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# Defence and adaptation mechanisms of the intestinal epithelium upon infection

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ABSTRACT

The intestinal epithelium is a monolayer of polarized columnar cells that act as a border between the host and its environment and are the first line of defence against the luminal microbes. In addition to providing a physical barrier, the epithelium possesses a multitude of active mechanisms to fight invading pathogens and regulate the composition and spatial distribution of commensals. The different epithelial cell types have unique functions in this context, and crosstalk with the immune system further modulates their intricate antimicrobial responses.

The epithelium is organized into clonal crypt units with a high cellular turnover that is driven by stem cells located at the base. There is increasing evidence that this anatomical organization, the stem cell turnover, and the lineage determination processes are essential for barrier maintenance. These processes can be modulated by microbes directly or by the immune responses to enteric pathogens, resulting in a rapid and efficient adaptation of the epithelium to environmental perturbations, injuries, and infections. Here we discuss the complex host-microbial interactions that shape the mucosa and how the epithelium maintains and re-establishes homeostasis after infection.

## 1. Introduction

The gastrointestinal epithelium is a vital interface between the host and the environment. Its main function is to absorb nutrients, which requires close contact with the luminal content of the gut. On the other hand, the epithelium acts as a barrier between the environment and the organism and is equipped to protect the host from invading pathogens and their virulence factors. We discuss here how the epithelium maintains this semi-permeable border, focusing on its anatomical structure and the various cell types that constitute the mucosa. Each cell type is equipped with unique defence mechanisms, which together maintain an efficient antimicrobial barrier. We describe here the diverse properties of different epithelial cell types that regulate commensals and defend against pathogens, highlighting new insights on the critical role of gastrointestinal stem cells. Stem cells are responsible for the rapid turnover of the epithelium, and also act as sensors and effectors during infection and injury. These cells have an exceptional ability to respond to a variety of environmental and internal niche signals. They modulate the epithelial barrier function by facilitating remarkably specific epithelial responses to environmental perturbations, thereby playing a key role in re-establishing and maintaining homeostasis. Commensal and pathogenic microbes have been shown to affect stem cell behaviour, which has an impact on the regenerative capacity of the epithelium, as well as the ability of the host to fight infectious agents.

## 2. Anatomical structure and turnover of the epithelium

The entire gastrointestinal epithelium is organized as invaginations called crypts, with protrusions called villi present only in the small intestine. This architecture serves to increase the surface area, allowing for maximum nutritional absorption by enterocytes located at the surface, while minimizing the exposure of the crypt base to the luminal content. The gastrointestinal epithelium is characterized by extremely high cell turnover, which is driven by long-lived stem cells located at the base of the crypts. They are marked by leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5) - the receptor for the Wnt-agonist R-spondin (Barker et al., 2007). Stem cells give rise to rapidly proliferating transit-amplifying (TA) cells in the lower part of the crypts, which subsequently differentiate into absorptive or secretory cell lineages. Except for the Paneth cells of the small intestine, which remain in the

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crypts, the other cell types differentiate into a mature phenotype as they migrate up from the crypts to replace extruded cells. The epithelium has a rapid turnover period of 3–5 days for the small intestine and 5–7 days for the large intestine (Barker, 2014), which helps expel any damaged or mutated cells (Fig. 1). The mucosa of the stomach is also organized as crypts, which due to their mainly secretory function are called glands, with stem cells located at both the base and the isthmus of the glands. Several key regulatory signals, such as the Wnt, epidermal growth factor (EGF) and Notch pathways, are active at the crypt base and are essential for the maintenance of the stem cell niche. In contrast, members of the transforming growth factor-beta (TGF-β) superfamily of ligands, such as bone morphogenetic proteins (BMPs), negatively regulate stemness and are active towards the upper part of the crypt (Gehart and Clevers, 2019). In healthy epithelium, a precise balance is maintained between cellular proliferation and cell death. As differentiated cells only have a lifespan of a few days, it is damage to the long-lived stem and progenitor cells at the crypt base that can critically impair epithelial integrity.

## 3. Stem cells

Stem cells are crucial for the epithelial turnover in the healthy state and for the regenerative response after epithelial injury. Recent insights suggest that they are not only master regulators of regeneration and maintenance, but also act as sensors and effectors during microbial infections, mediating epithelial defence and modulating immune responses.

Epithelial stem cells were originally considered sterile due to their location all the way at the base of the crypts and their proximity to secretory antimicrobial Paneth cells in the small intestine. It has now become clear that this notion does not hold true for the entire gastrointestinal tract. In the murine cecum and colon, a “crypt-specific core microbiota” has been demonstrated and regulation of stem cell activity by such bacteria has been suggested (Naito et al., 2017; Pedron et al., 2012). Using a murine model of infection with the gastric pathogen *Helicobacter pylori*, we were able to demonstrate that the bacteria are able to colonize deep inside the glands of the stomach and directly attach to stem cells (Sigal et al., 2015). This leads to a sequence of events, resulting in expansion of stem cells and increased proliferative activity. This is partially mediated by upregulation of the stem cell niche factor R-spondin 3, which is secreted by myofibroblasts directly below the gland base, and which controls the number of stem cells and their activity (Sigal et al., 2017). Strikingly, R-spondin driven responses to infection also result in differentiation of a subset of Lgr5+ cells in the gland base into secretory, antimicrobial cells which secrete a multitude of antimicrobial proteins to fight infection. We found that expression of some of these proteins, such as intelectin-1, is dependent on R-spondin 3 and depletion of R-spondin 3 or Lgr5+ stem cells leads to increased colonialization of gastric glands with *H. pylori*. We also observed that intelectin-1 binds to *H. pylori* in a calcium-dependent manner, impairing its motility (Sigal et al., 2019). These results demonstrate the remarkable ability of gastric stem cells and their immediate microenvironment to respond to an infection and to mediate an epithelial response to defend against the bacterial invasion and simultaneously enhance regeneration. Similar to the stomach, the role of R-spondin has also been demonstrated in intestinal infections. *Citrobacter rodentium* infection results in increased stromal expression of R-spondin 2, which causes a Wnt-mediated proliferative response of colonic crypt cells (Papapietro et al., 2013). This study showed that R-spondin can be harmful, leading to an overwhelming proliferative response that results in diarrhoea and increased mortality. Proliferation of stem cells has even been observed as a response to epithelial injury in the villus caused by rotavirus infection and it has been demonstrated that Wnt ligands secreted by the epithelium are essential for this regenerative process (Zou et al., 2018). Accelerated intestinal epithelial cell turnover has likewise been suggested as a mechanism for expulsion of the mucin-degrading bacteria *Akkermansia muciniphila* in the small intestine (Kim et al., 2020) and the parasite *Trichuris trichuria* in the large intestine (Cliffe et al., 2005). The number and proliferative activity of stem cells have also been shown to be altered by infection with a parasitic helminth, in this case leading to a loss of Lgr5+ stem cells, while simultaneously re-activating fetal-like stem cells that support the repair of injured tissue (Nusse et al., 2018). A similar process has also been demonstrated in the context of experimental colitis (Yui et al., 2018). In contrast, certain lactic-acid-producing bacteria have been shown to expand intestinal stem cells, Paneth cells, and goblet cells in response to injury (Lee et al., 2018). In addition to bacteria, their metabolites can also have an impact on stem cell proliferation. During homeostasis, bacterial fermentation of fibre-derived sugars produces short-chain fatty acids (SCFAs), such as butyrate, which reduces stemness and inhibits the expansion of the stem cell compartment (Kaiko et al., 2016). Overall, these data suggest that various microbes and their by-products can modulate the functions of stem cells, leading to expansion, loss, or reprogramming of these cells, which in turn has an impact on the composition and integrity of the epithelium.

