Epithelial Toll-like receptors and their role in gut homeostasis and disease

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Abstract | The human gastrointestinal tract is colonized by trillions of microorganisms that interact with the host to maintain structural and functional homeostasis. Acting as the interface between the site of the highest microbial burden in the human body and the richest immune compartment, a single layer of intestinal epithelial cells specializes in nutrient absorption, stratifies microorganisms to limit colonization of tissues and shapes the responses of the subepithelial immune cells. In this Review, we focus on the expression, regulation and functions of Toll-like receptors (TLRs) in the different intestinal epithelial lineages to analyse how epithelial recognition of bacteria participates in establishing homeostasis in the gut. In particular, we elaborate on the involvement of epithelial TLR signalling in controlling crypt dynamics, enhancing epithelial barrier integrity and promoting immune tolerance towards the gut microbiota. Furthermore, we comment on the regulatory mechanisms that fine-tune TLR-driven immune responses towards pathogens and revisit the role of TLRs in epithelial repair after injury. Finally, we discuss how dysregulation of epithelial TLRs can lead to the generation of dysbiosis, thereby increasing susceptibility to colitis and tumorigenesis.

The human gastrointestinal tract harbours approximately 4×10^{13} microorganisms, including bacteria, fungi, archaea and virus-like particles, that are separated from the largest immune compartment of the body by a 10-μm monolayer of intestinal epithelial cells (IECs)[1,2]. To avoid detrimental immune responses and to establish mutualistic relationships, the host and the gut microbial communities have mutually co-evolved and adapted via bidirectional communication that relies on microbial recognition and on the production of both host and microorganism-derived metabolites and peptides[3]. Efficient crosstalk between the host and the gut microbiota leads to a hyporesponsive state towards the resident microbiota that sustains homeostasis. However, dysregulation of this dialogue can lead to microbial imbalance, termed dysbiosis, or to a loss of immune tolerance, predisposing the host to inflammation and tumorigenesis.

Maintaining tolerance towards the commensal gut microbiota depends on constitutive and inducible defence mechanisms that cooperate to minimize exposure of the immune cells in the lamina propria to luminal antigens[4]. Constitutive mechanisms, such as the intestinal epithelial barrier, physically impede microorganisms from penetrating the host. Inducible mechanisms, which involve diverse immune and non-immune cell types, participate in strengthening the intestinal epithelial barrier function or promoting an immunomodulatory environment in the lamina propria. Activation of these inducible mechanisms requires microbial sensing and recognition by host cells, which is carried out by pattern recognition receptors (PRRs)[4].

PRRs are germ line-encoded, evolutionarily conserved receptors that recognize microbial-associated molecular patterns (MAMPs), which are molecular structures essential for microbial survival, and trigger diverse innate immune responses depending on the PRR-expressing cell type[4]. Furthermore, some PRRs can also recognize damage-associated molecular patterns, which are released during cellular stress or tissue injury, enabling cells to mount efficient repair responses to sterile inflammation[5,6]. Several families of PRRs have been described, including the Toll-like receptors (TLRs)[7,8], nucleotide-binding oligomerization domain (NOD)-like receptors[9], C-type lectin receptors[10], retinoic acid-inducible gene I (RIG-I)-like receptors[11], absence in melanoma 2 (AIM2)-like receptors[12], cyclic GMP–AMP synthase (cGAS)[13] and scavenger receptors such as receptor for advanced glycation end-products (RAGE)[14]. Despite recognizing different ligands, these receptors share signalling pathways that terminate in the activation of pro-inflammatory transcription factors, such as nuclear factor-κB (NF-κB) and interferon regulatory factor 3 (IRF3). Tight regulation of PRR-driven responses leads to elimination of the noxious stimuli, repair of the damaged structures and restoration of homeostasis. However, an excessive or defective function of PRRs in the gut can lead to dysregulated immune responses that increase susceptibility to infection, inflammatory diseases, such as Crohn's disease

