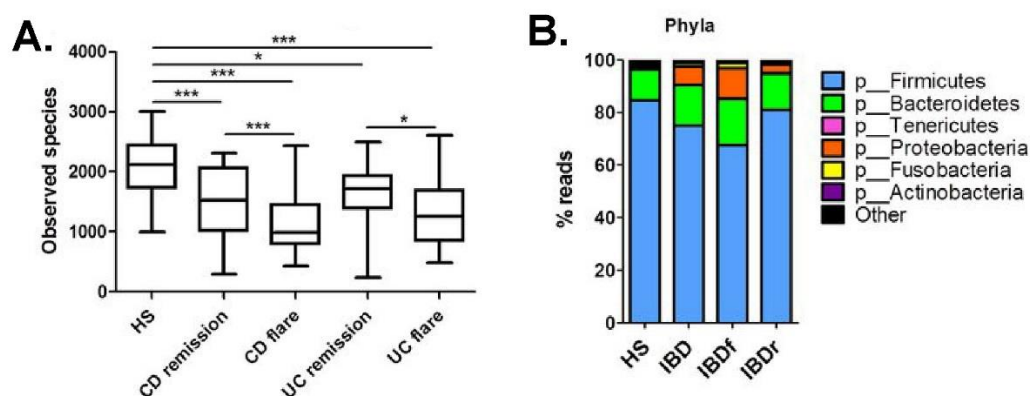


Figure 9. The biodiversity and composition of the intestinal microbiota is altered in IBD
A. Observed species number describing the diversity of the bacterial microbiota in the various groups studied. **B.** Global composition of bacterial microbiota at the phyla level. Healthy subjects (HS) and patient subgroups are labelled on the x-axis and expressed as the relative operational taxonomic unit (OTU) abundance for each group (IBD: inflammatory bowel disease, CD: Crohn's disease, UC=ulcerative colitis, IBDf= IBD flare, IBDr = IBD remission. Authorized modification from (Sokol et al., 2017).

Patients with IBD display reduced microbial biodiversity compared to healthy subjects, while patients in an active flare of inflammation having the lowest bacterial species count (**Fig. 9**). In addition, it is generally recognized that the Bacteroidetes and Firmicutes phyla are decreased while the Fusobacteria and Proteobacteria subgroups are more abundant in patients with IBD (Frank et al., 2007) (**Fig. 9**). However, the relationship between microorganisms and IBD is much more complex than that; the representation of bacterial populations will vary in every individual, according to diet, intestinal transit time, ethnicity, age, disease identity and disease state (Faith et al., 2013; Schloissnig et al., 2013; Flint et al., 2017; Yilmaz et al., 2019). Particularly, recent research uncovered additional differences between UC and CD: notably, short-chain fatty acid (SCFA)-producing bacteria are reduced in CD when compared to UC (Yilmaz et al., 2019). Microbiota-derived SCFAs, in particular butyrate, stimulate the development of Treg cells and mucus production to tune down inflammatory signalling

pathways and to strengthen the epithelial barrier (Furusawa et al., 2013; Gaudier et al., 2004). Thus, the loss of SCFA-producing bacteria may have detrimental effects in CD. Moreover, metagenomics sequencing of stool samples has shown that there is a marked separation between patients with CD and healthy subjects, whereas patients with UC were more heterogeneous and less well-defined from controls, but still showing a general trend towards loss of species diversity (Franzosa et al., 2019). Finally, a recent study could also derive a microbial signature associated with clinical response or remission in answer to anti-TNF therapy in CD, suggesting that the individual's microbiota might determine the clinical outcome to immunotherapy (Yilmaz et al., 2019; Neurath, 2020). In conclusion, while it still remains unclear whether the dysbiosis observed in IBD patients is a cause or a consequence of inflammation, it has now become absolutely clear that the microbiota is involved at multiple levels and that the targeting of the latter or its metabolome will emerge as an attractive novel approach for clinical therapy for patients with IBD.

4.2. Dysregulated molecular mechanisms in IBD

As one might expect from a complex multi-factorial disease such as IBD, many different signalling pathways have been shown to be dysregulated in IBD patients. The importance of the JAK/STAT and Toll-like receptors (TLR)/NF- κ B pathways will be discussed.

4.2.1. The JAK-STAT pathway

The Janus kinase (JAK) and signal transducer and activator of transcription (STAT) are part of a ubiquitous signalling pathway involved in a handful of pleiotropic cascades. Namely, they are critical constituents of many cytokine receptor systems that are known to regulate cell survival, proliferation, pathogen resistance and expression of pro-inflammatory cytokines. A number of ligand, particularly cytokines, can activate the JAK/STAT pathway (Mudter and Neurath, 2003).

Upon cytokine binding to its receptor, receptor-associated JAK proteins are trans-autophosphorylated and activated. JAK proteins phosphorylate residues in the cytoplasmic part of the receptor, thus creating binding sites for STAT proteins. STAT proteins are recruited to the receptor and phosphorylated by JAK kinases. Once phosphorylated, they form dimers that translocate to the nucleus and act as transcription factors for pro-inflammatory genes. A snapshot of the extent of the cytokines and their associated receptors engaging the JAK/STAT pathways is shown at **Figure 10**.

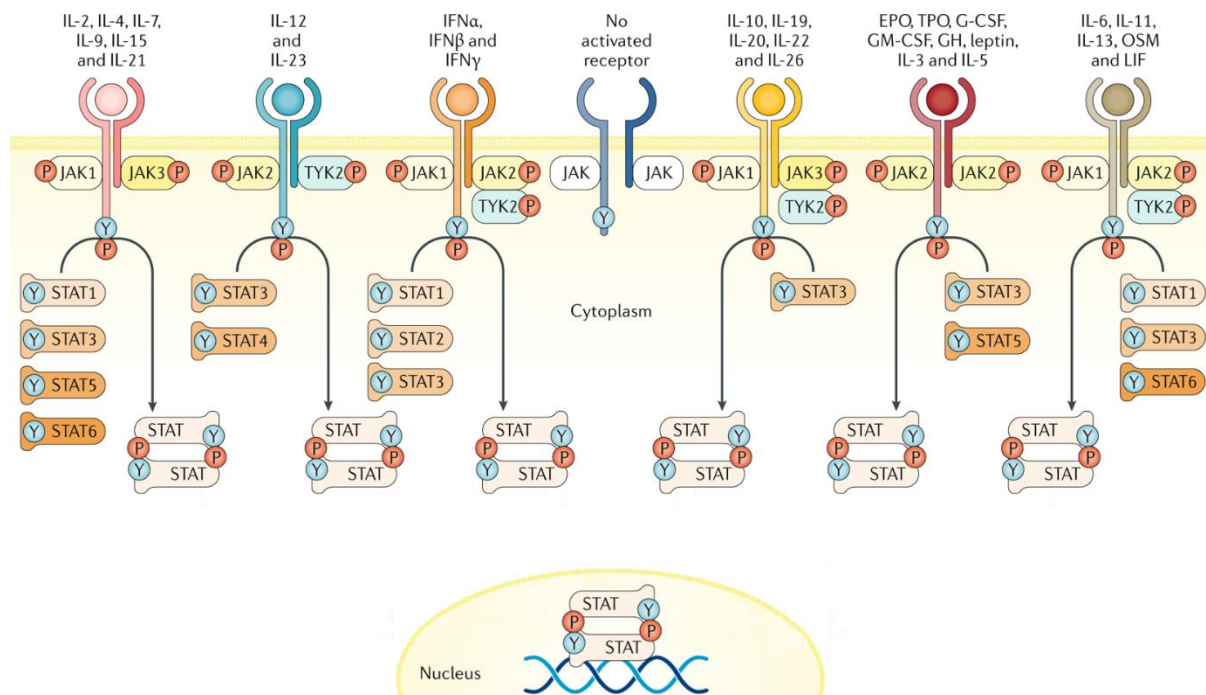


Figure 10. Intracellular signalling pathways mediated by JAK/STAT proteins

The biological effects of more than 50 cytokines and growth factors are mediated through the JAK/STAT pathway. The cytosolic domain of the transmembrane receptors includes tyrosine residues (Y) that can be phosphorylated (P) and act as docking sites for STAT proteins. Activated STAT proteins form dimers that act as nuclear transcription factors for genes involved in pro-inflammatory responses, proliferation and apoptosis. *EPO*, erythropoietin; *G-CSF*, granulocyte colony-stimulating factor; *GH*, growth hormone; *GM-CSF*, granulocyte–macrophage colony-stimulating factor; *IFN*, interferon; *IL*, Interleukin, *LIF*, leukaemia inhibitory factor; *OSM*, oncostatin M; *TPO*, thrombopoietin. Authorized adaptation from (Salas et al., 2020).

There are multiple lines of evidences that link the JAK/STAT signalling pathways to IBD. First, GWAS revealed that polymorphisms in *IL-23R*, *IL-12B*, *JAK2*, *TYK2* and *STAT3* genes are associated with an increased risk for developing IBD (Franke et al., 2010; Jostins et al., 2012; Anderson et al., 2011; Barrett et al., 2008). The locus of a negative regulator of the JAK/STAT pathways, suppressor of cytokine signalling 1 (*SOCS1*) is also associated with an increased susceptibility to both forms of IBD (Jostins et al., 2012).

Furthermore, multiple knock-out murine models have demonstrated the necessity for a functional JAK/STAT signalling in the protection of the epithelium against inflammation (Baird et al., 1998; Suzuki et al., 2000; Bollrath et al., 2009). This has been heavily reviewed through the years (Coskun et al., 2013; Schindler and Plumlee, 2008; Igaz et al., 2001; Kiu and Nicholson, 2012). Essentially, the JAK/STAT pathway has been shown to be essential for T and B cell development, activity and polarization of macrophages as well as barrier function and signal transduction of IEC (summarized in **Table 2**).

Cell type	STAT	Effects and functions in IBD
Lymphocytes	STAT1	Inhibition leads to amelioration of colitis, Important for Th1 function
	STAT3	Inhibition leads to amelioration of colitis, Important for Th17 differentiation
	STAT4	Deletion leads to protection against colitis, Drives Th1 differentiation, suppresses Treg function, Hyperactivation leads to spontaneous colitis
	STAT5	Drives Treg but limits Th17 differentiation
	STAT6	Deficiency leads to protection from colitis, Drives Th2 differentiation, limits Treg induction
Macrophages	STAT1	Reduction of pro-inflammatory cytokine production through IL-19
	STAT3	Inactivation leads to chronic colitis
	STAT6	Polarization of M2 macrophages
Intestinal epithelial cells	STAT1	Activation of IDO-1 (antibacterial/anti-inflammatory)
	STAT3	Promotion of goblet cells, mucus layer and IEC restitution through IL-22, Mucus production through IL-24, Maintenance of mucosal integrity through IL-11
	STAT5	Deficiency leads to increased colitis susceptibility through enhanced crypt apoptosis
	STAT6	Apoptosis through IL-13

Table 2. Summary of important STAT functions

Adapted from (Zundler and Neurath, 2016).

The pleiotropy of cytokines signalling regulated through STAT proteins, and consequently JAK proteins, highlight how important these proteins are for the maintenance of intestinal homeostasis. Importantly, many of the aforementioned cytokines have been shown to be elevated in IBD patients (Nguyen et al., 2015).

4.2.2. Toll-like receptors and NF-κB

Toll-like receptors (TLR) are involved in the recognition of bacterial motifs that work as the immune system's protective sentinels. They recognize pathogen-associated molecular patterns (PAMPs); highly conserved microbial structures such as lipopolysaccharides (LPS), lipoproteins and flagellin (Lim and Staudt, 2013). Upon activation, TLR engage downstream signalling cascades to maintain gut homeostasis by controlling immune response and shaping the microbiota. Activation and dimerization of TLR will activate various intracellular cascades such as NF-κB and mitogen-activated protein kinases (MAPK), known to consequently induce inflammatory cytokines gene transcription. Specifically, engagement of MAPK pathway upregulates the production of TNF-α, IL-1, IL-6, IL-8 and IL-12 while engagement of NF-κB produced anti-microbial factors, cytokines and chemokines that initiate innate and adaptive

immunity (Takeda and Akira, 2004; Wei and Feng, 2010). Activation of NF- κ B and MAPK can occur through other mechanisms than TLR, namely NF- κ B is activated downstream of TNF- α , a cytokine intimately linked with IBD (Gibson, 2004).

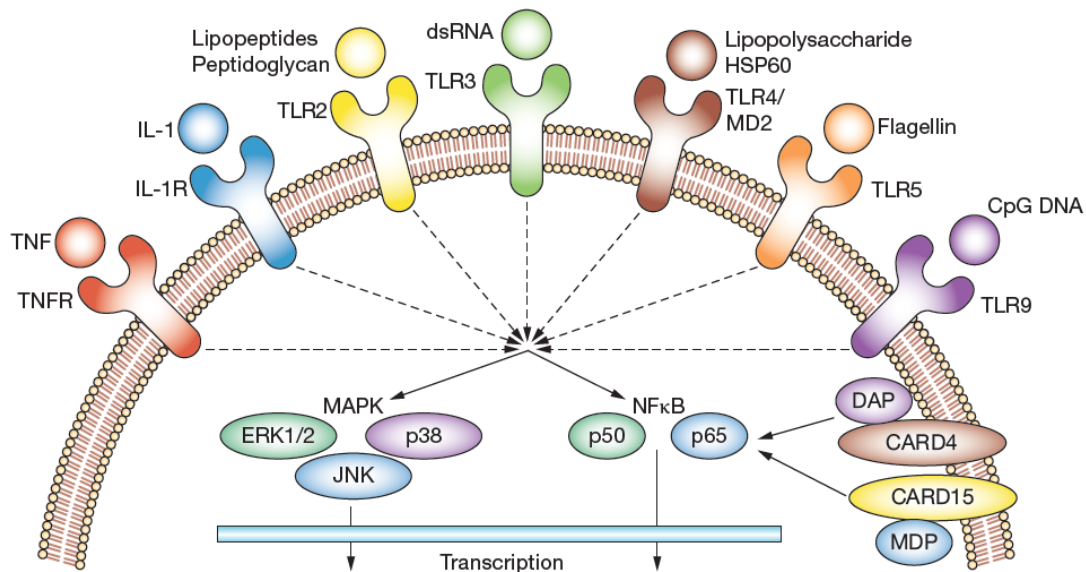


Figure 11. Intracellular signalling of Toll-like receptors

Toll-like receptors on the cell membrane selectively bind to various bacterial, viral or fungal components. This ligation activates conserved pathways that in turn activate NF- κ B and mitogen-activated protein kinases (MAPK) signalling. These transcription factors stimulate the expression of a number of pro-inflammatory and anti-inflammatory genes. CARD4 and 15 (NOD1 and 2) also recognize intracellular bacterial patterns and activate NF- κ B signalling. Other cytokines receptors, such as IL-1R and TNFR, are also known to engage the same pathways as TLR. *CARD*, caspase recruitment domain family member; *CpG DNA*, cytosine–guanine motifs DNA; *DAP*, diaminopimelic acid; *dsRNA*, double-stranded RNA; *ERK*, extracellular signal regulated kinase; *HSP60*, heat shock protein 60; *IL-1*, interleukin-1, *IL-1R*, interleukin 1 receptor; *JNK*, c-Jun amino-terminal kinase; *MAPK*, mitogen-activated protein kinase; *MDP*, muramyl dipeptide; *TLR*, Toll-like receptor; *TNF*, tumour necrosis factor; *TNFR*, TNF receptor. Authorized reproduction from (Sartor, 2006).

Patients with IBD show different patterns of TLR expression in IEC when compared to healthy subjects (Maynard and Weaver, 2009; Abreu et al., 2003; Frolova et al., 2008). Particularly, TLR4 has been shown to be essential to limit bacterial translocation in murine models of IBD (Fukata et al., 2005) and TLR4 gene polymorphisms have been associated with UC and CD (Török et al., 2004; Franchimont et al., 2004). Therefore, by allowing IEC to recognize bacterial patterns and shaping the appropriate tolerogenic or inflammatory immune responses to them, TLR play an important role in the maintenance of intestinal homeostasis.

4.3. Available treatments

Despite that there is currently no definitive cure for IBD available, a few treatments are able to manage the symptoms incurred by IBD; aminosalicylates, corticosteroids, immunomodulators

and biological drugs are the main options available to patients (**Table 3**). The localization and extent of the inflammation will decide which treatment is administered to the patient. Generally, practitioners will start with mild treatments such as aminosalicylates and will move on to corticosteroids or immunomodulators if aminosalicylates are inefficient. Biologicals, expensive monoclonal antibodies delivered intravenously, are usually kept for patients that are refractory to other treatment lines.

Treatment type	Drugs	Mechanism of action	References
Aminosalicylates	Mesalazine Olsalazine Balsalazide Sulfasalazine	Local inhibition of IL-1, TNF- α and PAF	(Wang et al., 2016b; Leighton et al., 2013)
Immunomodulators	Methotrexate Azathioprin	Blockage of <i>de novo</i> pathway of purine synthesis	(Feagan et al., 2014; Maltzman and Koretzky, 2003)
Corticosteroids	Prednisone Hydrocortisone Budesonide	Blockage phospholipase A2, Stimulation of apoptosis in lamina propria lymphocytes, Suppression of cytokines transcription	(Leonard et al., 2012; Reichel et al., 2010)
Biologicals	Infliximab Adalimumab Vedolizumab	Blockage of TNF- α interaction with TNFR: induction of apoptosis in pro-inflammatory cells Blockage $\alpha 4\beta 7$ integrin for leukocyte migration	(Kaser et al., 2001; Kam and Targan, 2000)

Table 3. Conventional treatments for patients suffering from IBD

Adapted from (De Mattos et al., 2015).

More recently, JAK inhibitors have emerged as a promising avenue for the treatment of IBD (De Vries et al., 2017). Indeed, despite the concerning safety profile of pan-JAK inhibitors, single JAK inhibitors have shown great efficacy and safety in clinical trials. Namely, Filgotinib, a small molecule developed by Galapagos that inhibits JAK1, is soon to be available for UC and rheumatoid arthritis patients¹.

However, despite the wide array of treatments available, a significant proportion of patients will fail to respond or develop resistance to these conventional therapies (Weissshof et al., 2018). As a last resort, surgery is necessary to remove the affected region of the GI tract. This

¹ Press release, <https://www.gilead.com/news-and-press/press-room/press-releases/2020/5/gilead-and-galapagos-announce-positive-topline-results-of-phase-2b3-trial-of-filgotinib-in-moderately-to-severely-active-ulcerative-colitis>. Last consulted July 14th, 2020.

strongly highlights the need for the development of novel treatments in IBD. However, IBD is still a poorly understood disease and the chance of developing new therapies is proportional to our level of understanding of its pathogenesis. We therefore need strong and relevant models to study and understand IBD to be able to develop new drugs that can tackle this challenging disease.

Models to study inflammatory bowel disease

The human intestine is a complex organ involving the interaction of multiple cell types together in order to maintain a strict balance between permission and restriction. Obviously, the incompletely described nature of IBD makes it even more challenging to create models that are able to grasp the complexity of the organ and the disease phenotype.

5.1. In vivo models

To this day, the study of IBD has mostly relied on murine models. A quick look into literature will automatically refer you to multiple murine models using genetically engineered mice or chemically-induced IBD, such as with dextran sodium sulfate (DSS) or trinitrobenzene sulfonic acid (TNBS) (Devoss and Diehl, 2014). So far, more than 50 experimental colitis models have been developed and they have informed us greatly into IBD physiology. There are good reasons why they are the preferred IBD study model so far; the murine GI tract closely resembles the human GI tract and offers a complex in vivo environment comprising all cell types that are important to the disease's pathophysiology.

Particularly, murine models have allowed the mechanistic characterization of genes known to be involved in IBD genetic susceptibility. For example, NOD2 an identified susceptibility locus in IBD, particularly in CD (Hugot et al., 2001), has been extensively studied in murine models. One of the first murine models studying NOD2 in IBD, which carries a *Nod2*2939insC mutation similar to the human CD-associated NOD23020insC frameshift mutation, is susceptible to DSS-induced intestinal injury (Maeda et al., 2005). Further murine models have informed us that *Nod2*-mutated mice present impaired Paneth cell function that leads to a reduced production of anti-microbial peptides, explaining the increased susceptibility to intestinal inflammation (Petnicki-Ocwieja et al., 2009). This kind of studies has allowed us to identify Paneth cells, the main producers of factors controlling the intestinal flora in the intestine as key regulators of intestinal homeostasis.

The scientific community has shown a growing enthusiasm towards the implication of the microbiota in IBD. A breakthrough in appreciating the major impact of gut microbiota on disease pathogenesis came by the observation that antibiotic treatment or germ-free breeding of various mouse models of IBD is associated with significantly less severe bowel inflammation and carcinogenesis. As for example, mice deficient for IL-10 expression do not develop intestinal inflammation when kept in germ-free conditions (Sellon et al., 1998). However, these mice show increased susceptibility to experimental colitis and show significant differences in microbiota composition when compared to control mice, which highlights the importance of microbiota in IBD development.

Even though murine studies have helped us tremendously in the comprehension of IBD mechanisms, they are, however, long-lasting studies that present high inter-individual variability. Furthermore, mice are complex organisms in which it is not possible to individually control each of the parameters involved in IBD pathogenesis which makes the interpretation of the results sometimes difficult. Finally, they do not fully recapitulate human IBD since often strong chemicals such as dextran sulfate sodium (DSS) or 2,4,6-trinitrobenzene sulphonic acid (TNBS) have to be used in high concentrations to induce intestinal inflammation (Kiesler et al., 2001). Therefore, the use of mice presents important limitations and better human models of human inflammation are strongly needed.

5.2. In vitro models

In an ideal situation, *in vitro* disease models would allow the integration of primary human disease-relevant cell types in a set-up that is close to the normal environment of the native organ and that supports the basic functions of the latter. Practically, *in vitro* models are often quite far from the physiology of the human body. In this section, past and current models of the human intestine as well as their application to IBD phenotype recapitulation will be reviewed in order to better understand what are their limitations and what further improvements are presently needed.

5.2.1. Cellular sources of intestinal epithelial cells

The intestinal epithelium is responsible for the segregation of the microbiota from our internal tissues and is often the first line of defense to be compromised in IBD. IEC are precisely at the center of this system because of their anatomical and functional position: they secrete and control the composition of the mucus layer on the luminal side, whereas they interact and crosstalk with other cell types on the basolateral side. It is therefore primordial to understand

the intrinsic differences between different IEC sources in order to carefully choose the appropriate cell model.

5.2.1.1. Cell lines

Cell lines are definitely the most represented intestinal cell type used in *in vitro* IBD studies because they are easy to culture, grow rapidly and can be cultured indefinitely. Cell lines are easy to establish from cells harbouring a cancerous origin because of their active proliferative and anti-apoptotic states. However, in the field of IBD, it is preferable to use cells from normal origin in order to model the patient's epithelial phenotype. Unfortunately, there are limited lines from normal origin. Establishing a cell line from normal cells may be challenging and immortalization of the cells could introduce pro-tumoral mutations, which is highly undesirable. The only few normal intestinal cell lines available are not well characterized; most of them were classified as epithelial cells because of their morphology but do not express epithelial markers such as cytokeratins.

The lack of commercially available reliable epithelial cell lines led the researchers to use cells from cancerous origin in IBD studies. Among those, the Caco-2 cell line is particularly attractive because of its human origin. Caco-2 cells are a heterogeneous hypertetraploid cell line originally isolated from a colorectal adenocarcinoma (Fogh et al., 1977). Although derived from a large intestine tumor, the cells differentiate and polarize when cultured under specific conditions in such a way that they resemble, both morphologically and functionally, the enterocytic lineage of the small intestine (Hidalgo et al., 1989). Upon confluence, adjacent cells will organize into a monolayer by forming cell-cell junctions called TJ, thus creating a selectively permeable barrier. Caco-2 cultured on extracellular matrix (ECM) also form villi-like structures that are polarized correctly; cells express polarization markers such as EZRIN, TJ proteins such as OCCLUDIN are localized at the cell membrane and transporters such as GLUT2 and SGLT1 are properly localized at the basolateral and apical membranes, respectively (Marino et al., 2005). Historically, Caco-2 cells have been largely used in drug absorption studies due to their enterocytic features and drug permeability of Caco-2 cells have been shown to correlate with oral drug absorption in humans (Artursson and Karlsson, 1991). As they do not reflect the whole intestinal lineage, they are regularly coupled with mucus-secreting HT-29 cells, that are also derived from a colorectal adenocarcinoma (Fogh and Trempe, 1975; Wikman-Larhed and Artursson, 1995). Despite the increased relevance of the latter co-culture, both cell types are of cancerous origin and are not reflective of the normal physiology of the gut. Indeed, although Caco-2 cells have been found to express a great number

of enzymes and transporters of the human intestine, studies suggest that there are variations between gene expression profiles of transformed epithelial cell lines such as Caco-2 and normal human IEC (Bourguine et al., 2012; Verhoeckx et al., 2015). Furthermore, Caco-2 cells show a blunted phenotype when it comes to IBD modelling; they express low levels of TLR1, TLR3 and TLR4, making them refractive to bacterial challenge (Furrie et al., 2005). As the microbiota is a key player in driving intestinal inflammation, *in vitro* models containing Caco-2 cells might not be ideally suited. Furthermore, the cells do not express some of the major inflammatory cytokines involved in IBD upon stimulation (Eckmann et al., 1993; Parlesak et al., 2004; Haller et al., 2000).

5.2.1.2. Explants

Intestinal primary cells can be isolated directly from a gut biopsy. After excision, the tissue is cultured as an explant, maintained *in vitro* in Ussing chambers or everted sacs (Rozehnal et al., 2012; Alam et al., 2012). Human primary cells closely mimic the physiological state of cells *in vivo* and generate relevant data representing living systems. However, they can only be maintained for a limited time *in vitro* (<8 hours) as the cells will gradually die without the adequate environment, making them unsuitable to study the normal physiology of the gut or host-microbiome crosstalk. Additionally, since you only get a limited number of cells per donor, many donors may be required to conduct experiments therefore increasing results variability. Recently, the culture time span of intestinal primary cells was found to be greatly improved by culturing them in ECM matrices such as Matrigel. In those matrices, primary cells form 3D structures called organoids, explained at [Section 5.2.2.2.](#)

5.2.2. In vitro culture systems

Conventional two-dimensional (2D) cell culture systems have already been used for more than a century (Harrison, 1910). Despite their established value in biomedical research, they cannot support differentiated cellular functions of a specific tissue needed to predict *in vivo* tissue functions and drug activities (Bein et al., 2018). These limitations have directed the development of more complex culture systems, such as those that incorporate multiple cell types in three-dimensional (3D) models that better represent the spatial and chemical complexity of living tissues.

5.2.2.1 Transwell

The Transwell culture has been developed over 25 years ago and is a widely used laboratory tool. Transwells are porous permeable supports inserted into cell culture plates allowing access

to both apical and basal compartments. Permeable support-based culture is the most common *in vitro* culture method for intestine models, especially to study barrier function or model drug absorption (Bein et al., 2018).

In the field of IBD, it is a useful system to obtain a monolayer of polarized IEC when the insert is coated with ECM components. It can be used to partially recapitulate *in vivo* environment by co-culturing IEC with immune cells and adding cytokines or inactivated bacteria to induce inflammatory immune responses. Immune cells can be embedded directly in the gel to mimic tissue-resident cells but can also be cultured in the basal compartment. However, the distance between the epithelium and the immune cells in the latter scenario is elevated and does not allow the interaction of IEC with immune cells. Interestingly, effluents from apical and basolateral compartments can be collected independently for downstream analyses.

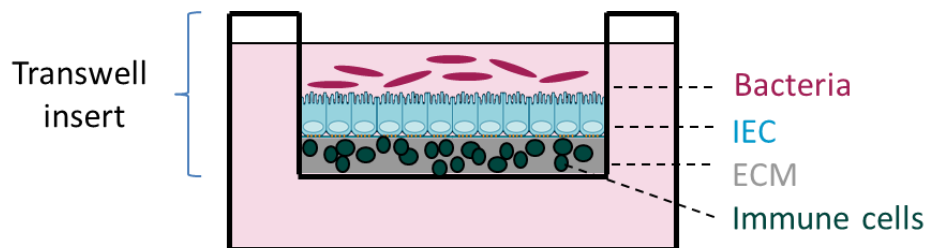


Figure 12. Typical epithelial co-culture in a Transwell system

A schematic representation of an IBD-like model using Transwell inserts. In that model, immune cells are embedded in an extracellular matrix (ECM). Intestinal epithelial cells (IEC) are seeded on the ECM matrix and will differentiate upon formation a confluent monolayer. Inactivated bacteria can also be added to the system to mimic the commensal microbiota.

Transwells have particularly been used to elicit crosstalk mechanisms between IEC, immune cells and bacterial strains. Inoculation of non-pathogenic *E.coli* on Caco-2 cells monolayers did not increase secretion of IL-1 β and TNF- α in the absence of peripheral blood mononuclear cells (PBMC) (Haller et al., 2000). When PBMC were added in the basolateral compartment, the basal production of IL-1 β and TNF- α was elevated by Caco-2 cells was elevated. Another similar study has established a co-culture of Caco-2 cells on a Transwell insert with human monocytic cell line THP-1 in the basolateral compartment (Kämpfer et al., 2017). The Caco-2 barrier was primed with IFN- γ while THP-1 cells were pre-stimulated with LPS and IFN- γ . Upon stimulation, a significant but temporary reduction in barrier integrity was measured as well as an upregulation of pro-inflammatory cytokines IL-1 β , TNF- α and IL-8 release. Interestingly, there was no effect of the inflammatory trigger in absence of THP-1 cells. For reasons previously explained, Caco-2 cells are not ideally suited for the recapitulation of IBD characteristics.

More recently, human primary IEC derived from organoids cultures ([Section 5.2.2.2](#)) could be integrated with monocyte-derived macrophages in a Transwell set up (Noel et al., 2017). Integration of macrophages enhanced barrier function and maturity of IEC monolayers as well as elevated cytokine production. Further infection by enterotoxigenic and enteropathogenic *E.coli* strains allowed macrophages to form intraepithelial projections involved in bacterial sensing but did not upregulate cytokine secretion of the co-culture, leading the authors to postulate that intestinal macrophages rather promote a supportive role in bacterial clearance.

Transwell models provide added physiological relevance compared to conventional 2D culture by combining polarized and accessible IEC with other cell types. However, it is still a static system that lacks organ-level features. Static cultures of IEC often fail to induce villi-like morphology as well as cytochrome P450-based drug metabolism (Bein et al., 2018). A lack of 3D microenvironment may lead to false results due to the poor cytodifferentiation of the cells. In addition, these conventional static models cannot support the co-culture of commensal microbiome with human IEC for more than a few hours, as bacteria rapidly overgrows and contaminates the system within a day (Kim et al., 2012; Kim and Ingber, 2013). Technically, inserts are challenging to image using high content imagers, making microscopy-based screening assays nearly impossible.

5.2.2.2. Organoids

In 2009, the team of Hans Clever adapted isolation and culture methods of human primary IEC in order to sustain long-term expansion and survival of the cells (Sato et al., 2011b, 2009). Stem cells were sorted from intestinal biopsies based on LGR5 expression and embedded in an ECM gel together with key soluble factors, such as epidermal growth factor (EGF), WNT3A, R-SPONDIN and NOGGIN supporting IEC proliferation and differentiation. Under these conditions, IEC will cluster in self-assembling cellular microstructures known as organoids. Using periodic cell dissociation and passaging, these cystic structures can be expanded, frozen and thawed just like regular cell lines would, which was not historically possible with the use of primary cells. Specifically, LGR5-positive stem cells divide to generate a self-renewing stem cell population but they are also able to differentiate towards the four main differentiated cell lineages of the intestine; enterocytes, enteroendocrine cells, Paneth cells and goblet cells. When they reached the apex of the villus-like domain, the terminally differentiated cells are extruded into the lumen, mirroring the biological turnover of the adult intestinal epithelium.

Human intestinal organoids (HIO) can be established from several parts of the GI tract and will adopt the morphology appropriate to the organ from which they were derived. Namely, small intestinal organoids will show centered villi-like domains as well as crypt-like domains that are budding out of the organoid structure (**Fig. 13**), containing enterocytes, Paneth cells, enteroendocrine cells and goblet cells. Oppositely, human colonic organoids will show a perfectly round structure with no discernable crypt- and villi-like structures, containing colonocytes, enteroendocrine cells and goblet cells. Since their establishment, murine and human organotypic cultures have been largely used in the field of IBD.

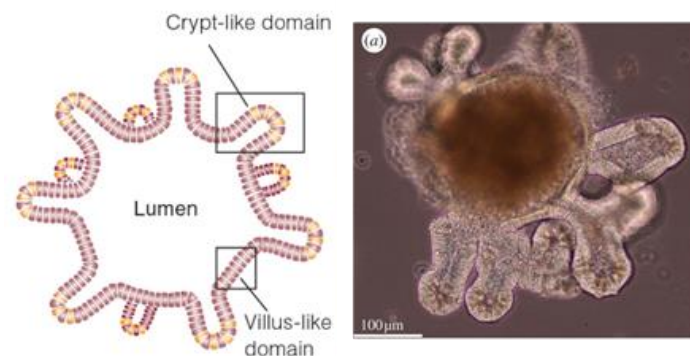


Figure 13. Morphology of small intestinal organoids.

Schematic of a mature intestinal organoid (left) and brightfield image of a mature murine intestinal organoid after 5 days of culture (right). The central lumen is surrounded by an IEC monolayer with budding crypt-like domains. Figure adapted with permission from (Fatehullah et al., 2013).

Hibiya *et al.* used organoids to assess the long-term effect of inflammation on IEC. IEC derived from murine intestinal biopsies were cultured with cytokines and bacterial products for over a year, leading to a notable activation of the NF- κ B pathway (Hibiya et al., 2017). After removal of the cytokines, IEC remained activated even 11 weeks after the removal of the inflammatory stimulus and still showed elevated expression of genes known to be related with colitis-associated cancer. Such long-term studies were not possible before the upraise of the organoids technology; explants cultures could not survive that long *in vitro* and commonly-used IEC lines are already cancerous and therefore cannot be used to study the development of inflammation-associated neoplasia.

Before the advent of the organoid technology, intraepithelial lymphocytes (IEL), a cell type important to IBD pathogenesis, could never be cultured *in vitro*. However upon culture with murine intestinal organoids, IEL could be maintained, stimulated and were exhibiting mobility in culture (Nozaki et al., 2016). Organoids therefore opened the window for detailed research on IEL implication in IBD. Similarly, co-cultures of intestinal organoids with fibroblasts and

enteric nerves have shown to modulate the intestinal stem cell niche (Pastula et al., 2016), highlighting the importance of incorporating multiple cell types to recreate an environment as close as possible to *in vivo* physiology.

Organoids could also help to bridge the gap between *in vitro* and *in vivo* models of IBD. In particular, *in vitro* cultured organoids had the capacity to reconstitute the damaged rectal epithelial layer in a DSS-colitis murine model by trans-anal transplantation (Yui et al., 2012). When those organoids were transplanted into mice, donor cells started to reconstitute the crypt structure and the overall inflammation was reduced, as depicted by the significant improvement of the clinical index of the colitis. Similar results were obtained with small intestinal organoids as well as colonic organoids (Fukuda et al., 2014; Sugimoto et al., 2018). Interestingly, murine small intestinal organoids could even regenerate the inflamed epithelium of the colon and gained expression of colonic marker CA2 (Fukuda et al., 2014). Recently, human colonic organoids could also regenerate the murine epithelium while retaining human characteristics, providing a new approach for therapeutic use of organoids (Sugimoto et al., 2018).

HIO have addressed many of the limitations associated with primary cells, principally their limited *in vitro* lifespan and offer a physiologically relevant option to the culture of immortalized cell lines. However, they do not implement physical cues to which the intestine is subjected and their enclosed lumen (**Fig. 13**) limits their value for drug exposure studies and co-culture with pathogens. As drugs are most frequently delivered orally in patients, it is important to develop models where the apical side of IEC, which would normally be oriented towards the intestinal lumen, is accessible.

5.2.2.3. Organ-on-a-chip

Organs-on-chips are microfluidic devices allowing the culture of multiple cell types together in continuously perfused chambers (**Fig. 14**). Generally, microfluidic devices are separators in chambers or microchannels where different cell types can be introduced. The microfluidic component of this technology allows modelling of physiological functions of tissues such as blood flow or peristaltic movement. Microchannels are often separated by porous membranes to recreate the interface between different cell types. Organs-on-chips can also incorporate physical forces such as fluid shear stress, mechanical compression or cyclic strain. In the past decade, various microfluidic platforms have emerged. Notably, the poly-dimethylsiloxane (PDMS) devices are the most commonly used because of their inexpensive, easy-to-produce and moldable character, which makes easier the adaptation of their size, shape as well as the

number of microfluidic channels used and their structure. However, the limited throughput level and adsorptive nature of the chips make them inappropriate for compound screening.

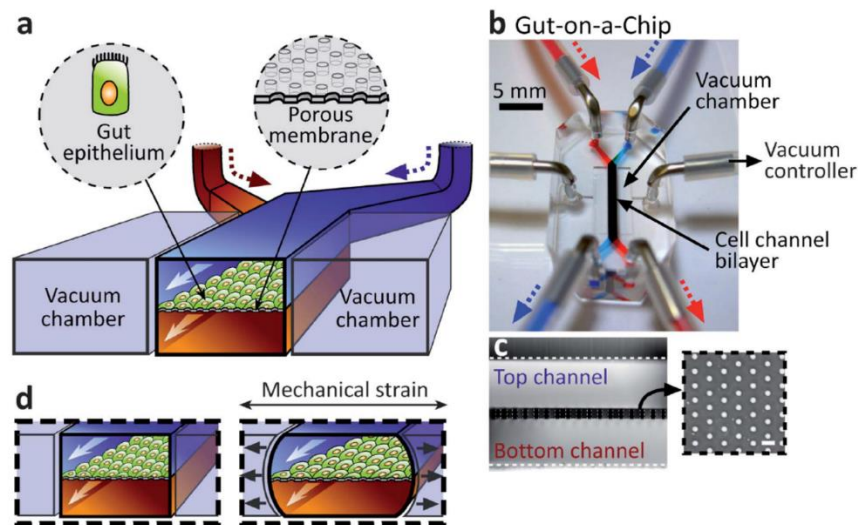


Figure 14. Representation of a PDMS-based organ-on-a-chip device

A. Transversal schematic view of a 2-channels device used for gut modelling. The continuously perfused upper (blue) and lower (orange) channels are separated by a flexible porous membrane. In this case, the membrane is coated with ECM before it is lined with gut epithelial cells. The microfluidic channels are flanked by full height vacuum chambers on each side to induce cyclic suction that will mimic peristaltic motions of the intestine. **B.** A photographic image of the organ-on-a-chip device composed of clear PDMS elastomer. A syringe pump perfuses (direction indicated by blue and red arrows) blue and red dyes through tubing to the upper and lower channels, respectively, to visualize these channels. Tubing connects a vacuum controller to vacuum chambers to create cyclic suction. **C.** Cross-sectional view of the microfluidic channels; square inset shows a top view of the porous membrane. **D.** Schematic illustration of the direction of fluid flow direction and mechanical strain. Authorized reproduction from (Kim et al., 2012).

The first demonstration of the Organ-on-a-chip technology applied to gut modelling came from the team of Kim *et al.* that lined PDMS devices with Caco-2 cells and subjected them to fluid flow and cyclic strain that mimics peristalsis-like movements (Kim et al., 2012). Upon only 3 days of microfluidic culture, cells displayed increased cell height compared to cells grown on Transwell inserts for 21 days. After 5 days, cells spontaneously formed crypt-villi structures with high aminopeptidase activity suggesting enterocytes' cytodifferentiation. They further investigated whether the gut-on-a-chip could support co-culture of microbial flora with the normal intestinal microbe, *Lactobacillus rhamnosus* GG, on the apical surface of the Caco-2 monolayer. The co-culture could be maintained for more than 6 days without viability nor transepithelial resistance values being affected. This is a major advancement; live bacteria could never be cultured with IEC for more than 24 hours in previous static setups. They further characterized the model and showed that Caco-2 cells could express markers associated with

differentiated cells types other than enterocytes; notably cells gained expression of the goblet cell marker MUC2 exclusively in the villi-like structures (Kim and Ingber, 2013). Finally, they showed that CYP3A4 activity and glucose uptake activity were largely upregulated when compared to static Transwell models. This is an unprecedented finding as Caco-2 were thought to be devoid of CYP3A4 activity before. These results definitely highlight the potential of the model in drug development and toxicity testing (Prueksaritanont et al., 1996; Kim and Ingber, 2013). The advent of Organ-on-a-chip models such as those allowed the uncoupling of each IBD causative factor, in this case the intestinal epithelium, the immune cells and the microbial cells and their cellular product, to assess their individual and combinational contribution to the IBD phenotype in a spatiotemporal manner. Such detailed analyses cannot be performed using *in vivo* models and provides great strength to the Organ-on-a-chip technology.

Recently, a few organ-on-a-chip models using human primary IEC have emerged. A first study have generated a gut-on-a-chip using duodenal organoids derived from human biopsies (Kasendra et al., 2018). Using PDMS devices where the porous membrane was coated with ECM components and the lower channel with microvascular cells, enteroids fragments were loaded in the device's upper channel. IEC derived from duodenal organoids formed crypt-villi structures in a flow-dependent manner and the addition of microvascular cells strengthened the barrier function of the epithelial monolayer. The study elegantly showed that IEC could differentiate towards all the expected differentiated intestinal cell lineages; absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells. The epithelial monolayer displayed correct polarization with apical expression of F-ACTIN, NHE3 and VILLIN, basolateral expression of Na⁺/K⁺-ATPase and basal expression of Integrin β 4. An important technological advancement of the Organ-on-a-chip technology is that the apical side of the cells form an open and accessible lumen in the upper channel, which can be exposed to drugs and microbiota and prevents the major technical difficulties linked with organoids. Finally, transcriptomic analyses revealed that the RNA expression profile of the duodenum-on-a-chip was closer to the organ's situation than the organoids used for chip seeding when looking at functional areas related to host defence response, digestion, response to nutrients, drug transport and cell proliferation (Kasendra et al., 2018). The same team further investigated the specific application of their model to compound testing (Kasendra et al., 2020). When focussing on the expression of genes involved in specific absorption and metabolism functions, the duodenum-on-chip displayed a transcriptomic profile much closer to the *in vivo* situation than static organoids. CYP3A4 expression, a member of the cytochrome P450 drug metabolizing enzymes family, was found to be high in the duodenum-on-chip and similar to

native duodenum levels, while the expression was almost absent in the Caco-2 gut-on-a-chip, supporting the higher relevance of using human primary material to study drug metabolism and transport. However, no study has yet studied the application of gut-on-a-chip models to the biotransformation of xenobiotics.

Finally, a team integrated the human induced pluripotent stem cell (iPSC) and organ-on-a-chip technologies together to create a gut-on-chip model (Workman et al., 2018). iPSC were directed towards HIO cultures, which were later used for chip seeding. They demonstrated that the gut-on-a-chip was responsive to exogenous stimuli by stimulating with IBD-upregulated cytokine IFN- γ . The iPSC-derived gut-on-a-chip demonstrated robust STAT1 phosphorylation in answer to trigger, as well as upregulation of IFN- γ target genes indoleamine 2,3-dioxygenase 1 (IDO1) and guanylate binding protein 1 (GBP1) in a stronger manner than in a Caco-2 gut-on-a-chip. A combined exposure to IFN- γ and TNF- α increased epithelial permeability without increasing cell death and recapitulates an important event in IBD pathogenesis. This model represents an important advance; iPSC can be noninvasively isolated from any given patient and have major implications for personalized medicine.

These models have already answered several limitations of other *in vitro* methods when it comes to IBD modelling. Firstly, Caco-2-based models display higher polarization and differentiation states while adopting an *in vivo*-like organization; they are able to organize into villi-like structures. Secondly, primary IEC-based models exhibit an accessible lumen that can be exposed to microbiota or drugs. Importantly, organ-on-a-chip culture allows, for the first time, bacteria and IEC to be co-cultured together for longer periods of time, without having the bacteria overtake and kill the epithelium. Furthermore, organ-on-a-chip models of the human intestine show higher transcriptomic and functional similarity to the native intestine. Notably, expression levels of genes involved in drug transport and metabolism are close to *in vivo* levels, suggesting that organ-on-a-chip models would be highly appropriate to question such mechanisms. However, no study has yet performed drug exposure studies on human primary gut-on-a-chip models. Furthermore, the application of these models to IBD modelling has not been thoroughly investigated. Notably, no study has investigated the crosstalk between IEC and immune cells in the development of inflammatory conditions. Finally, we are presently lacking models that have the potential to be applied to high throughput screenings that will become necessary in the search for novel IBD targets and treatments.

THESIS AIMS – CHAPTER I AND II

Clinical trials are a long and extensive process and most of the drugs entering this process will never make it to the patients. In fact, nearly 90% of drugs fail the preclinical to Phase I transition (Hay et al., 2014). From the 10% of drugs that successfully make it to Phase I, only 18% will make it through all 3 test phases when it comes to pharmaceutical drug development in CD (Parker and Kohler, 2010). While the later stages could be improved by implementation of more reliable biomarkers or by stratifying the patients, that will not increase the numbers of drugs effectively entering Phase I. Animal models have limited use in predicting the clinical relevance of therapeutic targets in humans. We lack strong and spontaneous colitis models, as most models are chemically-induced models which do not recapitulate the natural events occurring in IBD patients. On the other hand, current *in vitro* models lack the biological relevance incurred by the use of primary cells and fail to address the relationship between the intestinal epithelium and the adjacent immune cells, which are the main two cell types responsible for the maintenance of intestinal homeostasis. There is therefore a strong need for reliable and physiologically relevant *in vitro* models of the human intestine for the recapitulation and investigation of IBD. As up to 30% of IBD patients are refractory to treatment, there is an imminent urgency to grasp the molecular mechanisms of the pathology in a comprehensive manner. Furthermore, after our knowledge on the mechanisms is deepened, we will need to have suitable high throughput models to test the efficacy of the new compounds synthesized before moving to clinical trials.

The work presented in this thesis has therefore focussed on three major objectives, 2 of which will be deepened in Chapter I and II of this thesis:

1. Creation of a robust and simplified *in vitro* microfluidic model of the human intestine
 - a. Induce IBD-like conditions with a clean simple trigger
 - b. Validate the potential of the model for target and compound screening

In Chapter I and Appendices A-B, the development of an easy-to-use microfluidic gut-on-a-chip model is discussed. The goal was to specifically create a Caco-2-based model that would allow integration of the shRNA technology, to potentially be applied to larger screening campaigns to identify inflammatory targets. Furthermore, as Caco-2 are presently considered the ‘gold standard’ in drug absorption studies, its potential for drug exposure studies had to be validated.

2. Creation of a physiologically relevant *in vitro* microfluidic model of the human intestine addressing the immune-epithelial crosstalk
 - a. Confirm the increased biological relevance of the model
 - b. Induce intestinal inflammation resembling IBD
 - c. Apply this model to proof-of-concept experiments that evaluate compounds efficacy

In Chapter II and Appendices C-D, the development of a sophisticated gut-on-a-chip composed of a co-culture of human primary IEC and human primary macrophages is discussed. The goal was to validate the increased biological value brought by the microfluidic culture of IEC over standard static models, to apply this model to the recapitulation of known IBD events and to test a restricted set of compounds to evaluate whether this type of model could be useful to assess the efficacy of treatments.

CHAPTER I

Development of a gut-on-a-chip model for high throughput disease modeling and drug discovery

Preface

This chapter relates the development of the first microfluidic gut-on-a-chip model of this thesis, using the Caco-2 cell line subjected to an inflammatory cytokine cocktail. The results presented in this Chapter have been published in International Journal of Molecular Sciences (Beaurivage et al., 2019). *For continuity, figures and tables have been re-labelled to follow a continuous order throughout the thesis.*

Abstract

A common bottleneck in any drug development process is finding sufficiently accurate models that capture key aspects of disease development and progression. Conventional drug screening models often rely on simple 2D culture systems that fail to recapitulate the complexity of the organ situation. In this study, we show the application of a robust high throughput 3D gut-on-a-chip model for investigating hallmarks of inflammatory bowel disease (IBD). Using the OrganoPlate platform, we subjected enterocyte like cells to an immune-relevant inflammatory trigger in order to recapitulate key events of IBD and to further investigate the suitability of this model for compound discovery and target validation activities. The induction of inflammatory conditions caused a loss of barrier function of the intestinal epithelium and its activation by increased cytokine production, two events observed in IBD physiopathology. More importantly, anti-inflammatory compound exposure prevented the loss of barrier function and the increased cytokine release. Furthermore, knockdown of key inflammatory regulators *RELA* and *MYD88* through on-chip adenoviral shRNA transduction alleviated IBD phenotype by decreasing cytokine production. In summary, we demonstrate the routine use of a gut-on-a-chip platform for disease-specific aspects modeling. The approach can be used for larger scale disease modeling, target validation and drug discovery purposes.

Keywords

inflammation, inflammatory bowel disease, gut-on-a-chip, organ-on-a-chip, microfluidic, drug discovery, disease modeling

Introduction

Inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn's disease (CD), is a complex chronic idiopathic disease severely incapacitating the life of more than 2.5-3 million Europeans, bringing high socio-economic burden to society (de Souza et al., 2017; Kaplan, 2015; Burisch et al., 2013). The aetiology of the disease remains elusive, however, it is known to involve the interaction of genetic, environmental, microbiological and immunological factors (Burisch et al., 2013; de Souza and Fiocchi, 2016). There is a general consensus in the scientific community that IBD arises from an excessive activation of immune effectors in response to commensal microbiota, which is triggered by environmental factors in a genetically-susceptible host (Matsuoka and Kanai, 2015; Pedersen et al., 2014). Important and frequent primary events in IBD include loss of function of the epithelial barrier and an impaired balance between pro- and anti- inflammatory mediators secreted by intestinal epithelial cells (IECs) or immune cells (Atreya and Neurath, 2015; Pedersen et al., 2014).

The multifactorial nature of IBD makes it extremely difficult to develop realistic disease models that will be able to grasp and recapitulate the complexity of the disease development and progression. This is particularly important knowing that 25-30% of patients fail to respond to standard IBD therapy and more than 20% have to discontinue ongoing treatments due to unforeseen side-effects (Carter et al., 2004). The need for deeper understanding of the disease's mechanisms and for the development of new medicines is therefore urging.