Recent reports also suggest that stem cell responses to environmental alterations are regulated by the immune system. In the small intestine, T helper cells or their cytokines have been shown to interact with stem cells upon bacterial or helminth infection, suppressing stem cell renewal and promoting differentiation toward Paneth cells or tuft cells, respectively (Biton et al., 2018). By contrast, the anti-inflammatory cytokine interleukin-10 (IL-10) promotes stem cell self-renewal (Biton et al., 2018). Therefore, the immune system directs epithelial adaptation by modulating stem cell renewal or differentiation, in order to specifically target the respective infectious agent. Stem cells have also been shown to act as primary epithelial sensors and pro-inflammatory mediators of bacterial infections. In the context of *H. pylori* infection, both organoids and a polarized air-liquid interface model of the gastric epithelium have shown that Wnt-responsive stem cells induce high pro-inflammatory responses to infection, while differentiated surface epithelial cells do not (Bartfeld et al., 2014; Boccellato et al., 2018). Accordingly, the severity of gastritis has been shown to correlate with the ability of *H. pylori* to colonize deep in the gland (Sigal et al., 2015). Similarly, a study using intestinal organoids, which was recently published as a preprint, confirmed that also in the intestinal crypt base, stem cells appear to be the main responders to bacteria, through upregulation of the nuclear factor-κB (NF-κB) pathway (Meddens CA et al., preprint SSRN#3,485, 163). Thus, in addition to orchestrating epithelial homeostasis, once they come into contact with bacteria, stem cells appear to trigger an inflammatory reaction.

While stem cells are critical for homeostasis and important for defence, infection and the subsequent acute inflammation can lead to their depletion or reprogramming. Intestinal epithelial cells exhibit a high degree of cellular plasticity and de-differentiation of various different cell types can provide an alternative source of stem cells upon tissue damage. Inflammatory pathways can enhance Wnt-signalling, leading to the de-differentiation of epithelial non-stem cells (Schwitalla et al., 2013). Inflammation caused by administration of dextran sulfate sodium (DSS) has been shown to cause loss of stem cells in the small intestine of mice, which can be replaced by Paneth cells that have undergone de-differentiation (Schmitt et al., 2018). Bmi1-expressing cells in the small intestine that reside above the crypt base have also been shown to act as a reserve stem cell pool in case of injury (Tian et al., 2011; Yan et al., 2012). Additionally, secretory progenitor cells expressing the Notch ligand Dll1 revert to stem cells upon crypt damage in the small intestine (van Es et al., 2012). Alkaline phosphate intestinal (Alpi)-expressing short-lived enterocyte precursors can de-differentiate to replace lost Lgr5+ stem cells and Paneth-like cells in the small intestine (Tetteh et al., 2016). Similarly, Keratin-19+/Lgr5− cells above the crypt base in the small intestine and colon can replace Lgr5+ stem cells (Asfaha et al., 2015). In the colon, two stem cell populations have been identified, Lgr5+/Axin2+ cells at the base of the crypt, and secretory Lgr5−/Axin2+ cells just above the base. Administration of