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Key points

- The intestinal epithelium provides a physical and immune barrier between the host and the gut microbiota that is dynamically regulated through the production of metabolites and the signalling of pattern recognition receptors.
- Toll-like receptors (TLRs) are microbial-induced proteins that are expressed in most epithelial cell lineages and have important antimicrobial functions. Tight regulation mechanisms prevent excessive responses towards commensal microorganisms.
- Activation of TLRs controls crypt dynamics by altering proliferation and apoptosis in stem cells and transit amplifying cells. Differentiation into secretory lineages, particularly via the NOTCH pathway, occurs in a myeloid differentiation primary response protein 88 (MYD88) and TLR4-dependent manner.
- TLR recognition of microbial motifs enhances the intestinal epithelial barrier function by inducing the tightening of intercellular junctions, the secretion of mucus and antimicrobial peptides, and the production of reactive oxygen species.
- Microbial signalling through TLRs participates in intestinal epithelial repair after injury by inducing the production of trefoil factor 3, amphiregulin and prostaglandin E_2, which enhance migration, epithelial cell survival and proliferation, and by promoting the restitution of the normal epithelial architecture.
- Dysregulation of TLR signalling can lead to inefficient clearance of pathobionts and alterations in the normal microbial composition. Imbalances in microbial composition increase susceptibility to colitis and tumorigenesis.

and ulcerative colitis, or sporadic and colitis-associated colorectal cancer (CAC).

PRRs are expressed in most cell types of the gut and modulate the cell functions and interactions with neighbouring cells. Owing to space constraints, in this Review we strictly focus on how TLR signalling mediates the crosstalk between microorganisms and IECs, altering epithelial cell functions. In particular, we elaborate on the involvement of epithelial TLR-mediated microbial recognition in the structural and functional development of the intestinal epithelial barrier, as well as in the priming of immune cell responses in the gut mucosa. Furthermore, we discuss the consequences of dysregulation of epithelial TLR signalling on the generation of dysbiosis, inflammation, repair and tumorigenesis.

Epithelial barrier maintains homeostasis

The gastrointestinal tract consists of 30–40 m^2 of surface that is organized to maximize the area for digestion and absorption of dietary nutrients[15]. In the small intestine, finger-like projections called villi protrude to the lumen and lie near tubular invaginations that are known as the crypts of Lieberkühn. However, the colon, where absorption is restricted to water, electrolytes and products of microbial fermentation, has crypts but no villi[16]. At the base of the crypts, a cycling population of columnar stem cells gives rise to the rapidly dividing transit amplifying cells, which terminally differentiate into diverse epithelial lineages as they move up the crypt[17,18]. Differentiated cells comprise absorptive enterocytes, microfold cells and secretory lineages, such as goblet cells, enteroendocrine cells, tuft cells and either Paneth cells in the small intestine or deep crypt secretory cells in the colon[18–21] (FIG. 1). All of these cell types collaborate to build a functional intestinal epithelial barrier that stratifies microorganisms in the lumen, thereby limiting their interactions with the host.

Enterocytes are structurally polarized into an apical surface facing the intestinal lumen and a basolateral surface facing the lamina propria. Polarization is established by different families of proteins expressed on the lateral surface of IECs that form diverse intercellular junctions and regulate cell-to-cell interactions, paracellular permeability and transepithelial exchange of water and ions[22]. Tightening of the intestinal epithelial barrier is essential to prevent microbial colonization of the lamina propria due to a leaky gut, which is observed, for instance, in patients with IBD[23,24]. Absorptive enterocytes also express the polymeric immunoglobulin receptor (pIgR) and the transmembrane mucins that form the glycocalyx, a 3D matrix rich in carbohydrates that is associated with the membrane of IECs. pIgR mediates transcytosis of B cell-released IgA from the basolateral to the apical surface[25], and the glycocalyx reduces contact between pathogens and IECs at the apical surface[26].