Different *in vivo*, *ex vivo* and *in vitro* models are currently used to study IBD aetiology (Mizoguchi, 2012; Tsilingiri et al., 2012; Mokry et al., 2014; McKay et al., 1997). Despite the interspecies difference, animal models have proven to be extremely useful to understand IBD mechanisms such as the essential role of microbiota and T-cells (Eri et al., 2012; Roy et al., 2017). However, manipulation of individual IBD parameters, such as barrier function of the epithelium and cell-type specific activation, are difficult to achieve in these complex models which proves to complicate matters and in turn leads to poor prognosis of drug candidates (Prinz et al., 2011). Furthermore, from a drug discovery perspective, high throughput screening of compounds cannot be achieved in such models. On the other hand, *ex vivo* models such as intestinal explants come from a limited source with an extremely limited lifespan, making them unsuitable for drug discovery purposes (Tsilingiri et al., 2012; Fritschfredin et al., 2008; Vadstrup et al., 2016). Standard *in vitro* setups usually include the static culture of epithelial cells on rigid membranes separating two different chambers, sometimes comprising an immune component (Hilgers et al., 1990). Such conventional membrane insert based systems, often

called 2.5D or Transwell systems, are a useful tool with medium throughput but do not recapitulate 3D cell organization, which has proven to be important for increased physiological relevance (Edmondson et al., 2014). Given the limitations of existing models, there is a need for a tool that will mimic key aspects of IBD in a robust manner that is compatible with high throughput screening equipment.

The recently emerged field of Organ-on-a-Chip technology offers a potential alternative to traditional 2D cell cultures and animal models. Recent studies have shown enormous progress in both gut-on-a-chip and IBD research (Lee et al., 2016). In most cases, chips made out of a silicon rubber material are seeded with either cell lines, induced pluripotent stem cells (iPSCs) or Lgr5⁺-derived organoids (Kasendra et al., 2018; Lee et al., 2016). These studies are extremely valuable to assess the impact of micro engineering techniques on the physiological relevance of cell culture models. Nevertheless, there are some shortcomings associated with such silicon rubber chips including the lack of scalability and the reduction of bioavailability of some drugs and small molecules due to unspecific adsorption (Toepke and Beebe, 2006).

To overcome these limitations and to allow integration of Organ-on-a-Chip in the drug development process, we further adapted a recently published gut-on-a-chip system (Trietsch et al., 2017) to mimic inflammatory conditions. This model, based on the use of Caco-2 cells, has already been proven to be easy-to-use, robust and reproducible as well as relatively fast; Caco-2 cells form 3D leak-tight polarized tubules with accessible apical and basal sides after only 4 days of culture. The platform we used in this study, the OrganoPlate, offers a high throughput alternative to silicon rubber chips and allows compound screening. When compared to Transwell, the OrganoPlate offers a membrane-free handier system, with lower reagents consumption and high throughput real-time imaging capabilities.

To mimic inflammatory characteristics in this model, we applied an optimized immune-relevant cytokine trigger that mimics the effect of *E.coli*-activated DC on the IEC (Coler et al., 2011; Peral de Castro et al., 2012; Gad et al., 2011). We assessed the effect of this trigger on two main aspects of IBD; the integrity of the intestinal barrier as well as the cellular activation of IECs. To assess barrier integrity, we measured transepithelial electrical resistance (TEER) values of Caco-2 tubules in a high throughput manner and assessed the localization of cell junction-associated protein E-CADHERIN. The production of epithelial-relevant inflammatory cytokines by Caco-2 cells was used as a readout for cellular activation following trigger.

To assess the applicability of the model for target and drug discovery processes, we performed on-chip adenoviral transduction of validated shRNAs against proinflammatory targets RELA and MYD88. We further addressed the ability of the model to respond to well-known anti-inflammatory compound TPCA-1 (Podolin et al., 2005; Ivanenkov et al., 2011). Overall, our results show that this 3D model can be used as an *in vitro* tool for drug development in an accelerated manner and opens the way to more physiologically relevant models usable in high throughput experiments.

Methods

Ethics statement

The research described here has been performed according to applicable Dutch national ethics regulations and was conducted within Galapagos BV (Leiden, NL). Scientists from Galapagos BV are qualified to perform research using human material and have appropriate facilities and equipment available to comply with applicable laws, regulations and internal rules related to handling and storage of the material. The human material was obtained from Sanquin (Amsterdam, NL). The supplier has confirmed to Galapagos BV that informed consent from the donors to use the material for research purposes was received. The cells were solely used for target and drug discovery and were not used for human experimentation or therapy. All material is and will remain anonymized.

Cells

Human colon adenocarcinoma cell line Caco-2 (ECACC 86010202) was cultured in EMEM (ATCC, #30-2003) supplemented with 10% FBS (Sigma Aldrich, #F2442), 1% NEAA (Gibco, #11140050) and 1% Penicillin/Streptomycin (Sigma Aldrich, #P433). Cells were cultured at 37°C in a humidified atmosphere with 5% CO₂ up to 80% confluency and then either subcultured or used for the experiments. All experiments were performed on cells between passages 47 and 60.

OrganoPlate seeding and tubule formation

Detailed extracellular matrix (ECM) loading and seeding procedures of Caco-2 cells in three-lane OrganoPlate were performed as previously reported (**Fig. 15A-C.**) (Trietsch et al., 2017). In summary, 20 000 Caco-2 cells were seeded against a Collagen I gel (Cultrex, #3447-020-01) and let to attach against the gel for 4 hours (37°C, 5% CO₂). After attachment, EMEM medium was added to inlets and outlets and medium perfusion was started by putting the plate

on an interval rocker (Perfusion Rocker Mini, Mimetas) switching between a $+7^\circ$ and -7° inclination every 8 min (37°C , 5% CO_2).

Triggering of IBD-like conditions

Caco-2 cells were triggered basally with an adapted cocktail of cytokines (IL- 1β , IFN- γ and TNF- α at 2, 100 and 100 ng/mL respectively, all from ImmunoTools). Three trigger times were used throughout experiments; Day 7 to Day 11, unless stated otherwise.

Cytokine secretion of Caco-2 cells

The concentrations of Macrophage Inflammatory Protein-3 (CCL-20/MIP3A), IFN-Gamma-Inducible Protein 10 (IP-10/CXCL10), Interleukin-8 (IL-8/CXCL8), Interleukin-6 (IL-6), Interleukin-1-Beta (IL- 1β) and Tumor Necrosis Factor-Alpha (TNF- α) were quantified in the supernatants from both apical and basal channels using a human multiplex assay (Thermo Fisher) on a Luminex FlexMap 3D (Merck Millipore) according to manufacturer protocol.

TEER measurements

Transepithelial electrical resistance (TEER) was measured at different time points using an automated multichannel impedance spectrometer designed for use with the OrganoPlate (OrganoTEER, Mimetas). Before measurement, medium was added in the middle inlets and outlets and the OrganoPlate was returned in the incubator (37°C , 5% CO_2) to equilibrate for an hour. The electrode board of the OrganoTEER is matched to the OrganoPlate such that when an OrganoPlate is placed in the OrganoTEER, electrode pairs are dipped in the medium in all inlet and outlet wells connecting to the basal and apical side of all tubes. Point impedance measurements were performed by frequency sweep from 5Hz to 1MHz (75 points at precision 0) with TEER values going up to $1000\ \Omega\cdot\text{cm}^2$. Data was analysed using the OrganoTEER software, which automatically extracts the TEER contribution (in Ohm) from the measured spectra and normalizes it to $\text{Ohm}\cdot\text{cm}^2$ by multiplying by the tubule-ECM interface (estimated at $0.0056\ \text{cm}^2$).

Immunohistochemistry

Caco-2 tubules were fixed and prepared for immunohistochemistry as previously described (Trietsch et al., 2017). The primary antibody mouse α -E-CADHERIN (Abcam AB1416, 1:100) and the secondary antibody donkey α -mouse AlexaFluor647 (Molecular Probes A31571, 1:250) were used. Actin was stained using ActinGreenTM 488 ReadyProbesTM Reagent (Thermo Fisher, R37110) and nuclei was stained using NucBlueTM Fixed Cell ReadyProbesTM Reagent (Thermo Fisher, R37606), both reagents were added in the secondary antibody

solution according to manufacturer's instructions. All steps were performed at room temperature on an interval rocker (Perfusion Rocker Mini, Mimetas) switching between a +7° and -7° inclination every 1 min. Cells were imaged on the InCell 6000 (GE Healthcare Life Sciences) and Micro XLS-C HCI System (Molecular Devices, US).

The organization of the epithelial cell layer forming the Caco-2 tubule was quantified by segmentation of the E-CADHERIN (ECAD) positive cell junctions with Cell Profiler 3.0 (Carpenter et al., 2006). ECAD signals were enhanced using a neurite tubeness method to amplify the junction patterns. Subsequently the junctions were identified with a three classes adaptive Otsu threshold. To reduce fragmentation the objects were merged and filtered with area of > 600 pixels. The MeasureObjectSizeShape module was used to extract non-intensity related features of the ECAD objects and the MeasureImageIntensity module for extraction of intensity-related measurements from the ECAD objects. Input images and final output segmentation overlays are printed at **Figure 22**. Based on this segmentation process, two characteristics of the organizational pattern of the ECAD staining were quantified: compactness and major axis length of signal. The compactness represents the mean squared distance of the object's pixels from the centroid divided by the area. The major axis length represents the pixels' length of the major axis of the ellipse that has the same normalized second central moments as the region. A more tightly packed and organized epithelial cell layer will have a more continuous pattern of tight junctions leading to objects with a long length axis and a high compactness value.

Adenoviral preparation

For the generation of shRNA-carrying adenoviruses (AdV), pIPspAdapt-based constructs were transiently transfected with AdV5.Fib50 helper DNA into PER.C6/E2A producer cells (Crucell BV) as previously described (Arts et al., 2003; Michiels et al., 2002). The produced AdV were propagated by infecting PER.C6/E2A cells again. Throughout procedures, PER.C6/E2A cells were cultured in DMEM supplemented with 10% FBS and 10 mM MgCl₂. Finally, titres of the crude lysates were determined as described (Rippmann et al., 2009).

Insert name	Target sequence
AdV-shluc	GGTTACCTAAGGGTGTGGC
AdV_shmmNr1h3	CACACATATGTGGAGGCCC
AdV_shMYD88	GGTTCATCACTGTCTGCGA
AdV_shRELA	GATTGAGGAGAAACGTAAA

Table 4. shRNA adenoviruses selected for the study

For transduction efficiency determination, a knock-in AdV for ZsGreen was used; The ZsGreen reporter-gene construct was adapted from the pZsGreen-C1 vector (Clontech). A 3x NLS sequence was cloned directly downstream from the ZsGreen open reading frame for nuclear expression. The ZsGreen-3xNLS sequence was cloned into an AdV adapter plasmid containing a CMV promoter by standard restriction enzyme digestion and ligation. Viruses were selected based on their ability to reduce CCL20 production by Caco-2 cells (See **Fig. 23**).

Adenoviral transduction

One day after seeding, Caco-2 cells were exposed apically to different shRNA-carrying recombinant AdV for 6h (MOI of 5 IU/cell) in absence of Penicillin/Streptomycin on an interval rocker switching between a +7° and -7° inclination every 8 min (37°C, 5% CO₂). After transduction, medium was replaced with 50µl EMEM complete in all inlets and outlets and plates were put back on the interval rocker (37°C, 5% CO₂) for the rest of the experiment.

Transduction efficiency

Caco-2 cells treated with a ZsGreen-expressing virus were imaged 72h after transduction to determine transduction efficiency. Cells were stained for 30 min at 37°C with NucBlue™ Live ReadyProbes™ Reagent (Thermo Fisher, R37605) following manufacturer's instructions and were then imaged on the InCell 6000 (GE Healthcare Life Sciences). The transduction efficiency was calculated with an in-house algorithm quantifying the number of ZsGreen-positive cells relatively to the number of total nuclei.

Quantitative PCR

Total RNA was isolated from Caco-2 tubules using RNeasy Mini Kit (QIAGEN, # 74106) and converted to cDNA using TaqMan™ Reverse Transcription Reagents (Applied Biosystems, #N8080234), according to manufacturer's instructions. Real-time quantitative amplification (qPCR) was then performed using TaqMan® Fast Advanced MasterMix (Applied Biosystems, #4369016) on a LightCycler® 480 instrument. Probes for MYD88 (Life Technologies, Hs01573837_g1), RELA (Life Technologies, Hs00153294_m1) and GAPDH (Life Technologies, Hs02758991_g1) were used.

Compound exposure

Four days after seeding, Caco-2 cells were pre-treated apically and basally with TPCA-1 (Sigma Aldrich, Cas 507475-17-4, #T1452) compound in a concentration range from 0.005µM to 20µM in a final DMSO concentration of 0.22%. After a pre-exposure of 2h, basal medium was removed and replaced with cytokine-triggering medium (IL-1β, IFN-γ and TNF-α at 2,

100 and 100 ng/mL respectively, all from ImmunoTools) in presence of the compound for another 72h.

Viability assay

AlamarBlue Cell Viability Reagent (Thermo Fisher, # DAL1025) was pre-mixed in EMEM in a 1:10 ratio and 50µl was added to medium inlets and outlets. Plates were incubated at 37°C for 4h and fluorescence signals were measured (Excitation: 530-560nm, Emission: 590nm) with a multi-well plate fluorimeter (Fluoroskan Ascent FL, Thermo Fisher).

Statistics and data analysis

Data was analysed using GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA, USA). Unless stated otherwise, values are expressed as mean $\pm$ Standard Error of the Mean (SEM). When two groups of data were analysed, a two-tailed, unpaired Student's *t*-test was used to determine the statistical significance. When three or more groups were analyzed, parametrical or robust ANOVA were used to determine the statistical significance, depending on the presence of outliers. Differences with $p \leq 0.05$ were considered significant (*ns* $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). All graphs shown contain results from one representative experiment containing at least three technical replicates.

Results

Establishing leak-tight 3D Caco-2 tubules controlled by TEER measurements

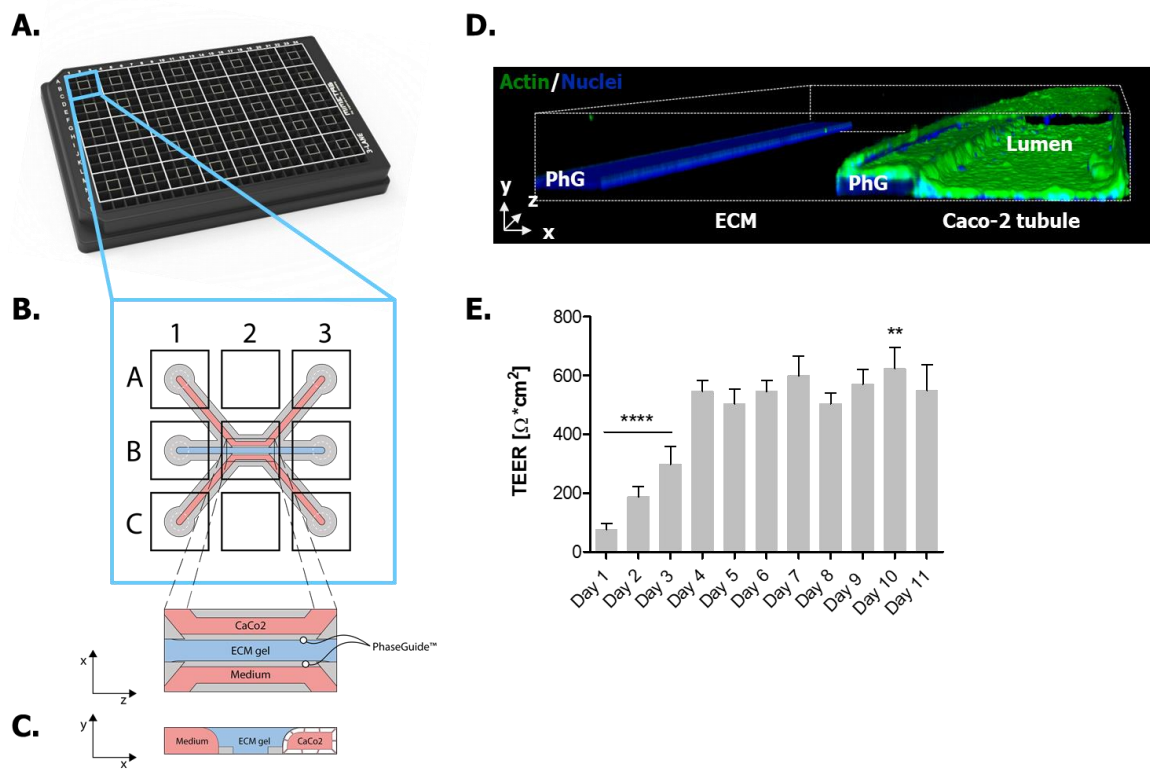


Figure 15. Caco-2 tubules in the 3-lane OrganoPlate.

A. Photograph of the 3-lane OrganoPlate. Each plate contains 40 individual microfluidic chips. **B.** Schematic representation of a microfluidic chip; each chip has three microfluidic channels each containing an inlet (A1, B1 and C1) and an outlet (A3, B3 and C3). Real-time imaging is done through the observation window (B2). **C.** Transversal view of a microfluidic view; Caco-2 cells adhere to the ECM meniscus created by the PhaseGuide™ technology. Upon medium perfusion, Caco-2 cells form tubules covering the walls of the top channel. **D.** 3D reconstruction image of a Caco-2 tubule at Day 4 stained for ACTIN (green) and DNA (blue), depicting the nuclei. **E.** TEER values of Caco-2 tubules over time until Day 11. Data is represented as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by one-way ANOVA with Dunnett's post-hoc test compared to Day 4 (n=13).

In this study, we employed the OrganoPlate platform in order to establish a 3D *in vitro* model recapitulating key IBD characteristics. We successfully established this IBD model by adapting our previously published gut-on-a-chip model where Caco-2 cells grow in 3D tubules following medium perfusion (Trietsch et al., 2017). **Figure 15** shows the 3-lane OrganoPlate where 40 microfluidic chips are organized at the bottom side of a standard 384-well plate format (**Fig. 15A**). Each of these chips has three microfluidic channels with dedicated inlets and outlets as well as an observation window allowing real-time monitoring of the 3D culture (**Fig. 15B**). An extracellular matrix (ECM) precursor is loaded into the middle channel and patterned with a surface tension technique called phaseguiding (Vulto et al., 2011). After gelation, fluids can be inserted in adjacent channels, allowing membrane-free co-culture of several cell types. In our study, we seeded Caco-2 cells in the top channel against an ECM gel while the bottom channel was kept free of cells (**Fig. 15C**). Data from our previous study

showed that, upon medium perfusion, Caco-2 cells form a complete and polarized leak-tight tubule after 4 days of culture in OrganoPlate (**Fig. 15D**). In those earlier studies, we determined the barrier integrity using a FITC-labelled dextran leakage assay (Trietsch et al., 2017). In the current study, we assessed the barrier integrity by measuring the TEER values of the Caco-2 tubules, leading to more sensitive and accurate data (Srinivasan et al., 2015). We show that the TEER values of Caco-2 tubules continuously increase and then stabilize after 4 days of culture until the end of the experimentation (**Fig. 15E**).

Induction of inflammatory state in Caco-2 tubules

Based on previous literature (Van De Walle et al., 2010), we optimized a cytokine cocktail that replicates the effect of E.coli-activated DC on the cytokine secretion of Caco-2 cells in the Transwell system. We first optimized the composition of the trigger and found that a combination of IL-1 β , TNF- α and IFN- γ led to the highest cytokine production in Caco-2 cells (**Fig. 20**). We then compared the trigger's effect to the effect of different concentrations of E. coli-activated DC on the cytokine production of Caco-2 cells (**Fig. 21**). The optimized trigger led to comparable cytokine production levels as the presence of E. coli-activated DC by Caco-2 cells, supporting the immunological relevance of the cytokine cocktail as an inflammatory trigger.

We consequently adapted the concentration of the trigger to the OrganoPlate platform. Because of the difference between the Transwell and the OrganoPlate systems, as well as the diffusion rate in the OrganoPlate (unpublished data from Mimetas) and in order to recapitulate the effects seen in the Transwell system, concentrations of 2, 100, 100 ng/mL were set for IL-1 β , TNF- α and IFN- γ respectively (results not-shown). Caco-2 cells were triggered basally at Day 4 or Day 7 leading to short or long trigger times. Overall, the morphology of Caco-2 cells did not change upon trigger. However, Caco-2 cells submitted to a prolonged inflammatory trigger frequently started to invade the ECM (**Fig. 16A**). As a change in the epithelium permeability is often an important event in IBD aetiology (Coskun, 2014), we assessed whether the cytokine trigger affected the TEER values of the tubules. The TEER values increased over time in untriggered Caco-2 tubules (**Fig. 16B**). However, upon triggering, the TEER values of the Caco-2 tubules decreased significantly for all trigger times when compared to untriggered conditions. The prolonged trigger resulted in the lowest TEER values (**Fig. 16B**).

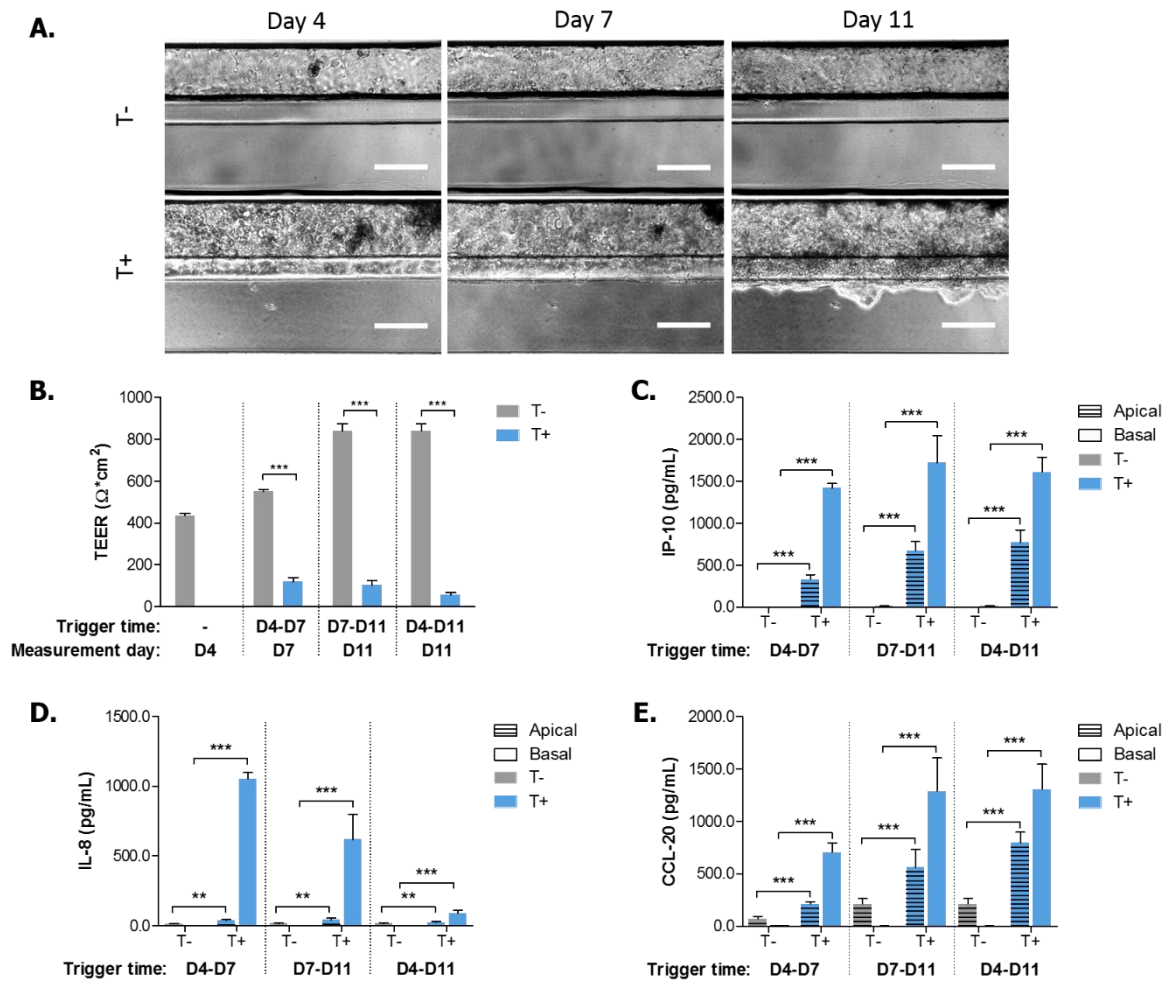


Figure 16. Effect of cytokine trigger on morphology and integrity of Caco-2 tubules.

A. Representative 4X phase contrast images of triggered (T+) and untriggered (T-) Caco-2 tubules at Day 4, Day 7 and Day 11. Scale bars=100μm. **B.** TEER values of triggered (T+) and untriggered (T-) Caco-2 tubules at Day 4, Day 7 and Day 11. Data is presented as mean ± SEM. *, P ≤ 0.05; **, P ≤ 0.01; ***, P ≤ 0.001; ****, P ≤ 0.0001 by two-way ANOVA with Bonferroni corrected post-hoc test compared to T- of each time point (n=3-10). **C-E.** Secretion of IP-10 (C.), IL-8 (D.) and CCL-20 (E.) in apical and basal compartments of triggered (T+) and untriggered (T-) Caco-2 tubules at Day 7 or Day 11. Data is represented as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001 by two-way ANOVA with arcsinh transformation and Holm corrected post-hoc test and compared to apical and basal T- of each time point (n=3-5).

To assess the effect of the inflammatory trigger on the cellular activation of Caco-2 cells, the production of epithelial cytokines IP-10, IL-8 and CCL-20 were quantified. Caco-2 cells secreted low amounts of these epithelial cytokines in untriggered conditions (**Fig. 16C-E**). After trigger, both apical and basal secretion of all analysed cytokines was increased significantly, with no major differences between short and long trigger times (**Fig. 16C-E**). However, the effect of the long trigger on secretion of IL-8 was marginal (**Fig. 16D**). In summary, both short and long inflammatory triggers induced a loss of barrier function of Caco-2 tubules as well as an increased cell activation, depicted with an elevated cytokine production in both apical and basal compartments.

In an attempt to further understand the impaired TEER values of the Caco-2 cells upon trigger, we investigated the expression levels and localization pattern of the *zonula adherens* protein E-CADHERIN (ECAD). It has been reported that *in vitro* wounded HT-29 monolayer models as well as CD and UC tissue have reduced levels of ECAD membranous expression (Karayiannakis et al., 1998; Zbar et al., 2004; Hanby et al., 1996).

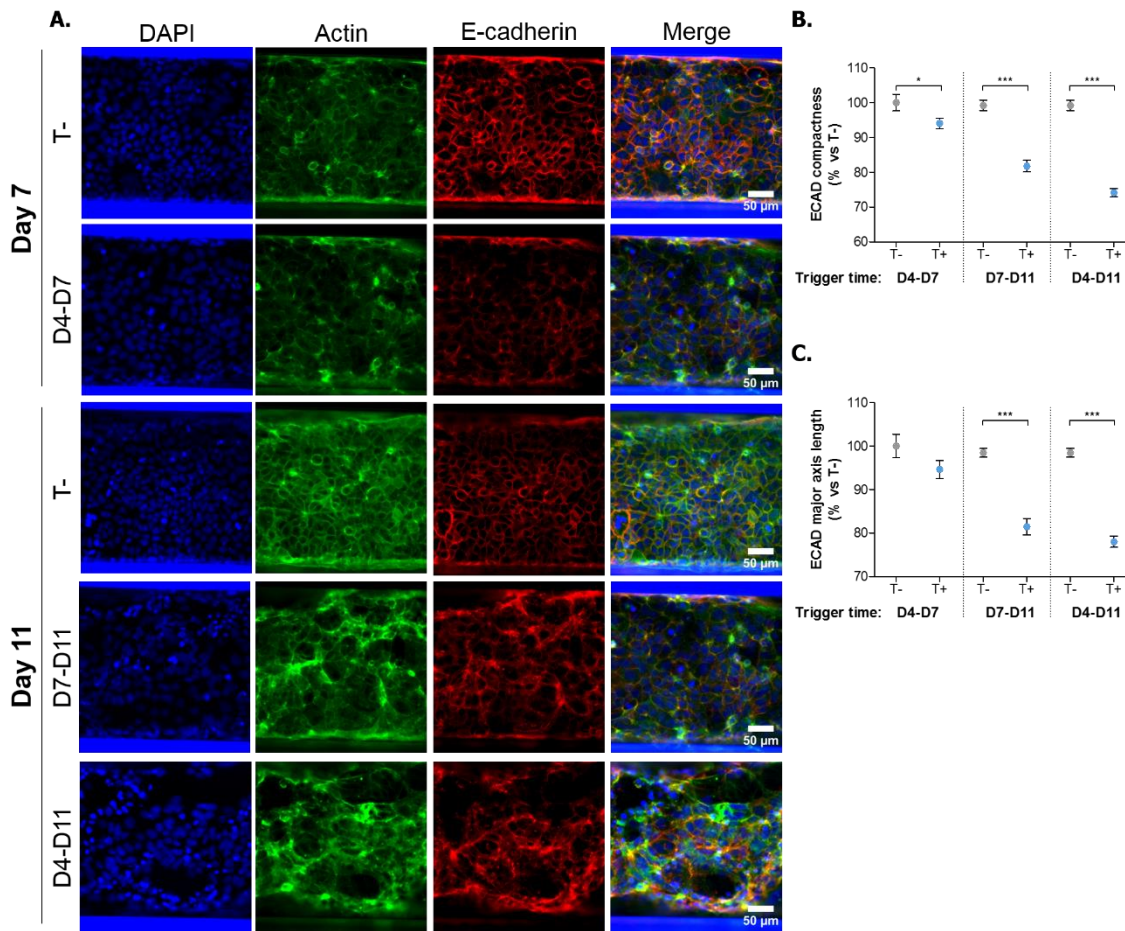


Figure 17. Cytokine trigger induces morphological changes in Caco-2 tubules

A. Representative 20X images of Caco-2 tubules stained for cytoskeleton marker ACTIN, *zonula adherens* marker E-CADHERIN and nucleus marker DAPI at Day 7 and Day 11 in untriggered (T-) or triggered (T+; D4-D7, D7-D11, D4-D11) conditions. Scale bars=50μm. **B-C.** Compactness (**B.**) and Major axis length (**C.**) of E-CADHERIN (ECAD) staining normalized to T- at Day 7. Data is represented as mean ± SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by two-tailed Student's *t* test and compared to T- of each time point (n=8-14). Segmentation process of ECAD staining is showed at Figure 22.

To determine if this also occurs in our model, we stained Caco-2 cells for ECAD and the cytoskeleton marker ACTIN (**Fig. 17A**). The organizational pattern of the ECAD staining was segmented and quantified based on two characteristics: compactness and major axis length of signal. A disorganized epithelial cell layer will display a fragmented ECAD phenotype with

short major axes and low compactness values. Short and prolonged triggers both induced a significant reduction of these two characteristics in Caco-2 cells (**Fig. 17B-C**). The compactness of the ECAD signal also showed a reduction following the early short trigger (D4-D7), but for this condition there was no significant effect on the length of the major axis. The reduction in epithelial cell layer organization confirmed the reduced TEER values of the triggered tubules. These results highlight that IBD-like conditions such as loss of barrier function and cytokine production can be induced in Caco-2 cells using a relevant cytokine trigger.

Exposure to TPCA-1 prevent the inflammatory state of Caco-2 tubules

In order to confirm the validity of our model, we treated Caco-2 cells to a well-known anti-inflammatory compound; TPCA-1, a selective inhibitor of human I κ B kinase-2 (IKK-2) (Podolin et al., 2005). Under normal conditions, IKK-2 phosphorylates the inhibitor of NF- κ B (I κ B α). When phosphorylated, I κ B α releases NF- κ B allowing its nuclear translocation to activate transcription of numerous genes involved in inflammation. Therefore, by inhibiting IKK-2, TPCA-1 prevents the nuclear translocation of NF- κ B, leading to an anti-inflammatory effect.

Cells were exposed to TPCA-1 for two hours prior to adding the inflammatory trigger at the basal side for 72 hours in the continued presence of the compound. We determined the cytokine levels in both the apical and basal supernatants. TPCA-1 induced a concentration-dependent inhibition of both apical and basal secretion of IP-10, IL-8 and CCL-20 by activated Caco-2 cells (**Fig. 18A-C**). At concentrations of 5 and 20 μ M, TPCA-1 could inhibit the secretion of all analytes to levels lower than untriggered cells (results not shown). However, these higher TPCA-1 concentrations also suppressed the barrier integrity (**Fig. 18D**) and the viability of the cells (**Fig. 18E**). At a concentration of 1.25 μ M, TPCA-1 led to a high percentage of inhibition (PIN) of cytokine production without significantly altering cell viability while restoring the barrier function of Caco-2 cells, when compared to triggered but TPCA-1 untreated tubule. These results clearly show that TPCA-1 exposure drastically decreases apical and basal cytokine secretion by Caco-2 cells and proves the suitability of the system to perform compound exposure studies.

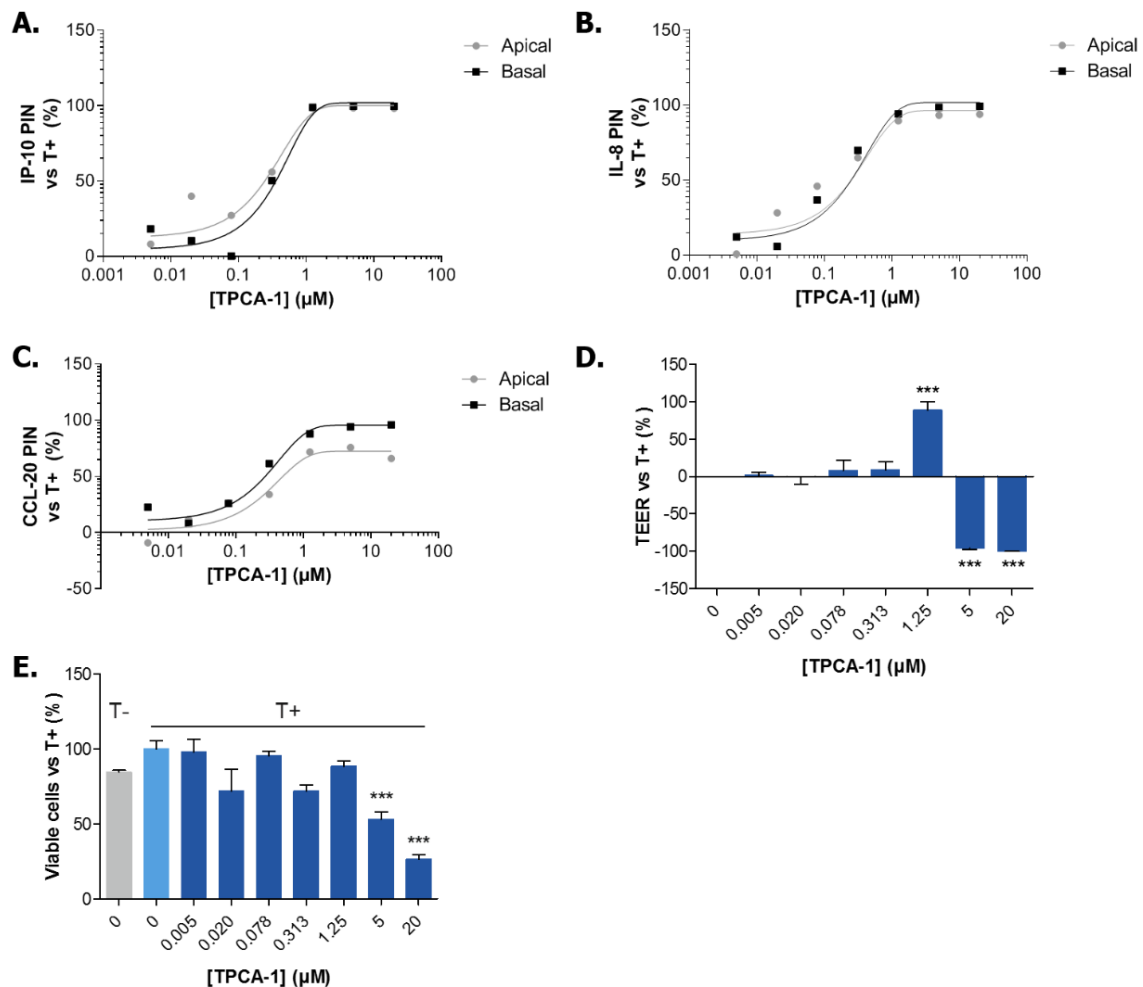


Figure 18. TPCA-1 exposure decreases cytokine secretion of Caco-2 tubules

A-C. Percentage of inhibition (PIN) of IP-10 (**A.**), IL-8 (**B.**) and CCL-20 (**C.**) secretion by Caco-2 cells following a 72h TPCA-1 exposure in apical and basal compartments. Dots represent the PIN mean normalized to triggered (T+) but TPCA-1 untreated tubules. The line depicts a non-linear regression between [TPCA-1] and cytokine secretion (n=4-5). **D.** TEER values of TPCA-1 treated tubules at Day 7. Data is represented as percentage of triggered (T+) but TPCA-1 untreated tubules $\pm$ SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by one-way ANOVA with Dunnett's post-hoc test compared to triggered (T+) but TPCA-1 untreated tubules (n=3-5). **E.** Percentage of viable cells after TPCA-1 treatment at Day 7. Data is represented as percentage of triggered (T+) but TPCA-1 untreated tubules $\pm$ SEM. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001 by one-way ANOVA with Dunnett's post-hoc test compared to triggered (T+) but TPCA-1 untreated tubules (n=3-4).

Adenoviral knockdown of inflammatory effectors prevents IBD-like phenotype

To further evaluate whether the inflamed state of Caco-2 cells could be prevented, we designed recombinant adenoviruses (AdV) expressing validated shRNAs (**Fig. 23**) against MYD88 (Ad-shMYD88) and RELA (Ad-shRELA). RELA is a subunit of NF- κ B, a main player in inflammatory pathways that controls the expression of various proinflammatory genes such as chemokines, cytokines and adhesion molecules (Lawrence, 2009). MYD88 is an adaptor protein downstream in the Toll-like receptor (TLR) and IL-1 signalling pathway involved in innate immune responses (Lawrence, 2009).

We chose to transduce cells directly in the chip. Recently, microfluidic transduction has been shown to be faster and more efficient than static transduction using clinically processed GFP-carrying lentiviruses (Tran et al., 2017). We demonstrate, for the first time, the use of on-chip adenoviral transduction to conduct knockdown studies. To evaluate the transduction efficiency of on-chip AdV-mediated delivery, Caco-2 cells were transduced with a recombinant AdV expressing a ZsGreen cDNA. Microscopic evaluation confirmed that the delivery of 5 infectious units (IU) of ZsGreen-carrying virus per cell resulted in almost 100% transduction of Caco-2 cells at Day 4 (**Fig. 19A-B**). We thus concluded that transduction experiments could be efficiently performed directly in the OrganoPlate.

The knockdown efficiency was further assessed on Day 11 and confirmed a 65% reduction of *MYD88* mRNA expression by AdV-shMYD88 and a 55% reduction of *RELA* mRNA expression by AdV-shRELA (**Fig. 19C**). Previous studies have shown that *Myd88*-knockout in murine bone marrow-derived macrophages show decreased levels of NF- κ B p65 subunit (RELA) activation following *Enterococcus faecalis* infection (Zou and Shankar, 2015). This suggests that MYD88 might be involved at some level in the regulation of RELA expression or activation, which could in turn explain the 28% reduction of *RELA* mRNA expression following MYD88 knockdown (**Fig. 19C**).

After having confirmed that the adenoviral technology induces effective knockdown, we investigated how the knockdown of *RELA* and *MYD88* could alleviate the IBD phenotype in Caco-2 cells. We could not determine whether the knockdown could prevent the loss of barrier integrity as the adenoviral delivery of non-targeting constructs affected the TEER values of the Caco-2 tubules (**Fig. 24**).

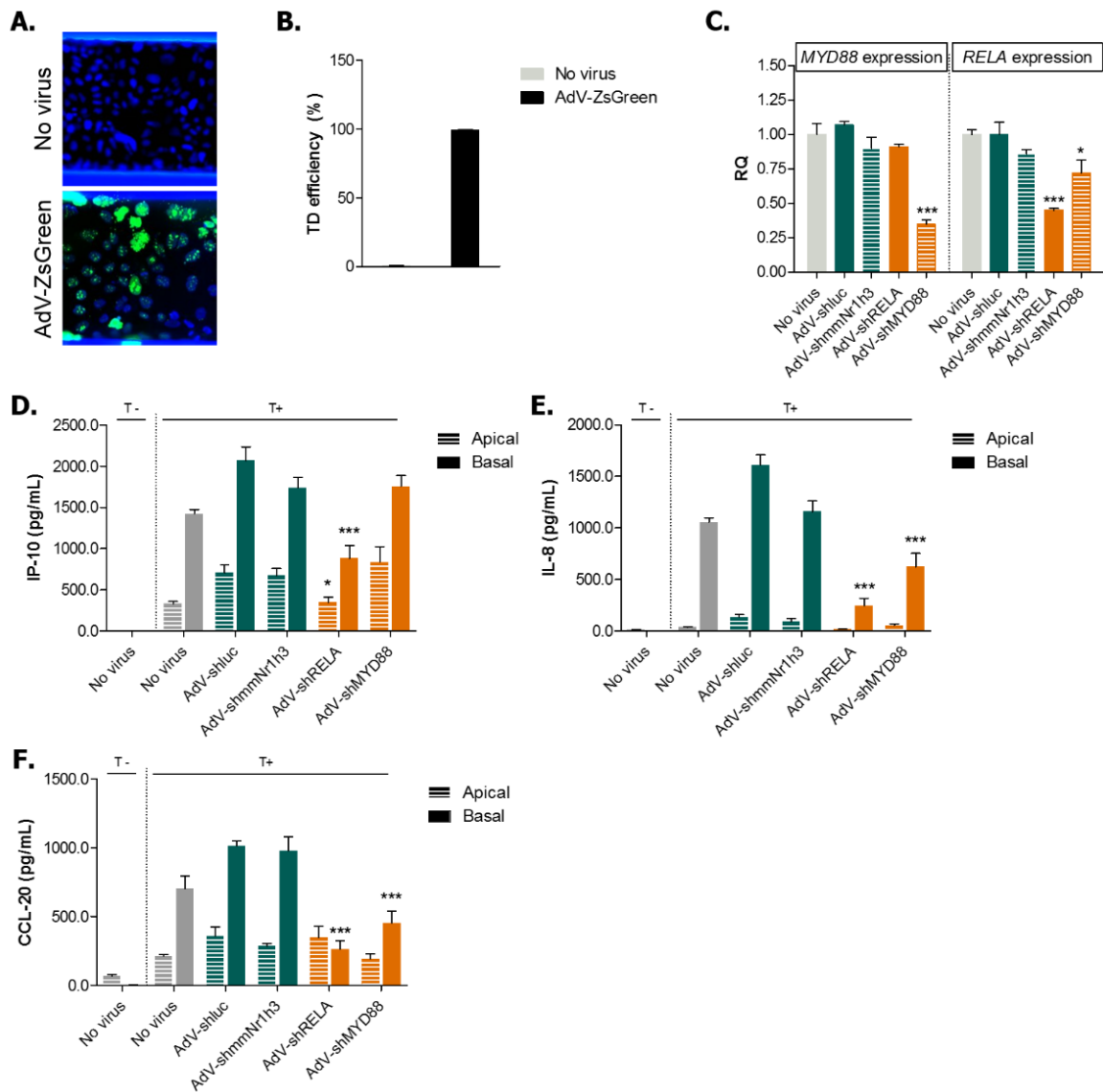


Figure 19. Efficient knockdown of inflammatory effectors decreases basal cytokine secretion in triggered Caco-2 tubules.

A. Representative 10X pictures of Caco-2 cells 72h after on-chip transduction with a ZsGreen-carrying virus (AdV-ZsGreen). Green shows ZsGreen signal and blue shows DAPI signal. **B.** On-chip transduction (TD) efficiency 72h post-transduction. The number of ZsGreen-positive cells was quantified in 2 fields of 10X per chip and normalized to the number of total nuclei. Data is represented as mean $\pm$ SEM ($n \geq 4$). **C.** Knockdown efficiency of *RELA* and *MYD88* at Day 11. Data is represented as mean $\pm$ SEM normalized to *GAPDH* expression. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by two-way ANOVA with Bonferroni post-test compared to no virus condition ($n=2-3$). **D-F.** Secretion of IP-10 (**D.**), IL-8 (**E.**) and CCL-20 (**F.**) in apical and basal compartments of triggered Caco-2 tubules at Day 7. Data is represented as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by two-way ANOVA with arcsinh transformation and Holm corrected post-hoc test compared to AdV-shluc ($n=3-5$). Untransduced condition is shown in grey, negative control viruses in green and viruses carrying shRNA for inflammatory effectors in orange.

We then assessed if the cytokine production of Caco-2 cells in untriggered conditions was affected. The adenoviral transduction itself induced the apical production of IP-10, CCL-20 and IL-8 by Caco-2 cells (**Fig. 19D-F and Fig. 25**). However, when compared to the negative control AdV-shluc, *MYD88* knockdown reduced both the apical and basal production of IL-8 as well as the basal production of CCL-20 in untriggered conditions (**Fig. 25**). The knockdown of *RELA* reduced the apical production of IP-10, both the apical and basal production of IL-8 but did not affect CCL-20 production. However, the production of these proinflammatory cytokines in Caco-2 cells is low in normal conditions (note the difference in the range of the Y-axis in **Figure 25** in comparison to the triggered samples in **Fig. 19D-F**). As we were particularly interested to know whether we could prevent the IBD phenotype establishment in our model, we analyzed cytokine production in triggered Caco-2 cells. Compared to AdV-shluc, AdV-shRELA significantly inhibited the apical and basal secretion of IP-10 (**Fig. 19D**) as well as the basal secretion of both IL-8 and CCL-20 (**Fig. 19E-F**). The reduced expression of *MYD88* also caused a decrease in secretion of basal IL-8 and CCL-20 (**Fig. 19E-F**). We did not observe a complete reduction of cytokine production after *RELA* and *MYD88* knockdown likely due to partial redundancy in underlying signalling pathways leading to cytokine production and due to incomplete knockdown. However, we show that our model could lead to potential target discovery, even by using a trigger composed of different cytokines.

Discussion

When developing a new 3D microfluidic model, users presently have to choose between the level of throughput they want to achieve and the physiological relevance of their model. From a drug development perspective, achieving a high level of throughput is an important factor to consider as it significantly decreases the time and costs associated with screening activities. In this study, we show a robust and reliable 3D gut-on-a-chip model that can be used in drug discovery. More importantly, by applying specific downstream cues of immune activation on Caco-2 cells, we were able to recapitulate key physiological aspects of IBD pathology; the loss of barrier integrity and an increased cytokine production.

For the first time, we could simultaneously monitor the barrier integrity of 40 membrane-free Caco-2 tubules in a real-time manner by measuring the TEER values of the tubules. In untriggered conditions, Caco-2 tubules could reach up to 600-800 $\Omega \cdot \text{cm}^2$ in TEER values after 11 days of culture. In the Transwell system, the TEER values of Caco-2 monolayers generally vary between 250-400 $\Omega \cdot \text{cm}^2$, but TEER values as high as 1200 $\Omega \cdot \text{cm}^2$ have also been reported (Maznah, 1999; Hellinger et al., 2012; Srinivasan et al., 2015). We have also observed slight

variations in the TEER values of untriggered Caco-2 tubules over different experiments. Variations in TEER values have been reported before due to factors such as temperature, medium formulation, cell culture period and passage number of cells (Srinivasan et al., 2015). This high variability in the range of TEER values of Caco-2 cells makes it difficult to fully integrate our results in the light of recent literature and to understand their physiological relevance. However, this novel method allowed us to constantly monitor the barrier integrity of Caco-2 tubules in order to further validate the Caco-2 gut-on-a-chip model previously described (Trietsch et al., 2017), before establishing IBD-like conditions.

It was previously shown that protein expression of IL-1 β (Ligumsky et al., 1990) and IFN- γ (Ghosh et al., 2005) as well as mRNA expression of *IFN- γ* (Dionne et al., 1997; Matsuda et al., 2009) are upregulated in the mucosa of patients with active IBD. Our data clearly demonstrates that an inflammatory state, reflected by an increased cell activation and a decreased barrier function, can be induced in the Caco-2 gut-on-a-chip model following trigger with immune-relevant cytokines IL-1 β , IFN- γ and TNF- α . Since cell activation and loss of barrier function are key inevitable events of IBD pathogenesis (Neurath, 2014), our findings showing increased cytokine release upon the IBD-like trigger as well as a drop in TEER values support the relevance of our model to study disease-specific mechanisms of IBD. Interestingly, Caco-2 cells secreted low cytokine levels in basal conditions as expected (Onyiah and Colgan, 2016). Upon inflammatory trigger, both apical and basal production of inflammatory cytokines IL-8, IP-10 and CCL-20 were increased. However, the basal secretion of these cytokines was consistently higher when compared to their apical production. As both cytokine receptors and pro-inflammatory molecules are localized and secreted basally in polarized IECs in order to recruit immune cells to the site of inflammation (Panja et al., 1998; Onyiah and Colgan, 2016), the increased basal cytokine production by Caco-2 cells upon trigger therefore reflects the *in vivo* physiology of the GI tract in inflamed conditions.

Furthermore, we showed that the decreased barrier function of Caco-2 cells was associated with a fragmented localization of *adherens* junction (AJ) protein ECAD upon inflammatory trigger. Mis-localization of ECAD has been observed in CD patients and may be linked with the decreased intestinal barrier function of these patients (Muisse et al., 2009). Tight junctions (TJ) have also been shown to be affected in IBD patients, with notably elevated levels of CLAUDIN-2 protein in colonic biopsies of UC patients (Heller et al., 2005). To this day, it is still unclear whether the loss of barrier function is a cause or a consequence of the establishment

of inflammation in IBD patients. It would therefore be interesting to use this model to investigate how and when AJ and TJ are affected in IBD pathology.

Throughout our research, we used different inflammatory trigger timelines to induce IBD characteristics. The initial rationale behind the idea was that using a short or a long trigger would induce different degrees of phenotype severity; the shorter trigger leading to a milder phenotype and the longer trigger leading to a more severe one. However, we did not observe any differences in cytokine production by Caco-2 cells between the short and the long triggers nor did we observe any striking differences in TEER values or ECAD localization. These results suggest that the increased cytokine production and the loss of barrier function in Caco-2 happen shortly after trigger and that the time of exposure does not further modulate these characteristics. In the future, the concentration of the cytokines used in the trigger could potentially be optimized in order to illustrate different levels of severity of the IBD phenotype in this gut-on-a-chip model. Nevertheless, we did observe that Caco-2 cells started to invade the ECM after a prolonged exposure to cytokines. It is known that many cytokines are able to regulate proliferation and invasion of Caco-2 cells and a prolonged exposure to IFN- γ , TNF- α and IL-1 β might affect those processes (Andrews et al., 2018).

Interestingly, we observed that a prolonged cytokine trigger did not increase IL-8 production by Caco-2 cells compared to untriggered conditions. This could be explained by the fact that the triggering medium was refreshed at Day 7. Indeed, it is known that IL-8 is rapidly produced after TNF- α triggering of Caco-2 cells and that the secretion is stopped 12h after trigger (Eckmann et al., 1993). It is plausible that, by refreshing the medium at Day 7, levels of IL-8 were back to normal and exhausted Caco-2 cells could not produce any additional IL-8.