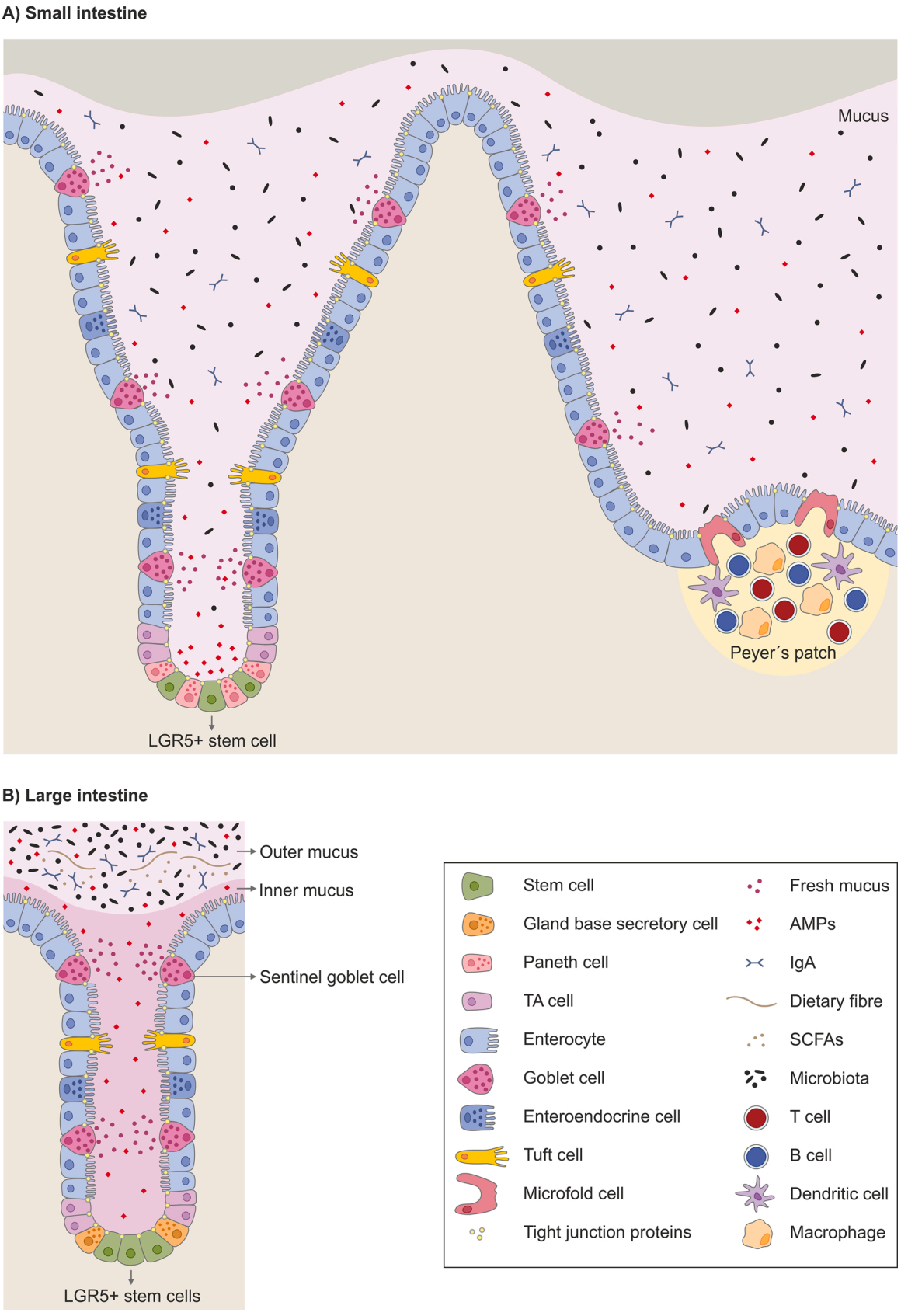


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**Fig. 1.** Anatomy and cell types of the intestinal epithelium.
A) The mucosa of the small intestine is organized into invaginations called crypts and protrusions called villi. LGR5 (leucine-rich repeat-containing G-protein coupled receptor 5) expressing stem cells and Paneth cells are present at the crypt base. Paneth cells secrete Wnt and are the main source of antimicrobial peptides (AMPs), which protect the stem cell niche and the mucosa from luminal microbes. Stem cells give rise to proliferating transit-amplifying (TA) cells. TA cells differentiate into the various functional cell types and replace the cells that are extruded at the tip of the villi. The turnover period of the small intestinal epithelium is 3–5 days. Goblet cells secrete mucus, the enteroendocrine cells secrete hormones, and tuft cells protect the host from parasitic infections. Enterocytes are the most abundant epithelial cell lineage and take up nutrients from digestive residues. Tight junction proteins seal the monolayer and prevent microbial invasion through the paracellular pathway. Microfold cells (M cells) cover underlying Peyer's patches that contain high numbers of dendritic cells, macrophages, and lymphocytes (T cells and B cells). M cells transport antigens and microorganisms to the immune cells beneath. Immunoglobulin A (IgA), which is secreted by subepithelial plasma B cells, is transcytosed by the epithelium into the lumen.
B) The large intestinal mucosa shares several characteristics with that of the small intestine, with some key differences. The epithelium of the large intestine consists of crypts and a flat luminal surface, lacking the villi found in the small intestine. LGR5+ stem cells are located at the base of the crypt and gland base secretory cells and transit-amplifying (TA) cells are present just above them. Gland base secretory cells represent a subpopulation of goblet cells that express antimicrobial markers, such as REG4. Paneth cells are absent. TA cells give rise to the differentiated cell lineages and epithelial turnover occurs every 5–7 days. Goblet cells secrete mucus, which consists of an inner firm mucus layer and an outer loose mucus layer. As the large intestine is more densely populated by microbes than the small intestine, the inner mucus prevents them from penetrating the crypts. Sentinel goblet cells guard the crypt entrance and extrude mucus in response to invading bacteria. A large number of intestinal microbes reside in the outer mucus layer and break down mucins and dietary fibre to produce short-chain fatty acids (SCFAs), an essential source of nutrition for the epithelium. Enterocytes take up nutrients and secrete AMPs.

DSS to mice leads to loss of Lgr5+ and Axin2+ cells, and differentiated Axin2−/Keratin-20+ enterocytes are then recruited to repopulate the injured crypt. This process is mediated by increased levels of stromal R-spondin 3 in response to the epithelial damage (Harnack et al., 2019). The epithelium is also known to repopulate itself through crypt fission if crypts die or are lost due to damage (de Sousa and de Sauvage, 2019). This remarkable plasticity of the intestinal epithelium ensures regeneration and restoration of tissue integrity in response to infection and injury. It will be important to explore to what extent members of the microbiota or pathogens are involved in these complex processes, and how transient loss of stem cells affects the ability of the epithelium to maintain a barrier and fight infections.