Goblet cells secrete gel-forming glycoproteins such as mucin 2 (MUC2) into the lumen of the crypt. These glycoproteins unfold and polymerize to generate the net-like structures that characterize the mucus layer[26,27]. In the small intestine, the mucus that diffuses out of the crypt mixes with antimicrobial peptides and IgA secreted by Paneth cells, absorptive enterocytes, intraepithelial lymphocytes and B cells to form a single, loose mucus layer. After mucus secretion, physical paths termed goblet cell-associated passages form within the mucus-depleted goblet cells, capturing and delivering small soluble luminal antigens to dendritic cells (DCs) in the lamina propria[28]. In the colon, where the microbial burden is 10^3 times higher than in the small intestine, a loose outer mucus layer reduces microbial physical contact with the firm inner mucus layer, which is impenetrable to particles >0.5 µm (REFS[26,29]). Thus, the inner mucus layer is relatively sterile compared with the outer mucus layer, and it prevents microbial colonization of the colonic crypts.

Paneth cells sustain stemness in the intestinal stem cell niche by providing crypt base columnar stem cells with essential growth factors[30] and participate in bacterial clearance by secreting antimicrobial peptides, such as α-defensins, lysozyme C, regenerating islet-derived IIIβ (RegIIIβ) and RegIIIγ, and angiogenin 4 (REF.[31]). Other secretory cell types, such as enteroendocrine cells and tuft cells, also contribute to mucosal homeostasis. Enteroendocrine cells secrete peptides and hormones, such as cholecystokinin and serotonin, which induce intestinal peristaltic movements that renew the mucus layer[32]. Tuft cells participate in the clearance of parasites from the intestinal lumen by synthesizing IL-25 and, subsequently, promoting T helper 2 (T_H2) cell immune responses[33]. Last, in the specialized epithelium covering the lymphoid structures of the gut-associated lymphoid tissue, microfold cells take up luminal antigens to transport them to the subepithelial regions, where they are captured and processed by DCs that subsequently migrate to the mesenteric lymph nodes to prime T cell and B cell responses[34,35].

TLRs and microbial recognition

Gnotobiotic models have emphasized the importance of the host–microbial crosstalk in the metabolic and immune maturation of the gut. Germ-free mice have