Finally, we showed the functionality of the simple model in IBD target discovery by reversing the effect of the IBD-like trigger through compound and gene knockdown intervention. We used direct on-chip transduction to knockdown the expression of *MYD88* and *RELA*. We showed that the increased cytokine production induced in triggered Caco-2 cells could be partially suppressed by *MYD88* and *RELA* knockdown, making the system applicable for large-scale knockdown screens. Once again, it was noticed that the basal production of the analytes was more affected by the knockdown than the apical production. As IECs generally recruit immune cells to the damaged epithelium by secreting proinflammatory cytokines in the subjacent intestinal mucosa (Panja et al., 1998; Onyiah and Colgan, 2016), these results support the physiological relevance of our model to study IBD mechanisms. Nevertheless, we observed residual effect of the trigger cocktail on cytokine production after *RELA* and *MYD88*

knockdown. This can first be explained by the partial knockdown induced by the shRNA-carrying viruses. Furthermore, the cytokines we quantified as readouts of the inflammation are involved in several of the pathways activated by the trigger cocktail. Nevertheless, we showed that this gut-on-a-chip is suitable for target discovery, even in a set-up using a mixture of different cytokines, reflecting the actual pathological situation in IBD.

The effect of the knockdown of inflammatory genes on barrier integrity could not be assessed as AdV transduction affected the TEER values of the Caco-2 tubules. The interaction between AdV and its receptors might have, amongst others, affected TJ or cell-cell interaction which may have impaired attachment of the cells and therefore, decreased TEER values. As MYD88 and RELA are immune regulators and are not expected to play a significant role in the maintenance of barrier integrity, it would be interesting to determine whether knockdown of genes involved in barrier function such as HNF4A or ECM1 could rescue the effect of AdV transduction on TEER values of Caco-2 tubules. Furthermore, we could potentially overcome this limitation of our model by establishing stable knockdown Caco-2 cell lines before seeding the cells in the OrganoPlate.

Finally, by using a compound treatment we could not only prevent the increased release of inflammatory cytokines but also the loss of barrier function in triggered Caco-2 tubules. Namely, Caco-2 tubules treated with 1.25 μ M of TPCA-1 retained their barrier integrity without affecting cell viability. The lower TPCA-1 concentrations did not significantly decrease cytokine release and the higher concentration caused cell death, which affected the barrier function of the epithelium. These results suggest that this model could be used to assess the effect of compounds on both cell activation and barrier integrity.

Our system shows the potential for further improvements by replacing Caco-2 cells with intestinal organoids or gut epithelium derived from iPSCs in the future. Moreover, the OrganoPlate platform is also ideally suited for co-cultures with immune cells, adding further complexity and physiological relevance. This would allow us to elicit epithelial-immune crosstalk mechanisms and could be of particular interest if microbial products are also added to the model. We also foresee application of this type of model in the field of personalized medicine. Key factor here would be to include patient-derived material in order to test and predict patient responsiveness to medication and find the optimal treatment for a given IBD patient.

In summary, this study establishes, for the first time, a robust and reliable gut-on-a-chip model allowing the recapitulation of few key aspects of IBD pathogenesis, in a high-throughput manner. This work provides a foundation for upcoming large-scale 3D microfluidic modeling of IBD and screening of relevant therapeutic targets, allowing us to further study and understand this idiopathic disease.

Author Contributions

C.B. and E.N. designed and performed experimental work and redacted the manuscript. Y.X.C. and G.V.M. helped with experimental work. E.D.E. designed algorithms and performed image analysis. HW helped with statistical analysis. A.N. and S.J.T. assisted with TEER measurements. J.S., H.L.L., D.K., K.S.E. and R.A.J.J. overviewed the research and contributed to writing the manuscript.

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Conflicts of Interest

EN, AN, DK, HLL, SJT, JJ and PV are employees of Mimetas BV, which is marketing the OrganoPlate and the OrganoTEER. PV, SJT and JJ are shareholders of that same company. OrganoPlate is a registered trademark of Mimetas BV. The authors have no additional financial interests.

APPENDIX A – SUPPLEMENTARY METHODS

Cells

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats (Sanquin) by standard Ficoll-Paque™ density centrifugation and CD14⁺ cells were then positively-isolated using LS Columns from MACS Cell Separation system (Miltenyi). Isolated CD14⁺ cells were cultured in RPMI supplemented with 10% heat-inactivated-FBS (Gibco), 1% Penicillin/Streptomycin (Sigma Aldrich), [60ng/mL] of GM-CSF (ImmunoTools) and [40ng/mL] of IL-4 (ImmunoTools) for 7 days to differentiate towards dendritic cells (DC).

Optimization of the immune-relevant trigger

An immune-relevant trigger was first set-up in the Transwell system (Corning, **Fig. 20** and **Fig. 21**). 42 900 Caco-2 cells were seeded on top of a 96-well plate Transwell insert and were triggered at Day 16 with human recombinant cytokines IL-1 β , IFN- γ and TNF- α (ImmunoTools). Basal supernatants were harvested at Day 19 and cytokine secretion was assessed by Luminex. The trigger leading to the highest secretion of cytokines by Caco-2 cells (IL-1 β , IFN- γ and TNF- α at 1, 50 and 50 ng/mL respectively) was then compared to the effect of UV-irradiated *E.coli*-activated DC. To do so, 42 900 Caco-2 cells were seeded on top of a 96-well plate Transwell insert and DC were added to the basal compartment in amounts ranging from 20 000 to 125 000 at Day 9. DC were triggered with UV-irradiated *E. coli* (ATCC, #25922) until Day 19 where apical and supernatants were harvested and cytokine secretion was assessed by Luminex.

Selection of viruses

Several putative negative- (**Table 5**) and positive-control (**Table 6**) AdV were screened for their ability to reduce CCL-20 secretion by Caco-2 cells seeded on top of a Transwell insert and triggered at Day 9 with a cytokine cocktail (IL-1 β , IFN- γ and TNF- α at 1, 50 and 50 ng/mL respectively, **Fig. 23**). Putative positive-control viruses reduce CCL-20 secretion by at least 40% compared to untransduced conditions whereas CCL-20 expression of Caco-2 cells transduced with putative negative-control viruses do not differ from untransduced conditions by more than 25%. Based on those criteria, luc_a and mmNr1h3_a were chosen as negative control AdV and MYD88_d and RELA_a as positive control AdV.

Insert name	Target sequence
aveGFP_a	GCCACAACGTCTATATCAT
fLuc_a	GCAGAAGCTATGAAACGAT
fLuc_b	GCATGCCAGAGATCCTATT
Luc_a*	GGTTACCTAAGGGTGTGGC
mmNr1h3_a*	CACACATATGTGGAGGCCC

Table 5. Putative negative-control shRNA viruses tested

* Virus selected for this study

Insert name	Target sequence
MYD88_a	GGAACAGACAAACTATCGA
MYD88_b	GGTTGTCTCTGATGATTAC
MYD88_c	GACTTCCAGACCAAATTTG
MYD88_d*	GGTTCATCACTGTCTGCGA
MYD88_e	CTGCTCTCAACATGCGAGT
RELA_a*	GATTGAGGAGAAACGTAAA
RELA_b	GTACCCTGAGGCTATAACT

Table 6. Putative positive-control shRNA viruses tested

* Virus selected for this study

APPENDIX B – SUPPLEMENTARY DATA

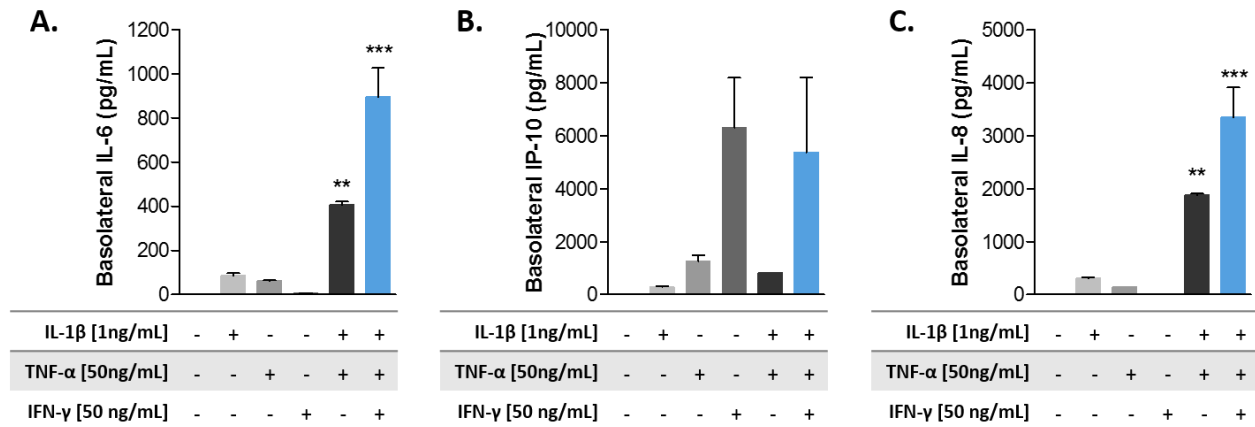


Figure 20. Optimization of the cytokine trigger composition in the Transwell system

Basal secretion of IL-6 (A.), IP-10 (B.) and IL-8 (C.) by Caco-2 cells at Day 19 after a 72h trigger with the indicated cytokines. Data is represented as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by one-way ANOVA with Dunnett's post-hoc test compared to untriggered condition (n=2).

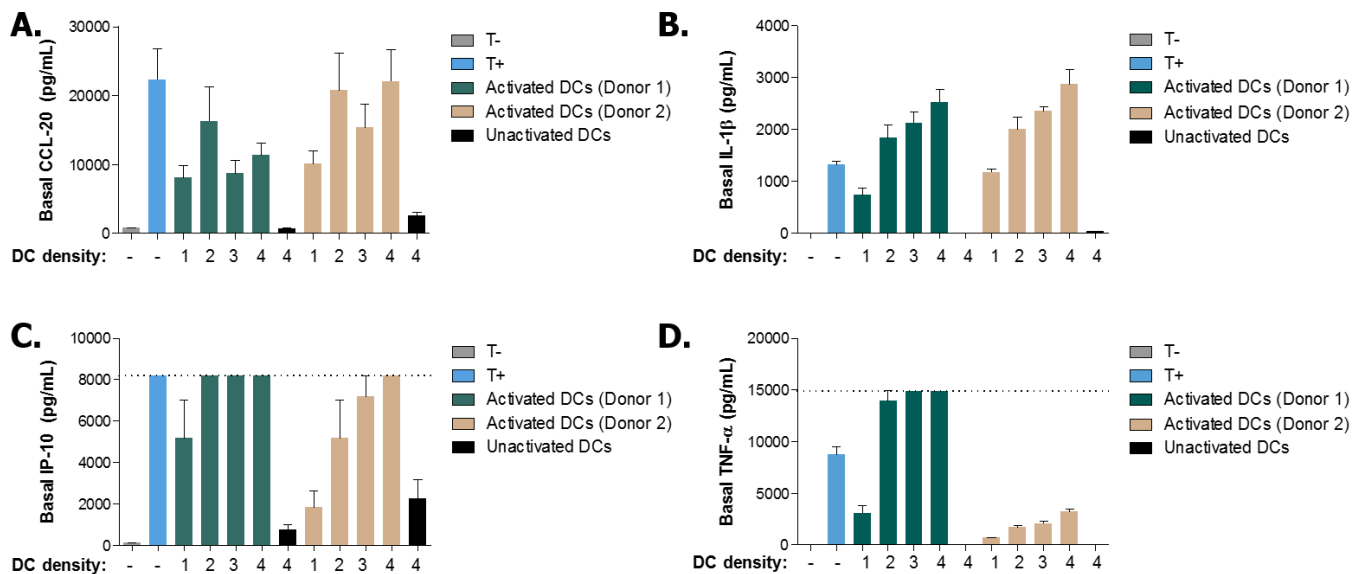


Figure 21. Effect of a cytokine trigger and the presence of activated-dendritic cells on the cytokine release of Caco-2 cells

Basal production of CCL-20 (A.), IL-1β (B.), IP-10 (C.) and TNF-α (D.) assessed by Luminex at Day 19. Caco-2 cells were either left untriggered (T-) or triggered (T+) with cytokines IL-1β, TNF-α and IFN-γ (1, 50, 50 ng/mL respectively) or co-cultured with *E.coli*-activated DC from two donors (DC density; 1=20 000, 2=55 000, 3=90 000, 4=125 000) or unactivated DC for 72h. Data is represented as mean $\pm$ SEM. Dotted line depicts the maximum detection range of the assay (n=2-4).

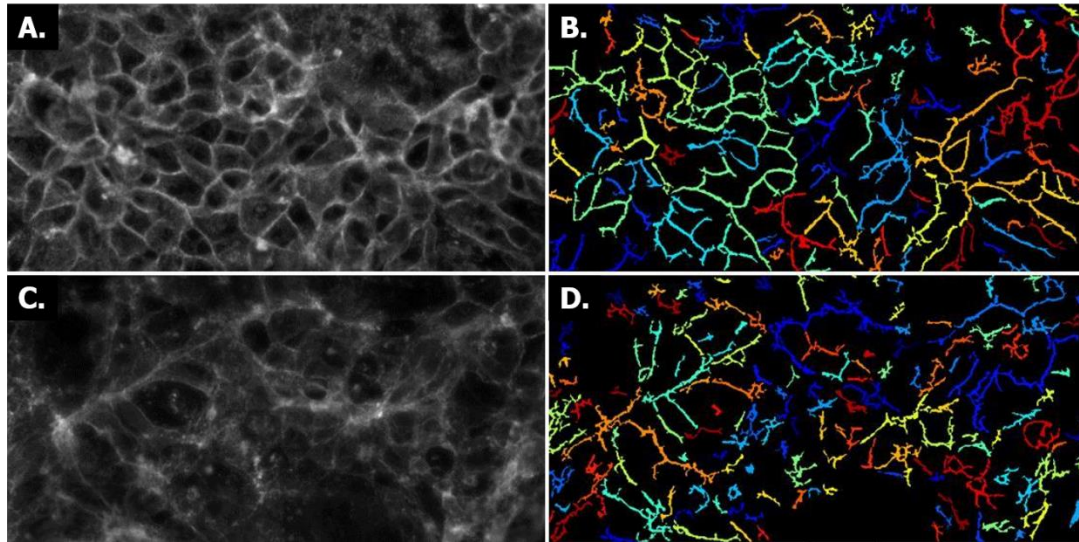


Figure 22. E-CADHERIN immunocytochemistry signal segmentation in Cell Profiler
Raw input images of untriggered (A.) and triggered (C.) Caco-2 tubules at Day 11 with their respective segmented masks (B and D) from the Cell Profiler analysis.

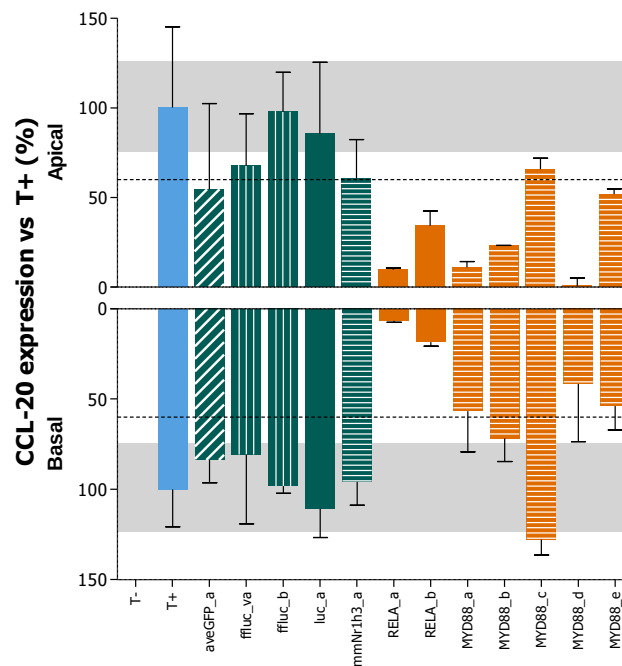


Figure 23. Selection of positive- and negative-control viruses

Apical and basal production of CCL-20 by transduced Caco-2 cells (MOI 8-65) assessed by Luminex at Day 12 after a 72h trigger with cytokines IL-1 β , TNF- α and IFN- γ (1, 50, 50 ng/mL respectively). Grey areas depict a 25% range from T+ and dotted lines depict a 40% reduction from T+. Data is represented as mean $\pm$ range of data (n=2-4). Untransduced conditions are shown in blue, putative negative control viruses in green and putative positive control viruses in orange.

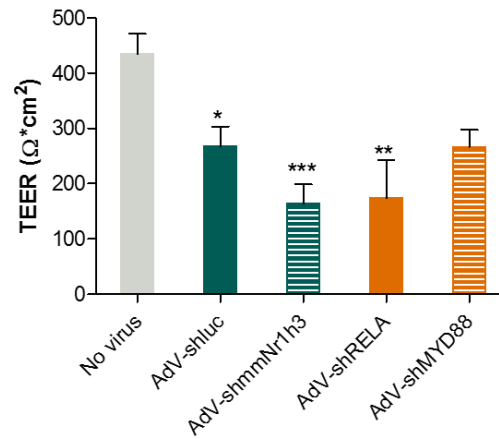


Figure 24. Adenoviral transduction of Caco-2 tubules causes TEER values reduction

TEER values of transduced Caco-2 tubules at Day 4. Data is presented as mean $\pm$ SEM. *, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$ by one-way ANOVA with Bonferroni post-hoc test compared to No virus condition (n=5-12).

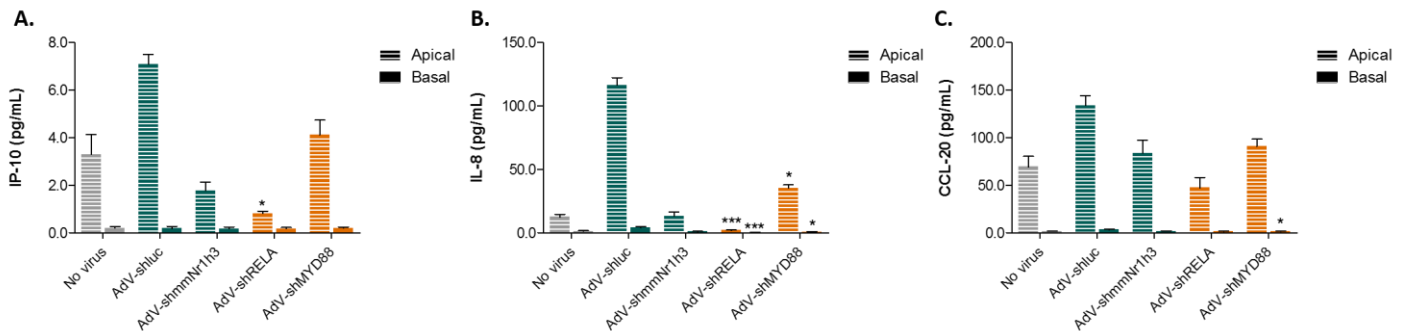


Figure 25. Knockdown of inflammatory effectors decreases basal cytokine secretion in untriggered Caco-2 tubules

Secretion of IP-10 (A.), IL-8 (B.) and CCL-20 (C.) cytokines in apical and basal compartments of untriggered Caco-2 tubules at Day 7. Data is represented as mean $\pm$ SEM. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ by two-way ANOVA with arcsinh transformation and Holm corrected post-hoc test compared to AdV-shluc (n=3-5). Untransduced condition is shown in grey, negative control viruses in green and viruses carrying shRNA for inflammatory effectors in orange.

CHAPTER II

Development of a human primary gut-on-a-chip to model inflammatory processes

Preface

This chapter relates the development of the second microfluidic gut-on-a-chip model of this thesis, using intestinal epithelial cells derived from human intestinal organoids in co-culture with monocyte-derived human macrophages, where IBD-like conditions are induced using a mixture of cytokine IFN- γ and bacterial component LPS. The results presented in this Chapter have been published in Scientific Reports (Beaurivage et al., 2020). *For continuity, figures and tables have been re-labelled to follow a continuous order throughout the thesis.*

Abstract

Inflammatory bowel disease (IBD) is a complex multi-factorial disease for which physiologically relevant *in vitro* models are lacking. Existing models are often a compromise between biological relevance and scalability. Here, we integrated intestinal epithelial cells (IEC) derived from human intestinal organoids with monocyte-derived macrophages, in a gut-on-a-chip platform to model the human intestine and key aspects of IBD. The microfluidic culture of IEC lead to an increased polarization and differentiation state that closely resembled the expression profile of human colon *in vivo*. Activation of the model resulted in the polarized secretion of CXCL10, IL-8 and CCL-20 by IEC and could efficiently be prevented by TPCA-1 exposure. Importantly, upregulated gene expression by the inflammatory trigger correlated with dysregulated pathways in IBD patients. Finally, integration of activated macrophages offers a first-step towards a multi-factorial amenable IBD platform that could be scaled up to assess compound efficacy at early stages of drug development or in personalized medicine.

Introduction

Inflammatory bowel disease (IBD), including Crohn's disease and ulcerative colitis, is a group of chronic relapsing inflammatory conditions of the gastrointestinal tract. Their pathogenesis is not fully understood yet. Nevertheless, they are thought to arise in genetically susceptible individuals in response to several factors such as diet, microbiota, smoking or environmental factors (Neurath, 2014; Strober et al., 2002a). Mechanistically, IBD patients may harbour genetic mutations in genes involved in the integrity of the intestinal epithelium, which allows microbiota to translocate in the intestinal tissue and initiate an inflammatory reaction leading to intestinal lesions showing excessive immune cell infiltration (Atreya and Neurath, 2015). Immune cells recruited to the intestinal mucosa are generally sufficient to dial down

inflammation and return the microenvironment to a normal status. However, IBD patients lack the required regulatory mechanisms or have aberrant activation of these immune cells, leading to a sustained inflammatory state (Atreya and Neurath, 2015; Neurath, 2014). Notably, the intestinal tissue of actively inflamed IBD patients show a drastic increase of activated macrophages; the number of pro-inflammatory M1 macrophages will increase as the number of anti-inflammatory M2 macrophages will decrease (Zhu et al., 2014; Lissner et al., 2015; Bouma and Strober, 2003). Obviously, macrophages are not the only cell type playing a role in the pathogenesis of IBD; dendritic cells (Hart et al., 2005), innate lymphoid cells (Forkel et al., 2019) as well as T helper cells (Bouma and Strober, 2003) have also been shown to be involved or disbalanced through the disease progression.

Trying to understand the mechanisms behind the establishment of these diseases is particularly challenging, as modeling multi-factorial diseases in an *in vitro* environment is difficult. Current *in vitro* IBD models mostly rely on adenocarcinoma-derived Caco-2 cells (Fogh et al., 1977; Engle et al., 1998). Caco-2 cells have provided great insight in the importance of the epithelial barrier function in intestinal inflammation (Shin and Kim, 2018; Kim et al., 2012), however they do not represent the cellular diversity of the intestinal epithelium and therefore poorly reflect *in vivo* physiology. Furthermore, they fail to express some of the major inflammatory cytokines involved in IBD upon stimulation (Eckmann et al., 1993; Parlesak et al., 2004). On the other hand, human primary intestinal epithelial cells (IEC) are a physiologically relevant option that can easily be cultured as human intestinal organoids (HIO). HIO contain multiple epithelial cell types derived from the intestinal stem cells and therefore faithfully recapitulate the cellular diversity of the intestinal epithelium *in vivo* (Sato et al., 2011c; VanDussen et al., 2015). However, one major drawback of HIO is their enclosed lumen because of the spherical nature of organoids, making luminal drug exposure difficult. Additionally, HIO cultures lack tissue-tissue interactions, mechanical tension, as well as the integration of other cell types important to intestinal physiology such as immune and vascular cells. Thus, there is a major need for more complex and physiologically relevant intestinal organ culture systems.

Recently, the organ-on-a-chip technology has emerged as an alternative approach that offers different perfusable microchannels in which cell types of interest can be cultured together in an organ-like orientation using the support of an extracellular matrix (ECM) gel (Bhatia and Ingber, 2014; Bein et al., 2018). A few gut-on-a-chip models have already been developed with adenocarcinoma-derived cell lines such as Caco-2 cells and HT-29 (Beaurivage et al., 2019; Kim et al., 2016, 2012; Kim and Ingber, 2013; Chen et al., 2015). Because of the limited

physiological relevance of these intestinal cell lines, a few studies have created gut-on-a-chip models using HIO or iPSC-derived organoids (Chen et al., 2017; Workman et al., 2018; Bein et al., 2018; Kasendra et al., 2018). These studies clearly showed the augmented relevance of human primary models over Caco-2 cells and even established co-cultures with microvascular endothelial cells and intestinal myofibroblasts (Kasendra et al., 2018; Chen et al., 2017). Importantly, the use of organ-on-a-chip technology transcends the limitation of ‘inside-out’ HIO as it allows the cells to grow in a monolayer that will cover all the surfaces of the microfluidic channel thus creating a tubular structure with an open lumen, allowing exposure to factors such as bacteria, cytokines or drugs in a localized manner. Despite the remarkable advance that these results represent, no study has yet applied their human primary gut-on-a-chip device for the recapitulation and investigation of disease-specific phenotypic characteristics of IBD. Furthermore, the lack of efficient treatment for IBD patients urges scientists to develop devices and models that allow screening of compounds in a relatively high-throughput manner. The commonly used PDMS-based chips lack the necessary scalability to perform such high throughput experiments and lead to absorption of small molecule drugs (Toepke and Beebe, 2006; Halldorsson et al., 2015), making these inadequate for drug screening.

Herein, we report the establishment of a gut-on-a-chip model composed exclusively of patient-derived human primary material. Monocyte-derived macrophages and HIO from different donors were integrated in the membrane-free 3-lane OrganoPlate platform, a higher throughput alternative to PDMS-based chips that would allow compound screening due to its glass composition. We show that the microfluidic culture of HIO leads to an increased polarization and differentiation profile of the cells over standard organoids culture. Importantly, the transcriptome of the microfluidic gut-on-chip resembles that of adult human normal colon *in vivo*. We induced IBD hallmarks by triggering the gut-on-a-chip with lipopolysaccharide (LPS) and interferon-gamma (IFN- γ), leading to the activation and increase cytokine production of both HIO and macrophages. Our model could individually pinpoint cell type-specific cytokine production, in both apical and basal channels simultaneously, allowing us to fully characterize the induction of the inflammatory phenotype. Induction of inflammation in HIO was also confirmed by transcriptomics of the microfluidic gut-on-a-chip. Finally, the suitability of the model to perform compound exposure experiments was confirmed. Our proof-of-concept experiments show that known anti-inflammatory compound TPCA-1 prevents the establishment of the inflammatory phenotype in a dose-dependent manner. In summary, our results highlight the suitability of our *in vitro* microfluidic gut-on-a-chip model to mimic key

physiological aspects of the intestine and further apply them to IBD modelling and potentially large-scale screening in the search for novel IBD treatments.

Methods

Ethics statement

The research described here has been performed according to applicable Dutch national ethics regulations and was conducted within Galapagos BV (Leiden, NL). The use of human cells and associated protocols were approved by the Galapagos biobanking committee. Scientists from Galapagos BV are qualified to perform research using human material and have appropriate facilities and equipment available to comply with applicable laws, regulations and internal rules related to handling and storage of the material. The human material was obtained from Sanquin (Amsterdam, NL), Tissue Solutions (Glasgow, UK) or the Hubrecht Institute (Utrecht, NL). The suppliers have confirmed to Galapagos BV that informed consent from the donors to use the material for research purposes was received. The cells were solely used for target and drug discovery and were not used for human experimentation or therapy. All material is and will remain anonymized.

In vitro culture of human intestinal organoids

Intestinal crypts were isolated from intestinal biopsies as previously described (Sato et al., 2011c, 2009) from 3 different donors (**Table 7**), resuspended in Matrigel (Corning) and polymerized at 37°C. Human intestinal organoids (HIO) were grown in Human intestinal stem cell (HISC) medium (Sato et al., 2011c, 2009), passaged at a ratio of 1:10 to 1:12 every 10-14 days and used between passages 4 and 15. The final composition of the HISC medium is shown at **Table 8**. HISC medium was produced in Advanced DMEM/F-12 (Gibco, #12634) supplemented with 1%v/v Glutamax (Gibco, #35050), 1%v/v HEPES (Gibco, #15630) and 1%v/v Pen/Strep (Gibco, #15140). 2X-concentrated HISC medium was diluted in a 1:1 ratio in Wnt3a conditioned medium. To produce Wnt3a conditioned medium, L Wnt3a cells (CRL-2647) were grown in Advanced DMEM/F-12 (Gibco, #12634) supplemented with 10% FBS (Bovogen, #SFBS), 1%v/v Glutamax (Gibco, #35050), 1%v/v HEPES (Gibco, #15630) and 1%v/v Pen/Strep (Gibco, #15140) in presence of 125 µg/mL Zeocin (Thermo Fisher, #R25001).

HIO donor	Internal nomenclature	Biopsy location	Gender donor	Age donor (years old)	Supplier
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1	HUB2	Rectum	Female	9	Hubrecht Institute
2	FCB1	Colon	Male	72	Tissue Solutions
3	FCB3	Sigmoid colon	Male	50	Tissue Solutions

Table 7. HIO donor information

Constituent	Supplier (Catalog number)	Final concentration
rh R-spondin 3	R&D systems (3500-RS/CF)	0.5 µg/mL
rh Noggin	PeproTech (120-10C)	0.1 µg/mL
B27 supplement	Gibco (17504)	1X
N-Acetyl-L-cysteine	Sigma Aldrich (A9165)	1.25 mM
Nicotinamide	Sigma Aldrich (N0636)	0.01 M
rh EGF	PeproTech (AF-100-15)	0.05 µg/mL
rh Gastrin 1	Tocris (3006)	0.005 µM
Primocin	InvivoGen (ant-pm)	0.1 mg/mL
A83-01	Tocris (2939)	0.5 µM
SB202190	Sigma Aldrich (S7067)	0.01 mM
Y-27632	Abmole (M1817)	0.01 mM
Wnt3a conditioned medium	-	50%

Table 8. Composition of the HISC medium

Isolation and in vitro culture of human macrophages

Human peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats using density gradient centrifugation on Ficoll-Paque according to manufacturer protocol. Monocytes were positively isolated using magnetic CD14 microbeads (Miltenyi, #130-050-201) and were cultured in a low-attachment flask for 5 days in RPMI (Gibco, #72400) supplemented with heat-inactivated FBS (Gibco, #F8067), Pen/Strep (Gibco, #15140) and [100ng/mL] rh M-CSF (ImmunoTools, #11343115) to differentiate towards macrophages.

Gut-on-a-chip coating, seeding and culture

Coating of gut-on-a-chip without macrophages

An ECM gel was prepared by mixing 1M HEPES (Gibco, #15630), 37 g/L NaHCO₃ pH9.5 (Sigma Aldrich, #S5761) and rat-tail Collagen I (Cultrex, #3447-020-01) at a 1:1:8 v/v/v ratio (HEPES/NaHCO₃/Collagen I). A 1.6 µL volume was dispensed in the middle channel of a 3-lane 400 µm OrganoPlate (Mimetas, #4003-400-B) and let to polymerize for 15 min in a 37°C environment in presence of 5%CO₂.

Coating of gut-on-a-chip with macrophages

Macrophages were harvested, pelleted and resuspended in the ECM gel at a concentration of 1.1x10⁶ cells/mL. Embedded macrophages were seeded in the middle lane of a 3-lane 400 µm OrganoPlate (Mimetas, #4003-400-B) in a 1.8 µL volume, thus making 20,000

macrophages/chip. Coated plates were placed in a 37°C incubator supplemented with 5% CO₂ for 15 min until the ECM solidified.

Gut-on-a-chip seeding

During ECM polymerization, HIO were retrieved from Matrigel droplets and made single cells using TrypLE Express (Gibco, #12605) and mechanical dissociation as previously reported (Sato et al., 2011c, 2009). 100,000 HIO cells were seeded in a 2 µL volume in the upper channel of the OrganoPlate and left to adhere against the gel for 45 min. After incubation, 50 µL of HISC medium devoid of A83-01 (attachment medium) was added to the upper inlet and the plates were returned to the incubator at a 70° angle for 4-6 supplementary hours. After 4-6 h, 50 µL of attachment medium was added to all remaining inlets and outlets and the plates were placed back in the incubator without medium perfusion for 24 h. On Day 1, plates were placed on an interval rocker (Perfusion Rocker Mini, Mimetas) switching between a +7° and -7° inclination every 8 min (37°C, 5% CO₂) leading to a 121.2 µL/hr perfusion rate (Vormann et al., 2018). Attachment medium was replaced with HISC medium two days after seeding, and HISC medium was refreshed every three days after that. During all experimentation, the lower channel medium was supplemented with [100ng/mL] rh M-CSF (ImmunoTools, #11343115) to maintain the macrophages differentiated.

Gut-on-a-chip activation

6 days after seeding, the co-culture was activated using [100ng/mL] LPS (Sigma Aldrich, #L3024) and [20ng/mL] rh IFN-γ (ImmunoTools, #11343534) in HISC medium in both upper and lower channels for 16-24 h. Effluents were collected from apical (upper channel) and basolateral (lower channel) compartments at specified times and kept at -20°C until downstream analyses were performed.

Gut-on-a-chip exposure to compound

6 days after seeding, the co-culture was pre-exposed to compound concentrations ranging from 0.02 µM to 20 µM in 0.22% DMSO in upper and lower channels for 2 h. After 2 h, the trigger (100ng/mL LPS, 20ng/mL IFN-γ) in presence of 0.02 µM-20 µM compound was applied in upper and lower channels. Effluents were collected from apical and basal channels 24 h and 72 h after compound exposure and viability was assessed 72 h after compound exposure.

Assessment of cytokine production

The concentration of Granulocyte-Macrophage Colony-Stimulating Factor (GM-CSF), Interleukin-12 unit p70 (IL-12p70), Interleukin-6 (IL-6), Interleukin-8 (IL-8/CXCL8), IFN-Gamma-Inducible Protein-10 (IP-10/CXCL10), Macrophage Inflammatory Protein-3 (CCL-20/MIP3A) and Tumor Necrosis Factor-Alpha (TNF-α) in collected effluents was determined

by a human multiplex assay (Thermo Fisher) on a Luminex FlexMap 3D (Merck Millipore) according to manufacturer protocol.

Assessment of viability

72 h after compound treatment, WST-8 reagent (Sigma Aldrich, #96992) was mixed in HISC medium in a 1:10 ratio and 25 µL was added to upper and lower inlets and outlets. Plates were incubated at 37°C for 4 h and absorbance signals were measured (450nm monochromator) with a multi-well plate fluorimeter (Envision, Perkin Elmer).

Immunocytochemistry

Cells were fixed and prepared for immunohistochemistry as previously described (Trietsch et al., 2017; Beaurivage et al., 2019). The primary antibodies used were anti-E-CADHERIN (Abcam AB1416, 1:100), anti-EZRIN (Invitrogen MA5-13862, 1:50), anti-MUC2 (Invitrogen MA5-12345, 1:50). Secondary antibody used was donkey anti-mouse AlexaFluor647 (Molecular Probes A31571, 1:250) in combination with ActinGreen™ 488 ReadyProbes™ Reagent (Thermo Fisher, R37110) and NucBlue™ Fixed Cell ReadyProbes™ Reagent (Thermo Fisher, R37606) for 1hr at room temperature as explained previously (Beaurivage et al., 2019). Images were acquired on the InCell 6000 (GE Healthcare Life Sciences).

RNA isolation

RNA was isolated from the microfluidic chips using TRI-reagent (Sigma Aldrich, #T9424). In a nutshell, 100 µL of TRI-reagent was added to the top inlet and 25 µL to the top outlet of the OrganoPlate. The plate was placed under an angle for 15 min. For standard HIO cultures, HIO were harvested from Matrigel droplets using cold PBS (Gibco, #10010), pelleted and resuspended in TRI-reagent. The lysed cells were harvested and a phenol/chloroform extraction was performed according to manufacturer's instructions. Isolated RNA was quantified on the Nanodrop 2000 (Thermo Fisher), was treated with amplification grade DNase I (Invitrogen, 18068015) for 15 min at room temperature and inactivated with incubation with the provided EDTA according to manufacturer specifications.

Reverse transcription and qRT-PCR

RNA quality control was performed using the 2100 Bioanalyzer microfluidic gel electrophoresis system (Agilent). Removal of gDNA contamination was performed using the Heat&Run gDNA removal kit (ArticZymes). cDNA synthesis was performed using the iScript Advanced cDNA Synthesis Kit (Bio-Rad) with 600ng of total RNA input. Each qPCR reaction

used 5ng cDNA equivalents of RNA as input and a SYBR Green I mastermix (Bio-Rad) in a total volume of 5µL. All qPCR reactions were run in duplicate in 384-well plates on a CFX384 instrument (Bio-Rad). All data presented was normalized to housekeeping genes *RPS18*, *HPRT1*, *GUSB* and *YWHAZ* (**Table 9**).

Gene	Ensembl ID	PrimePCR assay ID
<i>ALPI</i>	ENSG00000163295	qHsaCED0001947
<i>CDH1</i>	ENSG00000039068	qHsaCID0015365
<i>CDX2</i>	ENSG00000165556	qHsaCID0013185
<i>CHGA</i>	ENSG00000100604	qHsaCED0003225
<i>EZR</i>	ENSG00000092820	qHsaCID0016058
<i>GUSB</i>	ENSG00000169919	31189388
<i>HES1</i>	ENSG00000114315	qHsaCED0006922
<i>HPRT1</i>	ENSG00000165704	31189301
<i>KLF4</i>	ENSG00000136826	qHsaCED0044721
<i>LGR5</i>	ENSG00000139292	qHsaCED0034618
<i>LYZ</i>	ENSG00000090382	qHsaCID0008160
<i>MKI67</i>	ENSG00000148773	qHsaCID0011882
<i>MUC2</i>	ENSG00000198788	qHsaCID0011696
<i>OCN</i>	ENSG00000197822	qHsaCED0038290
<i>OLFM4</i>	ENSG00000102837	qHsaCID0021022
<i>PCNA</i>	ENSG00000132646	qHsaCID0012792
<i>RPS18</i>	ENSG00000231500	31189344
<i>SI</i>	ENSG00000090402	qHsaCID0008471
<i>TJP1</i>	ENSG00000104067	qHsaCID0018062
<i>VIL1</i>	ENSG00000127831	qHsaCID0017926
<i>YWHAZ</i>	ENSG00000164924	31189446

Table 9. PrimePCR probes

RNA-sequencing

Library preparations, sequencing reactions were conducted at GENEWIZ, LLC. (South Plainfield, NJ, USA) and have been described before (Lafita-Navarro et al., 2018; Tjitro et al., 2019). RNA samples were quantified using Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) and RNA integrity was checked with 4200 TapeStation (Agilent Technologies, Palo Alto, CA, USA). Ribosomal RNA depletion was performed using Ribozero rRNA Removal Kit (Illumina, San Diego, CA, USA). RNA sequencing library preparation used NEBNext Ultra RNA Library Prep Kit for Illumina by following the manufacturer's recommendations (NEB, Ipswich, MA, USA). Briefly, enriched RNAs were fragmented for 15 min at 94 °C. First strand and second strand cDNA were subsequently synthesized. cDNA fragments were end repaired and adenylated at 3'ends, and universal adapter was ligated to cDNA fragments, followed by index addition and library enrichment with limited cycle PCR. Sequencing libraries were validated using the Agilent TapeStation 4200 (Agilent Technologies,

Palo Alto, CA, USA), and quantified by using Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA) as well as by quantitative PCR (Applied Biosystems, Carlsbad, CA, USA). The sequencing libraries were multiplexed and clustered on one lane of a flowcell and loaded on the Illumina HiSeq instrument according to manufacturer's instructions. The samples were sequenced using a HiSeq 2x150 Paired End configuration. Image analysis and base calling were conducted by the HiSeq Control Software. Raw sequence data (.bcl files) generated from Illumina HiSeq was converted into fastq files and de-multiplexed using Illumina's bcl2fastq 2.17 software. One mis-match was allowed for index sequence identification.

Data and code availability

The datasets generated during this study are available at GEO [GSE153191].

Gene expression quantification and differential gene expression analysis

Quality reports for raw reads were generated using FastQC toolkit, followed by Trimmomatic trimming of raw reads using the following parameters (adapters: TruSeq3-PE.fa; sliding window:4; leading quality threshold:3). Obtained reads after trimming were checked for quality with FastQC and MultiQC toolkits and were mapped against the human genome (GRCh38.98) using STAR (Dobin et al., 2013) and Kallisto (Bray et al., 2016). Gene counts from each sample were used for subsequent analyses; STAR generated counts were investigated using DESeq2 package (Love et al., 2014) and Kallisto – with Sleuth package (Pimentel et al., 2017); this strategy was chosen to specifically compare for mapping accuracy and potential batch effects. DESeq2 was subsequently used to estimate variance-mean dependence in generated count data, followed by variance stabilizing transformation to test for differential expression using a model based on the negative binomial distribution. Fold changes and adjusted p-values (q-value) for sample comparison were calculated setting at $\alpha = 0.05$ and $\text{pAdjustMethod} = \text{"BH"}$. Differentially expressed genes (q-value < 0.05) were analysed based on log2fold change as well as rlog transformed variability across different conditions. Heatmaps were built selecting distance function: “euclidean” and hierarchical clustering function: “complete”. Statistical analysis and visualization of functional profiles for genes was performed with Clusterprofile package based on GO and KEGG datasets (Yu et al., 2012). All analyses performed with R for statistical programming (version 3.5.3) on the RStudio IDE (Version 1.2.5019). Additional datasets used for comparison: GSE109471 (Kasendra et al., 2018) (Caco-2 Transwell, Caco-2 gut-on-a-chip, duodenum-on-a-chip, duodenum organoid samples), GSE14938 (She et al., 2009) (human normal duodenum/jejunum/ileum samples), GSE9254 (LaPointe et al., 2008) (human normal colon samples). All additional samples were

analysed and normalized based on the platform used for read generation integrating into existing processing pipeline.

“MetaCore from Clarivate Analytics” was used to parse multiple disease and metabolic pathways against the gene set of interest (selecting upregulated genes above the threshold $p < 0.05$). Pathways were generated using the following parameters (network building: direct interactions, species: human, selecting for binding and functional interactions).

Investigation of inflammatory cytokine expression in IBD patients

Gene expression across patients samples was examined in curated databases using Genevestigator (Hruz et al., 2008). Dataset GSE59071 allowed the investigation of gene expression in the colonic mucosa of healthy and IBD patients (Vanhove et al., 2015).

Statistics

Data analysis was performed with GraphPad Prism software version 8 (GraphPad Software, La Jolla, CA, USA). All values are expressed as mean $\pm$ standard error of the mean (SEM), unless indicated otherwise. A two-tailed, unpaired Student’s t-test was used to determine the statistical significance when two groups of data were analysed. When more than two groups were analysed, parametrical ANOVA was used. Differences with p values ≤ 0.05 were considered significant (ns $p > 0.05$, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$). Results shown are from one representative experiment containing at least three technical replicates. The number of technical replicates is presented in the legend of each figure.

Results

Primary human gut-on-a-chip developed using biopsy-derived organoids

Presently, the unique source for primary intestinal cells are organoid cultures derived from patients' biopsies. We set out to develop a gut-on-a-chip model integrating human biopsy-derived epithelium under fluid flow to determine if such a model would better reflect the *in vivo* situation. HIO cultures were established using intestinal crypts derived from macroscopically normal regions of intestinal endoscopic biopsies (**Table 7**). After stabilization and expansion of the cultures, HIO were dissociated and seeded in the upper channel of the 3-lane OrganoPlate, on top of an ECM gel (**Fig. 26A**).

The composition of the human intestinal stem cell (HISC) medium previously published by Hans Clevers' group (Sato et al., 2009) was optimized to favour IEC attachment in the OrganoPlate and ALK5 inhibitor A83-01 was withdrawn from the formulation (attachment medium). IEC kept in attachment medium for the full duration of the experiment started to become mesenchymal as indicated by the upregulation of SM22 expression (**Fig. 32**). By switching back to HISC medium two days after seeding, IEC did not gain mesenchymal markers expression but did form epithelial monolayers in a more robust and reproducible way. The IEC first adhered to the ECM gel before they could cover all the walls of the upper microfluidic channel, therefore forming a polarized epithelial tubule, as previously reported in similar gut-on-a-chip models (Beaurivage et al., 2019; Trietsch et al., 2017).

After 10 days of microfluidic culture, the polarization status of the IEC was assessed (**Fig. 26B**). The localization of E-CADHERIN at cell-cell junctions indicated the formation of *zonula adherens* junctions. Similarly, EZRIN expression was present and its localization was restricted to the apical surface of IEC, confirming proper polarization of the cells. IEC showed unified organization of ACTIN filaments further supporting the conserved apical-basal polarity of the IEC. The expression level of polarization markers was also confirmed at the mRNA level by qPCR by comparing HIO grown in standard Matrigel droplets (static) and IEC grown in an OrganoPlate subjected to fluid flow (microfluidic). The microfluidic culture of HIO increased the expression of polarization markers *CDH1*, *TJP1*, *OCLN*, *EZR* and *VIL1* when compared to static HIO culture (**Fig. 26C**). These results were confirmed in additional HIO donor (**Fig. 33**).

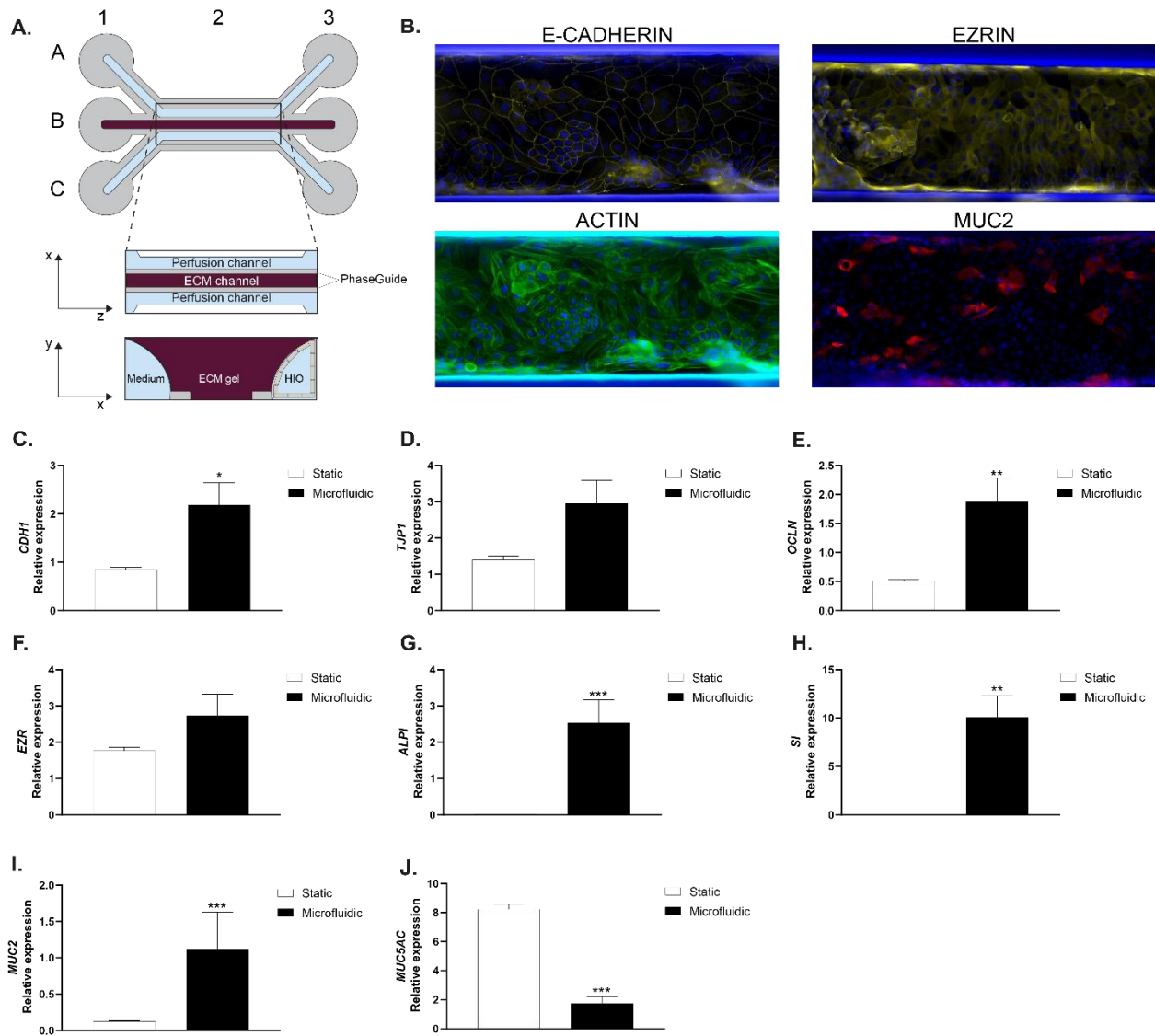


Figure 26. IEC cultured under microfluidic conditions show higher expression of polarization and differentiation markers

A. Schematic representation of the model. One microfluidic chip is composed of three channels: the upper channel (A), middle channel (B) and lower channel (C). Each channel has dedicated inlets (A1, B1, C1) and outlets (A3, B3, C3). Imaging is possible through the observation window (B2). The top view shows the two perfusion channels flanking the middle channel where an extracellular matrix (ECM) gel is seeded. The PhaseGuides allow the ECM gel to form a meniscus, allowing barrier-free culture. The transversal view shows the IEC forming an epithelial tubule in the upper channel against the ECM gel. Medium perfusion is started by placing the OrganoPlate on an interval rocker. **B.** Representative 20X stitched photographs of *zonula adherens* marker E-CADHERIN, apical polarization marker EZRIN, polarization marker ACTIN and goblet cell marker MUC2 in HIO grown in microfluidic conditions for 10 days. Blue depicts nuclei by DAPI staining. **C-J.** mRNA expression level of polarization markers *CDH1* (C.), *TJP1* (D.), *OCLN* (E.), *EZR* (F.) and differentiation markers *ALPI* (G.), *SI* (H.), and *MUC2* (I.) in HIO donor 3. HIO were grown in standard static culture or in monolayers subjected to fluid flow (microfluidic) for 8 days. Graph shows average relative expression values normalized to *RPS18*, *HPRT1*, *GUSB* and *YWHAZ* $\pm$ SEM with unpaired t-test compared to standard HIO culture (n=3).