## 4. Paneth cells and antimicrobial peptides

In the small intestine, Paneth cells, which are located in close proximity to crypt base stem cells, are a major source of antimicrobial peptides (AMPs), while in the colon, enterocytes secrete a variety of AMPs. These peptides are a crucial component of the epithelial barrier and have antimicrobial activity against a number of potential pathogens. Paneth cells secrete α-defensins, lysozyme, phospholipase A2, and RNases. Colonocytes secrete β-defensins and cathelicidins. C-type lectins are secreted by enterocytes in both the small and large intestine. Galectins, lipocalin, and peptidoglycan recognition proteins are further AMPs that are expressed throughout the intestinal tract (Gallo and Hooper, 2012). AMPs have different modes of action and can act on a broad range of microbes. The expression of some AMPs requires microbiota-derived signals: Ang4, an RNase expressed by Paneth cells, could be induced by infecting mice with the gut commensal *Bacteroides thetaiotaomicron*, showing that commensals shape innate immunity (Hooper et al., 2003). Similarly, RegIIIγ, a C-type lectin, is secreted by Paneth cells upon microbial colonization of germ-free mice (Cash et al., 2006), and is essential for maintaining an area directly above the epithelium ~50 μm deep, which is not colonized by the microbiota (Vaishnava et al., 2011). Furthermore, intestinal goblet cells have been shown to produce resistin-like molecule β (RELMβ) (Artis et al., 2004), which is induced by the microbiota and kills Gram-negative bacteria, and is essential for maintaining the spatial segregation of the intestinal microbiota (Propheter et al., 2017). Mice lacking an enzyme required for the processing of mouse α-defensins have microbial dysbiosis and enhanced inflammation (Salzman et al., 2010). Defensins were shown to undergo post-translational activation, such as reduction of disulphide bonds, which makes them more potent in the reducing environment of the intestinal lumen (Schroeder et al., 2011). Monocytes can induce the expression of defensins in Paneth cells by secreting Wnt ligands. However, expression of Wnt ligands in monocytes is compromised in patients with Crohn's disease (CD), resulting in a reduced ability to induce defensins (Courth et al., 2015). CD patients also show reduced expression of the Wnt signalling transcription factor Tcf-4, resulting in a decrease of Paneth cell α-defensins, further weakening the mucosal defence (Wehkamp et al., 2007). While Paneth cells are only present in the small intestine, unique populations of gland base secretory cells with antimicrobial properties can be found throughout the gastrointestinal tract, such as gastric gland base mucous cells (Sigal et al., 2019) and gland base secretory cells in the colon (Rothenberg et al., 2012). These cells rely on functional Wnt signalling, suggesting a critical role of this signalling pathway for mucosal antimicrobial defence. The release of defensins from Paneth cells has also been found to be stimulated in response to the pro-inflammatory cytokine interferon gamma (IFN-γ) (Farin et al., 2014). CpG-oligodeoxynucleotide (ODN), an agonist of TLR9, given orally to mice induced Paneth cell degranulation, but not in *Tlr9*$^{-/-}$ mice (Rumio et al., 2012). Other factors, such as acetylcholine-expressing T cells (Dhawan et al., 2016) and interleukins (IL-4 and IL-13) (Stockinger et al., 2014) have also been shown to induce Paneth cell degranulation and AMP secretion. Taken together, the bactericidal activity of AMPs regulates commensals and provides the epithelium with vital protection against pathogens.

## 5. Goblet cells

Goblet cells secrete mucus, a viscous fluid enriched in mucin glycoproteins, which form large net-like polymers, lubricate the epithelial surface, and generate a protective physical barrier against luminal microbes. The continuous production of mucus by goblet cells also creates an upward flow in the crypts, preventing bacterial infiltration (Pelaseyed et al., 2014). The mucus layer in the intestine is composed of gel-forming Mucin-2 (MUC2). In the colon, the mucus is composed of two layers, an inner firm mucus layer and an outer loose mucus layer. In the small intestine, this separation is less stringent. In humans, the inner layer is a dense, stratified layer that does not allow microorganisms to penetrate easily, thus keeping the epithelium free from intestinal microbes (Johansson et al., 2008). The outer mucus layer is less dense and is inhabited by a large number of intestinal microbes that utilize polysaccharides of MUC2 as an energy source (Okumura and Takeda, 2017). Commensal microbes have evolved mechanisms to utilize glycans (polysaccharides) from the diet and the mucus layer, resulting in the production of short-chain fatty acid (SCFA) metabolites, which in turn are essential nutrients for the intestinal epithelium (Koropatkin et al., 2012). *Muc2*-deficient mice exhibit increased colonic inflammation and spontaneously develop colitis (Van der Sluis et al., 2006). As the microbial density increases from the proximal small intestine to the distal colon, so does the percentage of goblet cells, from 4% in the duodenum to 16 % in the distal colon (Kim and Ho, 2010). Studies have shown that bacterial colonization of the intestinal tract plays an essential role in

mucus production. Germ-free mice have fewer goblet cells, lower expression levels of Muc2, and a very thin mucus layer (Allaire et al., 2018). These mice have a lower abundance of glycosyltransferase, an enzyme involved in Muc2 glycosylation, resulting in shorter Muc2 glycans and therefore thinner mucus layer (Arike et al., 2017). Once exposed to a conventional environment, the colonization by commensal bacteria results in increased numbers of goblet cells, higher expression of the goblet-specific protein RELMβ and thickening of the mucus layer (He et al., 2003). Goblet cell mucus secretion is regulated by the NOD-like receptor family pyrin domain–containing 6 (Nlrp6) inflammasome, and mice deficient for *Nlrp6* are unable to clear enteric pathogens and are highly susceptible to persistent colonic infection (Wlodarska et al., 2014). Sentinel goblet cells guard the colonic crypt entrance by activating the Nlrp6 inflammasome in response to bacterial intruders, triggering mucin exocytosis and inducing mucus secretion from adjacent goblet cells in the upper crypt, leading to expulsion of the bacteria (Birchenough et al., 2016).

Apart from mucus production for barrier maintenance, goblet cells also produce several important proteins such as intestinal trefoil factor 3 (TFF3) and resistin-like molecule-β (RELMβ). TFF3 stabilizes and aids the formation of the mucus gel layer by cross-linking mucin glycoproteins (Jarva et al., 2020; Kindon et al., 1995). Trefoil peptides play an important role in mucosal healing and intestinal trefoil factor deficient mice exhibit increased sensitivity to intestinal damage and reduced epithelial healing after oral DSS challenge (Mashimo et al., 1996). RELMβ is a cysteine-rich cytokine and studies in RELMβ-deficient mice have demonstrated that this protein is involved in the maintenance of colonic barrier integrity and regulates susceptibility to inflammation (Hogan et al., 2006). RELMβ protects against enteric pathogens by recruiting CD4+ T cells, which produce interleukin-22 (IL-22), a cytokine that directly increases epithelial cell proliferation (Bergstrom et al., 2015). In response to infection in mice, IL-22 also induces C-type lectin antimicrobial proteins, RegIIIβ and RegIIIγ in colonic epithelial cells (Zheng et al., 2008). Further, goblet cells form goblet cell-associated antigen passages (GAPs) that deliver luminal substances to underlying antigen-presenting cells (APCs) in the lamina propria, inducing adaptive immune responses (Knoop et al., 2015; McDole et al., 2012). Therefore, goblet cell functions play a fundamental role in innate and adaptive immunity.