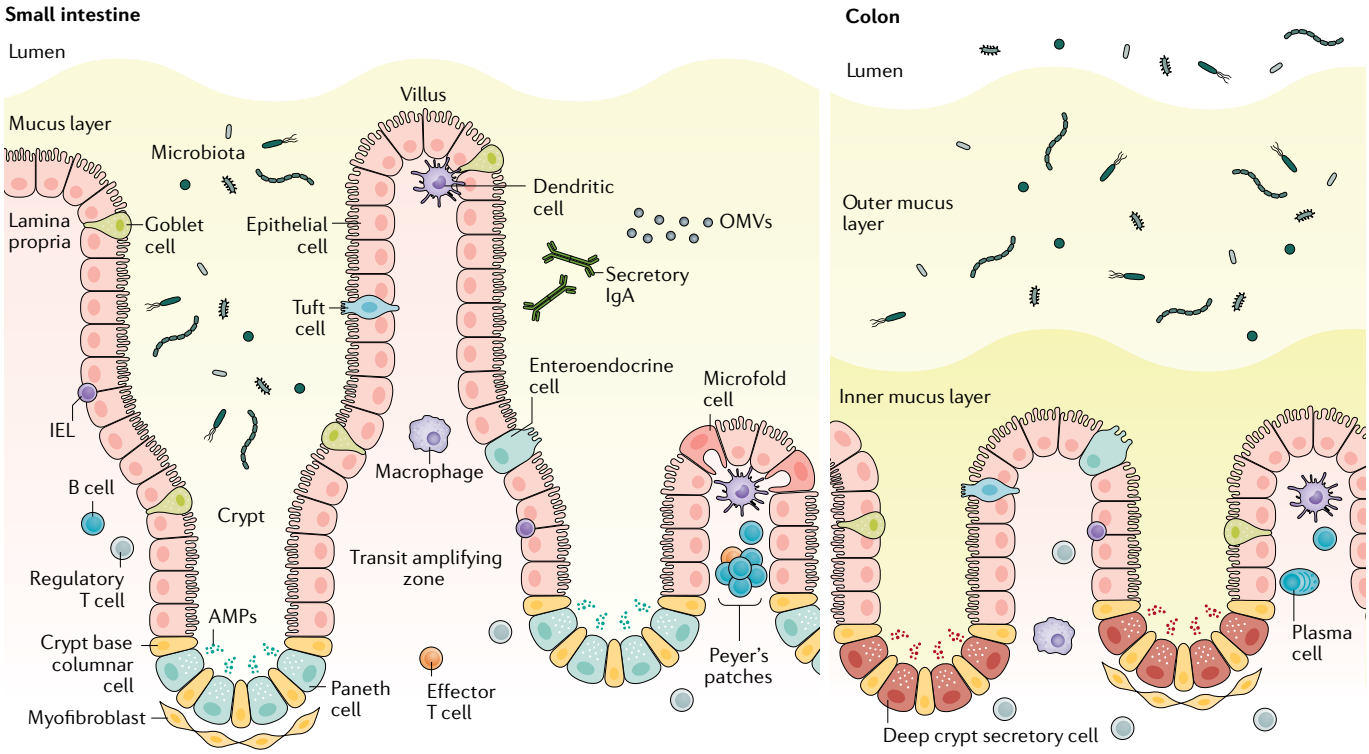

Fig. 1 | **Anatomy of the intestinal immune system.** The gastrointestinal mucosa is separated from the environment by a single layer of intestinal epithelial cells (IECs) that provides a physical and functional barrier. All of the lineages of IECs originate from the crypt base columnar stem cells, which divide to give rise to more proliferative daughter cells, the transit amplifying cells. As the transit amplifying cells proliferate, most daughter IECs move upwards to the crypt and differentiate owing to the decreasing gradient of growth factors that sustain stemness, which concentrate at the stem cell niche. IECs that reach the tip of the crypt (colon) or the villus (small intestine) undergo apoptosis and are then shed to the lumen. This entire cycle typically lasts 4–5 days. Throughout this migration, IECs differentiate into absorptive enterocytes, mucus-producing goblet cells, hormone-secreting enteroendocrine cells, antiparasitic tuft cells and Paneth cells (small intestine) or deep crypt secretory cells (colon). Paneth cells migrate downwards to the base of the crypt to provide stem cells with growth factors. Intermingled with IECs, intraepithelial lymphocytes (IELs) produce antimicrobial peptides (AMPs) and have cytolytic activity. Beneath the IECs, stromal cells (myofibroblasts), B cells and IgA-producing plasma cells, macrophages, dendritic cells and T cells dwell in the lamina propria, reinforcing the epithelial barrier by sampling luminal contents and maintaining a hyporesponsive state. Regional lymphoid structures, such as Peyer's (small intestine), caecum and colon patches, and the solitary isolated lymphoid tissues are overlaid by a specialized epithelium, known as follicle-associated epithelium, where microfold cells capture antigens and release them into the subepithelial dome. In the colon, the presence of a firm inner mucus layer reduces exposure to microorganisms. However, the microorganism-associated molecular patterns embedded in outer membrane vesicles (OMVs) can eventually reach the IECs. Adapted from REF.[2], Springer Nature Limited.

several deficiencies in gastrointestinal function compared with conventionally raised, specific pathogen-free mice: reduced epithelial paracellular permeability[36], higher mucus penetrability[37], reduced production of antimicrobial peptides[38,39], reduced IEC proliferation[40,41], underdeveloped lymphoid structures[42], reduced populations of intraepithelial lymphocytes[43], T helper 17 (T_H17) cells[44], regulatory T cells[45] and IgA-producing plasma cells[46], and slower motility[47]. Microorganisms communicate with the host by producing metabolites, such as short-chain fatty acids, aryl hydrocarbon receptor ligands and polyamines[3], by secreting outer membrane vesicles that contain MAMPs and can traverse the epithelial barrier[48], or by directly interacting with PRRs[4]. The host's PRRs recognize carbohydrates, lipoproteins and nucleic acids of bacterial, fungal, viral, helminthic and self origin, and determine whether the subcellular localization of microbial molecules is physiological or aberrant[5–7,9–11].