We further assessed the differentiation status of the IEC in the microfluidic chip. The presence of enterocytes, the predominant cell type of the intestinal epithelium, was confirmed by the successful mRNA detection of the brush border enzymes *ALPI* and *SI* (**Fig. 26D**). The presence of goblet cells was highlighted by a MUC2 staining of IEC grown under fluid flow for 10 days (**Fig. 26B**) and the mRNA levels of *MUC2* and *MUC5AC* expression (**Fig. 26D**). The microfluidic culture of IEC led to an increase of the expression of these markers at the mRNA level compared to the static HIO culture. As the cells used in this study are derived from distal colonic and rectal portions of the gastrointestinal tract (**Table 7**), limited expression of Paneth cell marker *LYZ* and enteroendocrine cell marker *CHGA* could be detected and was not significantly upregulated upon microfluidic culture of the cells (**Fig. 26D**). These results were confirmed in additional HIO donor (**Fig. 33**). Furthermore, the microfluidic culture seemed to increase the general differentiation state of IEC at the expense of proliferation as noted by the upregulation of expression of transcription factors involved in differentiation programs *HES1*, *CDX2* and *KLF4* and a decrease of expression of stem cell marker *LGR5* (**Fig. 34**). Interestingly, we found stem cell marker *OLFM4* expression to be upregulated in microfluidic-grown IEC (**Fig. 34**). Overall, these results indicate that IEC can be grown under microfluidic conditions and that fluid flow increases the expression of polarization and differentiation markers.

Transcriptomic comparison between gut-on-a-chip and standard HIO culture

To further determine whether the microfluidic culture of IEC affects the expression of physiologically relevant genes, we carried out transcriptome-wide analysis of IEC from three different donors. We performed a head-to-head transcriptomic comparison of the established gut-on-chip cultures with the original HIO cultures used for chip seeding. A non-biased analysis of all significantly up- and down-regulated genes was performed and the top 30 differentially expressed genes in microfluidic vs standard HIO culture conditions is shown at **Figure 27A** (PCA graph can be found at **Fig. 35**). In order to uncover the presence of physiologically relevant pathways, Gene Ontology (GO) enrichment analysis for all differentially expressed genes was executed. Interestingly, the analysis uncovered that gene sets involved in digestion, intestinal transport and hormone metabolic process were differentially expressed between microfluidic culture and static cultures (**Fig. 27B**). These results were confirmed by qPCR, where the microfluidic culture increased the expression of intestinal enzymes *SI* and *ALPI* (**Fig. 26G, 26H**).

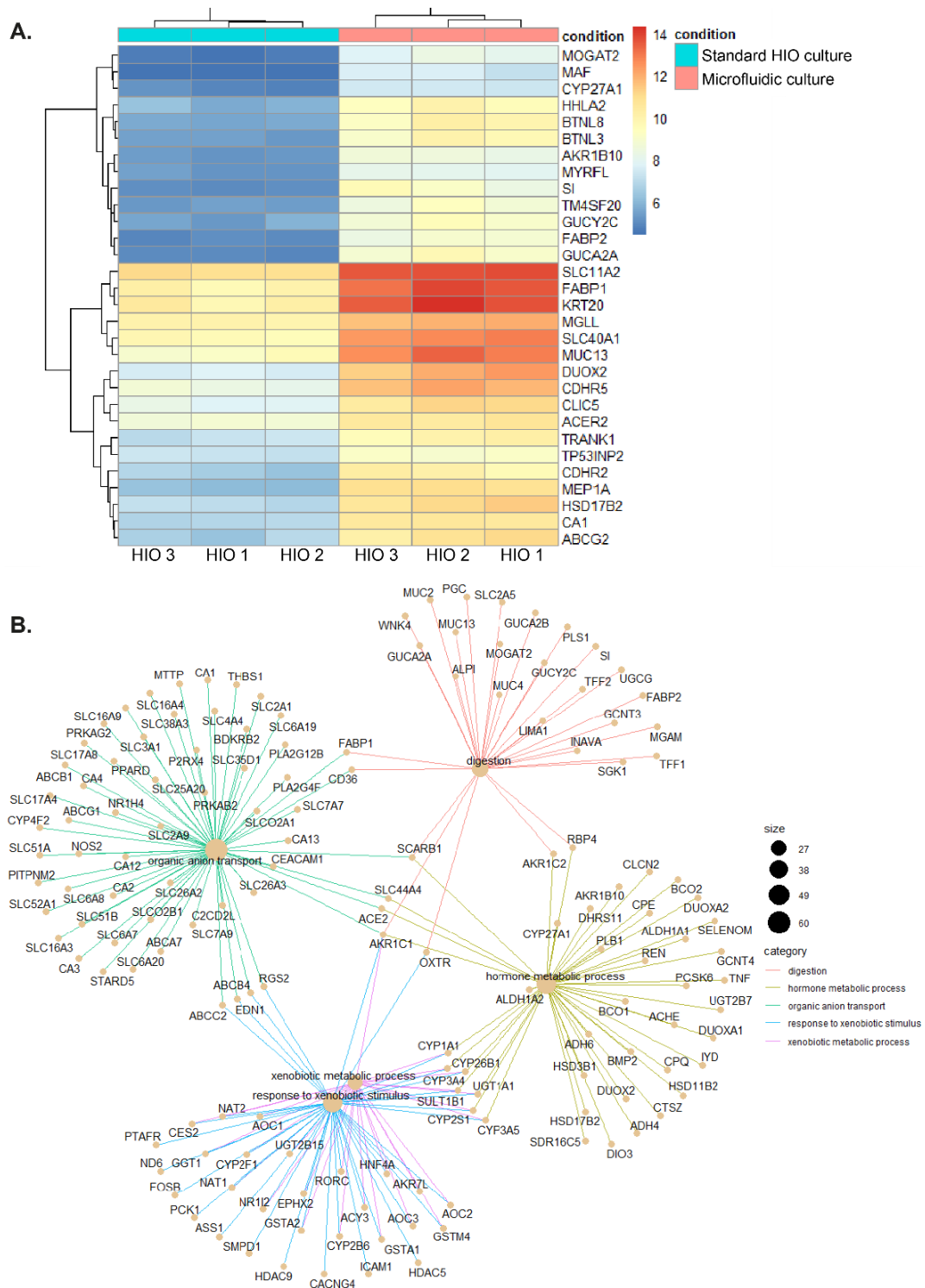


Figure 27. IEC cultured under microfluidic conditions show higher expression of genes involved in digestion, intestinal transport and hormone metabolic process

A. Heatmap representation of the top 30 differentially expressed genes between standard and microfluidic HIO culture in three HIO donors. Higher levels of expression are represented in red and lower levels in blue. **B.** Differentially-expressed genes between standard and microfluidic HIO culture were grouped under Gene ontology (GO) functional pathways. Circle size represents the number of genes differentially expressed in each GO pathway and lines link individual genes to their related GO pathways. Differentially expressed genes (q -value < 0.05) were analysed based on log2fold change as well as rlog transformed variability across different conditions.

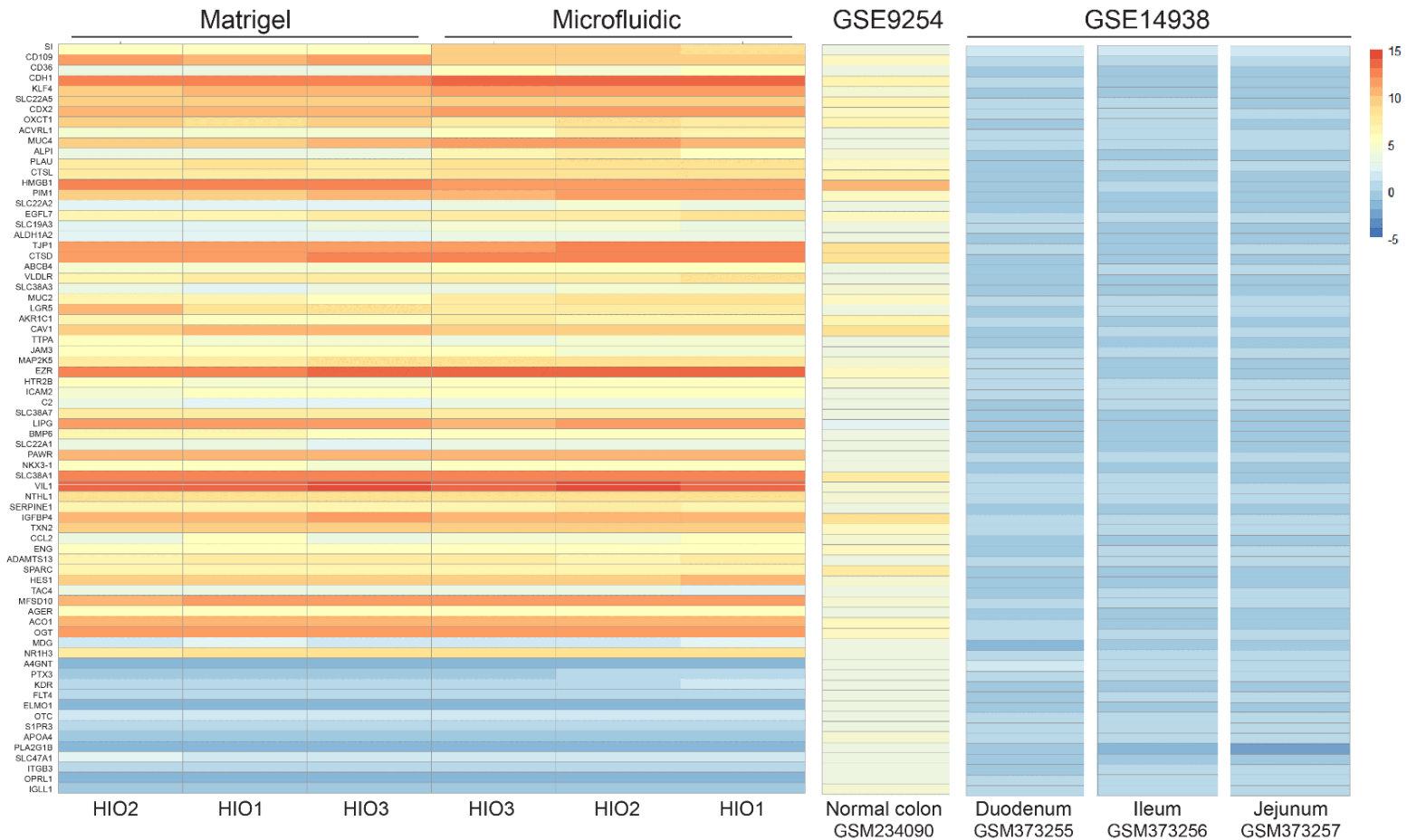


Figure 28. RNA expression profiles of HIO donors resemble human colonic mucosa

Heatmap representation of the RNA expression profile of 72 genes defining colonic identity in 3 HIO donors grown in microfluidic or standard HIO culture conditions for 8 days, compared with human normal colon from study GSE9254 (LaPointe et al., 2008) and human normal small intestine sections (duodenum, jejunum, ileum) from study GSE14938 (She et al., 2009). Genes with higher levels of expression are represented in red and genes with lower levels of expression in blue. Differentially expressed genes (q -value < 0.05) were analysed based on log2fold change as well as rlog transformed variability across different conditions.

Additionally, we compared the samples' transcriptomic results with gene profiles of distinct regions of the human GI tract (ileum, jejunum, duodenum and colon) (She et al., 2009; LaPointe et al., 2008). As the organoid cultures were derived from distal colon biopsies (**Table 7**), a panel of genes defining colonic tissue was selected for this purpose. Within this panel of genes, only subtle variations between static and microfluidic samples could be observed. Overall, the profiles resembled those of fresh human normal colon and the microfluidic culture did not alter tissue phenotype (**Fig. 28**). We also compared our datasets to a Caco-2 gut-on-a-chip and Transwell models published previously (Kasendra et al., 2018) and the differences were drastic; Caco-2 cells had lower expression for all tested genes and were more similar to small intestine tissue sections (**Fig. 36**). Once again, the microfluidic culture of Caco-2 cells did not have a striking effect on the expression of this specific set of genes over Transwell static culture. In conclusion, it appears that HIO have a higher expression of genes involved in

intestinal transport and digestion pathways when they are cultured under microfluidic conditions, while their overall colonic tissue phenotype is preserved.

Activation of the gut-on-a-chip towards an inflammatory state

Following the establishment of a physiologically relevant gut-on-a-chip, we wanted to determine whether our model could recapitulate main characteristics of intestinal inflammation. To do so, the microfluidic gut-on-a-chip was exposed to LPS and IFN- γ . IFN- γ is one of the most highly upregulated cytokines after microbial invasion and in chronic inflammatory diseases including IBD (Neurath, 2014; Verma et al., 2013). It is produced by innate lymphoid cells and has been shown to selectively alter the permeability of the intestinal epithelium, allowing the translocation of bacterial components to the intestinal tissue (Bruewer et al., 2005; Youakim and Ahdieh, 1999; Peterson and Artis, 2014). We have decided to apply the inflammatory trigger on both the apical and basolateral side in order to promote the complete activation and differentiation of macrophages into M1 macrophages, work that will be presented in the last section of this work. However, we did control asymmetric responses to the inflammatory stimuli in HIO (**Fig. 37**). Overall, there were no differences on cytokine production whether the LPS was applied apically or basally with IFN- γ kept on the basal side only. However, when LPS and IFN- γ were simultaneously applied on both apical and basal sides, the apical release of cytokines IL-6 and GM-CSF were increased, while IL12-p70 remained constant and cytokines IL-1 β and CXCL10 were decreased (**Fig. 37**).

The induction of inflammatory conditions in the gut-on-a-chip was first confirmed by RNA-sequencing of non-triggered and triggered samples from three HIO donors cultured in microfluidic conditions. The top 30 differentially expressed genes are shown at **Figure 29A**. All genes differentially expressed between the two experimental groups were assembled into functional GO pathways. When the gut-on-chip model was triggered with LPS and IFN- γ , pathways involved in the regulation of IFN- γ /TNF- α /IL-1 responses, in the activation of the innate immune response and in the response to bacterial factors were over represented compared to the non-triggered condition (**Fig. 29B**). We used Metacore to map the top 30 differentially expressed genes against available disease maps and observed that they correlated strongly with IBD (p-value 7.402E-6, FDR 5.796E-5) and, to a lesser extent, with gastrointestinal diseases in general (p-value 1.002E-2, FDR 1.917E-2). All top scoring pathologies did relate to the GI tract or inflammation (**Fig. 29C**) and more detailed analysis of the perturbed pathways further confirmed that the gene set identified in the triggered cultures is involved in immune regulation at many different levels (**Fig. 29D**). These results confirmed

that the trigger, composed of LPS and IFN- γ , successfully induced a pro-inflammatory state in the IEC and support the relevance of the model.

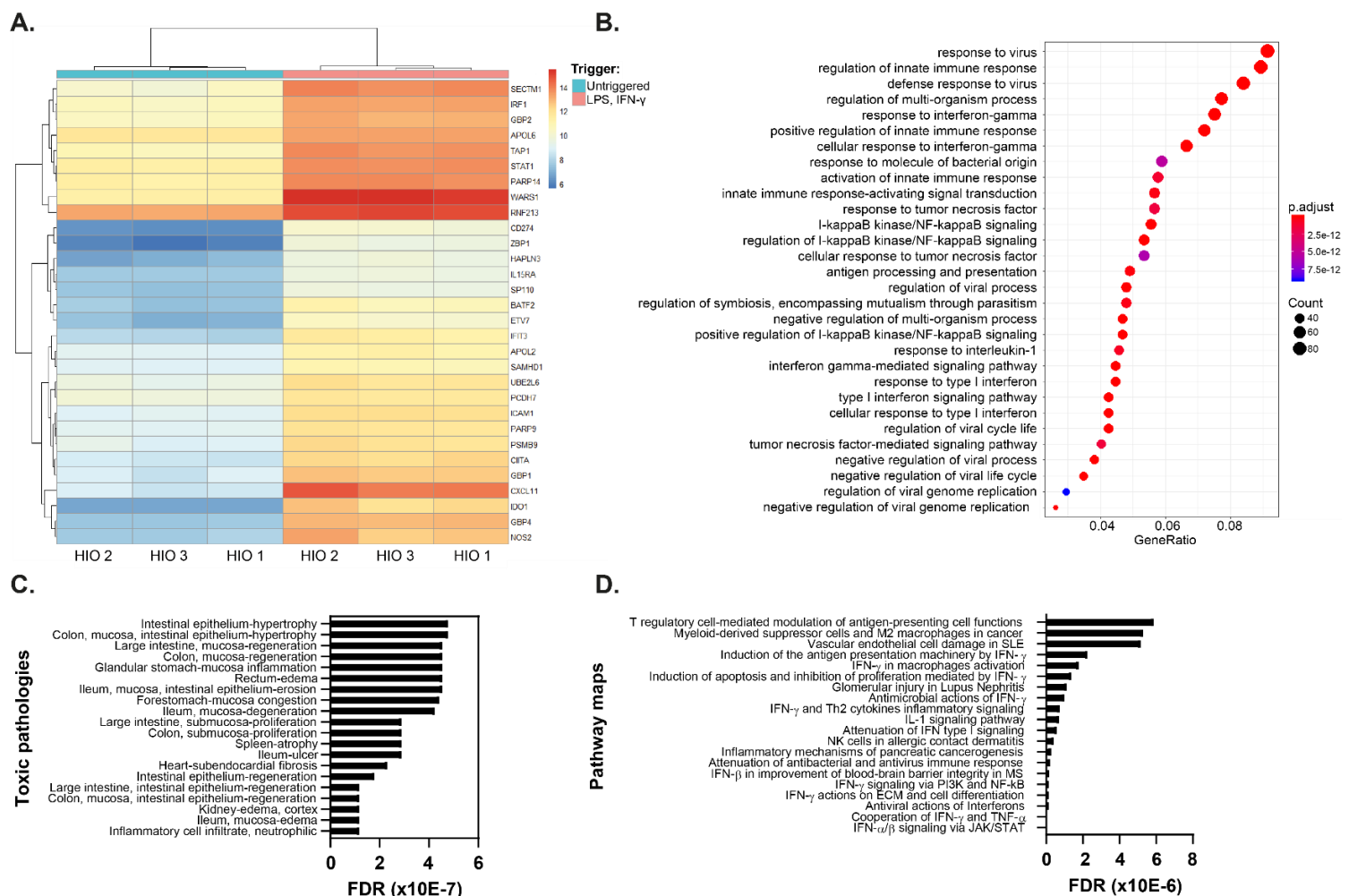


Figure 29. Induction of inflammatory conditions upregulates pathways involved in cytokine and immune signalling in IEC

A. Heatmap representation of the top 30 differentially expressed genes between normal (untriggered) and inflammatory conditions (LPS 100ng/mL, IFN- γ 20ng/mL) in 3 different HIO donors. IEC were grown under microfluidic conditions for 8 days and triggered for 24 h. Heatmap shows gene expression levels (adjusted p value). Genes with higher expression values are shown in red and genes with lower expressions values in blue. **B.** Based on significantly changed gene sets between normal and inflammatory conditions, functional Gene ontology (GO) pathways were isolated. Circle size represents gene counts and color gradation represents adjusted p values. **C-D.** The top 30 differentially expressed genes were queried against toxic pathologies (**C.**) and pathways (**D.**) in Metacore. Graphs show false discovery rate (FDR) values.

Anti-inflammatory compound exposure

In an attempt to tone down the inflammation and assess the potential of the gut-on-a-chip model for drug discovery, we used TPCA-1, a known anti-inflammatory compound (**Table 15**). TPCA-1 is a selective inhibitor of human I κ B kinase-2 (IKK-2) that inhibits production of pro-

inflammatory cytokines *in vitro* and *in vivo* and inhibits NF- κ B nuclear localization (Podolin et al., 2005; Beurivage et al., 2019; Ivanenkov et al., 2011).

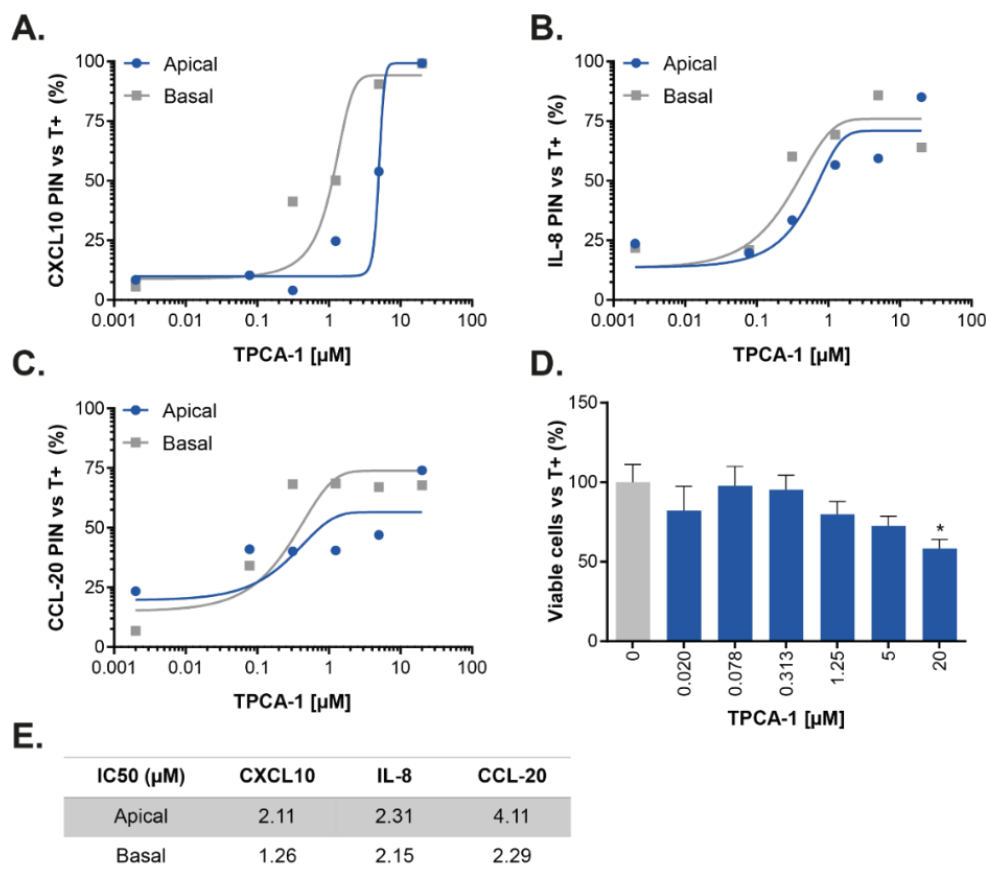


Figure 30. Exposure to TPCA-1 decreases inflammatory cytokine production by IEC

A-C. Apical and basal percentage of inhibition (PIN) of CXCL10 (**A.**), IL-8 (**B.**) and CCL-20 (**C.**) after TPCA-1 treatment. IEC from HIO donor 1 were grown under microfluidic conditions for 6 days before being exposed to TPCA-1 at the indicated concentrations. After 2 h of TPCA-1 exposure, cells were triggered for 24 h (LPS 100ng/mL, IFN- γ 20ng/mL) in the continuous presence of TPCA-1. Dots represent the PIN mean normalized to triggered but TPCA-1 untreated IEC (T+). The line depicts a non-linear regression between [TPCA-1] and cytokine secretion (n=4-5). **D.** Viability of IEC after 72 h of TPCA-1 exposure. Bars represent the percentage of viable cells average $\pm$ SEM with one-way ANOVA with Dunnett's post-hoc test compared to triggered but TPCA-1 untreated tubules (T+; n=4-5). **E.** Calculated CXCL10, IL-8 and CCL-20 IC50 values (μM) after 24 h of TPCA-1 exposure.

Apical and basal production of CXCL10, IL-8 and CCL-20 were efficiently inhibited in HIO in a dose-dependent manner in IEC (**Fig. 30A-C**). At IC50 concentrations (**Fig. 30E**), viability was not significantly altered and only the highest concentration of 20μM significantly decreased viability (**Fig. 30D**). Interestingly, TPCA-1 always decreased basal production of cytokines more efficiently than their apical production (**Fig. 30E**). These results demonstrate that the gut-on-a-chip model could be a suitable alternative to standard organoid technology, which is of limited use in compound evaluation due to its inside-out configuration.

Addition of a cellular immunological component

As previously reported, M1 macrophages are over-represented and activated in the *lamina propria* of IBD patients and have been shown to contribute to IBD physiopathology (Lissner et al., 2015; Zhu et al., 2014). To mimic this key feature of IBD, monocyte-derived macrophages were embedded in the ECM gel of our gut-on-a-chip and were allowed to directly interact with the intestinal epithelium due to the absence of physical barrier between the microfluidics channels (**Fig. 31A**). Once the IEC formed complete epithelial tubules, the co-culture was triggered with LPS and IFN- γ in both apical and basal channels, allowing the macrophages to polarize into M1 inflammatory macrophages, as demonstrated by an increase in TNF- α and IL-6 secretion by the macrophages (**Fig. 31B, 31D**).

In order to characterize the inflammatory state in our model in more detail, we assessed the effect of the trigger on cytokine production by the epithelium and macrophages individually, as well as on both cell types. The apical and basal production of TNF- α , CXCL10, IL-6, IL-12p70, GM-CSF and IFN- γ were assessed for each mono-culture and the co-culture (**Fig 31.B-F**). From the cytokines analysed, only CXCL10 production was not upregulated by macrophages after trigger (**Fig. 31C**). In IEC, production of TNF- α , IL-6, IL-12p70 and GM-CSF were upregulated only on the basolateral side upon trigger (**Fig. 31B, 31D, 31E, 31F**), while CXCL10 production was upregulated in both the apical and basolateral compartments (**Fig. 31C**). After triggering the co-culture, production of TNF- α and GM-CSF were increased only at the basolateral side (**Fig. 31B, 31F**), whereas both the apical and basolateral production of CXCL10, IL-6 and IL-12p70 were increased (**Fig. 31C, 31D, 31E**). Interestingly, production of IL-12p70 and IL-6 were synergized in the co-culture (**Fig. 31D, 31E**). On the contrary, IEC showed an immunoregulatory effect on the apical production of TNF- α and GM-CSF production by macrophages in the co-culture (**Fig. 31B, 31F**).

To support the relevance of these findings, we investigated the mRNA expression of all analytes in colonic mucosal biopsy samples of control and IBD patients from previously published datasets (Vanhove et al., 2015). The expression of all analytes, except *IL12A*, *IL12B* and *CSF2*, were found to be upregulated in the mucosa of patients with active UC or CD (**Fig. 38**). Overall, our results show that this trigger mixture could successfully induce an inflammatory state in the co-culture of HIO and macrophages, resulting in the upregulation of relevant epithelial and immune cytokines.

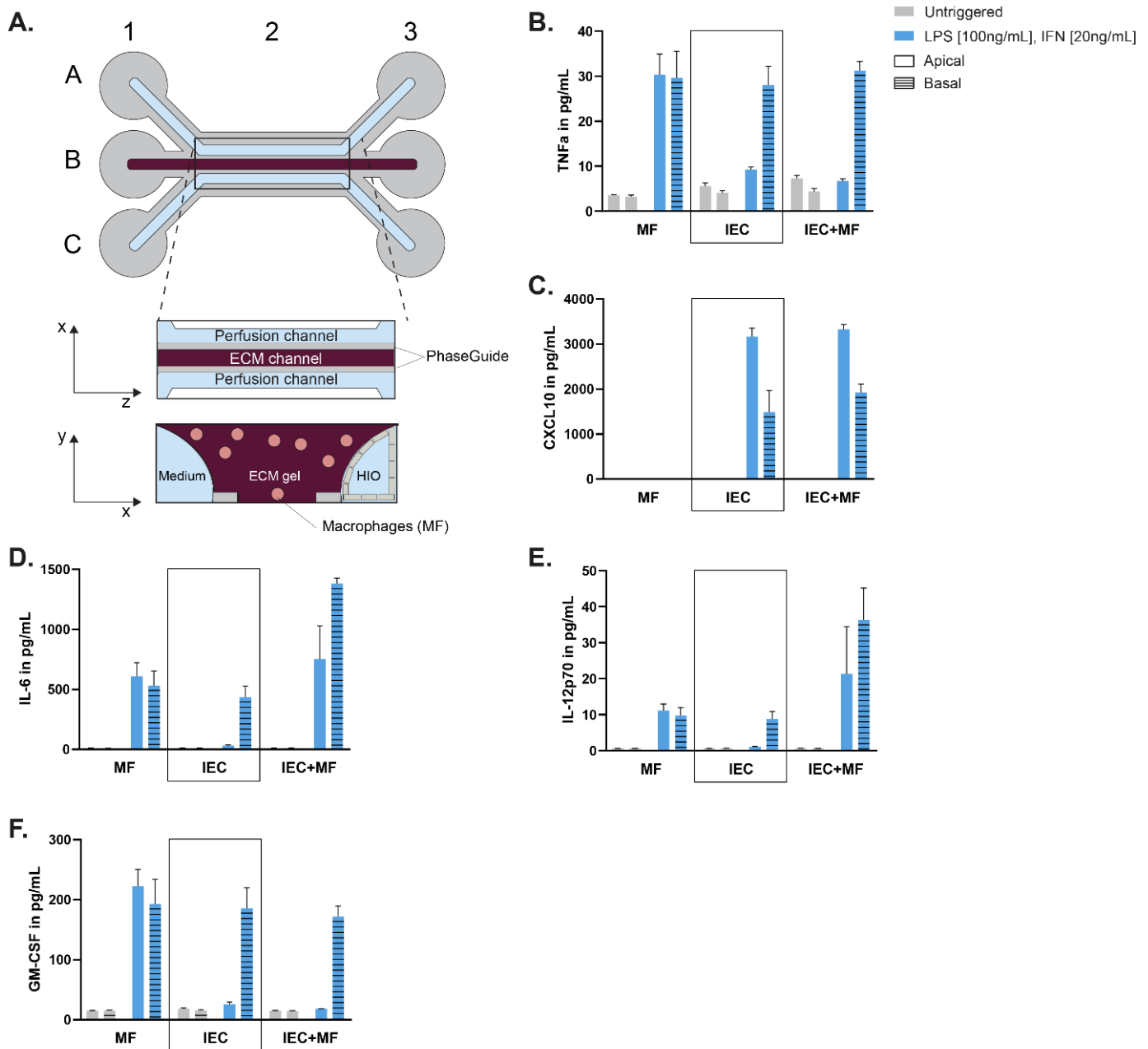


Figure 31. Inflammatory activation increases cytokine secretion of macrophages and IEC
A. Schematic representation from the microfluidic co-culture modified from Figure 26A. Macrophages are directly embedded in the ECM gel on which the epithelial tubule lay against. PhaseGuides allow a barrier-free culture. **B-G.** Apical and basolateral secretion of TNF- α (**B.**), CXCL10 (**C.**), IL-6 (**D.**), IL-12p70 (**E.**) and GM-CSF (**F.**) in macrophages (MF), IEC from HIO donor 1 and the co-culture of both at Day 8 after a 24 h trigger (LPS 100 ng/mL, IFN- γ 20 ng/mL). Bars represent average cytokine production [pg/mL] $\pm$ SEM (n=3-5).

Discussion

We report here the development of an *in vitro* model of the human intestine by integrating the organ-on-a-chip technology with organoid-based methods for culture of primary IEC from colorectal biopsies. A few gut-on-a-chip models integrating biopsy-derived or iPSC-derived IEC have been published recently (Kasendra et al., 2018; Workman et al., 2018; Kasendra et al., 2019; Chen et al., 2018a). However, none of them has addressed the importance of the

immune component nor its implication to the study of IBD. Our gut-on-a-chip model differs from previous models by not only integrating primary human macrophages together with IEC, but also by inducing an inflammatory state of the epithelium similar to the one observed in IBD patients.

Firstly, we confirmed that all differentiated cell types present in the HIO were also represented in the gut-on-a-chip model. Upon microfluidic culture, the mRNA expression of markers associated with enterocytes and goblet cell differentiated cell types of the intestinal epithelium, as well as transcription factors favouring differentiation were increased, while markers for proliferation were decreased. Interestingly, stem cell marker *LGR5* expression was decreased by fluid flow as observed by previous studies (Kasendra et al., 2018), while *OLFM4* expression was increased. Recent studies have demonstrated that *OLFM4* was expressed at very low levels in the foetal intestine and in HIO cultures, whereas it was expressed at higher levels in the adult intestine (Finkbeiner et al., 2015; Fordham et al., 2013). As it is unclear how a reduction in *LGR5* positive cells might be linked to this observation and that more investigation would be necessary, it could suggest that our gut-on-a-chip model has reached a higher level of maturity over static HIO culture.

Furthermore, IEC grown as a monolayer on an ECM gel under flow have higher expression of genes with a role in important intestinal functions such as digestion, intestinal transport and hormonal regulation over static HIO spheroids. This suggests that microfluidic gut-on-a-chip models might more adequately reflect organ-level functions over conventional organoids. Comparison of the expression level of a set of genes involved in colonic identity between microfluidic samples and *in vivo* intestinal segments revealed that there is no significant impact of microfluidic culture on intestinal phenotype. HIO donors, whether cultured in static or microfluidic conditions, highly resembled the human colonic mucosa. The differences between the general intensities of the signals could be explained by the different technologies used. Despite this difference in signal intensity, the relative gene expression profile was decidedly similar to that of genes in the human colon, making our model particularly appropriate for UC modelling, which mainly affects that area of the gastrointestinal tract. Previously published Transwell and gut-on-a-chip Caco-2 models, on the other hand, more closely resembled the human small intestine. One should therefore be careful using Caco-2 cells to study colon-specific mechanisms and pathologies such as UC, as our results suggest that they strictly represent cells from the small intestine, despite the fact that they have been described to express both enterocytes and colonocytes markers (Engle et al., 1998). Furthermore, in both the HIO

and Caco-2 case, the microfluidic culture did not significantly alter the expression of genes involved in intestinal phenotype. Despite the lack of regulation of RNA expression profiles of this specific set of genes, other genes involved in intestinal transport and metabolism were highly represented by microfluidic culture. Furthermore, other studies have also shown that HIO gut-on-a-chip models led to increased intestinal functions compared to organoids or Caco-2 gut-on-a-chip models (Kasendra et al., 2018, 2019). Thus, for assessments such as drug metabolism and response to nutrients, microfluidic models might be more informative of the *in vivo* situation.

In this study, we have used the original HISC medium recipe as originally published by Hans Clevers group presented at **Table 8** (Sato et al., 2009). To optimize IEC attachment in the microfluidic plate, ALK5 inhibitor A83-01 was withdrawn for the first 48 hours, as previous studies have also done (Wang et al., 2017). When withdrawn for 48 hours, we did not observe any upregulation of EMT markers. However, it cannot be excluded that the removal of A83-01 could be responsible for some molecular changes observed in our RNA-sequencing analyses. However, we did not observe an upregulation of pathways regulated by TGF- β such as EMT, proliferation or apoptosis. We have, in fact, observed a clear reduction in proliferation markers expression.

We next inquired whether the gut-on-a-chip could be applied to model inflammatory responses. To do so, we applied a mixed trigger composed of bacterial LPS and cytokine IFN- γ to the microfluidic model. We first confirmed the induction of an inflammatory state by RNA-sequencing of HIO. Pathways involved in cytokine regulation and bacterial response that are dysregulated in IBD patients were found to be overrepresented in triggered IEC, confirming the general inflammatory state of the model and supporting its physiological relevance.

Naturally, IBD is a multi-factorial disease that cannot be solely recapitulated by one cell type. Macrophages, which were shown to be overrepresented and activated in IBD (Zhu et al., 2014; Lissner et al., 2015), were therefore included into our model to increase its complexity. Upon trigger, macrophages were activated into pro-inflammatory M1 macrophages and secreted immune-relevant cytokines TNF- α and IL-6. The inflammatory trigger also activated IEC, which in turn, secreted epithelial cytokines. This was particularly the case for IL-12p70 and IL-6 whose secretion was synergized in the co-culture. Contrarily, IEC showed an immunoregulatory effect on the apical production of TNF- α and GM-CSF production by macrophages in the co-culture. IEC are capable of secreting modulators that can modulate the

cytokine secretion of macrophages, such as cholecystokinin and glucagon-like peptide (GLP) 1 and 2 (Luyer et al., 2005; Worthington, 2015). Particularly, IEC have been reported to induce differentiation of normal macrophage into a tolerogenic phenotype resembling intestinal macrophages (Kristek et al., 2015). In all cases, the basolateral production of inflammatory cytokines upon trigger was always greater than the apical production, suggesting that IEC respond to an inflammatory state by secreting analytes at the basolateral side. The reason for this could be that IEC are involved in recruitment of immune effectors residing in the underlying *lamina propria* or circulating in the blood through the secretion of chemokines and cytokines at the basolateral site (Onyiah and Colgan, 2016).

Our study did not assess whether other changes in the medium composition would further affect IEC differentiation and the cellular responses engaged. Research groups have now shown that innate immune responses of IEC are modulated by the removal of Wnt3a conditioned medium, which alters the state of differentiation of IEC. For instance, removal of Wnt3a was shown to induce MHC II expression which can in turn promote TLR-triggered innate immune responses (Wosen et al., 2019). Similarly, the presence of anti-oxidants in the HISC medium have also been shown to influence innate immune responses of IEC (Ruan et al., 2020). Notably, jejunal organoids cultured in medium devoid of antioxidants were more responsive to host and microbial inflammatory signals. While we demonstrate that innate immune responses were successfully induced in our model, it would become important in the future to assess whether the modification of the HISC medium composition alters those responses in IEC and whether the IEC-immune cell relationship is consequently affected. Furthermore, our study focused on the effect of microfluidic culture and pro-inflammatory stimulation on the intestinal epithelium, but we did not assess the contribution of individual cell types to the phenotype. It would be of particular interest to investigate cellular changes occurring in goblet and Paneth cells during co-culture with macrophages, as abnormal cell function has been described in IBD patients (Elphick and Mahida, 2005; Kim and Ho, 2010).

One of the drawbacks of the organoid methodology is their enclosed lumen, making drug exposure studies difficult. In addition, it does not allow to simultaneously measure the secretion of analytes at both the apical and basolateral site. The novel gut-on-a-chip model presented in this study transcends these limitations and allowed IEC exposure to TPCA-1 compound in a polarized manner. TPCA-1, by selectively binding the ATP pocket of IKK-2, prevents the phosphorylation of inhibitor of NF- κ B (I κ B), preventing NF- κ B nuclear translocation and the activation of genes involved in inflammation and other immune responses (Podolin et al.,

2005). TPCA-1 markedly reduced the epithelial production of CXCL10, IL-8 and CCL-20 by IEC. The compound has previously been shown to reduce IL-8 by HT-29 cells (Rauert-Wunderlich et al., 2013) as well as CXCL10, IL-8 and CCL-20 production by Caco-2 cells (Beaurivage et al., 2019). Furthermore, NF- κ B has been shown to be activated in mucosal biopsies of patients with active IBD and the use of steroids, by decreasing NF- κ B activity, have shown to reduce clinical symptoms of patients (Yamamoto and Gaynor, 2001). Hence, our proof-of-concept experiment show preliminary evidence that our model could potentially be used to assess the efficacy of drug candidates on cytokine secretion in the intestine. Importantly, our model offers numerous advantages over Caco-2 models currently used in drug absorption studies (Hilgers et al., 1990; Artursson, 1990). Indeed, although Caco-2 cells have been found to express a large number of enzymes and transporters present in the normal human intestinal epithelium, studies suggest that there are variations between gene expression profiles of transformed epithelial cell lines like Caco-2 and the normal human intestinal epithelium (Bourgine et al., 2012; Verhoeckx et al., 2015). This could, in turn affect the accuracy and relevance of results obtained in this system. Furthermore, while some studies show the limited presence of MUC2 expression in Caco-2 cells under specific conditions (Kim and Ingber, 2013), they are overall considered enterocyte-like only and do not reflect the diversity of the intestinal epithelium. By using human primary IEC derived from biopsies, we addressed the limitations incurred by the use of Caco-2 cells. In addition, culturing the IEC in microfluidic conditions corrected the inside-out configuration of HIO and increased the expression of intestinal transporters such as SLC superfamily members that are known to be important for the transport of drugs through the epithelium (Shugarts and Benet, 2009).

Our novel primary human gut-on-a-chip model could therefore not only be used to study drug transport, absorption and toxicity but could also be potentially of use in studying intestinal development, tissue-tissue interactions, host-pathogen connections as well as regenerative medicine. In the future, the implementation of other cell components participating in IBD pathogenesis, such as supplementary immune cell types to reflect the heterogeneity of the mucosal immune system, would allow us to reflect *in vivo* physiology more closely. Similarly, access to IBD-patient material could allow us to investigate patient-specific disease mechanisms and therapy response, opening the doors to personalized medicine. The advent of the organ-on-a-chip technology allows us to separately control physical and mechanical factors (e.g., fluid flow) as well as cellular components to better understand and model intestinal homeostasis and diseases of the gastrointestinal tract such as IBD.

Acknowledgments

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Author contributions

Conceptualization, C.B., C.L., J.S., R.A.J.J.; Data curation, C.B., A.K.; Formal analysis, C.B., A.K.; Funding acquisition, K.S.E., R.A.J.J.; Investigation, C.B.; Methodology, C.B.; Project administration and supervision, C.L., J.S., K.S.E., R.A.J.J.; Visualization, C.B.; Writing – original draft; C.B.; Writing – review & editing, C.B., A.K., C.L., J.S., K.S.E., R.A.J.J.

Competing interests

The authors declare no competing interests.

Data availability

The datasets generated during and/or analysed during the current study are available in the GEO repository, [[GSE153191](#)].

APPENDIX C – SUPPLEMENTARY DATA

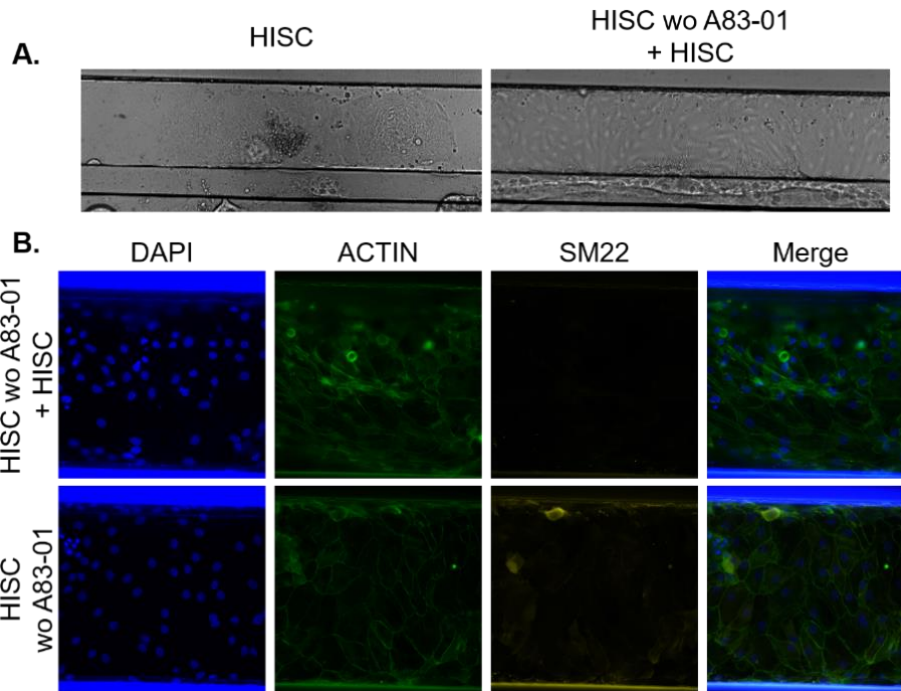


Figure 32. Optimized HISC medium favours IEC attachment

A. Representative 10X brightfield stitched images of HIO donor growing in microfluidic conditions for 8 days in HISC medium (left panel) or in HISC without A83-01 for 2 days followed by HISC for 6 days (right panel, wo=without). **B.** Representative 20X photographs of polarization marker ACTIN and EMT marker SM22 in HIO (donor 1) grown in microfluidic conditions for 8 days in HISC medium without A83-01 for 2 days followed by 6 days of HISC medium (top panel) or in HISC without A83-01 for 8 days (bottom panel). Blue depicts nuclei by DAPI staining. Images were created with the Image J software (version 1.47v, <https://imagej.nih.gov/ij/>).

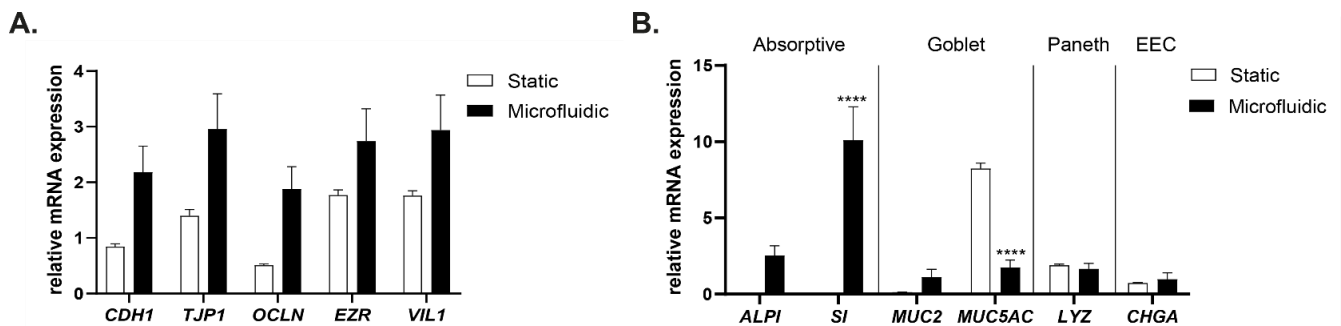


Figure 33. HIO cultured under microfluidic conditions show higher expression of polarization and differentiation markers

mRNA expression level of (A.) polarization markers *CDH1*, *TJP1*, *OCLN*, *EZR* and *VIL1* and (B.) differentiation markers *ALPI*, *SI*, *MUC2*, *MUC5AC*, *LYZ* and *CHGA* in HIO donor 3. HIO were grown in static condition or in monolayers subjected to fluid flow (microfluidic) for 8 days. Graph shows average relative expression values normalized to *RPS18*, *HPRT1*, *GUSB* and *YWHAZ* $\pm$ SEM with two-way ANOVA with Sidak's post-hoc test compared to the static condition (n=3).

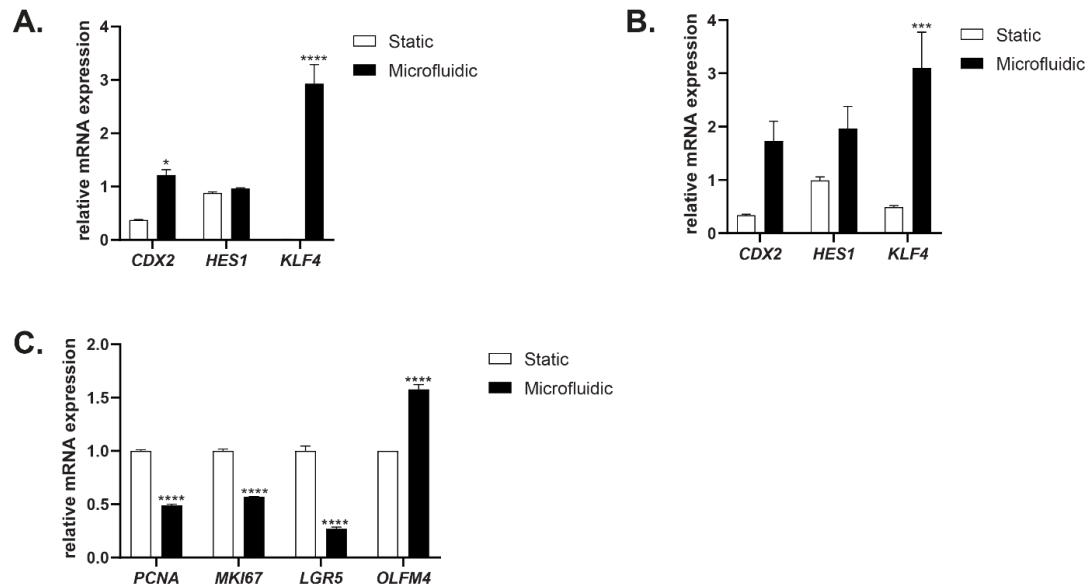


Figure 34. HIO cultured under microfluidic conditions seem to favour differentiation over proliferation pathways

A-B. mRNA expression level of differentiation transcription factors *CDX2*, *HES1* and *KLF4* in HIO donor 2 (**A.**) and HIO donor 3 (**B.**). **C.** mRNA expression level of proliferation markers *PCNA* and *MKI67* and stem cell markers *LGR5* and *OLFM4* in HIO donor 3. **A-C.** HIO were grown static condition or in monolayers subjected to fluid flow (microfluidic) for 8 days. Graph shows average relative expression values normalized to *RPS18*, *HPRT1*, *GUSB* and *YWHAZ* $\pm$ SEM with two-way ANOVA with Sidak's post-hoc test compared to the static condition (n=3).

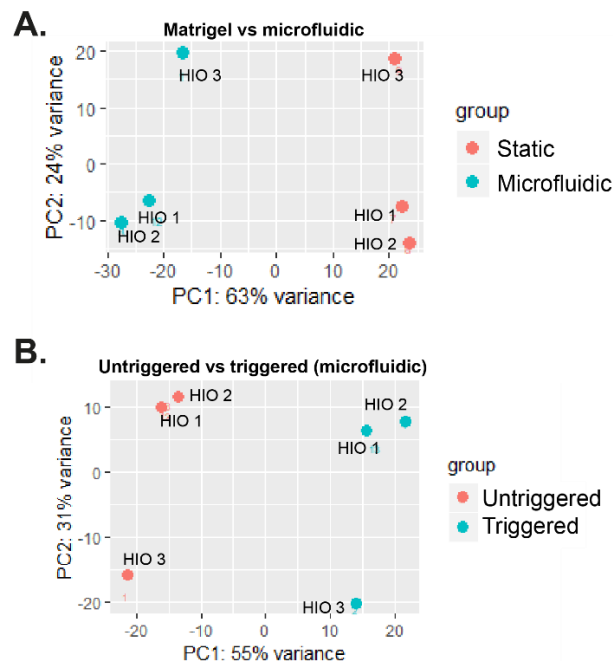


Figure 35. Principal component analysis (PCA) plots

A. 3 HIO donors were grown in static (red) or microfluidic conditions (blue). **B.** 3 HIO donors were grown in microfluidic conditions and triggered using LPS [100 ng/mL] and IFN- γ [20 ng/mL] for 24 h (blue) or left untriggered (red). In the 2 plots, a clear separation between the 2 experimental groups is observed. HIO donor 3 also shows a clear distinction but is segregated from HIO donor 1 and 2 in all groups.



Figure 36. Caco-2 gut-on-a-chip RNA expression profile resembles small intestine
Heatmap representation of the RNA expression profile of 72 genes defining intestinal identity in HIO from three donors grown in static or microfluidic conditions for 8 days, compared with human normal colon from study GSE9254 (LaPointe et al., 2008) and human normal small intestine sections (duodenum, jejunum, ileum) from study GSE14938 (She et al., 2009).