## 6. Enterocyte-microbiota homeostasis

During homeostasis, the colon microbiota is dominated by obligate anaerobic bacteria that convert fibre into fermentation products, such as butyrate, propionate, and acetate. These SCFAs are a critical energy source for the colon epithelium. Oxidation of SCFAs by enterocytes maintains epithelial hypoxia, which in turn ensures that the microbial community is dominated by the beneficial obligate anaerobes (Litvak et al., 2018). During homeostatic conditions, these metabolic processes support microbial diversity in the colon, which appears to be important for various physiological processes. Inflammation can disrupt this homeostasis, due to oxygenation of the colon by generation of reactive nitrogen species (RNS) and reactive oxygen species (ROS). Host-mediated inflammation causes overgrowth of resident or introduced facultative anaerobic bacteria in mice, particularly the Enterobacteriaceae family (Lupp et al., 2007). The nitrate generated as a by-product of the inflammatory response is host-derived, as mice deficient in inducible nitric oxide synthase do not support the growth of commensal *Escherichia coli* by nitrate respiration in the colon (Winter et al., 2013). Nutritional imbalance or enhanced inflammatory signals can shift the metabolic activity of the colonic epithelium from lipid oxidation toward anaerobic glycolysis, which increases the abundance of facultative anaerobic bacteria, a hallmark of dysbiosis (Litvak et al., 2018). One such facultative anaerobe, *Citrobacter rodentium,* causes crypt hyperplasia and expansion of undifferentiated Ki67+ cells in the colon. This leads to increased oxygenation of the mucosal surface, which reduces the density of obligate anaerobic commensal bacteria, and therefore indirectly further supports the expansion of this pathogen (Lopez et al., 2016). ROS generated during inflammation reacts with the luminal sulphur compound thiosulphate to form tetrathionate. *Salmonella* Typhimurium has the ability to use tetrathionate as an electron acceptor, giving it a growth advantage over the fermentation-dependent commensals (Winter et al., 2010). Similarly, depletion of butyrate-producing *Clostridia* through oral antibiotic treatment alters the epithelial metabolism to lactate production, which is utilized by *S.* Typhimurium as a nutrient, allowing the pathogen to outcompete other microbes (Gillis et al., 2018; Rivera-Chavez et al., 2016). The host receptor peroxisome proliferator-activated receptor-γ (PPAR-γ) senses butyrate, and a reduction in butyrate-producing bacteria by antibiotic treatment leads to decreased PPAR-γ signalling and increased expression of nitric oxide synthase. Conversely, microbiota-induced PPAR-γ signalling limits luminal oxygen bioavailability and prevents dysbiosis and expansion of *Escherichia* and *Salmonella* strains (Byndloss et al., 2017). SCFAs also play a role in adaptive immunity, as they regulate the proper development and function of regulatory T cells in the colon (Arpaia et al., 2013; Furusawa et al., 2013; Smith et al., 2013). Enterocyte metabolic processes that maintain epithelial hypoxia are therefore essential to restrict the growth of facultative anaerobes and pathogens.

## 7. Enteroendocrine cells

Intestinal enteroendocrine cells taken together constitute the largest endocrine system in the human body (Beumer et al., 2018). In addition to secretion of multiple regulatory peptide hormones that serve paracrine and endocrine functions that mediate digestion, these cells can also sense gut microbiota and their metabolites (Yu et al., 2020). Enteroendocrine cells are also known to secrete cytokines in response to microbial metabolites, thus directly influencing the intestinal mucosal immune system (Worthington et al., 2018). Spore-forming bacteria from mouse and human colon microbiota produce SCFAs that promote host serotonin biosynthesis by enteroendocrine cells (Reigstad et al., 2015), which has an impact on gastrointestinal motility and platelet function (Yano et al., 2015). Reduced expression of serotonin decreases secretion of antimicrobial peptides and increases susceptibility to colitis and sepsis after infection with *S.* Typhimurium (Essien et al., 2013). Enteroendocrine cells have cytokine receptors, and cytokines from activated CD4+ T cells interact with enteroendocrine cells to enhance the production of serotonin in the colon after infection of mice with the nematode *Trichuris muris* (Wang et al., 2007). Glucagon-like peptide-2 (GLP-2) is a hormone secreted by enteroendocrine cells, and in GLP-2 receptor deficient ($Glp2r^{-/-}$) mice Paneth cell antimicrobial activity was found to be reduced and bacterial colonization of the small intestine significantly increased (Lee et al., 2012). GLP-2 was also shown to enhance the small intestinal epithelial barrier function in mice (Benjamin et al., 2000). Enteroendocrine cells can sense lipopolysaccharides (LPS), and show enhanced secretion of glucagon-like peptide-1 (GLP-1), a hormone that attenuates inflammation (Lebrun et al., 2017). Ablated Paneth cells in the small intestine are replaced by enteroendocrine and tuft cells, and these cells serve as an alternative source of Notch signals, which are essential for Lgr5+ stem cell maintenance (van Es et al., 2019). The functional diversity of enteroendocrine cells and their role in epithelial homeostasis and immunity is currently under active investigation, driven by the broad availability of single-cell characterization approaches.

## 8. Mechanisms to maintain a physical epithelial barrier

As summarized above, a multitude of cellular responses prevent direct interaction between the epithelium and microbes. However, bacteria do have the ability to bypass these defence mechanisms to reach the epithelium. Therefore, the cells are additionally equipped to prevent penetration of bacteria through the epithelium, by forming a tightly

closed monolayer with intercellular junctions. Tight junction proteins link epithelial cells and physically hamper microbial invasion through the paracellular pathway. Tight junctional complexes are located at the apical pole of the epithelial cells and include occludins, zonula occludens, claudins, and junctional adhesion molecules to create a seal and regulate epithelial permeability (Ashida et al., 2011). Tight junctions are highly dynamic structures and several physiological and pathophysiological conditions regulate their permeability. Pro-inflammatory cytokines, such as interferon-gamma (IFN-γ), tumour necrosis factor-alpha (TNF-α), and interleukins, can disrupt tight junctions and impair gut barrier integrity (Heller et al., 2008; Rawat et al., 2020; Wang et al., 2006). In addition, some pathogens have developed mechanisms to breach the tight-junction integrity. For example, *Clostridium perfringens* produces an enterotoxin that can disintegrate claudins by binding to them, thereby increasing paracellular permeability (Saitoh et al., 2015). *Vibrio cholerae* produces zonula occludens toxin (ZOT), which interacts with occludins and zonula occludens 1 (ZO1) protein and increases mucosal permeability (Fasano et al., 1991). Enterotoxigenic *Bacteroides fragilis* produces *B. fragilis* toxin (BFT), which alters tight-junction integrity of intestinal epithelial cells by cleaving E-cadherin, the extracellular domain of the zonula adherens protein (Wu et al., 1998). *Salmonella* secretes specific effector proteins and pathogenic *E. coli* strains can synthesize a protein kinase, both of which disrupt host intestinal epithelial tight junctions (Boyle et al., 2006; Tomson et al., 2004). Junctional adhesion molecule-A (JAM-A) deficient mice have a compromised epithelial barrier and increased susceptibility to DSS induced colitis (Laukoetter et al., 2007). However, these mice do not develop spontaneous colitis, due to adaptive immune-mediated protection. Specifically, TGF-β-producing CD4+ T cells promote IgA secretion, protecting against colitis (Khounlotham et al., 2012). These data demonstrate the significant role of tight junctions for maintaining an intact epithelium, and pathogens that can circumvent this barrier can gain entry into the subepithelial host tissue and potentially cause systemic infection.