TLRs are transmembrane proteins, located in the cell membrane and in endosomes, that recognize Gram-positive and Gram-negative structures, flagellin, single-stranded and double-stranded RNA, unmethylated CpG DNA and various damage-associated molecular patterns[7,49,50]. Interaction of their extracellular leucine-rich repeat-containing domains with their cognate ligands induces the dimerization of the receptor, enabling the intracellular Toll/IL-1 receptor (TIR) domain to interact in a homologous manner with different cytosolic TIR-containing adapters, including myeloid differentiation primary response protein 88 (MYD88) and TIR-domain-containing adapter-inducing IFNβ (TRIF; also known as TICAM1)[51–54]. These adapters trigger signalling pathways that culminate in activation of NF-κB, activator protein 1 (AP-1) and IRF3 (REFS[49,51,53]). These transcription factors translocate into the nucleus and induce the synthesis of pro-inflammatory cytokines, such as IL-6, tumour necrosis factor (TNF) and the inactive precursor pro-IL-1β; induce the synthesis of antiviral type I interferons, such as IFNα and IFNβ; and activate anti-apoptotic or proliferative pathways[49,53,55,56]. TLR1–TLR9 are expressed in different cell types in the gut, including IECs[57], immune cells[58,59] and other stromal[60] and parenchymal non-immune[61] cells. Given that bacterial

populations outnumber other microorganisms by two orders of magnitude[1], we focus on the TLRs that specialize in bacterial recognition: TLR4, TLR5, TLR9, and TLR2 and its heterodimerizing partners, TLR1 and TLR6. Their principal bacterial ligands are presented in TABLE 1.

TLR expression is strongly influenced by the presence of bacteria. TLR1, TLR2, TLR5 and TLR9 are upregulated in specific pathogen-free mice when compared with germ-free mice[41,62,63], and expression of TLR1, TLR2, TLR4 and TLR5 is highest in the colon, which is consistent with increased bacterial burden in the distal gastrointestinal tract[57,64–66]. TLRs are expressed in most IEC lineages: stem cells[67], absorptive enterocytes[65,68–72], goblet cells[73–75], Paneth cells[38,57,76], enteroendocrine cells[77,78] and microfold cells[79,80] (TABLE 1). TLR-mediated recognition of MAMPs triggers lineage-dependent responses that regulate IEC proliferation and lineage fate, strengthen intestinal epithelial barrier function and shape the immune response.

TLRs regulate epithelial crypt dynamics. Regulation of crypt dynamics relies on proliferation, apoptosis and differentiation, which are strongly influenced by microbial metabolites[81–83] and epithelial recognition of MAMPs. Rakoff-Nahoum et al.[84] were the first to report that disruption of TLR signalling in MYD88-deficient mice elicits increased epithelial proliferation in the transit amplifying zone, which was later corroborated in TLR1-deficient and TLR9-deficient mice[64,85]. In addition, two other studies showed that lipopolysaccharide (LPS)-induced activation of TLR4 in intestinal stem cells reduced proliferation and enhanced apoptosis in small intestine[67] and colon[86] wild-type organoids. Conversely, *Tlr4*$^{-/-}$ organoids were protected from LPS-induced apoptosis. Similar findings were observed in vivo in the small intestine crypts of mice raised in conventional housing conditions[67] and in the colon crypts of germ-free mice monocolonized with select bacterial species of the genera *Acinetobacter*, *Stenotrophomonas* and *Delftia*[86]. These reports, which suggest that epithelial cycling and stem cell self-renewal are inhibited by TLR signalling, have been challenged by other in vivo data in mouse models that indicate that overactivation of TLR4 increases IEC proliferation in both the small intestine and the colon[87], and that epithelial TLR4 deficiency leads to downregulation of proliferating cell nuclear antigen (PCNA) in IECs from the small intestine[88]. These divergent results between different mouse models might be caused by differences in microbial communities in TLR-deficient and TLR-transgenic mice, highlighting the need for a standardized characterization of microbial populations in future studies.