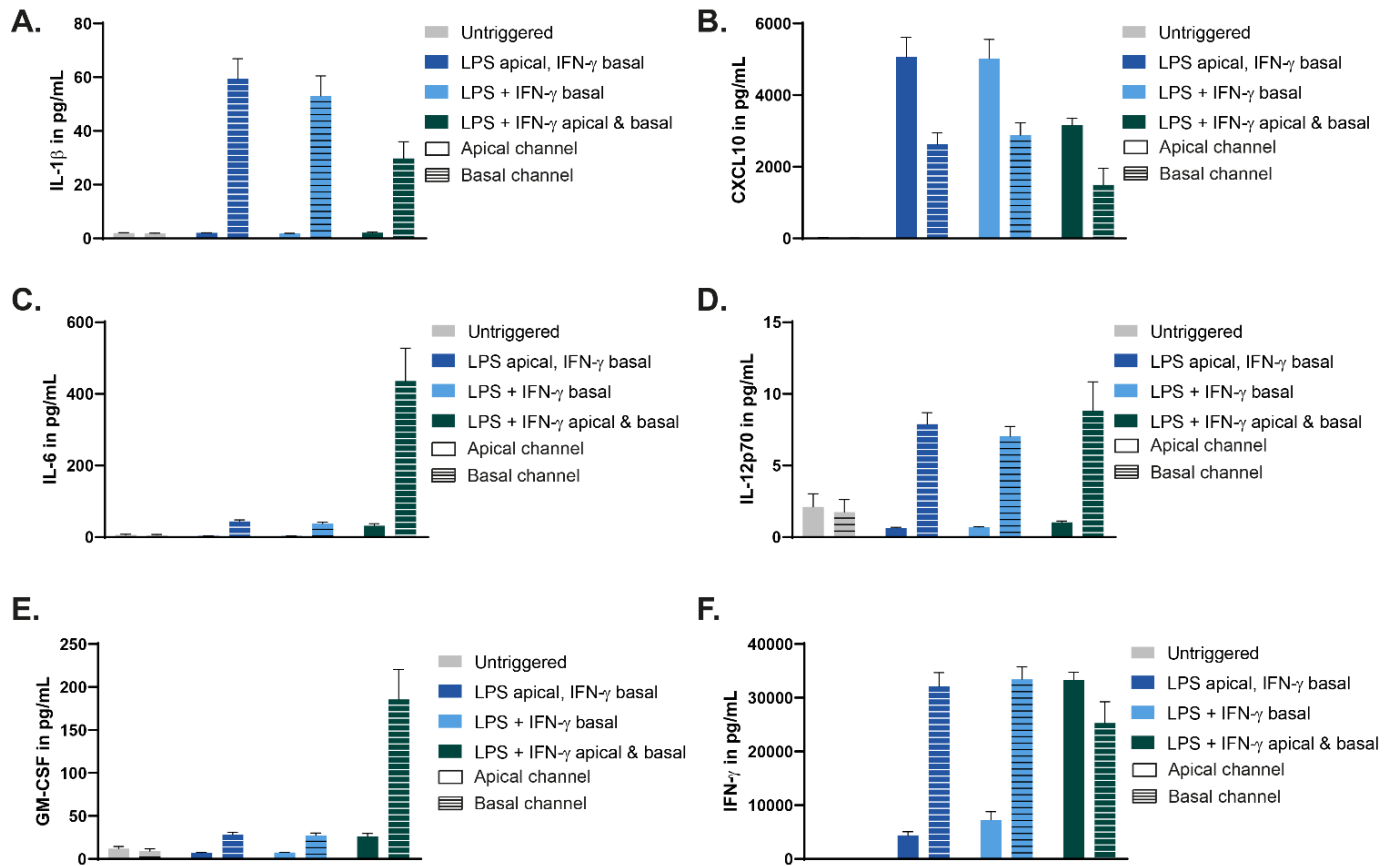


Figure 37. Asymmetric responses of IEC to inflammatory stimuli

A-F. Apical and basolateral secretion of IL-1 β (**A.**), CXCL10 (**B.**), IL-6 (**C.**), IL-12p70 (**D.**), GM-CSF (**E.**) and IFN- γ (**F.**) in IEC from HIO donor 2 at Day 8 after application of LPS [100 ng/mL] and IFN- γ [20 ng/mL] for 24 h. Trigger constituents were either segregated in different channels (LPS apical, IFN- γ basal; dark blue bars), applied together in the basal channel only (pale blue bars) or applied together simultaneously in the apical and basal channels (green bars). Bars represent average cytokine production [pg/mL] $\pm$ SEM (n=4-10).

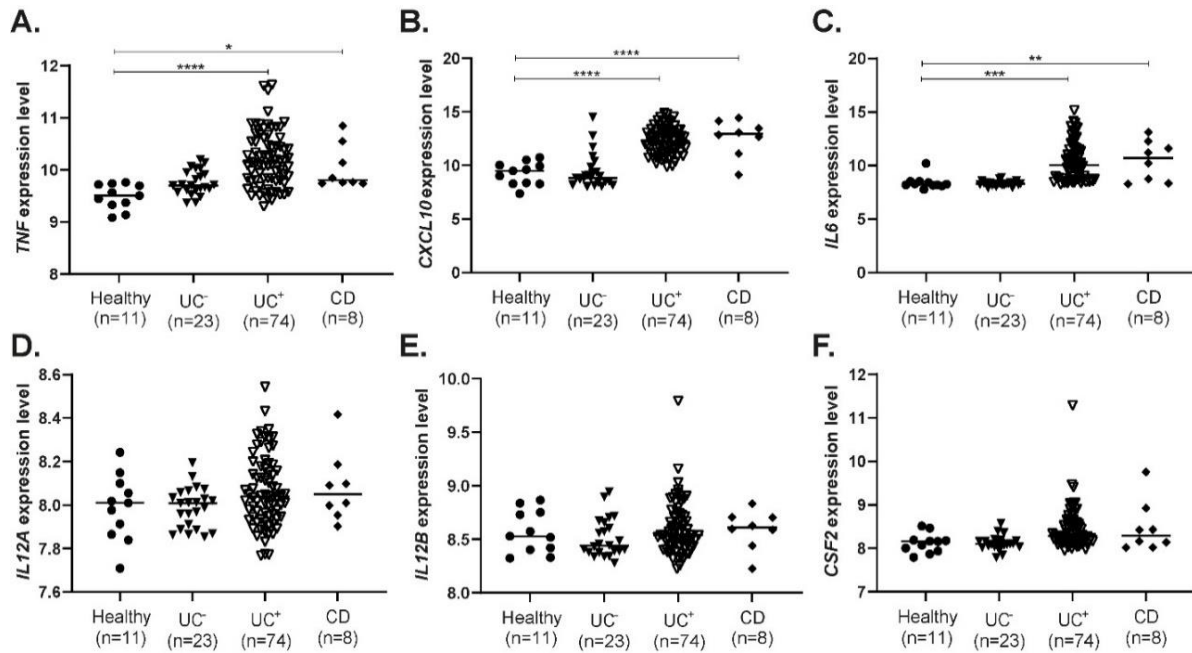


Figure 38. The expression of multiple inflammatory cytokines is overexpressed in the colonic mucosa of IBD patients

A-G. Gene expression of *TNF* (A.), *CXCL10* (B.), *IL6* (C.), *IL12A* (D.), *IL12B* (E.) and *CSF2* (F.) in mucosal colonic biopsies of human patients from GSE59071 (UC⁻= inactive ulcerative colitis, UC⁺=active ulcerative colitis, CD=Crohn's disease) (Vanhove et al., 2015). Each symbol represents expression data from one individual patient, horizontal bars denote median values. One-way ANOVA with Dunnett's post-hoc test compared to healthy samples (n=8-74).

APPENDIX D – EXTENDED COMPOUND EXPOSURE

Preface

Exposure to TPCA-1 (Chapter II) and GSK650394 (Chapter III) on the primary microfluidic model have been performed to validate the potential of the microfluidic model for compound testing and elucidate SGK2 implication in IBD, respectively. We extended the panel of compounds tested to include Bosutinib and Dasatinib. Bosutinib and Dasatinib are clinically approved Salt-inducible kinase (SIK) inhibitors used in the treatment of cancer, but further studies have shown that they to have an anti-inflammatory effect on activated macrophages (Ozanne et al., 2015). Their effect on the inflamed intestinal epithelium, however, remains unexplored.

Results

Treatment with Bosutinib led to a dose-dependent reduction of the release of TNF- α , IL-6, IL-8, IL-12p70, IL-23 and CCL-20 by macrophages without affecting viability (**Fig 39-40**). In HIO donor 1, the inhibition profiles were more modest, but generally followed a dose-response curve, except for IL-6 and IL-12p70 whose inhibition remained constant (**Fig. 39**). The effect of Bosutinib was greater in HIO donor 3, but apical release of IL-6 was not affected (**Fig. 40**). Bosutinib was particularly efficient on IL-8 and CCL-20 production by HIO, where even the smallest concentration (0.02 μ M) of compound led to an inhibition of about 50% of IL-8 and CCL-20 release. Bosutinib also led to dose-dependent inhibition of all analytes on the co-culture of HIO and macrophages in the two donors. However, Bosutinib led to toxicity at the two highest concentrations in HIO and the co-culture.

Similarly, treatment with Dasatinib efficiently reduced the production of inflammatory cytokines by macrophages, HIO donor 3 and the co-culture (**Fig. 41**). In macrophages, all analytes were inhibited by the compound without affecting viability (**Fig. 41F**). In HIO, only basolateral IL-12p70 and both apical and basal IL-6 production were not inhibited in a dose-dependent manner (**Fig. 41A and 41C**), and all analytes were inhibited. However, Dasatinib was very toxic to the cells; all concentrations significantly reduced viability (**Fig. 41F**). Interestingly, the two lowest concentrations of Dasatinib did not decrease viability in the co-culture, suggesting a protective effect of the macrophages on HIO. The inhibition profiles of the co-culture were, all and all, similar to those of HIO only.

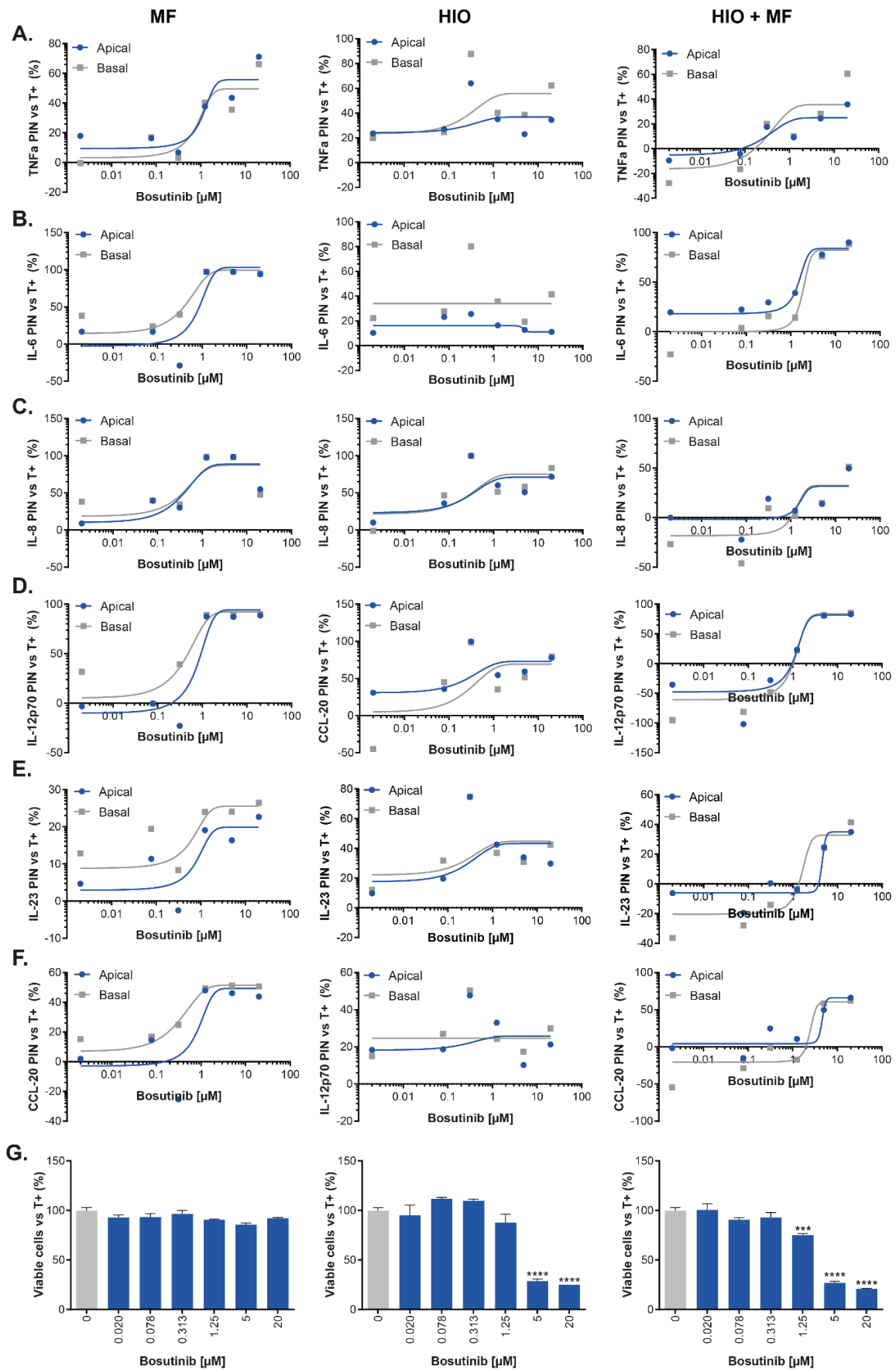


Figure 39. Bosutinib reduces cytokine release of macrophages and HIO donor 1

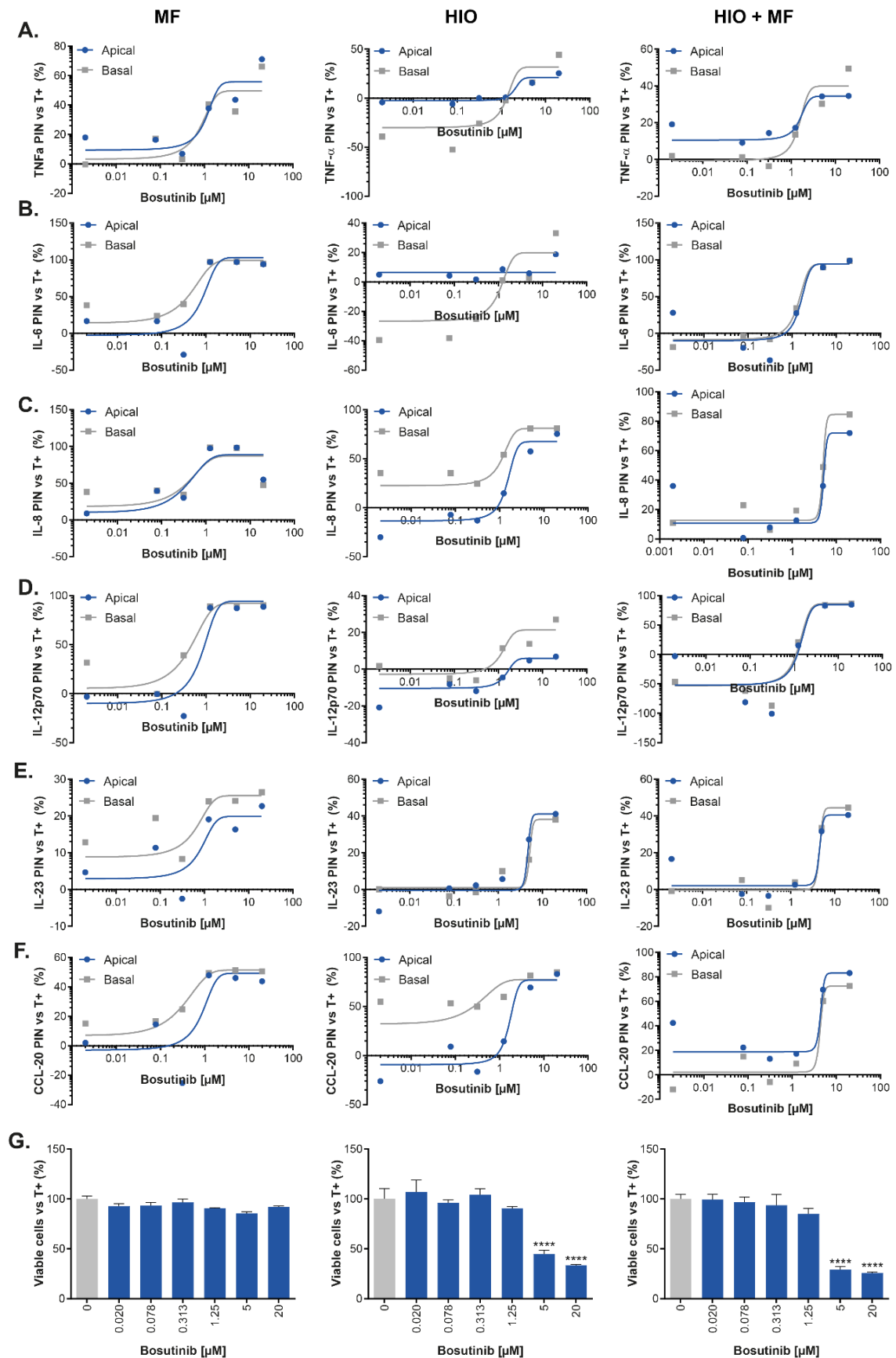


Figure 40. Bosutinib reduces cytokine release of macrophages and HIO donor 3

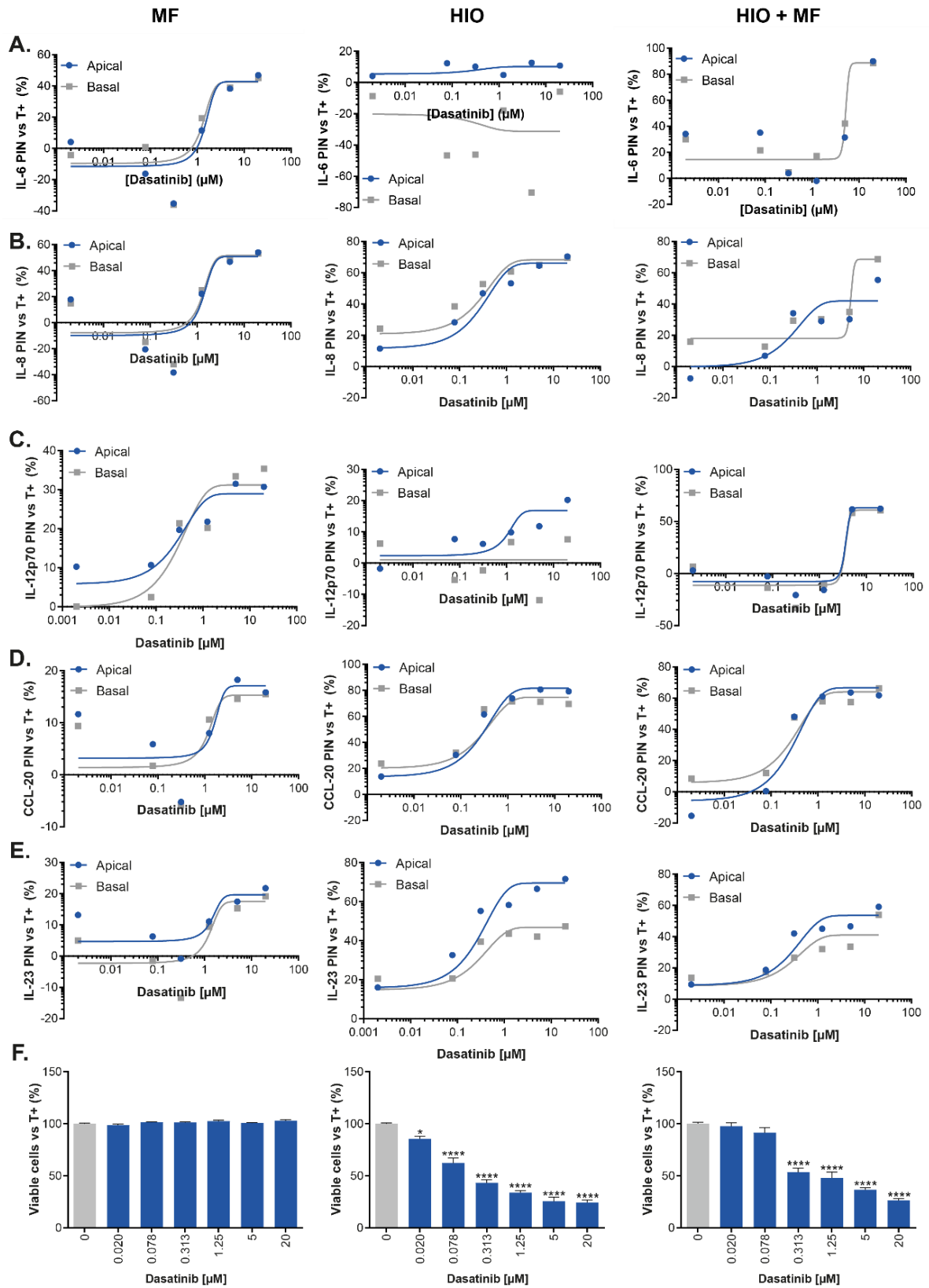


Figure 41. Dasatinib reduces cytokine release of macrophages and HIO donor 3

Figure 39. A-F. Apical and basal secretion of TNF- α (A.), IL-6 (B.), IL-8 (C.), IL-12p70 (D.), IL-23 (E.) and CCL-20 (F.) were assessed 72hrs after Bosutinib treatment at the indicated concentrations in macrophages (MF, left panel), HIO donor 1 (middle panel) or the co-culture (right panel). Dots represent the percentage of inhibition (PIN) mean normalized to triggered (LPS 100ng/mL, IFN- γ 20ng/mL) but Bosutinib untreated tubules. The line depicts a non-linear regression between [Bosutinib] and cytokine secretion (n=3-5). **G.** Viability of macrophages (MF, left panel), HIO donor 1 (middle panel) or the co-culture (right panel) after 72hrs of Bosutinib exposure. Bars represent the average of the percentage of viable cells $\pm$ SEM with one-way ANOVA with Dunnett's post-hoc test compared to triggered but Bosutinib untreated tubules (n=3-10).

Figure 40. A-F. Apical and basal secretion of TNF- α (A.), IL-6 (B.), IL-8 (C.), IL-12p70 (D.), IL-23 (E.) and CCL-20 (F.) were assessed 72hrs after Bosutinib treatment at the indicated concentrations in macrophages (MF, left panel), HIO donor 3 (middle panel) or the co-culture (right panel). Dots represent the percentage of inhibition (PIN) mean normalized to triggered (LPS 100ng/mL, IFN- γ 20ng/mL) but Bosutinib untreated tubules. The line depicts a non-linear regression between [Bosutinib] and cytokine secretion (n=3-5). **G.** Viability of macrophages (MF, left panel), HIO donor 3 (middle panel) or the co-culture (right panel) after 72hrs of Bosutinib exposure. Bars represent the average of the percentage of viable cells $\pm$ SEM with one-way ANOVA with Dunnett's post-hoc test compared to triggered but Bosutinib untreated tubules (n=3-10).

Figure 41. A-E. Apical and basal secretion of IL-6 (A.), IL-8 (B.), IL-12p70 (C.), CCL-20 (D.) and IL-23 (E.) were assessed 72hrs after Dasatinib treatment at the indicated concentrations in macrophages (MF, left panel), HIO donor 1 (middle panel) or the co-culture (right panel). Dots represent the percentage of inhibition (PIN) mean normalized to triggered (LPS 100ng/mL, IFN- γ 20ng/mL) but Dasatinib untreated tubules. The line depicts a non-linear regression between [Dasatinib] and cytokine secretion (n=3-5). **F.** Viability of macrophages (MF, left panel), HIO (middle panel) or the co-culture (right panel) after 72hrs of Dasatinib exposure. Bars represent the average of the percentage of viable cells $\pm$ SEM with Dunnett's post-hoc test compared to triggered but Dasatinib untreated tubules (n=3-10).

Conclusion

Herein, we tested whether the administration of Bosutinib and Dasatinib could successfully prevent the establishment of inflammation in the gut-on-a-chip. Bosutinib and Dasatinib, drugs used in the treatment of chronic myeloid leukaemia, showed a notable reduction of the inflammatory phenotype induced in macrophages. These results go along with previous findings indicating that both compounds are able to block pro-inflammatory cytokine production in inflammatory macrophages (Ozanne et al., 2015). We also assessed, for the first time, whether those compounds could inhibit inflammation of the intestinal epithelium. Similarly, both compounds reduced cytokine production of HIO as well as in HIO co-cultured with macrophages in a dose-dependent manner. Overall, a major effect on viability of the cells could be observed; Dasatinib cause high cell toxicity whereas Bosutinib only slight affected viability. The use of these compounds in the treatment of IBD should definitely be further investigated as they have shown to be immunoregulatory not only in macrophages but also in

DC (Sundberg et al., 2014; Ozanne et al., 2015), two important cell types in the aetiology of IBD (Lissner et al., 2015; Hart et al., 2005). Their direct and specific effect on the inflammation of the intestinal epithelium has never been carefully investigated, however a study shows that Dasatinib could protect the murine intestinal epithelium against radiation-induced damage by increasing mucosal thickness and maintaining intestinal epithelial integrity (Strup-Perrot et al., 2016). Combined with the latter study, our results show the potential of SIK inhibitors to be used in the treatment of IBD. However, they have also been showed to cause intestinal toxicity in patients affected with chronic myeloid leukaemia (Kantarjian et al., 2014; Chisti et al., 2013), but this toxicity could be reduced at lower doses of Dasatinib. Therefore, more comprehensive models showing multi-organ effect and toxicity should be used before assessing their efficacy in IBD patients.

These results show that our model has great potential for the assessment of compounds efficacy. However, more optimization will be needed before undertaking larger scale screenings. Indeed, as it is right now, HIO have proven to be very sensitive to compound exposure and the window before toxicity and effect could be improved. Perhaps, lowering the compound concentration and increasing the exposure time could help resolve the issue and obtain more reliable results out of the platform. However, these results suggest that this would be an interesting avenue to pursue.

CHAPTER III

Investigation of the role of SGK2 in intestinal inflammation

Introduction

Target discovery in inflammatory bowel disease

Before being commercialized and administrated to IBD patients, a drug needs to go through a long path of assessment and approval stages. After extensive laboratory research in human cells and animal models and after approval by competent authorities such as European Medicines Agency (EMA), the clinical evaluation process is launched [process reviewed in (Umscheid et al., 2011)]. First, Phase I assesses the safety of the drug studied in a small number of healthy individuals. Particularly, pharmacokinetics such as drug absorption, metabolism and excretion are determined. Phase II studies further test the efficacy of the drug in several hundred of patients divided in blinded groups receiving either placebo or standard treatment. When a drug moves to Phase III it goes through large-scale testing in hundreds to thousands of patients, providing solid evidence of the drug's efficacy and safety. The final phase of clinical trials happens when the drug is already on the market. Phase IV studies allow monitoring of long-term efficacy of the drug as well as its cost-effectiveness in comparison to other existing drugs.

1.1. General target discovery approach

Pharmaceutical and large biotech companies that carry drug discovery activities generally use a similar process (**Fig. 42**). The process starts with the identification of potential targets involved in a particular disease in a screening campaign. Specifically, Galapagos aims to develop assays that are able to recapitulate the biology of a disease as closely as possible by using human primary material derived from healthy and diseased patients. Once the assay is set-up with relevant disease phenotype and readouts, a patented arrayed library of >20,000 adenoviral shRNAs targeting the druggable genome is applied in the assay to induce gene knockdown. Hits are identified by the capacity of the knockdown to reduce disease markers and readouts. The selected hits are then 're-screened' in the same assay that was used in the screening campaign to confirm their potential implication in the diseased phenotype. Once the re-screen is completed, target candidates are validated by alternative knockdown or knock-out technologies, such as CRISPR-Cas9, lentiviral knockdown, siRNA technology, compounds or antibody-mediated protein knockdown. When the target's implication in the disease is supported by strong scientific evidence, competition in the field and chemical safety are assessed before lead discovery and medicinal chemistry phases are undertaken. Chemists will develop small molecules that will selectively target the protein-of-interest and these compounds will further be tested on *in vitro* and *in vivo* models before going into clinical trials.

Over the years, Galapagos has launched hundreds of screening campaigns in indications such as idiopathic pulmonary fibrosis, hepatitis B, cystic fibrosis, rheumatoid arthritis, scleroderma and IBD.

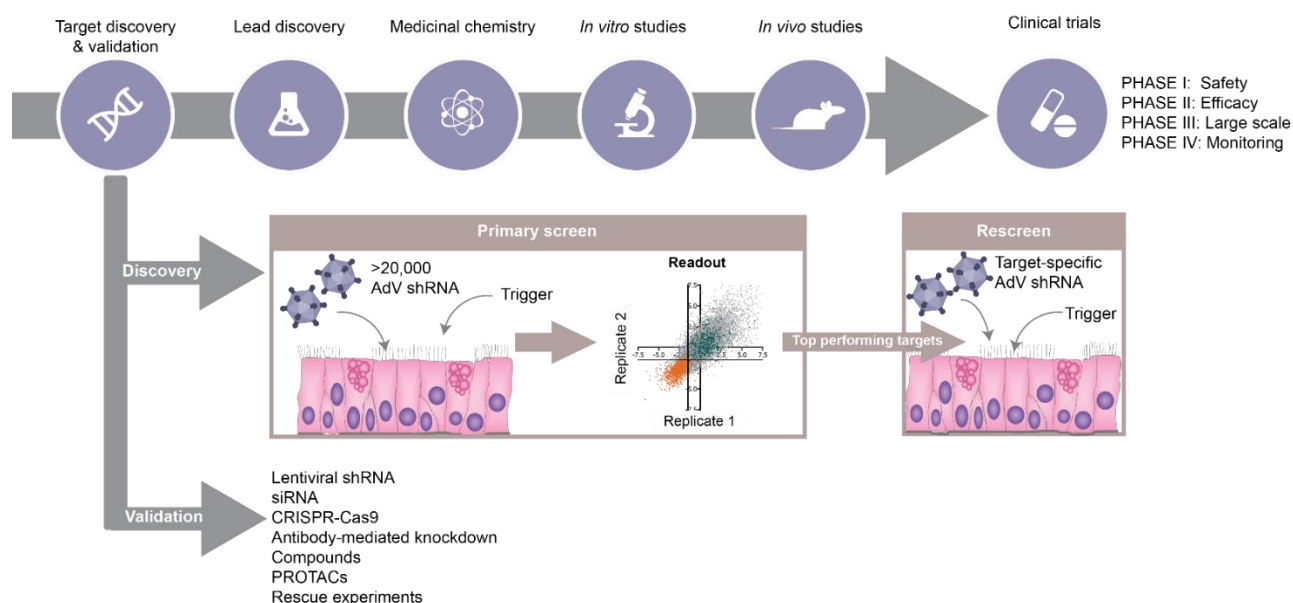


Figure 42. The clinical process in drug discovery

Representation of the general pipeline used in drug discovery, with a special focus on the target discovery and validation activities of Galapagos. After targets are identified in disease-relevant assays using the shRNA technology, they are validated using shRNA-independent technologies and lentiviral knockdown. After careful examination of *in-house* data and literature, drugs are developed against the identified targets. The efficacy as well as safety of the synthesized drugs are evaluated in *in vitro* and *in vivo* models, before entering the clinical trial stage. AdV = Adenovirus

1.2. Screening for targets in inflammatory bowel disease at Galapagos

In 2014, scientists from Galapagos started a screening campaign for target identification in IBD. As mentioned earlier, sources of human primary IEC that can generate enough cells for a screening campaign are limited. Therefore, Caco-2 cells were chosen as the epithelial cell type and were grown on top of Transwell filters (**Fig. 43**). Because of the limited throughput of the Transwell system, a reduced library of 7,054 shRNA was applied to the Caco-2 cells on the next day. 9 days after, the Transwell filters were transferred to receiver plates containing *E. coli*-activated DC. The cells were then triggered with an immune-relevant cytokine cocktail (IL-1 β , TNF- α and IFN- γ) for 3 days. The effect and relevance of this trigger on Caco-2 cells have been described previously (Beaurivage et al., 2019; Van De Walle et al., 2010). At the experiment end-point, the barrier integrity of the Caco-2 monolayers was assessed by FITC-dextran leakage assay and the apical and basal secretion of inflammatory cytokines was quantified.

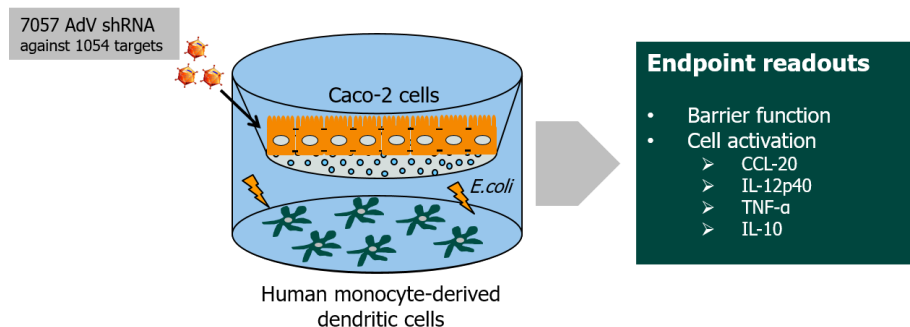


Figure 43. Assay used for target identification in IBD at Galapagos

42,900 Caco-2 cells were seeded on top of a Transwell insert. On Day 1, cells were transduced with MOI 8-35 per cell. On Day 9, seeded Transwell filters were co-cultured with 60,000 monocyte-derived dendritic cells with [100ng/mL] UV-irradiated *E. coli* and triggered with IL-1 β , TNF- α and IFN- γ (1-50-50 ng/mL respectively) for 3 days. Apical and basal effluents were collected at Day 12 to assess cytokine secretion and a FITC-dextran leakage assay was performed to assess barrier integrity.

1.3. Identification and validation of *SGK2* as a target in IBD

In **Figure 44**, the performance of all targets screened on CCL-20 reduction in the adenoviral screening assay (**Fig. 44A**), the adenoviral re-screening assay (**Fig. 44B**) and the lentiviral re-screening assay (**Fig. 44C**). A total of 203 targets were identified in the adenoviral screening assay. In those 203 targets, *SGK2* came out as a potential hit through its regulation of CCL-20 secretion. Upon adenoviral-mediated knockdown of *SGK2*, apical secretion of CCL-20 by Caco-2 cells was reduced by 4 out of 6 *SGK2*-targeting shRNAs in both the screening campaign (**Fig. 44B**) and the re-screen campaign (**Fig. 44D**). The team next performed a supplementary re-screen campaign where lentiviral shRNAs were delivered to the Caco-2 cells. Two out of 8 lentiviral shRNAs reduced CCL-20 secretion. This is in line with other in-house data; lentiviral knockdown generally requires the implementation of multiple shRNA versions in order to obtain a few that give a strong knockdown. Despite the fact that the team did not assess knockdown level in the lentiviral re-screen, they did correlate *SGK2* expression together with CCL-20 percentage of inhibition (PIN) in the adenoviral re-screen (**Fig. 45**). Constructs leading to a knockdown of *SGK2* expression also led to inhibition of CCL-20 production by Caco-2 cells.

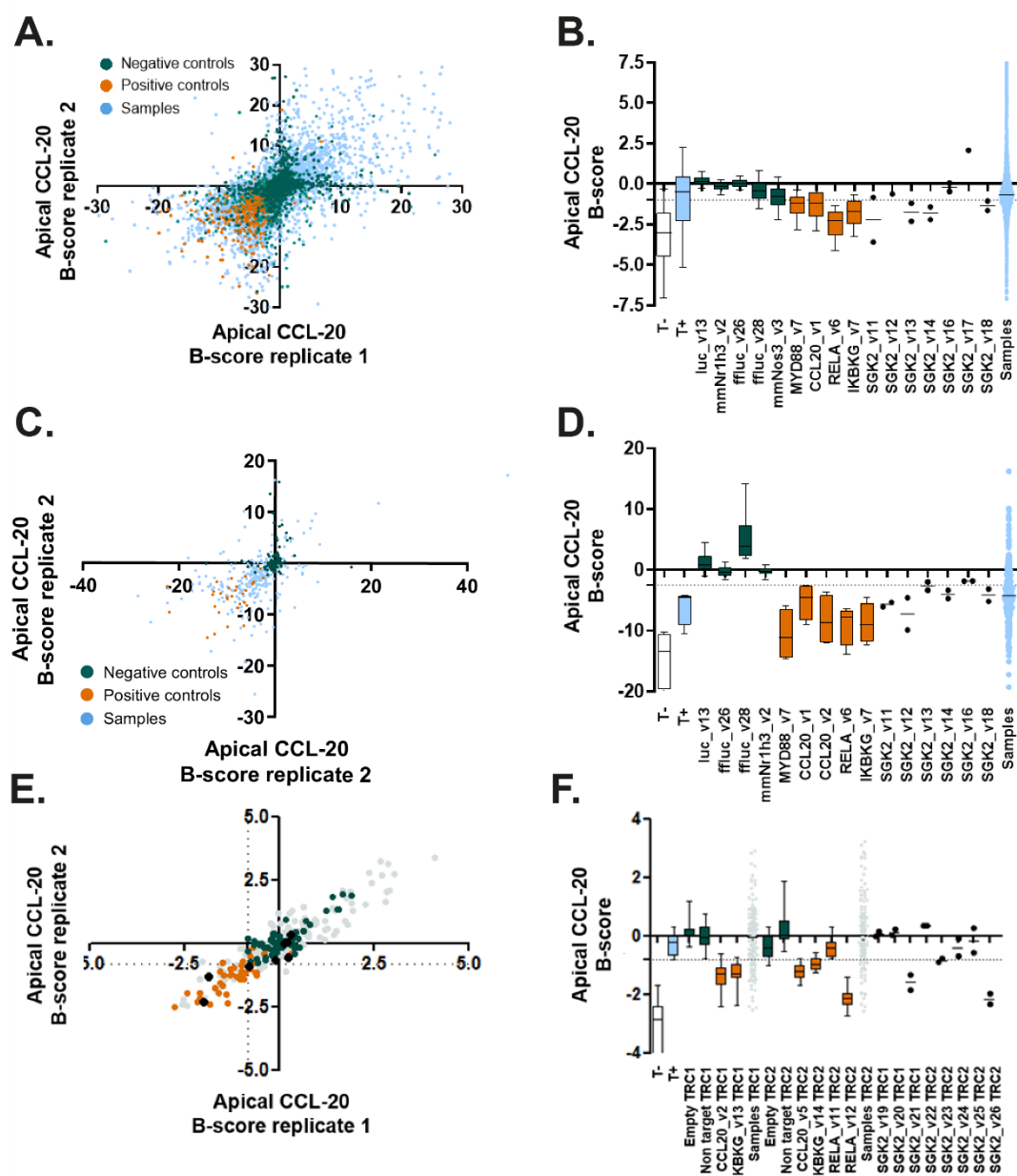


Figure 44. Performance of SGK2 in screening and re-screen assays

A-C-E. Correlation graphs of normalized B-scores (relative to 3 internal negative controls) of apical CCL-20 production by Caco-2 cells in replicates 1 and 2 of the screening assay (**A.**), adenoviral re-screen assay (**C.**) and lentiviral re-screen assay (**E.**). Each dot represents one duplicate; negative controls (green), positive controls (orange), samples (grey/blue). **B-D-F.** Normalized B-scores (relative to 3 internal negative controls) of apical CCL-20 production by Caco-2 cells in screening assay (**B.**), adenoviral re-screen assay (**D.**) and lentiviral re-screen assay (**F.**). Boxes extend from the 25th to 75th percentile (IQR); line represents the median; Tukey whiskers. Negative controls (green), positive controls (orange), samples (grey/blue), SGK2-interfering viruses (black). Each dot represents a replicate. Dotted line= Cut off (-1).

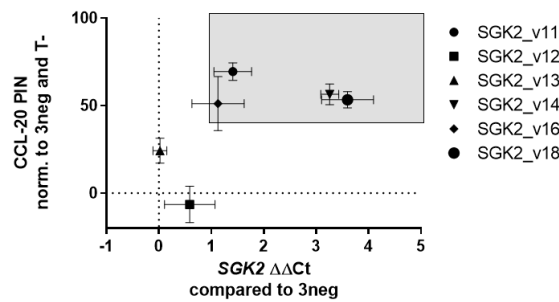


Figure 45. Knockdown of *SGK2* decreases CCL-20 production by Caco-2 cells

CCL-20 percentage of inhibition (PIN) vs $\Delta\Delta Ct$ values for *SGK2* expression 4hrs after trigger. CCL-20 expression was normalized for intra-plate variability using 3 internal negative controls (3neg) and compared to untriggered (T-) cells. $\Delta\Delta Ct$ values were calculated using *GAPDH* (n=2). The grey area depicts, on the y-axis, a range of CCL-20 inhibition between 40 and 100% and, on the x-axis, the fold change difference in *SGK2* expression between the different shRNA versions and the 3neg controls. It highlights the correlation between knockdown and inhibition of CCL-20 secretion.

Finally, a tool compound inhibiting SGK1 and SGK2 [IC_{50} = 62 nM (SGK1) and 103 nM (SGK2)] (Wang and Gray, 2015) was used to confirm the effects of SGK1/2 inhibition on CCL20 secretion. Application of the compound to the co-culture led to a strong inhibition of the apical release of CCL-20 by Caco-2 cells and to inhibition of basolateral IL-6, IL12-p40, IL-10 and TNF- α without affecting barrier integrity after 3 days of exposure (Fig. 46). However, internal investigation at Galapagos have determined that this compound inhibits several other kinases *in vitro* at an IC_{50} < 100 nM and cannot discriminate between SGK1 and SGK2. It is therefore not possible to use this compound to specifically investigate SGK2.

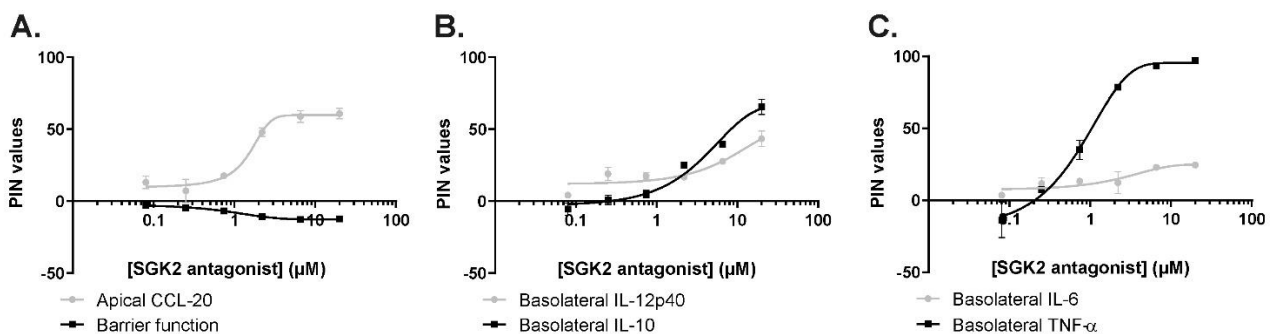


Figure 46. Exposure to GSK650394 inhibits cytokine release by Caco-2 cells and DC

Percentage of inhibition (PIN) of apical CCL-20 and barrier integrity (A.), basolateral IL-12p40 and IL-10 (B.) and basolateral IL-6 and TNF- α (C.) in Caco-2 cells co-culture with dendritic cells (DC; donor D16_030) after 3 days of compound treatment. Dots represent the mean, error bars the range of data and the line depicts a non-linear regression between PIN and compound concentration (n=2). Data from one representative DC donor.

These results were overall conclusive and suggested a potential role for SGK2 in the regulation of inflammatory processes; however, the target was dropped because another similar, more

promising target was already in the pipeline at the time. We therefore chose SGK2 as a target to investigate in this project due to the excellent preliminary data generated by Galapagos and also because it offered a potential for publication of the results since the target was not actively investigated by Galapagos.

SGK paralogs

Serum- and Glucocorticoid-Regulated Kinase 1 (SGK1) was the first paralog of the SGK subfamily identified. As its name suggests, it has been originally described as a gene transcriptionally stimulated by serum and glucocorticoids in rat mammary tumour cells (Webster et al., 1993a, 1993b). The human paralog of SGK1 was later identified in a screen for anisotonic and isotonic cell volume mediators in a human hepatoma cell line (Waldegger et al., 1997, 2000). In 1999, two novel paralogs of SGK, subsequently named SGK2 and SGK3, were identified (Kobayashi et al., 1999). The three SGK paralogs share 80% of homology within their catalytic domain (Kobayashi et al., 1999), but the homology is reduced to only 50% of amino acid homology in their COOH-terminal domain and <15% homology in their NH2-terminal domains (Pao et al., 2007). Their structure is shown at **Figure 47**. SGK paralogs belong to the AGC family of protein kinases, which, in addition to the SGK kinases, includes the PKB/Akt kinases, p70 ribosomal S6 kinase (S6K) and atypical forms of protein kinase C (PKC). SGK are protein kinases that phosphorylate serine and threonine residues that lie within Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr motifs (Kobayashi et al., 1999).

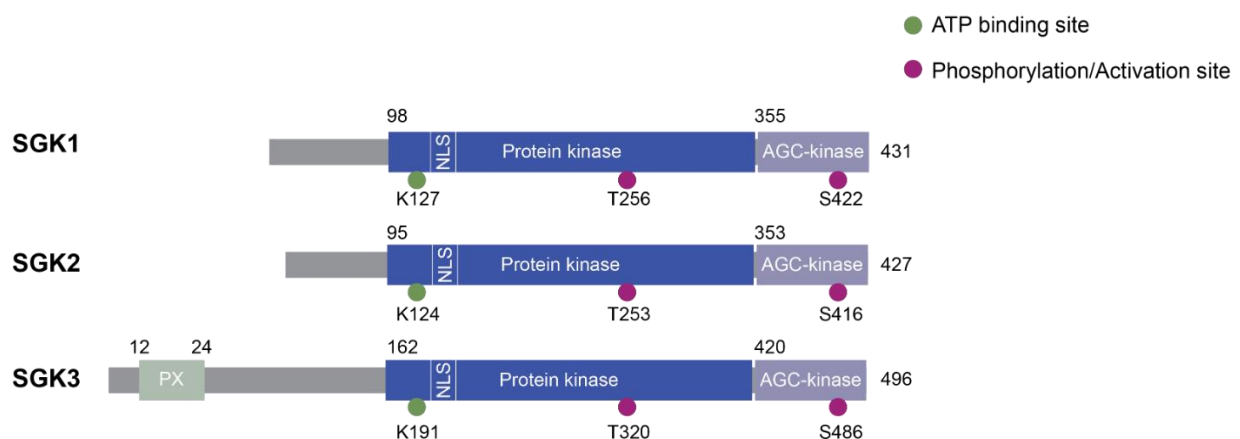


Figure 47. Structure of the human SGK paralogs

SGK paralogs all share a protein kinase domain, an AGC kinase C-terminal domain and a nuclear localization signal (NLS). SGK3 also possesses a N-terminal Phox homology (PX) domain. Important residues for SGK activation and activity are highlighted. All numbers shown are amino acids positions.

2.1. Genomic regulation

The three paralogs are products of distinct genes; the gene encoding human SGK1 has been localized to chromosome 6q23 (Waldegger et al., 1998), whereas the gene encoding SGK2 is

localized to chromosome 20q12 (Lang and Cohen, 2001) and SGK3 is on chromosome 8q12.3 (Dai et al., 1999; Liu et al., 2000). While the mRNAs encoding SGK1 and SGK3 are widely expressed, SGK2 has a more restricted distribution, being highly expressed in epithelial tissues such as liver, kidney, pancreas, brain and GI tract. In contrast to SGK1, the mRNA levels of SGK2 and SGK3 are not increased by cell stimulation with serum or glucocorticoids and are constitutively expressed (Kobayashi et al., 1999).

2.2. Activation

SGK paralogs require two main phosphorylation events in order to become active. The current consensus is that they are first phosphorylated by an unknown kinase on a COOH-terminal Ser (Ser⁴²² in SGK1, Ser⁴¹⁶ in SGK2, Ser⁴⁸⁶ in SGK3). Once phosphorylated in the COOH-terminus, their activation-loop is further phosphorylated (Thr²⁵⁶ in SGK1, Thr²⁵³ in SGK2, Thr³²⁰ in SGK3) by 3-Phosphoinositide-dependent protein kinase-1 (PDK1) (Kobayashi et al., 1999) and is therefore dependent on the PI3K pathway. Interestingly, equivalent residues are present in many protein kinases of the AGC family, such as p70 S6 kinase, and there is increasing evidence that PDK1 catalyses the activation of these enzymes *in vivo* (Le Good et al., 1998; Dutil et al., 1998; Williams et al., 2000; Stephens et al., 1998).

Activation of SGK paralogs can be obtained through various ways. Hydrogen peroxide is the greatest inducer of SGK activation known yet; all three paralogs have shown at least a 5-fold increase of their activation in 293 cells (Kobayashi et al., 1999). This activation is dependent on PI3K only for SGK1 as treatment with wortmannin and LY294002 totally abolished SGK1, but only partially SGK2 and SGK3 activation. All paralogs are also activated by IGF1, although SGK1 is stimulated more efficiently than the other paralogs.

2.3. General functions of SGK paralogs

All three kinases are potent regulators of ion channel activity, transport and transcription (Bhargava and Pearce, 2004; Fillon et al., 2001; Lang et al., 2003; Pearce, 2003; Loffing et al., 2006; Stockand, 2002; Verrey et al., 2003). Functional analysis of gene-targeted mice lacking SGK1 (Wulff et al., 2002) and SGK3 (McCormick et al., 2004) expression provided insight into the functional significance of SGK-dependent mechanisms. Interestingly, neither knockout of SGK1 (Wulff et al., 2002) or SGK3 (McCormick et al., 2004), nor knockout of both SGK1 and SGK3 (Grahammer et al., 2006a) leads to a severe phenotype, suggesting that neither of them is required for survival. However, closer inspection of the SGK1 knockout mice (*sgk1*^{-/-}) reveals that the function of the intestinal transporter SGLT1 in answer to

mineralcorticoids is fully dependent on SGK1 and that the function of intestinal NHE3 in response to mineralcorticoids is partly dependent on SGK1 (Grahammer et al., 2006b). *Sgk1*^{-/-} mice also displayed impaired Na⁺ reabsorption in distal renal tubules leading to low arterial pressure and decreased glomerular filtration rates when mice were kept on low levels of NaCl (Wulff et al., 2002). SGK3 knockout mice (*sgk3*^{-/-}), on the other hand, surprisingly displayed an impaired hair follicle morphogenesis (McCormick et al., 2004) and only a subtle but metabolically significant defect in sodium-driven intestinal glucose absorption (Sandu et al., 2005). The double knockout mutants recapitulated the phenotypes observed for both *sgk1*^{-/-} and *sgk3*^{-/-} animals, without further aggravation of the phenotype, which led the authors to postulate that SGK2, or another similar kinase such as AKT family members, might compensate for the loss of expression of the other SGK paralogs (Grahammer et al., 2006a). This hypothesis has, however, never been officially tested and transcripts levels of SGK2 appeared to remain the same between WT and *sgk1*^{-/-} (Grahammer et al., 2006b), *sgk3*^{-/-} (McCormick et al., 2004) or *sgk1*^{-/-}*sgk3*^{-/-} littermates (Grahammer et al., 2006a). Unpublished data states that *sgk1*^{-/-}*sgk2*^{-/-} mice had an impaired ability to reabsorb water, sodium, chloride, and potassium during salt deprivation compared to *Sgk1*^{-/-} or *Sgk2*^{-/-} mice and therefore suggests that SGK2 does compensate for the loss of SGK1 expression in *sgk1*^{-/-} mice (Guerriero et al., 2020).