## 9. Epithelium as an active interface between the environment and the immune system

### 9.1. Epithelium as immune sensor of microbes

In addition to being an efficient barrier effector, the epithelium performs the critical function of sensing pathogens and mediating immune responses. It is equipped with a variety of sensors to induce an immune response and act as an active interface that integrates environmental signals to either drive or suppress an immune reaction. Detection of bacteria is mediated *via* pattern recognition receptors (PRR). Epithelial PRRs sense and recognize both commensal and pathogenic microorganisms by their microbial-associated molecular patterns (MAMPs). There are two main categories of PRRs that induce transcriptional responses, Toll-like receptors (TLRs) and NOD-like receptors (NLRs).

TLRs activate four adaptor proteins: Myeloid differentiation primary response 88 (MYD88), Toll-receptor-associated activator of interferon (TRIF), MyD88-adaptor-like/TIR-associated protein (MAL/TIRAP), and Toll-receptor-associated molecule (TRAM), which leads to the activation of the transcription factor NF-κB (Beutler, 2004). TLR4 recognizes LPS, which is mainly present on the outer membrane of Gram-negative bacteria. TLR4 signalling through MyD88 is important in intestinal responses to injury and limiting bacterial translocation. *Tlr4*-deficient mice have defective innate immune responses and are more susceptible to DSS-induced colitis (Fukata et al., 2005). Paneth cells directly sense both commensal and pathogenic bacteria through cell-autonomous MyD88-dependent TLR activation, triggering expression of multiple antimicrobial factors (Vaishnava et al., 2008). Deletion of *Myd88* in intestinal epithelial cells causes a compromised immunological response, with more mucus-associated bacteria and increased bacterial translocation. These mice also show downregulated expression of Muc2, IgA transporter, and antibacterial peptides (Frantz et al., 2012). TLR5 detects bacterial flagellin and *Tlr5*-deficient mice harbour more proteobacteria and develop colitis (Carvalho et al., 2012). Due to changes in the composition of their gut microbiota, *Tlr5*-deficient mice also display classic features of metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity (Vijay-Kumar et al., 2010). TLR3 is a virus-specific PRR that has an asymmetric distribution towards the basolateral side. Using primary human intestinal cells, it was shown that this is a mechanism that promotes tolerance of apical commensals present in the lumen, while remaining fully responsive to invading pathogens (Stanifer et al., 2020).

NOD1 detects the DAP-type tri-peptide motif of peptidoglycan present on bacteria. Activation of small Rho GTPases in host cells by effector proteins of *Salmonella* triggers NOD1 and activation of the NF-κB pathway (Keestra et al., 2013). NOD2 recognizes muramyl dipeptide (MDP), another peptidoglycan motif present on bacteria. NOD2 both regulates commensal bacteria and depends on the presence of commensal bacteria for its expression. *Nod2*-deficient mice have an increased load of commensal bacteria and a diminished ability to prevent colonization with pathogenic bacteria (Petnicki-Ocwieja et al., 2009). Paneth cells of *Nod2*-deficient mice have lower expression of anti-microbial peptides and show increased susceptibility to the gastrointestinal pathogen *Listeria monocytogenes* (Kobayashi et al., 2005). Moreover, *Nod2* is known to be one of the susceptibility genes for inflammatory bowel disease (IBD), indicating a crucial role of PRRs in maintaining epithelial homeostasis, where their dysfunction can lead to a disbalance between microbes and the mucosa, resulting in chronic inflammation. Overall, these results indicate that PRRs are important for stimulating antimicrobial defences, shaping the microbiota, and protecting the epithelium from pathogenic invasion.

### 9.2. Tuft cells

Tuft cells play a vital role in the defence against helminths, as they promote type 2 immunity in response to intestinal parasitic infections (Howitt et al., 2016). Tuft cells secrete interleukin-25 (IL-25), thereby activating type 2 innate lymphoid cells (ILC2s). In the small intestine, interleukin 4 (IL-4) and interleukin 13 (IL-13) signals produced by ILC2s in response to parasitic infections cause stem cell differentiation towards goblet and tuft cells (Gerbe et al., 2016; von Moltke et al., 2016). Tuft cell hyperplasia is seen in the small intestine of mice infected with the tapeworm species *Hymenolepis diminuta* (Rajeev et al., 2020). Secreted IL-25 provides immunity against reinfection and contributes to infection resistance in the host (Schneider et al., 2018). Tuft cells also monitor microbial metabolites, such as succinate, to initiate type 2 immunity. Succinate receptor 1 (Sucnr1) is specifically expressed in small intestinal tuft cells and senses succinate secreted by the microbiota and helminths (Lei et al., 2018; Nadjsombati et al., 2018). In addition, doublecortin-like kinase 1 (Dclk1) expressed by tuft cells has been shown to play a role in epithelial restorative responses in mice after DSS treatment (Qu et al., 2015) and following radiation-induced injury (May et al., 2014). Thus, apart from protecting the host from helminth infections, tuft cells also contribute to recovery after epithelial damage.

### 9.3. Microfold cells

Microfold cells (M cells) are specialized absorptive epithelial cells present in the small intestinal mucosa, which play an important role in mucosal immune surveillance. They cover the underlying Peyer's patches - mucosa-associated lymphoid follicles that contain high numbers of lymphocytes, macrophages, and dendritic cells. M cells sample the intestinal lumen and transport antigens, immunoglobulin A (IgA)-containing immune complexes or whole microorganisms to the immune cells beneath, thereby initiating an immune response or tolerance. Glycoprotein 2 (GP2) expressed on the apical plasma membrane of

M cells binds to FimH, a type 1 pili component of commensal and pathogenic enterobacteria, to trigger an antigen-specific immune response (Hase et al., 2009). Uromodulin, a GP2 homolog present on M cells, can also bind to FimH-positive bacteria (Sato et al., 2013). M cell differentiation is dependent on receptor activator of nuclear factor-κB ligand (RANKL). They express osteoprotegerin (OPG), a soluble inhibitor of RANKL, which suppresses the differentiation of adjacent follicle-associated epithelial cells into M cells, thereby regulating M-cell density (Kimura et al., 2020). Treatment of intestinal organoids with RANKL induces expression of M cell-specific markers and increases M cell numbers (de Lau et al., 2012). Targeted delivery of vaccine antigens to M cells could be a potential means of inducing antigen-specific mucosal immune responses (Mabbott et al., 2013).