As described above, SGK2 was identified as a potential IBD target through the effect of its knockdown on CCL-20 production by Caco-2 cells at Galapagos in 2014. The next section will specifically deepen the knowledge on the latter paralog.

Serum- and Glucocorticoid-Regulated Kinase 2 (SGK2)

3.1. Generalities

SGK2 is the paralog of the SGK family that has been the lesser one described in literature. As highlighted in the previous section, its transcripts are constitutively expressed in a restricted set of tissues when compared to the other SGK paralogs. There are three transcript variants known for SGK2 gene to this day that all result from alternative RNA splicing. Transcript variant 1 encode the 367 amino acids (aa) isoform alpha, whereas transcript variant 2 encodes isoform beta which is a 427aa protein and transcript variant 3 encodes isoform 3, a 397aa protein.

The structure of the canonical isoform beta, encoded by transcript variant 2, is shown at **Figure 48**. Other than a kinase domain spanning most of its sequence (aa95-427), no domain other than a NLS motif (amino acids 128-138) is recognizable in SGK2 sequence. Within the kinase

domain, the ATP binding site is located on lysine 124 (K124) and is responsible for the catalytic activity of the enzyme. The mutation of the homologous residue on SGK1 into a methionine (K127M) leads to a catalytically inactive enzyme often referred as kinase-dead mutant (Mikosz et al., 2001). In contrast, the mutation S356D of murine Sgk2 (homologous human residue: S416) causes its constitutive activation (Pao et al., 2010).

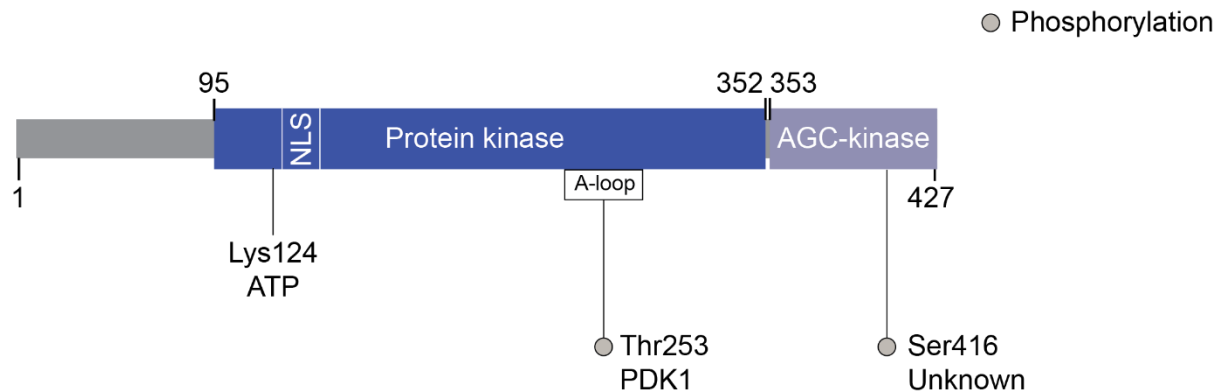


Figure 48. Main structural features of SGK2 isoform beta

The protein kinase domain of SGK2 extends from amino acid (aa) 95-352 and the ACG kinase domain from aa353-427. SGK2 becomes activated by phosphorylation by an unknown kinase on Ser416. PDK1 can subsequently phosphorylate Thr253 for SGK2 to be fully activated. Mutation of Ser416 into an aspartic acid constitutively activates SGK2. A nuclear localization sequence (NLS) is present from aa128-138 and an ATP binding site is localized at aa124.

3.2. Regulation of the expression and activity of epithelial transporters

As the other SGK paralogs, SGK2 has been characterized to regulate multiple epithelial transporters in the kidney. Co-expression of SGK2, as well as SGK1 and SGK3, and epithelial sodium channel ENaC in *Xenopus* oocytes up-regulated renal ENaC (*SCNN1A*) protein levels (Friedrich et al., 2003). The mutation of the SGK consensus phosphorylation site on ENaC (ENaC^{αS622A} mutant) revealed that this regulation is not mediated through direct phosphorylation of ENaC (Friedrich et al., 2003). In a further study investigating the relationship between SGK1 and ENaC function, SGK1 was shown to phosphorylate ubiquitin ligase NEDD4-2, which ubiquitinates ENaC and thus prepares the channel for subsequent internalization and degradation (Debonneville et al., 2001). As a result, NEDD4-2 accelerates the clearance of ENaC from the cell membrane. The phosphorylation by SGK1 inactivates NEDD4-2 leading to persistence of ENaC channel protein in the cell membrane (Debonneville et al., 2001). Accordingly, SGK1 enhanced the abundance of the ENaC channel protein in the plasma membrane (De La Rosa et al., 1999). SGK1 was also shown to modulate the glutamate transporter EAAT1 (*SLC1A3*) (Boehmer et al., 2003a) and potassium channel hERG (*KCNH2*) (Lamothe and Zhang, 2013) through regulation of NEDD4-2. Given the resemblance between SGK1 and SGK2, it is not to be excluded that SGK2 could regulate epithelial channels in similar ways as SGK1. Furthermore, SGK2 has also been shown to directly interact with and

modulate NEDD4-2 ubiquitin-ligase ability, most likely through phosphorylation, leading to the stimulation of hOAT4-mediated estrone sulfate uptake in the kidney (Wang et al., 2016a).

SGK2 has also been shown to regulate the activity of Na^+/H^+ exchanger NHE3 (*SLC9A3*) in the kidney (Pao et al., 2010). Notably, SGK2, but not SGK1, controlled the activity and cell surface expression of NHE3 in a PI3K-dependent mechanism. As PI3K appears to stimulate NHE3 activity (D'Souza et al., 1998) and since insulin, a hormone dependent on PI3K activity, enhances NHE3 cell surface abundance and activity in kidney cells (Kliscic et al., 2002), it was postulated that SGK2 could potentially function as an effector for insulin-mediated stimulation of NHE3 activity. The mechanistic basis of this regulation was, however, not elucidated.

3.3. *SGK2 in the intestinal epithelium*

Despite the fact that members of the SGK family have been identified to regulate the activity and expression of multiple epithelial receptors, a direct link between SGK2 and intestinal transporters has never been described. There is, however, some evidence that suggests that SGK2 might play an important role in the intestinal mucosa.

Firstly, elevated SGK2 expression in the GI tract, together with the fact that NHE3 and ENaC are also expressed in the human intestine, suggest that SGK2 might also regulate the activity of these channels in the gut. Furthermore, members of the ACG subfamily of kinases have been shown to regulate the activity of intestinal transporters. Notably, SGK1, SGK3 and PKB were shown to regulate Na^+ -coupled glucose transporter SGLT1 (*SLC5A1*) in *Xenopus* oocytes, once again through the phosphorylation of NEDD4-2 (Dieter et al., 2004). The team of Boehmer has demonstrated the regulation of glutamate and amino acid transporters EAAT1 (*SLC1A3*), EAAT2/GLT-1 (*SLC1A2*), EAAT4 (*SLC1A6*), EAAT5 (*SLC1A7*) and SN1 (*SLC38A3*) by SGK1, SGK3 and in some cases PKB (Boehmer et al., 2003a, 2003b; Böhmer et al., 2004; Boehmer et al., 2005, 2006). SGK1 has further been shown to stimulate Na^+/K^+ -ATPase (Henke et al., 2004; Setiawan et al., 2002; Zecevic et al., 2004), which is required for Na^+ gradient homeostasis, the second driving force for Na^+ -coupled nutrient transport (Schultz, 1981). However, these studies have systematically left SGK2 out of the experimental design. Whether SGK2 was not included by lack of interest as it is the least characterized paralog or whether it gave inconclusive results and was later left out of the publications is unknown and in turn, complicate matters. However, SGK paralogs have historically exerted similar functions and have often shown compensatory roles, which suggests that SGK2 might also be involved in the regulation of these receptors and channels. Finally, further studies suggest a larger role

for the ACG subfamily in the regulation of intestinal transport. As exposed earlier, PDK1 phosphorylates and activates ACG subfamily members such as the SGK paralogs and PKB. PDK1 hypomorphic mice (*pdk1^{hm}*), which express only 10-25% PDK1, showed reduced jejunal electrogenic transport of phenylalanine, cysteine, glutamine, proline, leucine and tryptophan compared to *pdk1^{wt}* mice (Rexhepaj et al., 2006). The authors postulated that the reduced expression of PDK1 led to a reduced activation of SGK paralogs, which in turn affected the activity of Na⁺ and K⁺ channels and therefore the driving force of the epithelium and the coupled transport of amino acids. Notably, *pdk1^{hm}* mice displayed reduced expression of SLC6A19 (Rexhepaj et al., 2006), a neutral amino acid transporter which is also expressed in the intestine and have been showed to be regulated by all SGK paralogs in *Xenopus* oocytes (Böhmer et al., 2010).

In conclusion, although there is only limited *in situ* evidence that SGK2 regulates the activity of transporters in the intestine, there is increasing supporting information that allow us to hypothesize that SGK2 might play a crucial role in intestinal homeostasis.

3.4. SGK2 in pathophysiology and therapy

There are no known associations between single nucleotide polymorphisms (SNP) in *SGK2* gene and diseases. However, the online platform Open Targets Platform offers genomic association, text mining and RNA expression tools to help rapidly uncovering associations between SGK2 and diseases (Carvalho-Silva et al., 2019). Notably, Open Targets Platform uses a machine learning method to link GWAS-identified loci to most likely causal genes. The strongest association score between SGK2 and pathologies is found in cell proliferation disorders, followed by nervous system diseases, gastrointestinal diseases and immune system diseases².

SGK2 has indeed been mechanistically linked to cell proliferation disorders such as cancer. Its expression was found upregulated in both hepatocarcinoma (HCC) and renal cell cancer (RCC) tissues when compared to adjacent non-cancerous tissues (Liu et al., 2019, 2017a). Downregulation of *SGK2* expression by siRNA reduced invasion and migration in HCC cells and led to reduced levels of inactivated GSK-3 β and reduced levels of active β -catenin, implying that SGK2 might modulate HCC progression through GSK-3 β inactivation (Liu et al., 2017a). In RCC cell lines, SGK2 promoted cell proliferation, migration, invasion and colony formation and mediated activation of AKT and ERK1/2 by phosphorylation (Liu et al.,

² Open Targets Platform, <http://www.targetvalidation.org>, last consulted 13th April 2020.

2019). The same team has also identified an oncogenic role for SGK2 in bladder cancer cell lines presumably via modulating β -catenin expression (Chen et al., 2018b). Oncogenic functions for SGK1 and SGK3 have also been described (Sun et al., 2016) and the SGK paralogs have even been suggested as the second AKT in cancer (Bruhn et al., 2010). Although the specific mechanistic remains to be elucidated, these studies suggest that SGK2 favours oncogenic characteristics by regulating key cellular functions.

On the same note, GSK650394, a SGK1/2 antagonist (IC₅₀ of 62 nM and 103 nM for SGK1 and SGK2 respectively), quantitatively blocked the effect of androgens on prostate cancer cell growth (Sherk et al., 2008) and also prevented the proliferation and migration of colorectal cancer cell lines (Liang et al., 2017a). QGY-5-114-A, an analog of GSK650394, also proved to reduce oncogenic characteristics of colorectal cancer cell lines (Liang et al., 2017b). The two antagonists have never been compared side-to-side and have not been further investigated in clinical trials.

SGK2 has also been predicted to be linked to nervous system diseases. Recently, the peptoid BN201, a SGK2 agonist, has demonstrated good safety and tolerability in Phase I clinical trials and is presently awaiting the beginning of Phase 2 in acute optic neuritis and multiple sclerosis. In pre-clinical trials, BN201 prevented axonal and neuronal degeneration and promoted remyelination in models of multiple sclerosis and glaucoma (Villoslada et al., 2019). Interestingly, the activation of SGK2 by BN201 also caused increased expression levels of multiple inflammatory cytokines in splenocytes of treated mice. This increased production of pro- and anti-inflammatory cytokines by splenocytes did not, however, enhance the encephalitogenicity of T cells, suggesting a homeostatic effect on the immune system (Villoslada et al., 2019). The mode of action of BN201, other than physically preferably binding to SGK2/1/3 (by order of efficacy: 6.8%, 25% and 33% of competitive binding vs control respectively), was not further clarified (Villoslada et al., 2019).

It therefore seems like SGK2 has a pleiotropic role in pathophysiology; its pharmacological inhibition reduces oncogenic cellular features while its activation leads to neuroprotection. Its mechanism of action remains unclear, but evidence shows potential involvement of PI3K and GSK-3 β / β -catenin pathways.

Thesis aims – Chapter III

3. Deepen the knowledge on a novel target for inflammatory bowel disease, SGK2

The last objective deviated slightly from the two first presented in Chapter I and II. In Chapter III, I investigated how SGK2, a potential IBD target identified in a screening assay in Galapagos, might be linked with the regulation of inflammatory processes. As there is little to nothing known on the subject, the objective was to first confirm SGK2 involvement in inflammation as well as to uncover potential mechanisms that might explain the effects observed. Mainly, the effect of the modulation of its expression on cytokine production of IEC, its activation status in answer to trigger as well as identification of potential interaction partners were investigated. Furthermore, the microfluidic from Chapter II was used to assess the effect of a SGK1/2 antagonist on cytokine secretion of the cells.

Methods

Expression of SGK paralogs in healthy and diseased human tissues

Genevestigator was used to query the expression of SGK1, SGK2 and SGK3 in publicly available datasets (Hruz et al., 2008). The studies presented at **Table 10** were used for expression analysis of colonic mucosa samples of IBD patients. For tissue distribution of the SGK paralogs, all the studies using healthy tissues deposited on Genevestigator in date of the 17 May 2020 were queried (66,949 samples).

Study	Details	Reference
GSE59071	- Colonic mucosa biopsies - 97 UC, 8 CD, 11 controls - Biopsies taken at the most affected sites but at a distance of ulcerations	(Vanhove et al., 2015)
GSE11831	- Descending colon mucosa biopsies - 17 controls	(Olsen et al., 2009)
GSE9452	- Colonic mucosa biopsies - 8 inflamed UC, 13 uninflamed UC, 5 controls - Biopsies taken at sites with and without macroscopic inflammation	(Olsen et al., 2009)
E-TABM-118	- Descending colon mucosa biopsies - 46 UC (33 uninflamed area, 18 inflamed area)	(Csillag et al., 2007)

Table 10. Curated datasets investigated in this study

Transient overexpression of SGK2 in Caco-2 cells

96-well plates were coated with 5µg/cm² of rat-tail Collagen I (Cultrex, #3447-020-01) following manufacturer's instructions. 7,000 Caco-2 cells per well were seeded in a 100µl volume. The day after seeding, cells were transduced with adenoviral particles (**Table 11**) at a MOI of 52.5 infective units (IU) per cell for 24 hours. The preparation of viral particles has

been previously described (Beaurivage et al., 2019). 48 hours after transduction, cells were triggered with IL-1 β , TNF- α and IFN- γ (1,50,50 ng/mL respectively) for 10 min, 30 min or 960min. Effluents were collected for cytokine secretion assessment and cells were lysed for protein extraction.

Name virus	AdV type	Fiber variant	Promoter	Backbone attenuation	Comments
WT SGK2	5	50	CMV	Δ E2a	WT SGK2
K124R SGK2	5	50	CMV	Δ E2a	Kinase dead mutant
AcGFP	5	20	CMV	Δ E2a	GFP

Table 11. Adenoviruses used for transient overexpression

Stable knockdown generation in human intestinal organoids

25,000 single organoids cells were seeded in suspension in 125 μ l of Human intestinal stem cell (HISC) medium per well of a 48-well plate. On the same day, cells were transduced with lentiviral particles (Mission® particles, Sigma Aldrich, **Table 12**) at a MOI of 40 IU per cell in presence of 8 μ g/mL polybrene. Cells were centrifuged at 32°C for 1 hour and incubated at 37°C for an additional hour. Viruses were removed, cells were pelleted, resuspended in Matrigel and reseeded. One week after transduction, selection with 4 μ g/mL of puromycin was carried for 7 days. Cells were expanded and when reached sufficient confluency, triggered with LPS [50 μ g/mL] + IFN γ [5ng/mL] or with IL-1 β , TNF- α , IFN- γ [0.1, 5, 5 ng/mL respectively] for 16hrs. Supernatants were harvested and viability was assessed.

Name virus	TRC clone	Promoter	shRNA target sequence	Supplier
SGK2_v22	TRC 1	U6	GCACTCCCTCAACATCATTTA	Sigma Aldrich
SGK2_v23	TRC 1	U6	ACACGTGGTGCTGACGGATTT	Sigma Aldrich
SGK2_v25	TRC 1	U6	TCTACTTCGTGCTCGACTATG	Sigma Aldrich

Table 12. Mission® lentiviruses used for stable knockdown

Transfection of human intestinal organoids

500,000 single organoids cells were seeded on a 10% Matrigel (Corning, # 356231) layer in a 12-well plate. 24 hours later, cells were transfected with 2 μ g of endotoxin-low DNA (**Table 13**) with TransIT-X2 transfection reagent (Mirrus, #MIR6000) at a 1:3 ratio. Transfection mixes were prepared according to manufacturer instructions. Medium was refreshed 24 hours after transfection. Transfection efficiency and downstream analyses were performed 72 hours after transfection.

Construct name	Encoded protein	Tag	Vector backbone	Features
pEGFP	EGFP	EGFP	pcDNA3.1(+)	Used to assess transfection efficiency
pMiniTurbo	MiniTurbo	3xHA	pcDNA3.1(+)	Biotin-ligase
pTurbo	Turbo	3xHA	pcDNA3.1(+)	Biotin-ligase
pSGK2MiniTurbo	SGK2-MiniTurbo	3xHA	pcDNA3.1(+)	Fusion protein between SGK2 and biotin-ligase
pSGK2Turbo	SGK2-Turbo	3xHA	pcDNA3.1(+)	Fusion protein between SGK2 and biotin-ligase
pSGK2	SGK2	-	pcDNA3.1(+)	For overexpression of wild-type (WT) SGK2
pmycSGK2	mycSGK2	MYC	pcDNA3.1(+)	For overexpression of MYC-tagged SGK2

Table 13. DNA constructs used in transfection experiments

Isolation and quantification of proteins

Extraction of proteins for proximity labelling experiments is described in the section below. Other proteins extractions were carried in the 96-well plate format. Briefly, cells were rinsed with 100µl cold PBS (Gibco) per well before 7.5µl of RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 0.5% SDS, 0.5% sodium deoxycholate, 1% NP-40) per well was added. Plate was incubated on ice for 5 min and three wells of each condition were pooled in a single microtube. Lysates were centrifuged for 15 min at 10,000RCF at 4°C. Proteins were quantified with the Pierce BCA Protein Assay kit (Thermo Fisher, #23225) according to manufacturer's instructions.

Proximity labelling in human intestinal organoids

72 hours after transfection with biotin-ligase constructs (**Table 13**), human intestinal organoids were exposed to 500µM biotin (Sigma Aldrich, #B4501) for 45 minutes. Cells were rinsed with PBS to stop the biotinylation reaction and lysed with RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 0.5% SDS, 0.5% sodium deoxycholate, 1% NP-40). Biotinylated proteins were enriched by overnight incubation with Pierce NeurAvidin Agarose Resin (Thermo Fisher, #29201) at 4°C (tumble end-over-end program, 25 RPM). Three fractions were collected for Western Blot analysis; total lysate (input fraction), non-biotinylated proteins (unbound fraction) and enriched biotinylated proteins (pulldown fraction).

Proximity labelling in Caco-2 cells

2,000,000 Caco-2 cells were seeded in 10cm dishes. The day after seeding, cells were transfected with 24µg of endotoxin-low DNA (**Table 13**) using 50µl of Lipofectamin Stem (Thermo Fisher, #STEM00015) for 24 hours. 48 hours after transfection, Caco-2 cells were

triggered with IL-1 β , TNF- α and IFN- γ (1,50,50 ng/mL respectively) for 16 hours. At the end of the trigger, cells were exposed to 500 μ M biotin (Sigma Aldrich, #B4501) for 10 minutes. Cells were rinsed with PBS to stop the biotinylation reaction and lysed with RIPA buffer (50mM Tris pH7.4, 150mM NaCl, 1mM EDTA, 0.5% SDS, 0.5% sodium deoxycholate, 1% NP-40). Three fractions were collected for Western Blot analysis; total lysate (input fraction), non-biotinylated proteins (unbound Biotinylated proteins were enriched by overnight incubation with Pierce NeurAvidin Agarose Resin (Thermo Fisher, #29201) at 4°C (tumble end-over-end program, 25 RPM). fraction) and enriched biotinylated proteins (pulldown fraction).

Lysates were washed on Wizard Minicolumns (Promega, #A7211) with, in order: 2% SDS, RIPA buffer, 0.5M NaCl, 2M Urea/50mM ammonium bicarbonate, 50mM ammonium bicarbonate. Samples were reduced with 50mM tris(2-carboxyethyl)phosphine) (TCEP, Sigma Aldrich, #646547) for 15 min at 37°C, followed by an alkylation step with 50mM iodoacetamide (IAA, Sigma Aldrich, #I6125) for 15 min at 37°C before being digested with 1 μ g of trypsin for 4 hours at 37°C. Peptides were then acidified with trifluoroacetic acid (TFA, Sigma Aldrich, #T6508) and desalted using C18 tips (Thermo Fisher, #SP301). The retained peptides were washed with 0.1% TFA and eluted in 0.1%TFA/50% acetonitrile.

Mass spectrometry analysis

Samples were analysed by nanoflow LC-MS/MS using an Orbitrap Elite (Thermo Fisher) hybrid mass spectrometer equipped with a nanospray source, coupled to an Ultimate RSLCnano LC System (Dionex). The system was controlled by Xcalibur 2.1 (Thermo Fisher) and DCMSLink 2.08 (Dionex).

Immunoprecipitation

Caco-2 cells were seeded, transfected, triggered and lysed as mentioned (Proximity labelling). 200 μ l of lysate was diluted to 450 μ g/ml and incubated with magnetic Protein A (Cell Signaling Technologies, #73778) or Protein G beads (Cell Signaling Technologies, #70024) for 20 min at 4°C before 1 μ g of capture antibody (**Table 14**) was added to the mixture and incubated overnight at 4°C under rotation. The next day, the immunocomplex was pelleted, washed and denatured before the samples were probed for protein expression using detection antibodies (**Table 17**) on the Simple Western system.

Construct	Capture antibody	Detection antibodies	
Endogenous SGK2	SGK2 (Cell Signaling #5595)	SGK2 (Cell Signaling #7499, 1:50)	STAT3 (Cell Signaling #12640, 1:50)
WT SGK2	SGK2 (Cell Signaling #5595)		
TurboSGK2	HA (Abnova #MAB7911)		

Table 14. Antibodies used in immunoprecipitation experiments

Pharmacological inhibition

Seeding of the OrganoPlate was performed as described in Chapter II. 6 days after seeding, the co-culture was pre-exposed to compound concentrations ranging from 0.02 μ M to 20 μ M in top and bottom channels for 2hrs. After 2hrs, the trigger (100ng/mL LPS, 20ng/mL IFN- γ) in presence of 0.02 μ M-20 μ M compound was applied in top and bottom channels. Effluents were collected from apical and basal compartments 24hrs and 72hrs after compound exposure and viability was assessed 72hrs after compound exposure. Compounds tested are presented in

Table 15.

Name	Target	CAS	Concentration	Diluent	Supplier
GSK650394	SGK1/2	890842-28-1	10 mM	DMSO	Aldrich Market Select
TPCA-1	IKK-2	507475-17-4	10 mM	DMSO	Aldrich Market Select
Bosutinib	Src/Abl	380843-75-4	10 mM	DMSO	Sigma Market Select
Dasatinib	Bcr/Abl	302962-49-8	10 mM	DMSO	Fluorochem

Table 15. Pharmacological compounds

Viability assessment

For stable SGK2 knockdown experiments, CCK8 reagent (Sigma Aldrich, #96992) was diluted 1:10 in human intestinal stem cell (HISC) medium, 350 μ l was pipetted per well of a 12-well plate and left to incubate for 4 hours at 37°C. For compounds experiments in the OrganoPlate, CCK8 reagent was diluted 1:10 in HISC medium and 25 μ l was added to all inlets and outlets (100 μ l total) and incubated for 4 hours at 37°C on the interval rocker (Perfusion Mini, Mimetas). Absorbance (450nm) was recorded on an Envision plate reader (Perkin Elmer).

Detection of proteins by Western blot

Whole cell lysates were fractionated by SDS-PAGE and transferred to a 0.2 μ m nitrocellulose membrane (GE Healthcare) using a transfer apparatus according to the manufacturer's protocols (Bio-Rad). After incubation with either 3%BSA in TBST (50 mM Tris pH 7.4, 150 mM NaCl, 0.1% Tween 20) or PBST (2.73M NaCl, 55 mM KCl, 35mM KH₂PO₄, 200mM Na₂HPO₄.12H₂O, 0.05% Tween 20) or with 5% non-fat milk in TBST or PBST for 60 min, membranes were incubated with primary antibodies (**Table 16**) overnight at 4°C.

Target	Antibody used	Dilution	Diluent
B-tubulin	Sigma Aldrich #T4026	1:5000	5% milk in PBST
HA	Cell Signaling #2367	1:1000	5% milk in TBST
SMAD2/3	Cell Signaling #8685	1:1000	5% milk in TBST
Streptavidin	Invitrogen #S32358	1:4000	3% BSA in TBST

Table 16. Primary antibodies used for Western blotting

Membranes were washed three times for 10 min with TBST or PBST and incubated with a 1:5000 dilution of DyLight 800-conjugated goat anti-mouse IgG (Invitrogen, SA535521) or with a 1:10000 dilution of AF680-conjugated goat anti-rabbit IgG (Invitrogen, A21076) antibodies for 1h at room temperature. Blots were washed with TBST or PBST three times, let to dry for 15 min and imaged on the Odyssey SA (LI-COR Biosciences).

Detection of proteins by Simple Western (capillary immunoassay)

Whole cell lysates were analysed on the Wes or PeggySue system (Simple Western) according to manufacturer's instructions. Deviations from the standard protocol on the PeggySue included a separation time of 40 min and primary antibody incubation time of 60 min. On the Wes, separation time was 35 min and primary antibody incubation time was 90 min.

Target	Condition	Protein amount (µg/µl)	Antibody used	Dilution
SGK2	Endogenous	1.00	Cell Signaling #7499	1:100
SGK2	Overexpressed	0.25	Cell Signaling #7499	1:100
STAT3	Endogenous	0.25	Cell Signaling #12640	1:50
pSTAT3 Ser727	Endogenous	1.00	Cell Signaling #9134	1:50
NEDD4-2	Endogenous	0.25	Cell Signaling #4013	1:50
pNEDD4-2 Ser448	Endogenous	1.00	Cell Signaling #8063	1:50
GAPDH	Endogenous	0.25	ProteinTech #6004-1	1:500
B-ACTIN	Endogenous	0.25	Cell Signaling #8457	1:100

Table 17. Antibodies used in Simple Western capillary immunoassays

Assessment of cytokine secretion

The concentrations of Macrophage Inflammatory Protein-3 (CCL-20), IFN-Gamma-Inducible Protein 10 (IP-10), Interleukin-1-Beta (IL-1β), Interleukin-6 (IL-6), Interleukin-10 (IL-10), and Tumor Necrosis Factor-Alpha (TNF-α) were quantified in the supernatants using a human multiplex assay (Thermo Fisher) on a Luminex FlexMap 3D (Merck Millipore) according to manufacturer protocol.

Immunostaining

Glass coverslips were coated with 5µg/cm² rat-tail Collagen I (Cultrex, #3447-020-01) according to manufacturer's protocol and placed in 12-well plates. 100,000 Caco-2 cells were

seeded per well. 72 hours after seeding, cells were triggered with IL-1 β , TNF- α , IFN- γ (1,50,50 ng/mL respectively) for 16-24hrs. Cells were fixed with paraformaldehyde (PFA) 4% for 15 min, rinsed with PBS, quenched for 5 min (0.1M Glycine in PBS), permeabilized (0.3% Triton-X100 in PBS), blocked for 45 min (2% FBS, 2% BSA, 0.05% Tween20 in PBS) and incubated overnight with the primary antibody at 4°C. Next day, cells were rinsed with PBS, incubated with secondary antibody and Hoechst (1:20 000) for 1 hour at room temperature, rinsed with PBS and water and mounted with ProLong Gold Antifade Mountant (Thermo Fisher, #P10144). Images were acquired on UltraVIEW VoX spinning disk microscope (Perkin Elmer).

Isolation of RNA

Total RNA was isolated from human intestinal organoids with TRI Reagent (Sigma Aldrich, #T9424). The collected solution was extracted using phenol-chloroform according to manufacturer's instruction. Purity and yield of extracted RNA was assessed on a NanoDrop 2000 (Thermo Fisher).

Real-time polymerase chain amplification

Total RNA was converted to cDNA using TaqMan Reverse Transcription Kit (Applied Biosystems, #N8080234) according to manufacturer's instructions. Real-time polymerase chain amplification (qPCR) was performed using TaqMan® Fast Advanced MasterMix and TaqMan FAM-VIC probes (Applied Biosystems, #4369016) (**Table 18**) on a LightCycler® 480 instrument (Roche) following the program at **Table 19**.

Gene acronym	Gene name	Assay ID
CGA	Chromogranin A	Hs00985275_g1
LGR5	Leucine-rich repeat-containing G-protein coupled receptor 5	Hs00969422_m1
LYZL1	Lysozyme 1	Hs00378982_m1
MUC2	Mucin 2	Hs00894052_g1
SGK1	Serum and glucocorticoid-regulated kinase 1	Hs00178612_m1
SGK2	Serum and glucocorticoid-regulated kinase 2	Hs00367639_m1
SGK3	Serum and glucocorticoid-regulated kinase 3	Hs00179430_m1
SGLT1	Sodium/glucose cotransporter 1	Hs01573793_m1
TFF3	Trefoil factor 3	Hs00902278_m1
TLR4	Toll like receptor 4	Hs00152939_m1
VIL1	Villin 1	Hs01031739_m1

Table 18. TaqMan qPCR probes

Programs	# of cycles	Temperature	hh:mm:ss	Ramp rate (°C/sec)
1: UNG	1	50°C	00:02:00	4.4
2: Enzyme Activation	1	95°C	00:10:00	4.4
3: Amplification	45	95°C	00:00:03	4.4
		60°C	00:00:10	2.2
4: Cooling	1	37°C	00:00:30	2.2

Table 19. qPCR cycling conditions

SGK2 kinase activation

12-well plates were coated with 5µg/cm² rat-tail Collagen I (Cultrex, #3447-020-01) according to manufacturer's protocol and seeded with 100,000 Caco-2 cells per well. 48 hours after, cells were triggered with IL-1β, TNF-α, IFN-γ (1,50,50 ng/mL respectively) for 2, 10, 30, 180 or 960 minutes. Cells were rinsed with cold PBS and lysed in 50µl of M-PER mammalian protein extraction buffer (Thermo Fisher, #78501) using a cell scraper. Lysates were centrifuged for 15 min at 10,000RCF at 4°C. Proteins were quantified with the Pierce BCA Protein Assay kit (Thermo Fisher, #23225) according to manufacturer's instructions. 1µg of protein was loaded per array of a STK PamChip on a PamStation 12 instrument (Pamgene) following manufacturer's instructions.

Results

Tissue expression of SGK paralogs

We first confirmed the tissue distribution of the SGK paralogs as well as their levels of expression in human tissues. *SGK1* showed the highest levels of expression in all tissues queried, whereas the expression of *SGK3* was slightly lower and *SGK2* expression was the lowest overall (**Fig. 49A** and **49B**). However, as depicted in **Figure 50**, the GI tract is where *SGK2* mRNA expression was found to be the highest in the human body, which suggests that *SGK2* might have important functions in that set of organs.

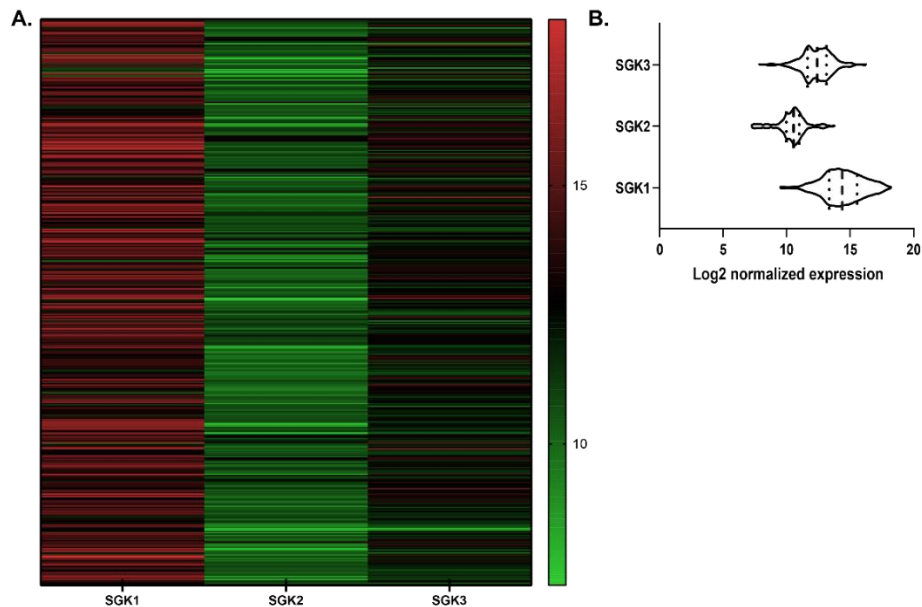


Figure 49. Gene expression of SGK paralogs

A. Log2 normalized expression of *SGK1*, *SGK2* and *SGK3* in human tissues (66,949 samples from healthy tissues were queried in Genevestigator). **B.** Violin plots depicting the distribution of *SGK1*, *SGK2* and *SGK3* expression levels in all tissues. Dashed line represents the median and dotted lines the quartiles.

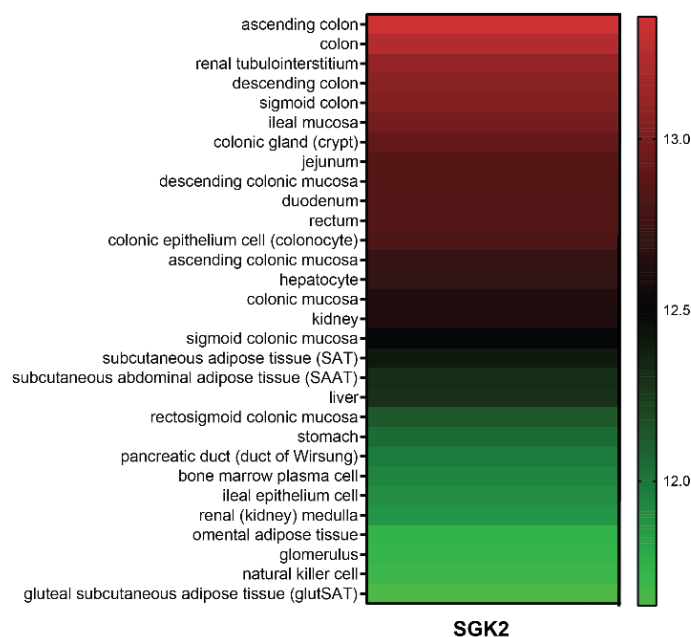


Figure 50. SGK2 is highly expressed in the gastrointestinal tract

Log2 normalized expression of *SGK2* in the top 30 highest-expressing *SGK2* tissues (66,949 samples from healthy tissues were queried in Genevestigator).

Investigation of SGK2 expression in the intestinal mucosa of IBD patients

In order to further support the hypothesis that SGK2 is involved in the regulation of inflammation in IEC, its expression was investigated in tissues derived from IBD patients using Genevestigator. Genevestigator presents publicly available datasets that have been manually

curated and controlled by experts. The mucosal expression of the SGK family members were investigated in studies GSE59071 and GSE11831/GSE9452/E-TABM-118 (Vanhove et al., 2015; Olsen et al., 2009). In GSE59071, *SGK2* expression was significantly reduced in the diseased colonic mucosa of IBD, the greatest reduction being in UC patients suffering from active inflammation (**Fig. 51B**). The portrait is similar in the second study, *SGK2* expression is significantly decreased in inflamed intestinal mucosa of UC and CD patients (**Fig. 51D**). When looking at the expression of *SGK1* and *SGK3* in the same studies, their expression remains unchanged independently of the tissue state, suggesting that SGK2 is the only family member affected. *SGK3* expression levels were unavailable in GSE59071. These results suggest that SGK2 might be involved at some level in the regulation of inflammatory processes. However, at this point, it is unclear whether decreased *SGK2* expression is a cause or a consequence of inflammation.

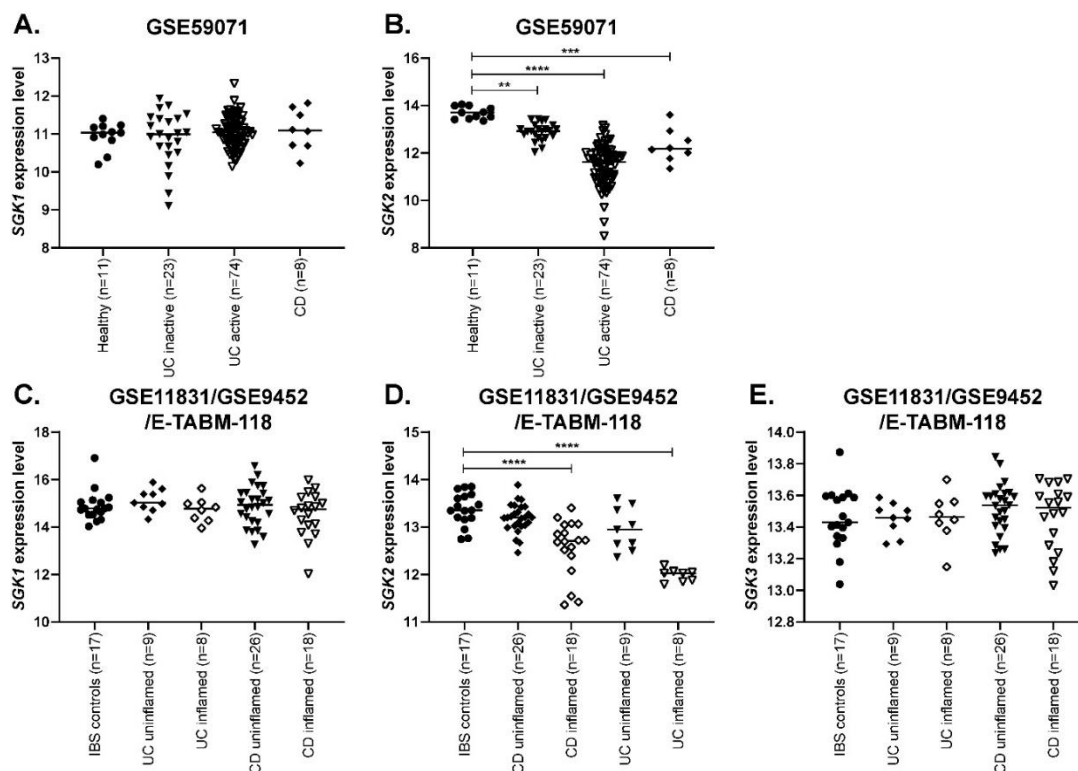


Figure 51. SGK2 expression is decreased in the colonic mucosa of IBD patients suffering from active intestinal inflammation

A-B. Gene expression of *SGK1* (**A.**) and *SGK2* (**B.**) in mucosal colonic biopsies of human patients from GSE59071. Each symbol represents expression data from one individual patient, horizontal bars denote median values. One-way ANOVA with Dunnett's post-hoc test compared to healthy samples (n=8-74). **C-E.** Gene expression of *SGK1* (**C.**), *SGK2* (**D.**) and *SGK3* (**E.**) in descending mucosal colonic biopsies of human patients from GSE11831/GSE9452/E-TABM-118. Each symbol represents expression data from one individual patient, horizontal bars denote median values. One-way ANOVA with Dunnett's post-hoc test compared to healthy samples (n=8-26).

Establishment of relevant cell models to recapitulate inflammation in IEC

Throughout this study, two cell models of IECs have been used; Caco-2 cells and patient-derived HIO. Caco-2 cells are known to be an enterocytic-like lineage, meaning that they display typical markers of small intestine enterocytes (Mahraoui et al., 1994). The HIO used in this study were extracted from different regions of the GI tract out of different patients (**Table 7**). Established HIO cultures were first characterized through the expression of cell type-specific markers, in order to grasp their intra-individual differences. At a glance, it is noticeable that the HIO display variable representations of each cell type, which could certainly affect our future results. The FCB1 donor, per example, displayed higher expression of crypt base columnar stem cells (CBC) marker *LGR5* and lower expression of differentiated cell types markers such as *KLF4*, *MUC2* and *CHGA* (**Fig. 52**). The two other donors, HUB2 and FCB3, overall display very similar profiles, with only a significant difference in *VIL1* and *MUC2* expression.

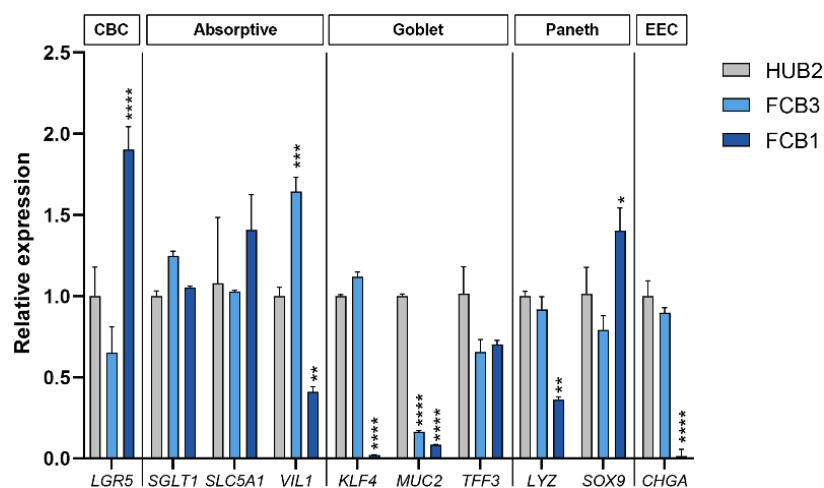


Figure 52. HIO donors exhibit variations in cell lineages representation

Expression of markers for crypt base columnar cells (CBC; *LGR5*), absorptive cells (*SGLT1*, *SLC5A1*, *VIL1*), goblet cells (*KLF4*, *MUC2*, *TFF3*), Paneth cells (*LYZ*, *SOX9*) and enteroendocrine cells (EEC; *CHGA*) in HIO from three donors (HUB2, FCB3, FCB1). Graph shows average RQ values ($2^{-\Delta\Delta Ct}$) normalized to *RPS18*, *HPRT1*, *GUSB* and *YWHAZ* $\pm$ SEM. Two-way ANOVA with Dunnett's post-hoc test compared to HUB2 (n=2).

In order to induce an inflammatory state, cells were triggered with pro-inflammatory trigger cocktails. Specifically, Caco-2 cells were triggered with a mixture of IL-1 β , TNF- α and IFN- γ that has previously been shown to induce an inflammatory state similar to the one induced by activated immune cells (Beaurivage et al., 2019; Van De Walle et al., 2010). Two different concentrations of this trigger were used; the concentration used in the screening assay that initially identified SGK2 as a potential IBD target (later referred as 1-50-50ng/mL; see Section 6.2) and the concentration used in the microfluidic gut-on-a-chip model (Beaurivage et al.,

2019) (later referred as 2-100-100ng/mL; See [Chapter II](#)). As for HIO, they were also triggered with the cytokine mixture of IL-1 β , TNF- α and IFN- γ at lower concentrations of 0.1-5-5ng/mL, respectively. Previous experiments have determined that HIO are more sensitive to trigger than Caco-2 cells and were ongoing heavy cell death when triggered at the same concentration (results not shown). However, a 10-fold reduction of the trigger concentration allowed a sustained CCL-20 production (**Fig. 53A**) while maintaining cell viability. HIO, comparatively to Caco-2 cells, express TLR4 (**Fig. 53B**), which allowed us to use *E.coli* UV-irradiated LPS in combination with IFN- γ to trigger inflammatory responses in HIO. This is also the trigger that was used to activate the microfluidic primary gut-on-a-chip model (see [Chapter II](#)).

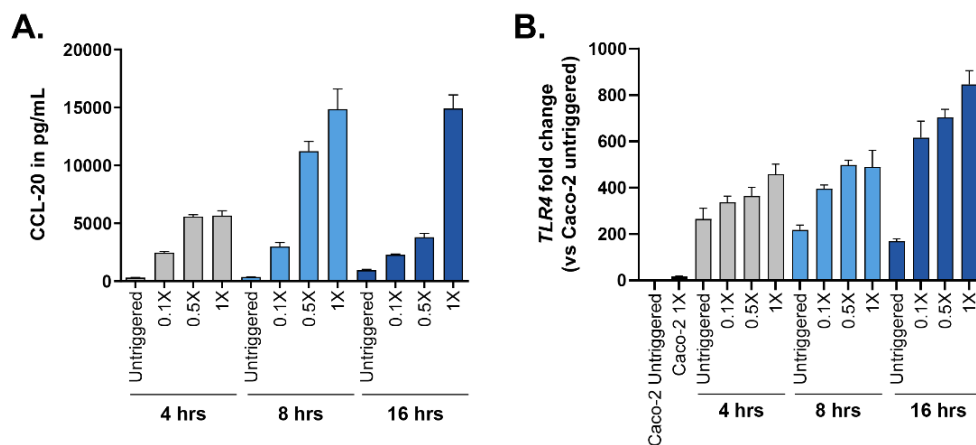


Figure 53. HIO are responsive to inflammatory trigger and express higher levels of *TLR4* when compared to Caco-2 cells

A. CCL-20 production of HIO (HUB2 donor) after 4, 8 and 16hrs of IL-1 β , IFN- γ , TNF- α exposure at 1X, 0.5X and 0.1X concentration. The 1X concentration corresponds to the concentration used in the Caco-2 cells screening assay (1-50-50ng/mL respectively). **B.** Fold change of *TLR4* expression in Caco-2 cells triggered with IL-1 β , IFN- γ , TNF- α (1-50-50ng/mL respectively) for 24hrs or in HIO (HUB2 donor) after 4, 8 or 16hrs IL-1 β , IFN- γ , TNF- α exposure at 1X, 0.5X and 0.1X of the Caco-2 concentration. Graph shows RQ values ($2^{-\Delta\Delta Ct}$) normalized to *GAPDH* from each condition normalized to RQ values of Caco-2 Untriggered $\pm$ SEM (n=3).

Investigation of SGK2 expression in intestinal epithelial cells

The first step was to investigate the expression of the SGK paralogs in normal and inflammatory conditions to see if the situation of IBD patients would be reflected in our IEC models using the triggers explained in the previous section. In HIO, both triggers significantly decreased *SGK2* mRNA expression in all HIO donors after a stimulation of 16 hrs (**Fig. 54B**). Similarly, *SGK3* expression was also repressed, but to a lesser extent (**Fig. 54C**) in all donors. *SGK1* expression was, however, upregulated in response to the trigger, particularly after LPS-IFN- γ exposure (**Fig. 54A**).

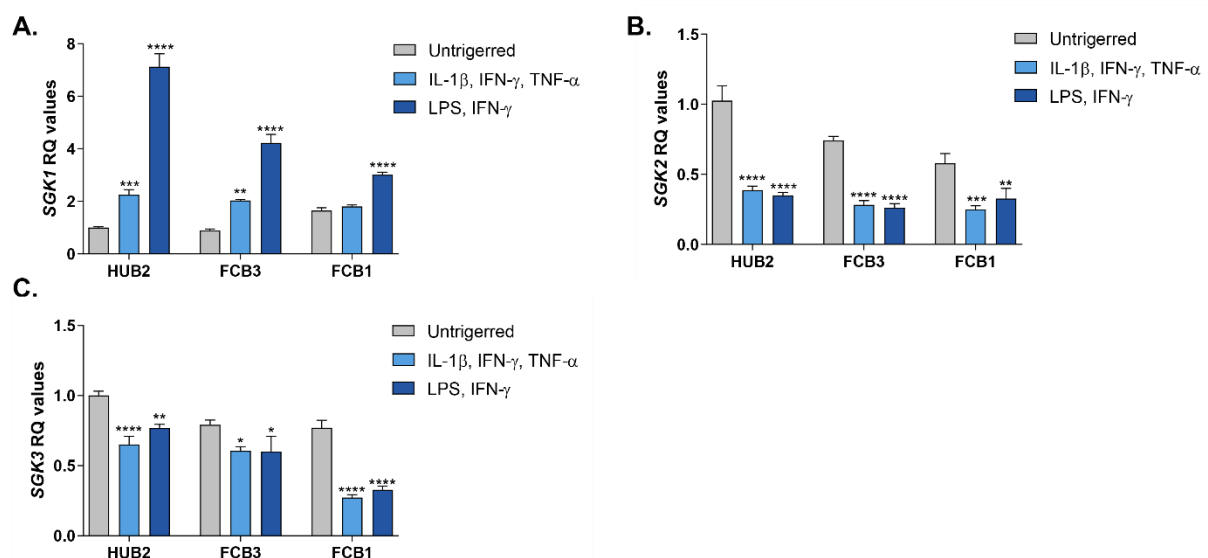


Figure 54. SGK2 and SGK3 expression are downregulated while SGK1 expression is upregulated by inflammatory triggers in HIO

HIO from 3 different donors (HUB2, FCB3, FCB1) were grown in Matrigel droplets and triggered with IL-1 β , IFN- γ , TNF- α [0.1, 5, 5ng/mL respectively] or LPS [50 μ g/mL], IFN- γ [5ng/mL] for 16hrs. Graph shows the average RQ values (2- $\Delta\Delta$ Ct) from one representative experiment for SGK1, SGK2 and SGK3 normalized to TBP expression $\pm$ SEM (n=3 ran in technical duplicates). Two-way ANOVA with Dunnett's post-hoc test compared to HUB2 untriggered.

In Caco-2 cells, SGK2 and SGK3 expression were significantly decreased upon inflammatory trigger at both concentrations, while SGK1 expression remained unchanged after a 24 hrs exposure (Fig. 55).

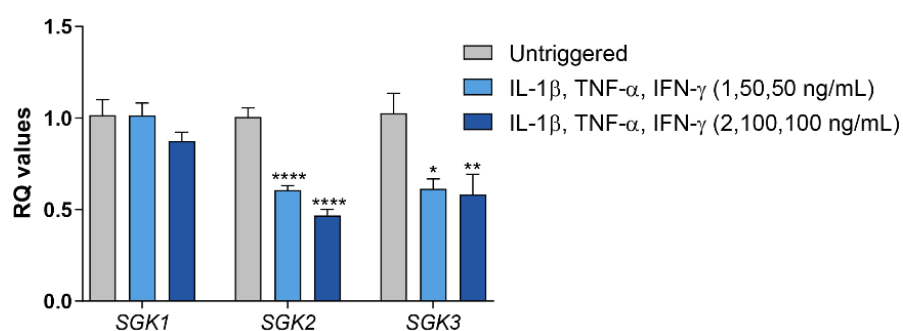


Figure 55. SGK2 and SGK3 expression are downregulated after trigger in Caco-2 cells

Caco-2 cells were seeded on a Collagen gel (5 μ g/cm²) and were triggered with the indicated cytokine cocktails at Day 2 for 24hrs. Graph shows the average RQ values (2- $\Delta\Delta$ Ct) from one representative experiment for SGK1, SGK2 and SGK3 normalized to TBP expression $\pm$ SEM (n=4 ran in technical duplicates). One-way ANOVA with Dunnett's post-hoc test compared to untriggered.

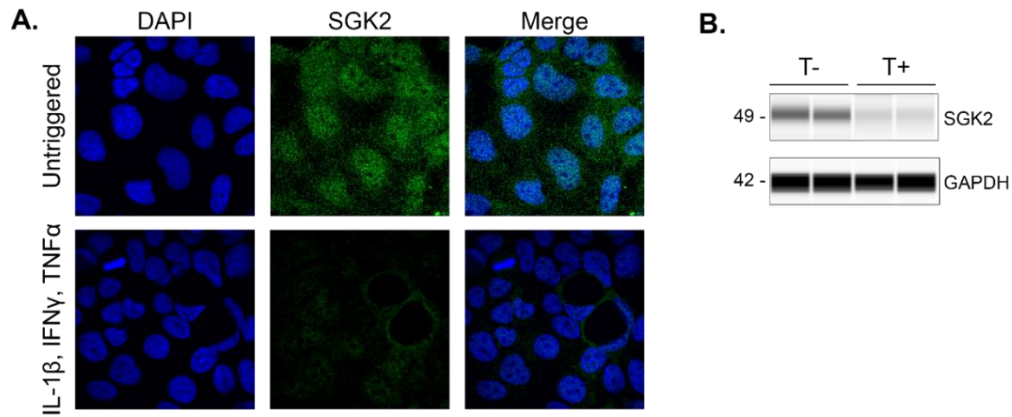


Figure 56. Protein levels of SGK2 are reduced in inflammatory conditions in Caco-2 cells
A. Representative 60X images of SGK2 immunostaining in Caco-2 cells following a 24hrs trigger with IL-1 β , TNF- α , IFN- γ (1,50,50 ng/mL respectively). DAPI is used to depict nuclei (n=6). **B.** Capillary immunoassay for SGK2 and GAPDH expression in Caco-2 cells after a 24hrs trigger with IL-1 β , TNF- α , IFN- γ (1,50,50 ng/mL respectively; T- = untriggered, T+ = triggered, 0.64 μ g protein per lane).

Immunostaining allowed us to further inquire the localization and the protein expression levels of SGK2 in Caco-2 cells (**Fig. 56A**). In untriggered conditions, SGK2 could be detected both in the nuclei through its NLS sequence as well as in the cytoplasm of Caco-2 cells. After a 24hrs trigger, a marked reduction in SGK2 signal was noted, but the remaining SGK2 signal was found both in the nuclei and the cytoplasm of the cells. It therefore seems like the inflammatory state does not affect SGK2 localization but does reduce its protein expression in Caco-2 cells. Those results were confirmed by capillary immunoassay, where SGK2 expression was also downregulated after inflammatory trigger (**Fig. 56B**). In conclusion, despite differences in *SGK1* and *SGK3* expression, *SGK2* expression was systematically reduced upon inflammatory trigger, both on the mRNA and protein level in Caco-2 cells, HIO and IBD patients.

Identification of potential interaction partners of SGK2

In order to identify interaction partners that might explain how SGK2 regulates inflammatory processes, an enzyme-catalysed proximity labelling approach was set-up using the properties of biotin-ligase TurboID (Branon et al., 2018), generated by the directed evolution of *E. coli* biotin ligase (BirA). A fusion protein between TurboID in N-terminal and SGK2 in C-terminal separated by a flexible linker allowing the correct folding of the protein was created. In order to facilitate detection of the fusion protein a 3xHA tag was added at the N-terminal end of the protein. The principle of proximity labelling (**Fig. 57**) resides in the biotin-ligase function of TurboID; upon addition of biotin, all proteins in close proximity to TurboID, and therefore SGK2, will be biotinylated and can be further enriched and eluted for analysis on a mass spectrometer.

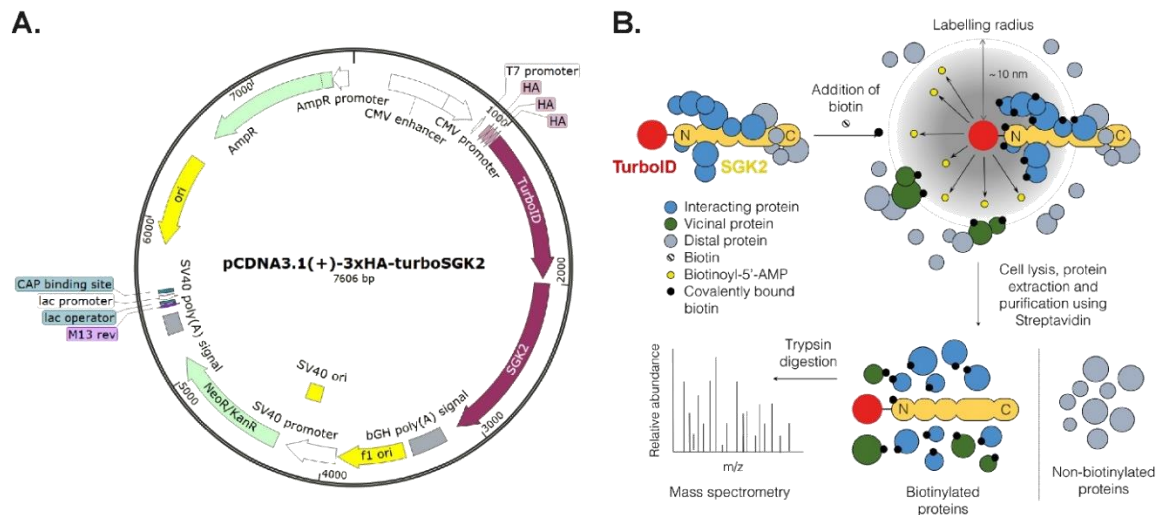


Figure 57. TurboID-based proximity labelling approach

A. Map of pCDNA3.1(+)-3xHA-turboSGK2 (TurboSGK2). **B.** Schematic representation of the proximity labelling process. The fusion between TurboID and SGK2 (TurboSGK2) is transfected into the cells. Biotin is added for 10 min, allowing biotinylation of vicinal proteins within a 10nm radius. Total proteins are extracted and biotinylated proteins are enriched using a streptavidin resin and purified before they are digested and ran on a mass spectrometer. Figure modified with permission from (Varnaitė and MacNeill, 2016).

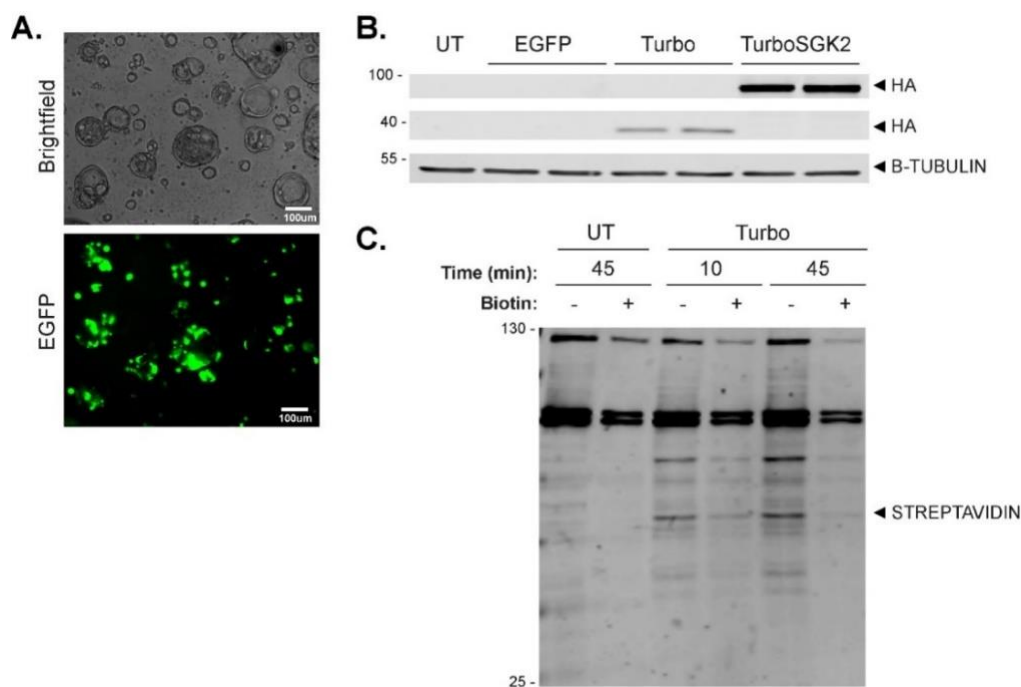


Figure 58. Turbo constructs are expressed in HIO but do not lead to protein biotinylation

A. Representative images depicting transfection efficiency of HIO (FCB3 donor) 72hrs after transfection with pCDNA3.1(+)-EGFP construct. Scale bars = 100µm. **B.** Immunoblotting for HA and B-TUBULIN expression in HIO lysates 72hrs after transfection (FCB3 donor). Each lane contains 50µg of protein (*UT* = Untransfected). **C.** Immunoblotting for STREPTAVIDIN expression in HIO lysates (FCB3 donor). 72hrs after transfection with TurboID, cells were treated with biotin (500µM) for the indicated times. Each lane contains 10% of protein lysate (*UT* = Untransfected).

The initial goal was to perform proximity labelling in HIO. However, despite good transfection efficiency (**Fig. 58A**) and sufficient expression of Turbo constructs at expected molecular weight (Turbo=38kDa, TurboSGK2=80 kDa) (**Fig. 58B**), no increased of biotinylation upon addition of biotin could be observed (**Fig. 58C**).

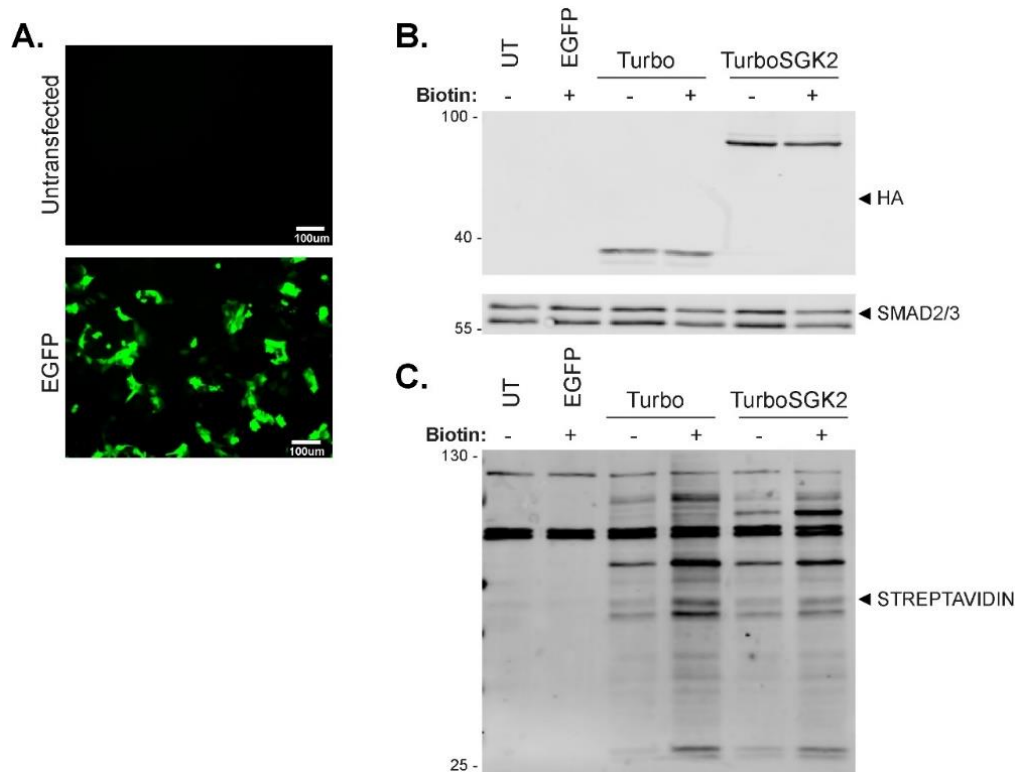


Figure 59. Expression and activity of TurboID constructs in Caco-2 cells

A. Representative images of untransfected or pCDNA3.1(+)-EGFP-transfected Caco-2 cells 48hrs after transfection. Scale bars = 100µm. **B.** Immunoblotting for HA and SMAD2/3 expression in Caco-2 cells lysates 48hrs after transfection. Each lane contains 30µg of protein (UT = *Untransfected*). **C.** Immunoblotting for STREPTAVIDIN expression in Caco-2 cells lysates. 48hrs after transfection with Turbo constructs, cells were treated with biotin (500µM) for 10min. Each lane contains 10% of protein lysate (UT = *Untransfected*).

Therefore, Caco-2 cells were selected to perform further proximity labelling experiments. Transfection of Caco-2 cells led to successful expression of TurboID constructs (**Fig. 59B**) and increased protein biotinylation upon biotin addition (**Fig. 59C**). Pilot experiments allowed us to confirm that known interactors of SGK2 were picked up and confirmed the approach. As the goal was to investigate potential interaction partners of SGK2 in the intestinal epithelium but particularly in inflammatory conditions, proximity labelling was performed in normal and inflammatory conditions (IL-1 β , TNF- α , IFN- γ at 1,50,50 ng/mL respectively) leading to four experimental groups [*oo*; *no trigger no biotin*, *oB*; *no trigger biotin*, *To*; *trigger no biotin*, *TB*; *trigger biotin*]. To compensate for the fact that no enrichment was notable for Biotin-streptavidin capture groups, the total proteome was reported. When comparing untriggered and

triggered samples, a total of 124 proteins were found to be differentially represented in the samples, of which the top 30 is shown in **Figure 60**.

Computation of the totality of these 124 hits allowed their integration into biological functions that were further organized into networks (**Fig. 61A**). At a glance, networks involved in IFN- γ response pathways, metabolic processes, as well as cellular organization can be discerned. When only the top significantly regulated proteins are considered (**Fig. 61B**), pathways involved in the regulation of interferon-gamma are mainly overrepresented, together with tricarboxylic acid metabolic process in a smaller measure. These results confirmed that the trigger worked efficiently and that inflammatory processes were engaged in Caco-2 cells.

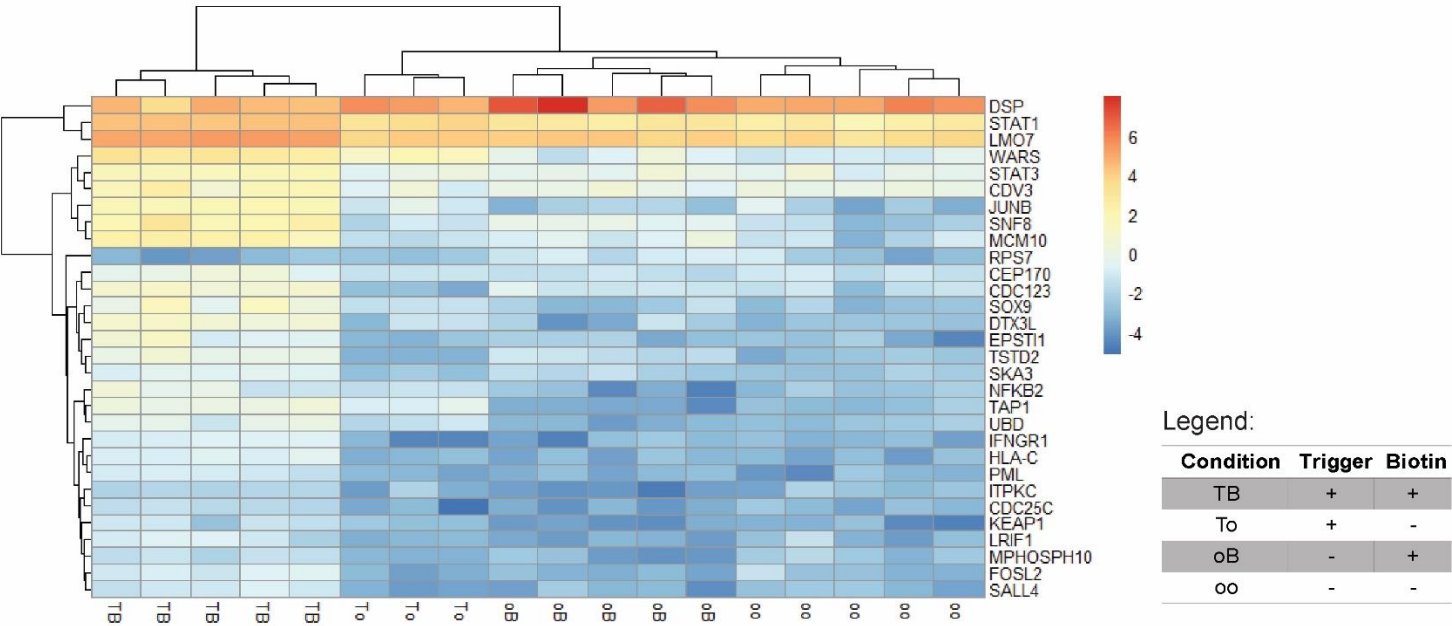


Figure 60. Identification of potential interaction partners of SGK2 in Caco-2 cells
Heatmap representation of the top 30 significantly expressed genes with the most significant adjusted p-value between oo and To groups, using log2 transformed counts normalized per protein (mean-based normalization); gene names reported. Upregulated genes are shown in red and downregulated genes in blue.

Individual top hits were entered into the STRING database to reveal existing or predicted protein-protein interactions. Some of the hits were known to interact with each other already (**Fig. 62**). SGK2 was not predicted to interact with any of these proteins. As little is known on SGK2 nor on its interactions partners, there is a strong possibility that SGK2 might interact with one of these proteins, therefore explaining why the whole complex was picked up. However, the physical interaction between SGK2 and any of these proteins have not been confirmed yet.

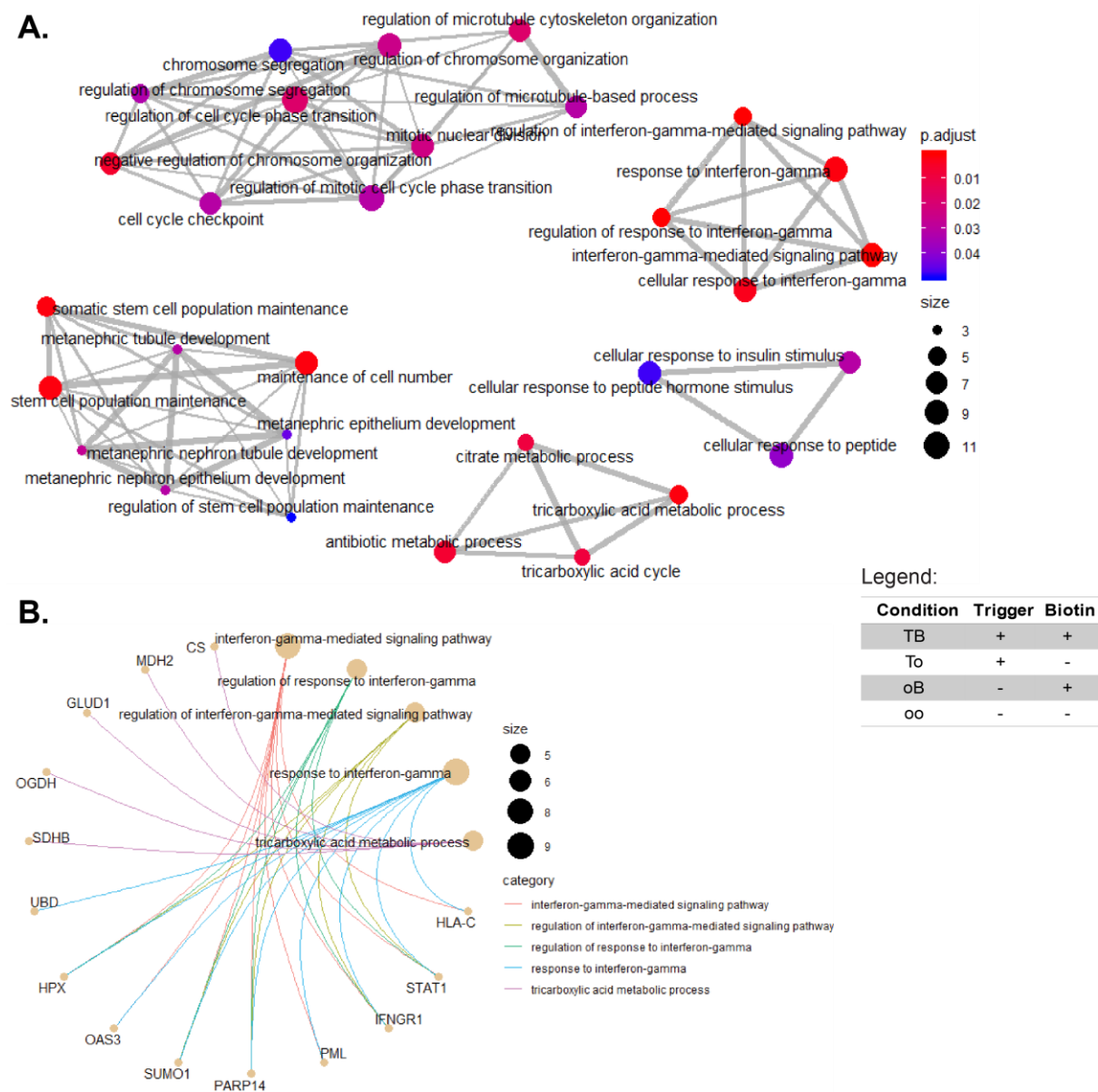


Figure 61. Computation of proteomics datasets into biologically meaningful networks
 Based on comparison between untriggered and triggered Caco-2 cells in absence of biotin (oo-To comparison), 124 proteins had most significant change. **A.** Enriched overrepresented pathways were integrated into networks and **B.** Most significantly regulated genes were linked to biological concepts (Gene Ontology terms/KEGG pathways). Enrichment counts represent circle size.

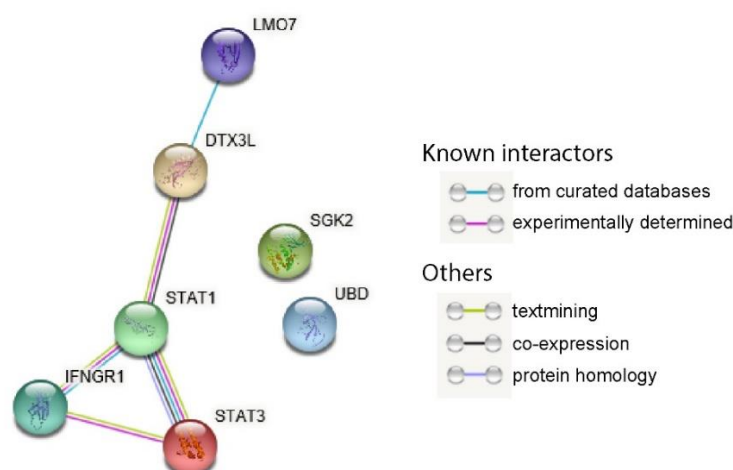


Figure 62. STRING analysis of top proteomics hits reveals potential SGK2 interactors

Top differentially enriched proteins were computerized into the STRING pathway analysis tool to reveal existing or predicted protein-protein interactions (Snel, 2000).

Confirmation of proteomics hits by immunoprecipitation

As proximity labelling does not guarantee physical nor functional interaction, immunoprecipitation was used to try to confirm the interaction of SGK2 and the potential hits identified in proteomics (**Fig. 62**). In a first time, the transfection of TurboSGK2 and WT SGK2 constructs in Caco-2 cells (**Fig. 63A**) as well as the successful immunoprecipitation (**Fig. 63B**) were confirmed by capillary immunoassay. The presence of all putative SGK2 interactors was queried in the input samples and the immunoprecipitated fractions. Of all the proteins, only STAT3 could be picked up in both the input lysates and the immunoprecipitated fraction of TurboSGK2-transfected cells but could not be observed in WT SGK2-transfected cells.

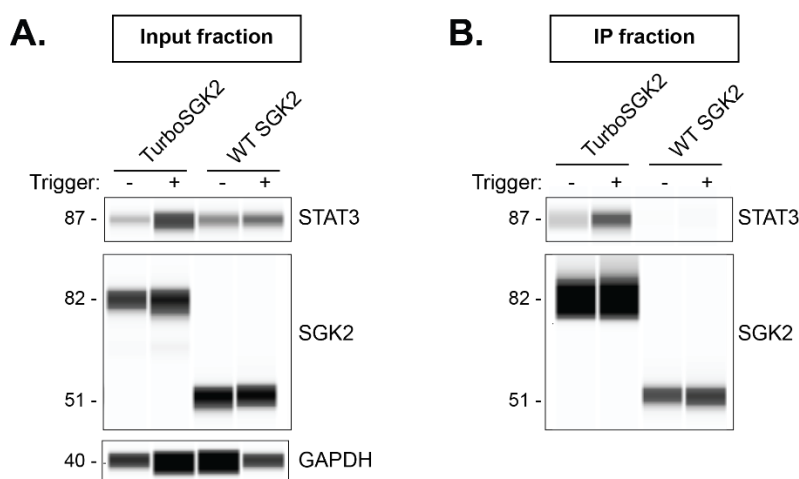


Figure 63. TurboSGK2 physically interacts with STAT3

Capillary immunoassay for STAT3, SGK2 and GAPDH expression. Each lane contains 0.32µg of protein lysate (Input fraction) or 12% of immunoprecipitated proteins (IP fraction). Results from one representative experiment are shown.

SGK2 activation in inflammatory conditions

In an attempt to understand further SGK2 function and as expression is not representative of the activity of a kinase, we investigated whether SGK2 was activated or repressed downstream of the inflammatory trigger in Caco-2 cells. The effect of the inflammatory trigger on the phosphorylation status of target peptides was assessed at several time points and results were integrated and compared against public repositories in order to assess which kinases' activity are most likely responsible for the observed effect. Specifically, the kinase activity of SGK2 was inhibited 2 minutes after subjecting Caco-2 cells to an IL-1 β , TNF- α , IFN- γ inflammatory trigger (**Fig. 64**). SGK2 activity profile switched to an activation state at 10 and 30 minutes after trigger, with the strongest activation observed 10 minutes only after cytokine exposure. After that, SGK2 activity is gradually repressed to reach a strong inhibition 960 minutes after the induction of inflammatory conditions.

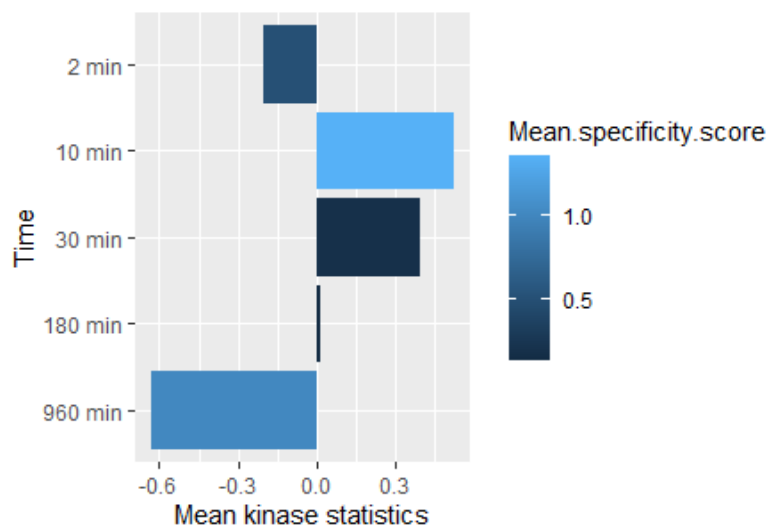


Figure 64. SGK2 is quickly activated by the inflammatory trigger in Caco-2 cells

Heatmap representation of the activation status of SGK2 following trigger. Caco-2 cells were seeded on a Collagen gel (5 μ g/cm²) and were triggered with IL-1 β , TNF- α , IFN- γ (1,50,50 ng/mL respectively) 48 hrs after seeding for the indicated times. Mean kinase statistics depicts the difference in peptide phosphorylation between the different trigger times (negative=inhibition, positive=activation) whereas mean specificity score reflects the significance of the results (paler shades reveal higher specificity).

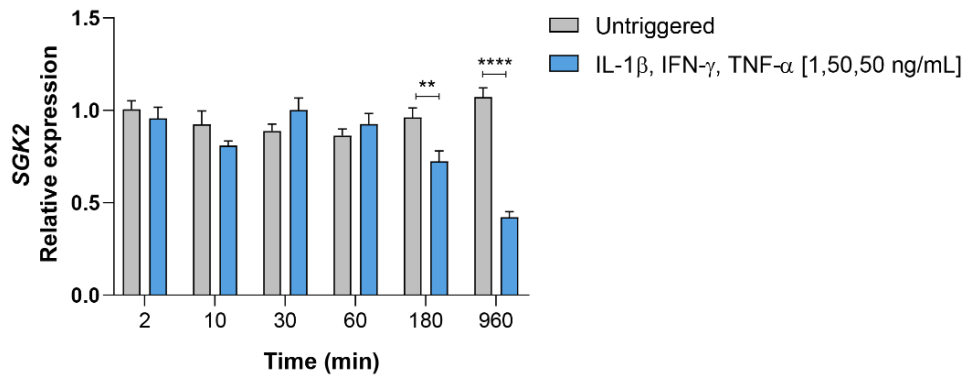


Figure 65. SGK2 expression is not downregulated as an early response to inflammation
Caco-2 cells were seeded on a Collagen gel (5μg/cm²) and were triggered with the indicated cytokine cocktail at Day 2 for the mentioned times. Graph shows the average RQ values (2-ΔΔCt) from one representative experiment for SGK2 expression normalized to TBP expression ± SEM (n=7). One-way ANOVA with Dunnett's post-hoc test compared to untriggered of each time point.

Following these results, the mRNA expression of SGK2 in Caco-2 cells was investigated once again, but this time following the same time course as in the activation experiment (**Fig. 65**). Interestingly, SGK2 expression was only reduced 180 and 960 minutes after trigger. In previous experiments, SGK2 expression was always quantified at the end of the 16hrs trigger, which did not allow us to realize that SGK2 expression was only downregulated at a later stage of the trigger. It therefore seems like SGK2 is activated as an early response to inflammation, while its expression remains constant. When the inflammation is sustained, the activity of SGK2 is gradually repressed together with its mRNA expression.

Modulation of SGK2 expression in intestinal epithelial cells

Stable knockdown of SGK2 in HIO

In order to obtain a stable SGK2 knockdown, HIO were transduced with shRNA-containing lentiviruses and transduced cells were selected using puromycin. A 7 days puromycin selection led to the death of untransduced cells, while cells that integrated the shRNA constructs grew back in cystic structures (**Fig. 66**). Cells that integrated the non-targeting shRNA and some shRNA versions did not survive further passaging, freezing and thawing. Only the cell cultures with the shRNA versions that survived the entirety of experimentation (v22, v23, v25) are shown at **Figure 66**.

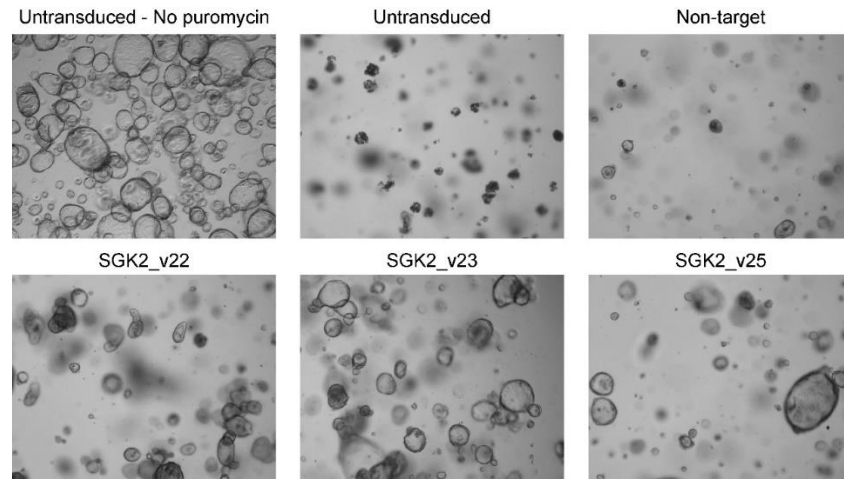


Figure 66. Establishment of stable SGK2 knockdown HIO

Representative 5X brightfield images of transduced HIO (MOI 80-100 per cell, HUB2 donor). Top left image is in absence of puromycin, all other images show HIO after 7 days of puromycin selection (4 μ g/mL). Cells resistant to puromycin were then further expanded and frozen.

SGK2 knockdown efficiency was assessed by qPCR in the remaining cell lines. SGK2_v23 and SGK2_v25 led to a 60.5% and 39.5% knockdown respectively, while SGK2_v22 did not affect SGK2 expression (**Fig. 67B**). SGK2 knockdown did not affect the expression levels of SGK1 and SGK3, except for shRNA SGK2_v25 that caused a 42% reduction in SGK3 expression (**Fig. 67C**).

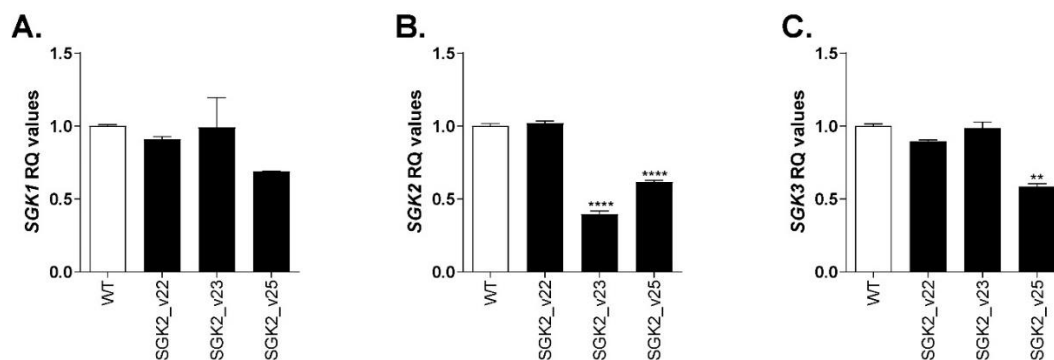


Figure 67. Lentiviral delivery of shRNA leads to SGK2 knockdown in HIO

Expression of SGK1 (A.), SGK2 (B.) and SGK3 (C.) following stable shRNA lentiviral knockdown in HIO (HUB2 donor) normalized to *TBP* expression. Graph shows average RQ values ($2^{-\Delta\Delta Ct}$) $\pm$ SEM. One-way ANOVA with Dunnett's post-hoc test compared to WT HIO (n=2-4).

HIO cells treated with lentiviruses were difficult to culture and grew out slowly even at very low passage ratios, it was therefore impossible to obtain enough cells to grow in the OrganoPlate platform. Nevertheless, in order to assess the knockdown's effect on the cytokine production of HIO, cells were triggered directly in Matrigel droplets in their inside-out configuration. The LPS-IFN γ trigger used in the OrganoPlate platform would not have

performed well in Matrigel-grown HIO because of their inverted configuration and only the cytokine cocktail trigger was used. Perhaps because of the apparent exhaustion of the cells, the trigger resulted in a lower-than-normal increase in cytokine production of HIO and the window between untriggered and triggered cells was small. However, the production of CCL-20, IP-10 and IL-8 were upregulated upon trigger. SGK2_v23 and SGK2_v25, which had an efficient *SGK2* knockdown, also caused a decreased production of the three cytokines analysed compared to WT HIO (**Fig. 68**). SGK2_v22, which did not show any knockdown, showed an increase of cytokine release comparatively to WT HIO. In conclusion, these results show that the knockdown of *SGK2* in HIO leads to reduced cytokine production, similarly to what was observed in the original screening assay.

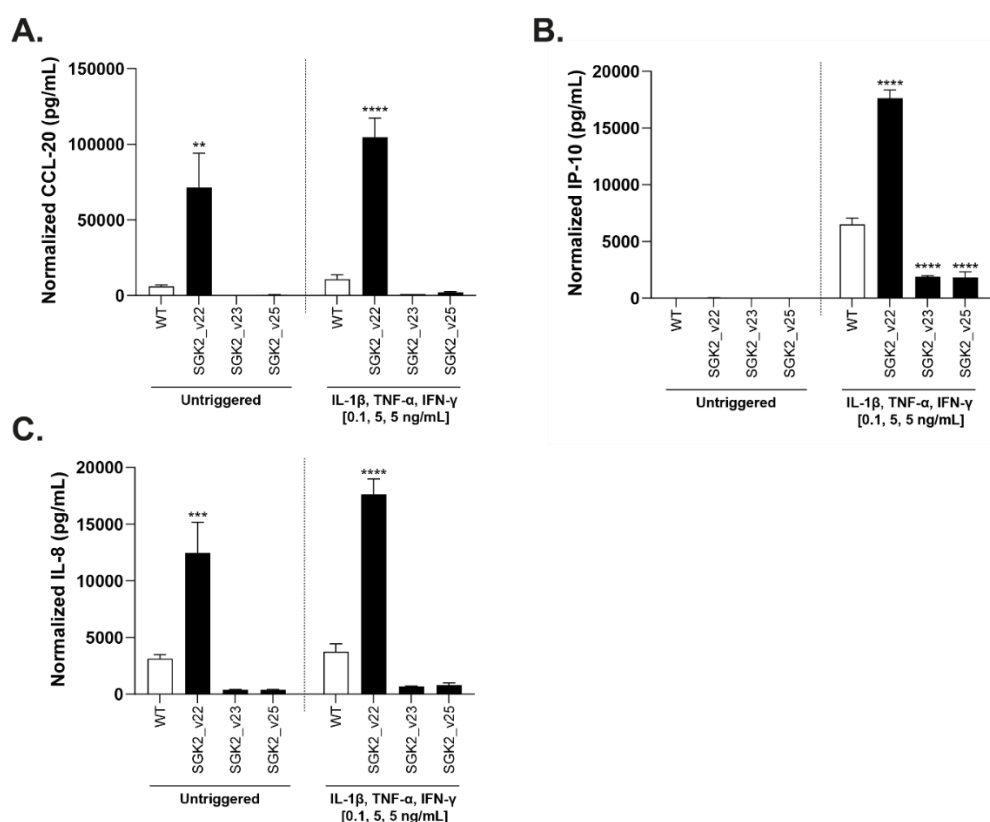


Figure 68. Knockdown of *SGK2* decreases cytokine production by HIO

Secretion of CCL-20 (A.), IP-10 (B.) and IL-8 (C.) by transduced HIO growing in Matrigel droplets triggered with the indicated cytokine cocktail for 24hrs (HUB2 donor). To compensate for different number of cells per well, results were normalized to a WST-8 viability assay. Graphs show average cytokine production normalized for viability $\pm$ SEM. Two-way ANOVA with Dunnett's post-hoc test compared to WT HIO (n=2-3).

Overexpression of *SGK2* in Caco-2 cells

Previous screening results have demonstrated that the transient knockdown of *SGK2* drives a reduction of cytokine release in Caco-2 cells upon inflammatory trigger. Stable knockdown of *SGK2* in HIO also reduced the production of inflammatory cytokines after stimulation. The

effect of transient SGK2 overexpression on the cytokine levels of Caco-2 cells was assessed. Both the overexpression of WT SGK2 and TurboSGK2 (construct used in proteomics, see **Table 13**) caused a significant increase in cytokine production by Caco-2 cells after a 24hrs trigger with IL-1 β , TNF- α , IFN- γ (1,50,50 ng/mL respectively). Notably, the production of CCL-20 (**Fig. 69A**), IL-12p70 (**Fig. 69B**) and IFN- γ (**Fig. 69C**) were the most affected by the overexpression of SGK2 constructs. These results suggest that, while SGK2 knockdown reduces the inflammatory phenotype in IECs, its overexpression intensifies it.

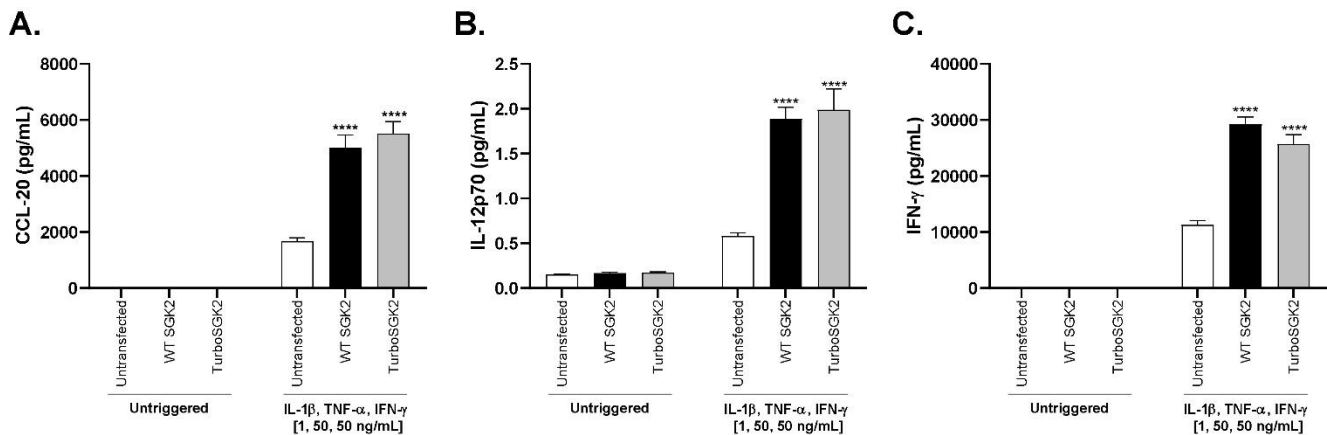


Figure 69. Overexpression of SGK2 increases cytokine production of Caco-2 cells

Secretion of CCL-20 (**A.**), IL-12p70 (**B.**) and IFN- γ (**C.**) following transient overexpression of SGK2 constructs by Caco-2 cells triggered with the indicated cytokine cocktail for 24hrs. Graphs show average cytokine production $\pm$ SEM. Two-way ANOVA with Dunnett's post-hoc test compared to Untransfected (n=8).

Investigation of the role of SGK2 kinase's function in inflammation

As the kinase activity of a protein is easily inhibited by small molecules, it was investigated whether the kinase function of SGK2 is necessary for the regulation of inflammation. In order to do so, a mutated version of SGK2 harbouring a K124R point mutation was overexpressed in Caco-2 cells in an adenoviral backbone. Transduction of both SGK2^{WT} and kinase-dead SGK2^{K124R} led to successful overexpression of SGK2 (**Fig. 70A, 70B**), but with high intra-well variability. The trigger successfully upregulated CCL-20 production after 960min of stimulation. No differences on CCL-20 production between the different constructs could be observed in untriggered conditions (**Fig. 70C**). Interestingly, overexpression of AcGFP increased CCL-20 production of Caco-2 cells when compared to untransduced cells (**Fig. 70C**). Since the transduction process itself affected CCL-20 values, AcGFP was chosen as the negative control to use as a reference. The overexpression of SGK2^{WT} led to an increase of approximately 40% of CCL-20 production by Caco-2 cells when compared to AcGFP overexpressing cells (**Fig. 70C**). The overexpression of the SGK2^{K124R} mutant did not significantly alter CCL-20 production when compared to AcGFP cells, despite higher levels of

SGK2 expression than SGK2^{WT} (**Fig. 70B**). Overall, these results are suggesting that the kinase function of SGK2 is important for the regulation of CCL-20 production by Caco-2 cells.

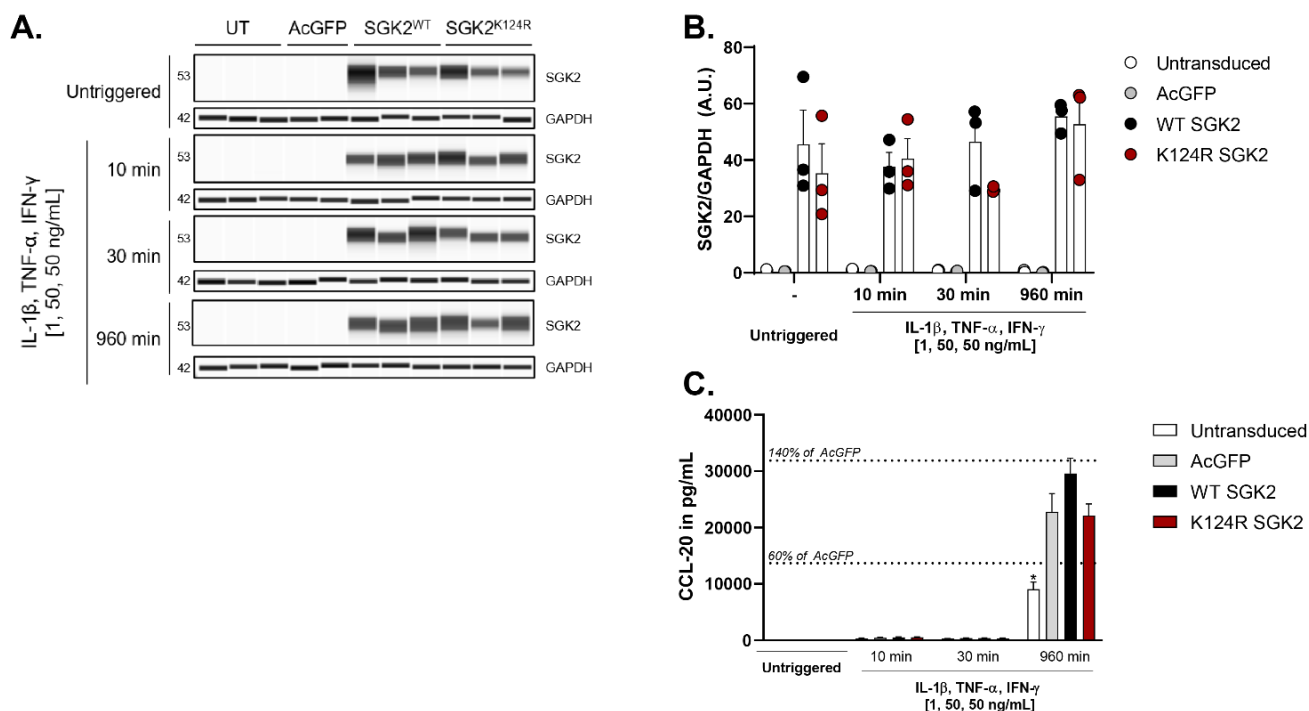


Figure 70. SGK2 kinase function is important for the regulation of CCL-20 production

A. Capillary immunoassay for SGK2 and GAPDH expression in Caco-2 cells overexpressing AcGFP, SGK2^{WT} or kinase-dead SGK2^{K124R} or left untransduced (UT, 0.13µg of protein per lane). The day after seeding, Caco-2 cells were transduced with adenoviruses containing the indicated DNA constructs for 24hrs (MOI 52.5). 48hrs after transduction, cells were triggered with IL-1β, TNF-α, IFN-γ (1,50,50 ng/mL respectively) for 10, 30 and 960 min. **B.** Quantification of SGK2 expression normalized to GAPDH expression. Bars show average signal intensity ± SEM while dots highlight individual values (n=3). **C.** CCL-20 production by Caco-2 cells overexpressing AcGFP, WT SGK2 or K124R kinase-dead SGK2 mutant. Graph shows average CCL-20 production ± SEM at Day 4. Lines depict a 40% range from the triggered AcGFP condition at 960min. Two-way ANOVA with Dunnett's post-hoc test compared to AcGFP at 960min (n=12).

Pharmacological inhibition of SGK2 in intestinal epithelial cells

Previous work from Galapagos have shown that exposure to GSK650394, a SGK1-SGK2 antagonist, could inhibit apical CCL-20 production as well as basolateral IL-12p40, IL-10, IL-6 and TNF-α production in a Caco-2/DC Transwell model (**Fig. 46**). As HIO represent a physiologically relevant alternative to the use of Caco-2 cells but have an inside-out configuration making drug inoculation difficult, further experiments were carried in the OrganoPlate platform. The effect of the compound was assessed on the microfluidic gut-on-a-chip model composed of HIO and macrophages described in Chapter II. The applicability of the model to assess compound efficacy by using known anti-inflammatory drugs Bosutinib, Dasatinib and TPCA-1 was already discussed in **Chapter II** and **Appendix D**. The application

of GSK650394 did not lead to cytokine inhibition profiles as pronounced as these anti-inflammatory compounds. After a 24hrs exposure period, the secretion of IP-10, IL-8 and CCL-20 were slightly inhibited in HIO and the co-culture of HIO and macrophages (**Fig. 71A-C**). At the highest concentrations of GSK650394, both apical and basal secretion of IP-10, IL-8 and CCL-20 were inhibited by 20 to 40% without the compound being toxic to the cells (**Fig. 71D**). The other cytokines assessed were not inhibited by the compound (results not shown).