### 9.4. Immunoglobulin A transcytosis

In addition to maintaining the barrier in an autonomous manner, the epithelium actively communicates with immune cells. One example of this is their active role in immunoglobulin A (IgA) delivery to the lumen. IgA constitutes the main antibody isotype secreted in the gut and plays an essential role in maintaining homeostasis between host tissues and intestinal microbial communities (Macpherson et al., 2000). IgA antibodies are secreted from subepithelial plasma cells and transcytosed across the epithelium into the lumen. Luminal IgA provides an antigenic barrier by binding to bacteria and preventing colonization by pathogens and overgrowth of pathobionts. Clumping of bacteria by IgA-mediated cross-linking accelerates pathogen clearance from the gut lumen (Moor et al., 2017). The active transport of IgA is mediated by the epithelial polymeric Ig receptor (pIgR). *pIgR* knockout mice that are unable to trancytose IgA into the intestinal lumen have been shown to have increased mucosal leakiness and increased presence of bacteria in the mesenteric lymph nodes (Johansen et al., 1999). In primary colonic epithelial cell monolayer cultures, pIgR expression and IgA transcytosis can be stimulated by LPS, TNF-α, IL-1β, IL-17, and heat-killed *E. coli* (Moon et al., 2014). Dendritic cells can retain live commensal bacteria for several days and interact with naive B cells in the mesenteric lymph nodes to induce production of IgA, thus preventing mucosal penetration by commensals. These commensal-carrying dendritic cells are restricted to the mucosal immune compartment, therefore ensuring a local immune response (Macpherson and Uhr, 2004). In germ-free mice production of intestinal plasma cells and secretion of IgA is induced in response to exposure to commensals (Hapfelmeier et al., 2010). B-cell development has been shown to occur in the intestinal mucosa, where it is regulated by extracellular signals from commensal microbes, which were again absent in germ-free mice (Wesemann et al., 2013). Mice that have impaired capacity of B cells to undergo somatic hypermutation, thereby limiting the diversity of their secretory IgA repertoire, were shown to have reduced mucosal defence against toxins and pathogens (Suzuki et al., 2004; Wei et al., 2011). In summary, homeostatic barrier function requires a functional immune system and close co-operation with the epithelium that mediates the immune response through processes such as IgA transcytosis.

## 10. Organoids as an infection model

Organoids are three-dimensional primary epithelial cell cultures that closely mimic the structure and physiology of the source tissue (Sato et al., 2009). While initially used to study epithelial stem cell biology and development, they have recently proven to be a valuable tool for investigating host-microbial interactions in the gastrointestinal tract, as they enable infection of untransformed, healthy epithelial cells *in vitro*. Several studies have used gastric organoids to study epithelial responses to the gastric pathogen *H. pylori*. Infection of human gastric organoids with *H. pylori* was shown to activate the NF-κB inflammatory pathway (Bartfeld et al., 2014). Epithelial responses to *H. pylori* were also studied by infecting primary cells that were first grown as organoids and then transferred to 2-dimensional as well as air-liquid interface cultures (Boccellato et al., 2018; Morey et al., 2018; Schlaermann et al., 2016). In addition, chemoattraction of *H. pylori* to urea from gastric epithelial cells was demonstrated using organoid cultures (Huang et al., 2015). The ability to infect untransformed primary epithelial cells is particularly useful for studying *H. pylori* driven carcinogenesis. For example, a host tumour suppressor, apoptosis-stimulating protein of p53 2 (ASPP2), was found to play a vital role in the disruption of cell polarity by CagA during *H. pylori* infection of gastric organoids (Buti et al., 2020).

Intestinal organoids have also been used for infection studies: *S.* Typhimurium was shown to invade epithelial cells of intestinal organoids, disrupt tight junctions, reduce expression of stem cell markers, and trigger inflammation (Forbester et al., 2015; Zhang et al., 2014). In an alternative approach, the epithelial polarity of organoids was reversed by manipulating extracellular matrix (ECM) proteins. Infection of these apical-side-out enteroids with *S.* Typhimurium showed that this pathogen invades the apical surfaces by inducing cytoskeletal rearrangements, whereas *Listeria monocytogenes*, which binds to basolateral receptors, invades apical surfaces at the sites of extruding cells (Co et al., 2019). Infection of intestinal organoids with *Clostridioides difficile* or the purified toxin from the bacteria was shown to disrupt epithelial barrier function (Leslie et al., 2015). In addition, reduction in colonic organoid formation was observed from crypts isolated from *C. difficile* infected mice (Mileto et al., 2020). Intestinal organoids have also been used to study the interplay between microbial and immune factors. For example, interferon-gamma (IFN-γ) was shown to trigger degranulation and luminal extrusion of Paneth cells in intestinal organoids following addition of microbial antigens to the culture medium or infection with *E. coli* (Farin et al., 2014).

Additionally, intestinal organoids have been used to study the role of genotoxic bacteria in inducing mutations and transforming epithelial cells: *S.* Typhimurium has been shown to activate the MAPK and AKT pathways and induce cellular transformation in gallbladder organoids with pre-existing mutations affecting p53 and c-Myc (Scanu et al., 2015). *S.* Typhimurium can also induce DNA double-strand breaks in gallbladder organoids, and primary cells intoxicated with the typhoid toxin were shown to proliferate despite the presence of DNA damage (Sepe et al., 2020). Repeated luminal injection of genotoxic colibactin-producing *pks+ E. coli* in human intestinal organoids was shown to give rise to a specific mutational signature that was also detected in a subset of human colorectal cancer (Pleguezuelos-Manzano et al., 2020). Similarly, infection of Caco-2 cells with *pks+ E. coli* showed an enrichment of DNA double-strand breaks at AT-rich hexameric sequence motif, and this motif was also detected to be enriched in colorectal cancer (Dziubanska-Kusibab et al., 2020). We utilized colon organoids to study the transformation potential of *pks+ E. coli* upon short-term infection. After infection, organoids were observed to increase their proliferative activity and grow independent of exogenous Wnt ligands (Iftekhar et al., 2021), a characteristic of colorectal cancer organoids (Drost et al., 2015; Matano et al., 2015). DNA sequencing revealed that these Wnt-independent colon organoids had major chromosomal aberrations, and chromosomal instability (CIN) (Iftekhar et al., 2021), resembling early events in colorectal cancer (Stoler et al., 1999). The Wnt-independent organoids harboured several mutations, including *miR-34a*, which acts downstream of p53 and downregulates the Wnt signalling (Kim et al., 2011). We demonstrated a functional interplay between the p53 and Wnt pathways, as knockout of *Trp53* or *miR-34* in organoids resulted in Wnt-independent growth (Iftekhar et al., 2021).