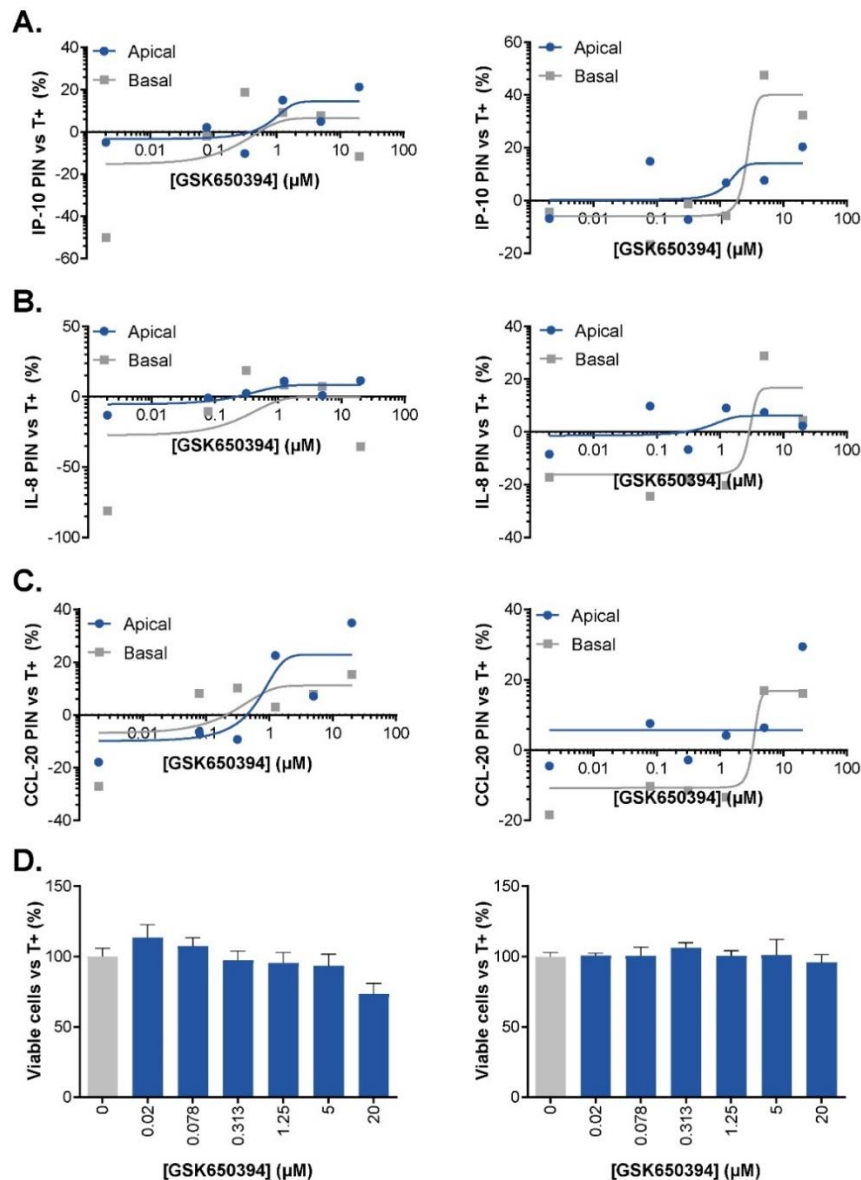


Figure 71. SGK1-2 antagonist leads to slight cytokine release inhibition by HIO

A-C. Apical and basal secretion of IP-10 (**A.**), IL-8 (**B.**) and CCL-20 (**C.**) were assessed 24hrs after GSK650394 treatment at the indicated concentrations in HIO (HUB2 donor; left panel) or in HIO+macrophages (right panel). Dots represent the percentage of inhibition (PIN) mean normalized to triggered (LPS 100ng/mL, IFN- γ 20ng/mL) but GSK650394 untreated tubules. The line depicts a non-linear regression between [GSK650394] and cytokine secretion (n=3-5). **D.** Viability of HIO (Donor HUB2; left panel) or in HIO+macrophages (right panel) after 72hrs of GSK650394 exposure. Bars represent the average of the percentage of viable cells $\pm$ SEM with Dunnett's post-hoc test compared to triggered but GSK650394 untreated tubules (n=3-5).

kinase activity (SGK2^{K124R}) as well as the wild type SGK2 (SGK2^{WT}) were overexpressed in Caco-2 cells. The inflammatory trigger increased expression of STAT3 and its phosphorylation on Ser727, especially after a 960min trigger (**Fig. 72A-D**). The overexpression of SGK2^{WT} and SGK2^{K124R} did not seem to affect total protein levels for STAT3 (**Fig. 72E**). Interestingly, the kinase-dead mutant showed a trend towards decreased levels of STAT3 phosphorylation on Ser727 when compared to WT SGK2 (**Fig. 72F**). These preliminary results suggest that SGK2, directly or indirectly, could lead to the phosphorylation of STAT3, which might shed a light on how SGK2 regulates cytokine release in Caco-2 cells.

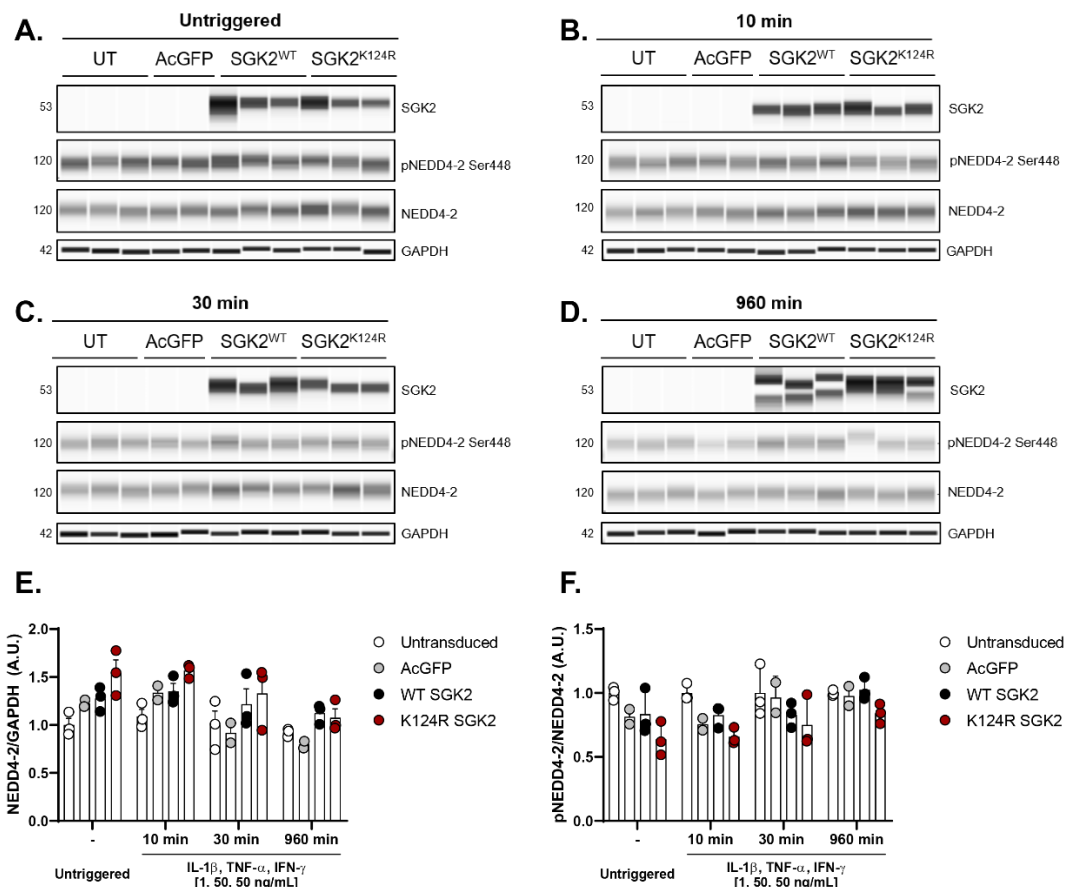


Figure 73. SGK2 kinase activity seems required for NEDD4-2 Ser448 phosphorylation
A-D. Capillary immunoassay of for pNEDD4-2 Ser448, NEDD4-2, SGK2 and GAPDH expression in Caco-2 cells overexpressing AcGFP, SGK2^{WT} or kinase-dead SGK2^{K124R} or left untransduced (UT, 0.13µg of protein per lane). The day after seeding, Caco-2 cells were transduced with adenoviruses containing the indicated DNA constructs for 24hrs (MOI 52.5). 48hrs after transduction, cells were left untriggered (**A.**) or triggered with IL-1β, TNF-α, IFN-γ (1,50,50 ng/mL respectively) for 10 (**B.**), 30 (**C.**) and 960 min (**D.**). **E.** Quantification of NEDD4-2 expression normalized to GAPDH expression. Bars show average signal intensity ± SEM while dots highlight individual values (n=3). **F.** Quantification of pNEDD4-2 Ser727 expression normalized to STAT3 expression. Bars show average signal intensity ± SEM while dots highlight individual values (n=3).

As exposed earlier, most of the described actions of SGK2 are mediated through its regulation of NEDD4-2 (Wang et al., 2016a; Boehmer et al., 2006; Böhmer et al., 2004). We therefore

investigated whether this pathway was affected in cells overexpressing a catalytically inactive form of SGK2. The overexpression of SGK2^{WT} did not seem to affect total protein levels for NEDD4-2, but interestingly the overexpression of SGK2^{K124R}, in untriggered condition and early trigger points of 10 and 30 min, did seem to slightly increase NEDD4-2 levels (**Fig. 73E**). Of this slightly higher NEDD4-2 pool, less NEDD4-2 is present in its phosphorylated form on Ser727 (**Fig. 73F**), while the overexpression of SGK2^{WT} did not seem to alter phosphorylated levels of NEDD4-2. As phosphorylation inactivates NEDD4-2 (Dieter et al., 2004), it can be speculated that the overexpression of K124R leads to an enriched pool of active NEDD4-2 comparatively to WT SGK2 overexpression.

Discussion & Conclusions

Investigation of the role of SGK2 in inflammatory IECs

SGK2 (Serum- and Glucocorticoid-Regulated Kinase 2) is a serine-threonine kinase known to be involved in the regulation of a wide variety of ion channels and membrane transporters mainly in epithelial cells of the kidney and liver. Earlier, we identified SGK2 as a regulator of inflammatory processes in IEC. In a Caco-2/DC co-culture assay, the apical secretion of CCL-20 in Caco-2 cells was reduced upon SGK2 knockdown. No study has yet explored the role of SGK2 in the intestinal epithelium nor its relation to inflammatory processes. In order to understand how SGK2 knockdown ultimately leads to the reduction of inflammatory cytokines production in IECs, SGK2 biology was first investigated in normal and inflammatory conditions of HIO, Caco-2 cells and in the colonic mucosa of IBD patients.

We found SGK2 expression to be systematically reduced in the colonic mucosa of IBD patients suffering from active inflammation, while SGK1 and SGK3 expression levels were not affected. After a 16hrs inflammatory trigger, SGK2 and SGK3 expression were downregulated in all HIO donors, while SGK1 expression was upregulated. In Caco-2 cells, SGK2 and SGK3 expression were downregulated in inflamed cells whereas SGK1 expression levels did not vary. The results in HIO and Caco-2 cells did not fully recapitulate the events observed in the colonic mucosa of IBD patients. These divergences could simply be explained by the intrinsic differences between the different cell types, donors and tissues used. Indeed, biopsies isolated from IBD patients are a complex mix primarily composed of IEC but also other cell types such as fibroblasts, tissue-resident immune cells and endothelial cells that might contribute to SGK expression levels. In our case, the use of IECs might not reflect the organ situation where other cell types can contribute to the global levels of gene expression. Furthermore, Caco-2 cells are strictly enterocytic-like, whereas HIO are composed of multiple differentiated cell types. We

have shown that these cell types are differentially represented in the three HIO donors used, which could explain the differences in SGK paralog expression between donors. The upregulation of *SGK1* expression might be caused by a differentiated cell type not present in the Caco-2 cell line. Furthermore, Caco-2 cells are initially derived from the small intestine whereas the HIO used in this study were derived from distal parts of the GI tract; regional differences of SGK paralogs expression are not to be excluded. In addition, the HIO used in this study were derived from non-IBD individual and the use of a cytokine trigger cocktail to induce IBD-like conditions certainly does not reflect the genetic and environmental complexity of the disease and SGK paralogs might be differently involved. Finally, the repeated upregulation of *SGK1* expression in HIO might be an early event of compensation for the loss of *SGK2* expression, which might return to normal levels on the long term to reflect what was observed in the colonic mucosa of IBD patients. In all cases, despite differences in *SGK1* and *SGK3* expression, the downregulation of *SGK2* expression upon inflammatory activation of IECs was a consistent event observed in all cell lineages studied and suggest that SGK2 regulates or is regulated by inflammatory processes.

Identification of potential interaction partners of SGK2

Since most know how regarding SGK2 stems from association with the most characterized family paralogs SGK1 and SGK3, proximity-labelling mass spectrometry was performed in order to identify potential interaction partners of SGK2. The initial goal was to perform proximity labelling in HIO. However, despite good expression of Turbo constructs in cells, no biotinylation activity was observed upon addition of biotin. To perform biotinylation experiments, HIO were seeded on top of a soft layer of 10% Matrigel. At the end of the experiment, it could be observed that HIO were capable to enter inside the gel and form spheroid-like structure. This could explain the inefficient biotinylation process. Indeed, uptake of free biotin by human IEC occurs via an efficient Na⁺-dependent through the polarized epithelium and involves biotin movement from the apical brush border membrane to the basolateral membrane (Said et al., 1987). HIO growing in spherical structures have an inverted polarization; their apical membrane is enclosed inside the lumen, making it inaccessible to biotin. Furthermore, biotin uptake occurs majorly in the small intestine; absorption being at its highest level in proximal sections of the small intestine over distal sections (Said et al., 1988). As HIO were derived from distal parts of the colon or rectum, it could explain why biotin absorption was inefficient. Finally, the possibility of receptor saturation, even before biotin addition, cannot be excluded as saturation has been observed at the micromolar range in IEC before (Said, 2009) and the medium used in the experiments already contains biotin in the low

micromolar range. This could have probably been prevented by growing the cells in a monolayer and/or overexpressing Sodium-dependent multivitamin transporter (SMVT), the transporter responsible for biotin absorption at the brush border of HIO.

However, it was decided to switch our proximity-labelling experiments to small intestine Caco-2 cells. The medium used for Caco-2 cells experiments is devoid of biotin and the addition of supplementary biotin did efficiently increase biotinylation and mass spectrometry experiments could be performed in this cell type. To do so, TurboSGK2 was overexpressed in Caco-2 cells that were later triggered with a mixture of IL-1 β , TNF- α and IFN- γ in an attempt to identify inflammatory interaction partners of SGK2. Importantly, known interactors of SGK2 were successfully picked up. Upon induction of inflammatory conditions, proximity labelling of TurboSGK2 fusion protein led to overrepresentation of proteins involved in regulation of IFN- γ signalling, response to insulin, tricarboxylic acid metabolism as well as cell cycle and cytoskeleton reorganization processes. The overrepresentation of IFN- γ signalling confirmed that the trigger successfully induced inflammatory pathways in Caco-2 cells. SGK2 has been shown to co-activate PXR-mediated *trans*-activation of gluconeogenic genes at Insulin Response Sequence (IRS) sites (Gotoh and Negishi, 2015, 2014), it is therefore not surprising to see potential interaction with proteins involved in metabolism and insulin response.

A STRING pathway analysis (Snel, 2000) was performed on the top 30 differentially represented proteins to uncover previously curated interaction pathways that might lie within the set of potential interactors identified. From this list, it was uncovered that five of these proteins were already known to interact together: IFNGR1, STAT3, STAT1, DTX3L and LMO7. It was therefore decided to focus our subsequent investigation on this restricted set of proteins.

Confirmation of proteomics hits by immunoprecipitation

Based on the list of potential interactors generated with proximity labelling mass spectrometry, we pursued the validation of a physical interaction using immunoprecipitation. Importantly, we could confirm the interaction between TurboSGK2 and STAT3. However, we could not confirm this interaction with a WT SGK2 construct nor with the endogenous protein. This could be due to the capture antibody used in the immunoprecipitation; TurboSGK2 was immunoprecipitated using an antibody directed against the HA tag while WT and endogenous SGK2 were precipitated with an antibody directed against SGK2. We observed that the immunoprecipitation efficiency was higher with the HA antibody. If the interaction with

STAT3 is transient or happening at a low frequency, it might be that the SGK2 antibody did not pull down enough SGK2 to be able to visualize STAT3 at sufficient levels. However, we cannot rule out that the interaction observed is an artefact inherent to TurboSGK2 transfection in Caco-2 cells and further optimization of immunoprecipitation will need to be performed to validate the interaction at the endogenous level. Nevertheless, we describe, for the first time, a potential interaction between SGK2 and STAT3. It is now well established that STAT3 is an important player in the regulation of inflammatory processes through the JAK-STAT pathway (Hillmer et al., 2016). Through its interaction with STAT3, SGK2 might participate in inflammatory responses at some level.

SGK2 activation in inflammatory conditions

As expression is not always a direct readout of the activity of a protein, we investigated SGK2 activation in answer to the inflammatory trigger. To do so, protein lysates from inflamed and uninfamed Caco-2 cells were applied into chips printed with phosphorylable peptides. The peptides being phosphorylated by the kinases present in the lysate are integrated to highlight the kinases most likely responsible for the effect observed. An inflammatory time-course revealed that kinase activity, including SGK2 activity, was generally reduced 2 minutes after trigger. However, SGK2 quickly switched to an activated state 10 minutes after induction of inflammatory conditions. SGK2 activity was then slowly repressed and reached a strong inhibition 16 hours after inflammatory trigger. Reflecting on these results, we assessed the mRNA expression of SGK2 following the same timeline. Interestingly, SGK2 expression remained stable in the early stages and only decreased at a later stage, 3 and 16 hours after trigger. These results suggest that SGK2 is quickly activated after establishment of inflammation, but that a negative feedback mechanism might be engaged to lead to the repression of its activity. However, the peptides phosphorylation results are derived from literature or computational predictions and are correlated with one or multiple upstream kinases. There is therefore no absolute certainty that the increase in peptide phosphorylation observed is caused by SGK2. A more direct readout of SGK2 activation, its phosphorylation on residues Thr253 and Ser416, could confirm that SGK2 is indeed activated as an early response to an inflammatory trigger. However, such readout is dependent on the development of specific and reliable antibodies, which are lacking presently.

As we learned by investigating SGK2 activation at a later stage in the project, SGK2 is most likely to play a role in the first instances of inflammation as it is quickly activated after an inflammatory trigger. After a sustained trigger of 16 hours, SGK2 activity and expression are

strongly downregulated, likely by negative feedback mechanisms. Therefore, the trigger time used in the mass spectrometry experiments might not have been adapted to reflect the pro-inflammatory role of SGK2. Perhaps, a trigger time of 10 or 30 minutes might have been more informative about how SGK2 can mechanistically be involved in the regulation of inflammation.

Modulation of SGK2 expression in intestinal epithelial cells

To understand further how SGK2 might be linked to inflammation, gain- and loss-of-function studies were performed. The screening results from Galapagos reveal that transient knockdown of SGK2 by adenoviral and lentiviral vectors lead to a reduction in apical secretion of CCL-20 but also basolateral secretion of IL-12p40 and TNF- α in Caco-2 cells. We then established stable knockdown of SGK2 expression by shRNA-expressing lentiviral vectors in HIO. For 2 out of the 3 constructs tested, the secretion of inflammatory cytokines CCL-20, IP-10 and IL-8 was reduced by HIO in answer to trigger. The third construct, SGK2_v22, upregulated production of these cytokines, but did not lead to SGK2 knockdown. In this set of experiments, the non-targeting control did not survive puromycin selection and subsequent passaging. We previously observed, in Caco-2 cells, that transduced cells show elevated cytokine secretion upon trigger (Beaurivage et al., 2019). There is a possibility that the construct leading to upregulation of cytokine production, when compared to untransduced cells, would still offer a reduction over the non-targeting control, but it could also be caused by off-target effects of the construct. On the other hand, the delivery of expression vectors for WT SGK2 and TurboSGK2 used in proteomics experiments both elevated cytokine production of Caco-2 cells, particularly CCL-20, IL-12p70 and IFN- γ . In both HIO and Caco-2 cells, SGK2 knockdown alleviated the inflammatory phenotype while SGK2 overexpression aggravated cytokine production of Caco-2 cells.

SGK2 kinase's function in the regulation of inflammatory processes

When considering pursuing compound discovery for a potential target, the druggability of the latter is an important factor to take into consideration. As SGK2 is a protein kinase and since small molecules can easily inhibit kinases, we investigated the necessity of the kinase activity of SGK2 to regulate inflammation. Our preliminary results show that the catalytically inactive mutant (SGK2^{K124R}) was unable to regulate CCL-20 secretion whereas SGK2^{WT} upregulated its secretion by Caco-2 cells, suggesting an important role for the kinase domain of SGK2 in this process. However, the adenoviral-mediated overexpression of these constructs leads to variable expression levels of SGK2 with high intra-experiments and intra-well variation, which

made interpretation of these results difficult. Ectopic gene expression using promoters such as CMV leads to high transgene expression levels that are variable and far from the endogenous protein levels. Other techniques such as CRISPR activation (CRISPRa), that increases endogenous gene expression rather than an exogenous transgene, might be far more suitable to study such mechanisms. Additionally, our dominant negative experiments were not conducted in a knock-out or knock-down background, meaning that endogenous SGK2 was still contributing to the effect observed, which might have further contributed to the variable portrait. In the future, it would be relevant to overexpress SGK2^{WT} and SGK^{2K124R} in cells devoid of SGK2 expression, in order to be able to clearly assess the contribution of the kinase domain.

Additionally, we also conducted experiments where Caco-2 cells were exposed to GSK650394, a SGK1/2 antagonist. Notably, exposure to GSK650394 decreased the production of IP-10, IL-8 and CCL-20 in the microfluidic culture of HIO and the co-culture of HIO with macrophages without causing toxicity. However, the inhibition profiles were subtle and the highest concentrations (5 and 20µM) only resulted in 20 to 40% inhibition of cytokine release. These percentages of inhibition were smaller than in the Caco-2/DC screening assay performed in Galapagos, where the highest concentrations of compound led to 50% to 100% inhibition of cytokine release. Internal investigation in Galapagos determined that GSK650394 was rather unspecific for SGK1/2 inhibition as multiple other kinases were inhibited at an IC₅₀<100nM and was therefore not the best compound to use to assess the particular contribution of SGK2 to inflammation. Other compounds such as BN201, a specific SGK2 agonist (Villoslada et al., 2019), might help shed more light on the latter question. In conclusion, these results suggest that the kinase activity of SGK2 might be important in the regulation of inflammation. However, it will become crucial to confirm these results using specific SGK2 compounds in the future.

Preliminary investigation of SGK2-related pathways in intestinal epithelial cells

Unfortunately, we could not pinpoint the exact mechanism by which SGK2 might be able to regulate inflammatory processes in IEC. However, our results allow us to hypothesise on two potential mechanisms through which SGK2 might act.

Hypothetic model 1 – SGK2 regulates inflammation through interaction with STAT3

The first hypothesized model comes from the interaction with STAT3 we described. Specifically, we observed that TurboSGK2, the construct used in our proteomics experiments,

could physically interact with STAT3 in Caco-2 cells, by using immunoprecipitation methods. Furthermore, our preliminary results show that the kinase dead version of SGK2 seems to lead to have lower levels of pSTAT3 Ser727, when compared to WT SGK2.

STAT3, Signal transducer and activator of transcription 3, is a transcription factor that relays the signal of diverse membrane receptors to modulate gene expression. Precisely, STAT3 plays a role in the response to a pleiotropy of mediators, such as IL-6 and IL-10 cytokine families. Its implication in inflammatory diseases such as IBD is now undisputable (Sugimoto, 2008; Leppkes et al., 2016). Namely, GWAS have linked an intronic polymorphism in *STAT3* gene (rs744166) to an increased risk to develop UC (Franke et al., 2008). Also, germline mutations in STAT3 coding regions have been observed in patients with an infantile-onset multi-organ autoimmune disease, that included gastrointestinal inflammation (Cénié et al., 2010).

Mechanistically, upon cytokine binding to its receptor, JAK proteins will be activated and will phosphorylate STAT3 which will later form homodimers that will translocate to the nucleus, bind to IFN- γ (Gamma-Activated Sequence [GAS]) DNA consensus sequences and act as a transcription factor of inflammatory, anti-apoptotic and pro-proliferative genes (Yu et al., 2009). There are two main phosphorylation events leading to the full activation of STAT3; the first phosphorylation is on Tyr705 residue is mediated through Jak1, Jak2, and Tyk2 and is necessary for the transcriptional activation of STAT3 (Rincon and Pereira, 2018). The second phosphorylation event, on Ser727, is only required for enhancing STAT3 transcriptional activity (Rincon and Pereira, 2018). The latter phosphorylation is thought to be mediated through Protein Kinase C (PKC) (Gartsbein et al., 2006; Macias et al., 2014), a member of the AGC kinase family. Members of the AGC family share a highly homologous kinase domain and have been shown to phosphorylate the same substrates (Pearce et al., 2010). The hypothesis is that, upon binding with STAT3, SGK2 phosphorylate STAT3 on Ser727 (**Fig. 74**). The homodimer of activated STAT3 will then translocate to the nucleus to engage transcription of *STAT3* expression itself. The unphosphorylated STAT3 (uSTAT3) will also form homodimers and translocate to the nucleus and bind DNA at GAS promoter regions to activate transcription of inflammatory genes (Timofeeva et al., 2012; Decker et al., 1997). Alternatively, uSTAT3 can also form a dimer with an unphosphorylated form of NF- κ B (uNF- κ B) that will bind DNA at NF- κ B binding sites (Yang et al., 2007; Yoshida et al., 2004) and engage transcription of inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, CXCL10 and TNF- α (Liu et al., 2017b). Concomitantly, SGK1 was identified as the facilitating kinase for PI3K-initiated

phosphorylation of IKK α Thr23, which in order allows the phosphorylation of IKK α Ser180 and the consecutive transcriptional activation of NF- κ B (Zhang et al., 2005).

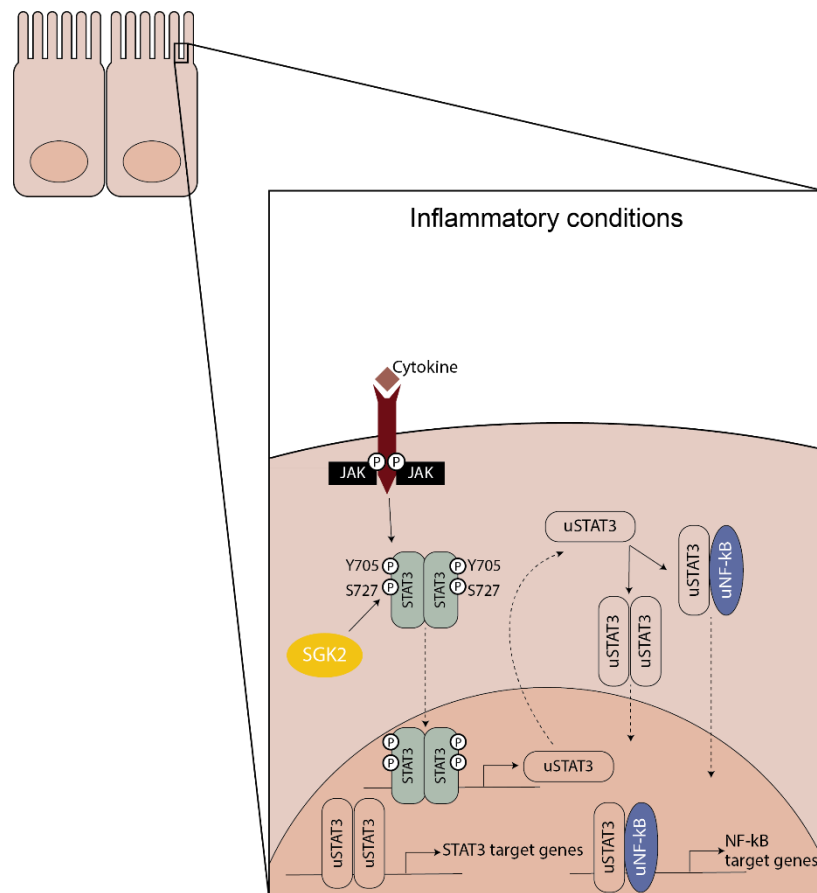


Figure 74. Hypothetic model - SGK2 mediates the phosphorylation of STAT3 on Ser727

Upon liaison between a cytokine and its receptor, JAK proteins are activated and lead to the phosphorylation of STAT3 on Tyr705. SGK2, through its serine/threonine activity, could lead to the phosphorylation of STAT3 on Ser727, which enhances STAT3 transcriptional activity. STAT3 homodimer translocates to the nucleus and engages the transcription of STAT3, leading to an augmented pool of unphosphorylated STAT3 (uSTAT3). uSTAT3 can form homodimers which will act as transcription factors at GAS sequences to upregulated STAT3 target genes. Alternatively, uSTAT3 can form heterodimers with unphosphorylated NF- κ B. The complex will translocate to the nucleus to engage transcription of NF- κ B target genes, leading to a sustained inflammatory state.

Multiple avenues would be suitable to confirm this hypothesis. Firstly, once the immunoprecipitation of endogenous SGK2 is optimized, an *in vitro* kinase activity assay combining the immunoprecipitated SGK2 and a STAT3 peptide containing Ser727 could assess whether SGK2 is responsible for STAT3 phosphorylation. The same assay could perhaps be performed with a recombinant SGK2 protein. After the direct phosphorylation is confirmed, mutation of Ser727 and its effect on cytokine production and on STAT3/NF- κ B target genes transcription would provide phenotypical evidence that this pathway is partly or totally responsible for SGK2 regulation of inflammatory processes. Evidently, as SGK2 is

quickly activated after inflammatory trigger, it will be of utmost importance that the timing used in this assay reflects SGK2 activation.

Furthermore, our proteomics analyses revealed an overrepresentation of proteins involved in the tricarboxylic acid (TCA) cycle in proximity of TurboSGK2 when cells are subjected to an inflammatory trigger. The TCA cycle, which essentially takes up acetylCoA produced by glycolysis to generate NADH/FADH₂ and support the oxidative phosphorylation pathway, has been linked to inflammation. Especially, pro-inflammatory M1 macrophages induced by LPS/IFN- γ have compromised mitochondrial metabolic activity of TCA cycle (Koo and Garg, 2019). It has been described that STAT3 plays a role in the process; importantly, its phosphorylation on Ser727 has been reported to lead to mitochondrial translocation of STAT3 (Tammineni et al., 2013) where STAT3 is further acetylated to participate in the TCA cycle (Xu et al., 2016). The export and breakdown of mitochondrial citrate has been associated with the production of numerous central pro-inflammatory mediators in macrophages, such as NO, ROS and prostaglandin E₂ (Williams and O' Neill, 2018). If SGK2 is indeed able to phosphorylate STAT3 on Ser727, it might participate in the regulation of inflammation through the TCA cycle.

Hypothetic model 2 – SGK2 regulates inflammation through NEDD4-2 phosphorylation

We cannot exclude that the regulation of inflammatory processes by SGK2 could go through other mechanisms that are not necessarily upregulated upon trigger and have therefore not been picked up by our proteomics approach. Indeed, it is not because a certain pathway is not upregulated upon stimuli that this pathway does not regulate the answer to this specific stimulus. Specifically, NEDD4-2 was picked up by our proteomics approach but its interaction with SGK2 was not upregulated upon inflammatory trigger. SGK2 has been previously shown to interact with NEDD4-2 in an *in vitro* binding assay (Persaud et al., 2009). Our results show that SGK2 overexpression leads to decreased levels of NEDD4-2 protein in Caco-2 cells. NEDD4-2, an E3-ubiquitin ligase, is fully active in its unphosphorylated form. Upon phosphorylation, NEDD4-2 ubiquitin ligase activity is compromised and the protein is targeted for proteasomal degradation (**Fig. 75**). NEDD4-2 is known to be involved in the degradation of several proteins. Namely, when unphosphorylated, NEDD4-2 leads to the degradation of SGLT1, a sodium-dependent glucose transporter. This is particularly interesting in this project as SGLT1 has been shown to protect against intestinal inflammation through the uptake of glucose (Yu et al., 2005; Palazzo et al., 2008) and the effect of SGK2 on receptors' activation

and expression status have also been reported to be mediated through NEDD4-2 (Wang et al., 2016a).

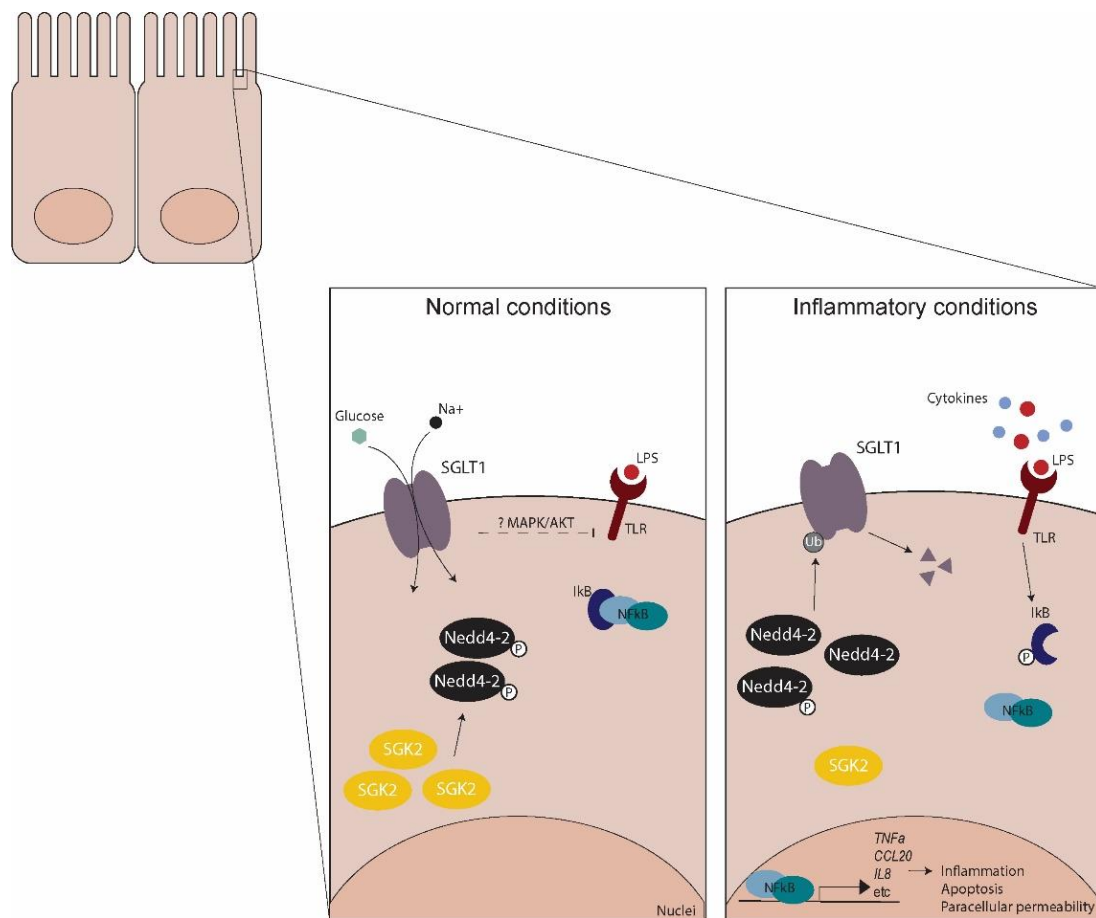


Figure 75. Hypothetic model - SGK2 regulates TLR signalling through NEDD4-2 phosphorylation

In normal conditions, SGLT1 is shown to protect against inflammation through sodium coupled glucose transport. The regulatory mechanism leading to inhibition of TLR signalling is thought to involve the MAPK-AKT pathway. In a sustained inflammatory state, the expression and activation of SGK2 are repressed, leading to increased levels of activated and unphosphorylated NEDD4-2. Active NEDD4-2 leads to SGLT1 ubiquitination and degradation. As SGLT1 cannot down regulate TLR signalling anymore, the latter is engaged by ligands such as LPS and engage inflammatory pathways. Namely, NF-κB can translocate to the nucleus and promote the transcription of pro-inflammatory genes.

Additionally, SGLT1 expression and activation have been shown to be regulated by highly-similar AGC kinases SGK1, SGK3 and PKB (Dieter et al., 2004). It can therefore be hypothesized that SGK2, through its interaction with NEDD4-2, is able to regulate SGLT1 expression and activity. We might not be able to visualize the effect of the transient and rapid activation of SGK2 and its effect on NEDD4-2 phosphorylation and degradation in the early stages of the trigger. However, SGK2 expression and activation are downregulated after a sustained exposure to pro-inflammatory cytokines. In such a case, less phosphorylated NEDD4-2 is present leading to an increased pool of activated NEDD4-2. Subsequently,

NEDD4-2 leads to the proteasomal degradation of SGLT1, which is required for tuning down TLR responses. Consequently, downstream effectors such as NF- κ B are upregulated to promote inflammatory processes. Our analyses confirmed that the overexpression of catalytically inactive SGK2^{K124R} decreased levels of NEDD4-2 Ser448 in Caco-2 cells. Nevertheless, we could not correlate these results to decreased levels of SGLT1 as we could not detect the latter membrane protein. Overexpression of SGK2^{K124R} and SGK2^{WT}, combined with proteasome inhibitor treatment, could confirm those preliminary observations.

With the latter hypothesis, it is important to realize that glucose levels play a role in the extent of protection that SGLT1 confers to IEC. Indeed, the team that identified a protective role of SGLT1 against LPS-induced apoptosis in Caco-2 cells did observe protection only in the high glucose medium (Yu et al., 2005). Subsequently, only cells cultured in a low glucose medium were sensitive to the LPS trigger. The effect of the LPS trigger on the cytokine production of Caco-2 cells was not assessed in that specific study. However, internal experience informed us that LPS do not upregulate cytokine secretion in Caco-2 cells as they express TLR4 only at low levels. Simultaneous administration of glucose and LPS protected mice and colorectal cancer cell line HT-29 against apoptosis and inflammation when compared to LPS treated counterparts (Palazzo et al., 2008). Specifically, glucose administration reduced secretion of pro-inflammatory cytokine IL-8 and increased production of anti-inflammatory cytokine IL-10. As all experiments presented in this study were performed in high glucose containing medium, it can be debated whether the effect of SGK2 might have been easier to appreciate if we would have compared low and high glucose media side-by-side. It would be an interesting avenue to pursue in the future.

Alternatively, other mechanisms that were not tackled in this study might also contribute to the inflammatory phenotype modulation by SGK2. This is perhaps the case with the autophagic pathway regulated by FOXO1 and FOXO3. Autophagy is a catabolic process intended to restore energy homeostasis through intracellular proteins and organelles self-digestion to survive under stress conditions (Lambelet et al., 2018). Autophagy is induced by IFN- γ , IL-1, TNF- α , IL-17 and IL-6 and facilitates the production of IFN- γ , TNF- α , IL-1 β (Ge et al., 2018). SGK2 deletion in HeLa cells has been shown to result in increased autophagy (Baldwin et al., 2010). Mechanistically, after activation through PI3K, AKT and SGK protein kinases phosphorylate FOXO1 and FOXO3 at defined sites, which increase their association with 14-3-3 proteins. FOXO proteins further translocate from the nucleus to the cytoplasm leading to their transcriptional inactivation (Brunet et al., 1999, 2001; Zheng et al., 2000). It is therefore

possible, that the decreased SGK2 expression induced by a prolonged inflammatory trigger could play a role in the impairment of autophagy. Importantly, studies revealed that variants of genes involved in autophagy are associated with compromised clearance of harmful bacterial species, impaired antigen presentation and with the higher production of pro-inflammatory cytokines involved in the pathogenesis of CD, making it especially important to elucidate the connection between SGK2 and autophagy in the context of IBD.

When thinking about pharmacological modulation of SGK2 activity, we will have to first pinpoint its exact mechanisms of action. Given that the STAT3 hypothesis is valid, then an antagonist inhibiting the kinase activity of SGK2 would be required to suppress STAT3 phosphorylation. If the NEDD4-2 hypothesis is correct, then an agonist activating the kinase activity of SGK2 would be required to inactivate NEDD4-2 through phosphorylation, therefore preventing SGLT1 from being degraded, allowing SGLT1 to downregulate TLR signalling through glucose transport.

Concluding remarks

Despite the fact that the exact mechanisms remain to be elucidated, we show an inflammatory role for SGK2 for the first time. We show that its knockdown alleviates cytokine production in Caco-2 cells and HIO. Its overexpression, on the other hand, aggravates cytokine secretion of IEC. Experiments using dominant negative versions of SGK2 and compound experiments suggest that the kinase activity of SGK2 is necessary for the regulation of inflammation. In answer to a cytokine pro-inflammatory trigger, SGK2 is quickly activated while its mRNA expression remains constant. After a sustained inflammatory state, SGK2 activity is switched to a repressed state and its mRNA expression decreases simultaneously, potentially the target of a negative feedback loop. Importantly, SGK2 expression was also found to be downregulated in the colonic mucosa of IBD patients, while the expression of the other SGK paralogs was not affected. Finally, our investigations reveal an interaction between STAT3 and TurboSGK2, a fusion between SGK2 and a biotin-ligase protein and suggest a novel mechanism of action for SGK2.

FUTURE DIRECTIONS

This thesis reports the development of two distinct microfluidic models of the human intestine that can uniquely contribute to the advancement of research in the IBD field. The first one, reported in Chapter I, is an easy-to-use and reliable model using the colorectal cancer cell line Caco-2 that is compatible with compounds as well as adenovirus-mediated knockdown. This model could easily, given the proper automation systems, be applied to high throughput screening campaigns in the search for IBD targets or their validation. The second model is more sophisticated and integrates primary IEC and macrophages of human origin in a co-culture that is more suited for target validation purposes due to technicalities involved in the culture of human intestinal organoids and their use in the microfluidic platform.

We have chosen the OrganoPlate technology from Mimetas as a starting point for both our models. Despite the clear advantage of the technology in terms of throughput and screening capacity, the system does have some important limitations that need to be addressed. Firstly, it uses an interval rocker to create fluid perfusion; it therefore does not create a unidirectional fluid flow as observed in the GI tract, but rather a bidirectional flow where the same medium goes back and forth in the microfluidic channels. Notably, a study using human umbilical vein endothelial cells have demonstrated that cells displayed a Gaussian distribution of F-actin filaments under unidirectional flow whereas cells exposed to bidirectional flow displayed a broader distribution of F-actin filaments (Yang et al., 2019). They have also enquired the cytokine secretion profiles of the cells; the bidirectional flow caused elevated levels of IL-6 and IL-8 in untriggered cells when compared to unidirectional flow. As IBD is a disease where the structural integrity of the epithelium as well as its cytokine production are key players of the disease's aetiology, it might become important to investigate whether the direction of the fluid flow is of importance for the full recapitulation of the disease's phenotype and its level of severity. Secondly, while the platform allows an interesting membrane-free technology allowing the cell types in different microfluidic chambers to be in direct contact with each other, it does not allow to integrate stretchable chambers that can mimic important physiological cues such as peristalsis motions. Studies have shown that integration of a periodic strain further increased barrier function in presence or absence of bacterial co-culture as well as aminopeptidase activity when compared to cells cultured only fluidic flow only (Kim et al., 2012).

Another important factor to consider is the magnitude of shear stress to which the intestine is subjected. Reports have shown that the luminal shear stress of the intestine is around

0.025dyne/cm² (Lentle and Janssen, 2008; Kim et al., 2017). With the settings used in this study, we have calculated that the shear stress imposed on the cell monolayers was 0.13dyne/cm² on average in the OrganoPlate (Vormann et al., 2018). The levels of shear stress varied through time; they were higher in the minutes following the rocking motions (1.6dyne/cm²) and were lower in between rocking motions. The shear stress levels used were on average 5 times higher than those of the GI tract. Fluid shear stress has been shown to affect cellular processes in IEC such as autophagy (Kim et al., 2017), an important mechanism for the homeostasis of the intestinal epithelium. Its dysregulation has been linked with aberrant cytokine secretion in IBD (Ge et al., 2018). We have not been particularly interested in autophagy in the scope of this work. However, it is important to realize that the levels of shear stress used might have impacted this process, which in turn, impacted the phenotype of our gut-on-a-chip models. It would be interesting to modify the angle and interval of the rocking motion of the OrganoPlate, in order more closely recapitulate physiological levels of shear stress. Finally, when thinking about the lumen of the intestine, one can observe that the lumen of the intestine is not filled with liquid but is rather in contact with air and chyme. However, the nature of the OrganoPlate technology makes it difficult to create an air-liquid interface (ALI). Indeed, the technology requires that the cells are initially seeded in liquid and the small size of the capillaries make it difficult to withdraw the liquid after initial seeding. Therefore, we were unable to create an ALI as other studies were able to (Ootani et al., 2009; Yissachar et al., 2017). Interestingly, studies using porcine and murine IEC have shown that ALI culture increased the polarization, differentiation and functional state of the cells (Nossol et al., 2011; Wilke et al., 2019), whether this leads to a more physiologically relevant state remains to be elucidated.

The ultimate goal of any *in vitro* model is to be as close as possible to human physiology. In our case, we wanted to establish a relevant model in order to identify targets and develop drugs that are safe and efficient for the patient. In that optic, we developed the microfluidic model presented in Chapter II, where only human primary material was used. This model represents a significant improvement over the use of ‘gold standard’ Caco-2 cells and we have showed the model’s RNA expression profile similarity to the human intestine. Other than the limitations inherent to the technology addressed above, a few optimizations could further increase the relevance of the model. First, the cells do not spontaneously form crypt-villus axes like it is observed in other microfluidic technologies such as PDMS devices (Kasendra et al., 2018; Workman et al., 2018). Perhaps the lack of mechanical strain in the OrganoPlate, as it is created by vacuum chambers in the PDMS devices, could be responsible for that. Furthermore,

with the settings used in this study, the liquid flow is calculated at 121.2 μ l/hr (Vormann et al., 2018), which is much more than other studies that have used 60 μ l/hr with human duodenal organoids (Kasendra et al., 2018) and 30 μ l/hr with iPSC-derived small intestinal organoids (Workman et al., 2018). This high fluid flow rate might annihilate our chances of having crypt-villi axes being formed.

Furthermore, we did not determine whether the removal of the WNT3A conditioned medium, once IEC are seeded in the OrganoPlate, helps to further improve the differentiation state of the cells. Indeed, we carried all experiments using standard HISC medium, also referred to as expansion medium (Sato et al., 2009). Some studies have, a few days after seeding of the microfluidic chips, switched from expansion medium to differentiation medium, which is HISC devoid of WNT3A conditioned medium, nicotinamide and SB202190 (Kasendra et al., 2018). Nevertheless, it has yet to be determined whether the switch to differentiation medium actually leads to a state that is closer to *in vivo* physiology.

Naturally, attempting to recreate a situation close to the GI tract will necessitate the integration of multiple other cell types. Herein, we report the successful integration of two cell types: HIO-derived IEC together with macrophages. This has proven to be difficult, as those are two really different cell types normally requiring totally different culture media. Optimization experiments revealed that IEC could not survive when the medium for macrophages was introduced in the lower channel of the OrganoPlate and inversely, the macrophages could not differentiate properly when cultured in HISC medium. As a trade-off, isolated monocytes were differentiated into M0 macrophages in their optimal medium in flasks before being seeded in the OrganoPlate, in presence of HISC medium supplemented with M-CSF, until the IEC formed a complete tubule. They were then activated into M1 macrophages using LPS and IFN- γ . This highlights the fact that creating complex culture models with multiple cell types will require a lot of optimization. The differentiation and functionality of each individual cell type has to be monitored closely; it is not sufficient to include as many disease relevant cell types as possible, they have to maintain their function and express the right markers. We chose to use macrophages in the scope of this study because of their important contribution to the IBD pathophysiology. However, when trying to recreate the human gut, inclusion of supportive stromal fibroblasts, vascular endothelial cells, other immune cell types such as intraepithelial lymphocytes, T cells and DC as well as microbiota is only a few of the cell types to be considered. Their incorporation together into an *in vitro* model of the gut will definitely be a challenge of the organ-on-a-chip field in the upcoming years.

The *in vitro* models we develop can help us understand normal physiology but more importantly, can also be applied to understand the mechanisms of unresolved diseases such as IBD. In this study, we have decided to induce an inflammatory state in cells derived from non-IBD subjects in order to obtain a consistent phenotype that is independent of the genetic background of the patient. Mutations in IBD-causative genes such as *NOD2*, *ATG16L1* or *CDH1* using technologies such as CRISPR-Cas9 could certainly ameliorate our model and our understanding of the disease. It could indeed be used to investigate the function of specific genes in the aetiology of IBD. Particularly, we have been interested by the function of SGK2 in intestinal inflammation in the scope of this work. Unfortunately, because of technical challenges and time constraints, we have mostly used Caco-2 cells in our experimentations. While we are aware of their limitations in terms of biological relevance for IBD, we confirmed that SGK2 is involved in the regulation of inflammatory processes in IEC and have suggested novel mechanisms of action for SGK2. We are presently confirming whether SGK2 is the only SGK paralog to have such function by using CRISPR-Cas9 and TRIM-away silencing technologies for SGK1, SGK2 and SGK3 in Caco-2 cells. The results we obtained certainly have to be repeated in cell models that are relevant to IBD such as HIO or iPSC-derived epithelium. Interestingly, a team has recently directed the differentiation of iPSC into IEC in the OrganoPlate platform, which could offer a nice alternative to time-consuming HIO culture (Naumovska et al., 2020). Assessing SGK2's role in an epithelium containing not only enterocytes, but also Paneth cells and goblet cells which are crucial cells for modulating the microflora through the production of anti-microbial peptides and inflammatory cytokines, will provide us a greater insight into the specific contribution of proteins such as SGK2 than Caco-2 cells would.

Finally, an important step forward in the IBD field will be to expand the model to integrate IEC or immune cells derived from diseased patients in the microfluidic model. Our first tests with HIO derived from IBD patients were inconclusive, as HIO derived from diseased patients were hard to keep in a proliferative state and did not attach to the OrganoPlate properly. Despite the high number of publications using HIO, only a few of them have used inflamed HIO and none of them have successfully implemented diseased HIO in a microfluidic technology yet. Conceivably, there are technical challenges that we do not fully grasp yet. However, these HIO cultures could be crucial in finally pinpointing the exact mechanisms of IBD or even the discovery of novel biomarkers for IBD. Presently, the diagnosis of IBD is rather an elimination process where patients are tested for several complications such as celiac disease and irritable bowel syndrome. When all the tests come back negative, a colonoscopy generally reveals the

presence of inflammation and a diagnostic of IBD is given. However, there is no diagnostic test for IBD detection and the discovery of biomarkers could remediate the situation. Furthermore, having access to material from patients could open unprecedented opportunities in terms of personalized medicine. A model such as the one we developed, with its high throughput level and its non-absorbent material, could be used to screen for larger scale compound testing. Reduction of the inflammatory phenotype or restauration of the epithelial barrier's function are certainly easy ways of monitoring the responsiveness to treatment. The model could be used to find the ideal treatment for a given IBD patient; no more trial-and-error cycles causing unnecessary suffering from potential side effects for the patient. Not only could we test drugs that are commercially available, but pharmaceutical companies could use such platforms to test the efficacy of panels of newly developed drugs to only engage the ones giving the most promising results in their clinical pipeline. Ultimately, the goal would be that a greater number of safe medicines reach the patient while simultaneously decreasing the number of animals used to achieve that.

In conclusion, despite the fact that the models presented in this thesis are far from perfection, they represent a good basis on which more sophisticated models of the human intestine can be built on. The organ-on-a-chip technology answers many of the limitations incurred by our current IBD *in vitro* and even *in vivo* models. It is a safe bet to say that organs-on-a-chip will play a major role in stepping up IBD research, whether it is by offering a controllable multi-factorial platform to help us gain a deeper understanding of the disease itself or by supporting target and drug discovery pipelines in the search of better treatments for patients.

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