Organoids have also been utilized to study infection by parasitic worms. In response to infection by intestinal nematodes, tuft cells produce IL-25, a cytokine that stimulates secretion of IL-13 by innate lymphoid cells, and IL-13 treatment of intestinal organoids markedly increases tuft-cell abundance (Luo et al., 2019). Intestinal organoids generated from mice infected with *H. polygyrus* formed spheroids that were devoid of crypt budding and exhibited characteristics of proliferative fetal intestinal epithelium (Nusse et al., 2018). Extracellular

vesicles secreted by *Trichuris muris* and *Nippostrongylus brasiliensis* were demonstrated to be internalized by murine intestinal organoid cells (Eichenberger et al., 2018a, b). The protozoan parasite *Cryptosporidium*, which could so far not be cultured *in vitro*, was shown to grow and complete its life cycle in differentiated human intestinal organoids (Heo et al., 2018). Similarly, intestinal organoids and monolayers derived from them also support the replication of enteric viruses that have been difficult to culture in cell lines, such as rotavirus and norovirus (Ettayebi et al., 2016; Finkbeiner et al., 2012). More recently, it was observed that some COVID-19 patients experience gastrointestinal symptoms, and human intestinal organoids were successfully used to show that SARS-CoV-2 receptor angiotensin-converting enzyme 2 (ACE2) is highly expressed on differentiated enterocytes. In these cells, SARS-CoV-2 can actively replicate and give rise to large numbers of new infective virus particles (Lamers et al., 2020). Similar results were also shown for intestinal organoids derived from horseshoe bats (Zhou et al., 2020). We demonstrated using human colon organoids that IFN-γ promotes differentiation of enterocytes, increasing SARS-CoV-2 susceptibility (Heuberger et al., 2021). Organoids are therefore a promising new *in vitro* tool for disease modelling and for conducting mechanistic studies to better understand host-pathogen interactions and the response of primary epithelial cells to infection.

## 11. Outlook

As elucidated in this review, the gastrointestinal mucosa is much more than a physical border between the host and the environment. Its dynamic turnover kinetics, controlled by stem cells, enable a rapid and targeted adaptation of the epithelium to environmental perturbations. The epithelium represents an active hub that integrates and transmits a variety of signals, both from the microbiota as well as from the endogenous niche, in order to mediate immune responses, which in turn affect mucosal defence, epithelial turnover, and cell composition. It is composed of a variety of cell types, each of which contributes to the barrier function and is equipped with a specific set of sensors and effectors to fight infections and regulate the microbiota. This concept could be significantly expanded by integrating the role of the healthy microbiota in shaping mucosal homeostasis and providing critical signals that control epithelial behaviour and its adaptation capacity. In contrast, altered microbiota composition, so-called dysbiosis, can be both cause and result of altered epithelial homeostasis, initiating pro-inflammatory trajectories and enhancing the risk of chronic gut disorders such as inflammatory bowel diseases or gastrointestinal cancers. While it is well-established that genetic predispositions, such as lack of defensins or other antimicrobial compounds, are linked to inflammatory and malignant disorders, it will be important to investigate whether and to what extent an altered epithelial adaptation capacity contributes to pathogenesis of gastrointestinal disorders. In this context, new technologies such as single-cell sequencing and spatial transcriptomics will be useful to resolve tissue responses at single-cell resolution. This may provide new clinical intervention strategies for re-setting the homeostasis between the microbiota and the mucosa, which could potentially be achieved by targeting both the microbial composition, as well as intrinsic signals that control epithelial differentiation.

Advances in genome sequencing have also enabled the identification of specific colorectal cancer (CRC) signatures, and one specific signature found in CRC has been associated with colibactin-producing *E. coli*, which are frequent constituents of the human intestinal microbiota. Therefore, in addition to altering mucosal differentiation and proliferation, both pathogens and microbiota-derived toxins can directly damage epithelial cells and their genomic integrity, which may have a profound, long-term impact on epithelial behaviour. Further such studies will help to provide a clearer picture of disease-specific microbiota composition and to identify other pro-carcinogenic gut bacteria.

The emergence of new animal models for lineage tracing has additionally revealed aspects of cell fate and plasticity of the epithelium, which will be important to further understand the dynamic cellular responses to infections. Cells from animals with specific genotypes or from human biopsies can be cultivated as organoids and primary epithelial monolayer cultures for *in vitro* investigation of stem cell biology, cancer mutations, infection biology, and intrinsic defence mechanisms of intestinal epithelial cells. Furthermore, targeted gene editing of organoids using CRISPR/Cas9 technology has helped to uncover the specific role of genes and cellular pathways. Likewise, *in vitro* models such as organs-on-chips, microfluidic primary human cell culture systems that recapitulate the physiological and functional characteristics of cells, are a promising tool for disease modelling, drug discovery, and personalized medicine. Other cell types, such as stromal cells and immune cells, can be co-cultured with primary epithelial cells in these culture systems to analyse the interactions between different cellular compartments. Using picoliter droplets, droplet microfluidics has provided a platform for isolating microbiota members from stool samples in an anaerobic chamber, making it possible to study their metabolic functions and susceptibility to antibiotics. Metabolites generated by bacterial species in the microbiota can also be analysed by high-throughput metabolomics. Overall, aided by these enhanced molecular biology techniques, future findings will further unravel the intricate relationship between the epithelium, the immune system, and the gut microbiota. These insights will contribute to a better understanding of complex epithelial biological processes and to the development of improved therapeutic solutions for inflammatory bowel disease, gastrointestinal malignancies, and enteric infections.

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## Declaration of Competing Interest

The authors declare no conflict of interest.

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