Stemness and differentiation in the stem cell niche are regulated by several signalling pathways[89,90]. Of these pathways, NOTCH has been identified as a major determinant of IEC fate. Activation of NOTCH in mice represses the master regulator for secretory differentiation, atonal homologue 1 (ATOH1), directing differentiation towards absorptive lineages[91,92]; conversely, inhibition of NOTCH supports the development of secretory IECs[88]. TLRs have been shown to modulate NOTCH activity, but whether they have a stimulatory or inhibitory effect is not completely understood. On the one hand, a study demonstrated that genetic ablation of TLR4 or the signalling adapter TRIF in IECs was associated with increased frequency of goblet cells and expression of ATOH1 in vivo in mice[88]. Furthermore, silencing of TLR4 in the TLR4-expressing rat cell line IEC-6 induced the production of MUC2, whereas overexpression of TLR4 in human Caco-2 cells, which

Table 1 | **Ligands, expression and functions of TLRs in the gut**

TLR	Bacterial ligands	Expression in small intestine IECs	Expression in colon IECs	Functions	Refs
1	Triacyl lipopeptides (Pam3CSK4)	Moderate: PCs; microfold cells	High: colonocytes; EECs; GCs	IL-8, TNF, iNOS expression; IEC proliferation; tight junction regulation; GC maturation; MUC2 secretion	57,64,70,74,75,78, 80,99,138,218
2	Diacyl and triacyl lipopeptides (Pam2CSK4, Pam3CSK4); peptidoglycan; lipoteichoic acid	Low: enterocytes; PCs; microfold cells (A/B)	High: colonocytes (A); EECs; GCs	IL-8, TNF, iNOS expression; tight junction regulation; MUC2 secretion; TFF3 secretion; microparticle uptake	57,70,72–75,78–80, 99,138,218
4	Lipopolysaccharide	Low: SCs; enterocytes; PCs; microfold cells	High: colonocytes; EECs; GCs	TNF, iNOS, APRIL, CCL20, CCL28 expression; IEC proliferation; IEC apoptosis; CCK secretion; MUC2 secretion; GC differentiation; PC degranulation	57,65,67,70,74–78, 80,88,183
5	Flagellin	Low: enterocytes; GCs; PCs	High: colonocytes (B); GCs	IL-8, TNF, iNOS expression; CCK secretion; MUC2 secretion; PC degranulation; microparticle uptake	57,66,69,70,72, 74–77,127,218
6	Diacyl lipopeptides (Pam2CSK4); lipoteichoic acid	Low to none: enterocytes; PCs	Low to none: colonocytes	Not described	57,70,138,218
9	CpG unmethylated oligonucleotides	Low: enterocytes; PCs	Low: colonocytes (A/B)	IL-8 production; IEC proliferation; CCK secretion; PC degranulation	57,70,72,76, 77,85,218

Only direct evidence of Toll-like receptor (TLR) expression in primary intestinal epithelial cells (IECs) was considered for the preparation of this table. A, apical; APRIL, a proliferation-inducing ligand; B, basolateral; CCK, cholecystokinin; CCL, CC-chemokine ligand; EEC, enteroendocrine cell; GC, goblet cell; iNOS, inducible nitric oxide synthase; MUC2, mucin 2; PC, Paneth cell; SC, stem cell; TFF3, trefoil factor 3; TNF, tumour necrosis factor.

typically do not express TLR4, caused a loss of their normal MUC2-producing phenotype[88]. On the other hand, experiments in zebrafish models determined that the presence of gut microbiota increases the number of secretory cells in the gut via MYD88-dependent inhibition of NOTCH[93]. Consistently, different groups observed that epithelial TLR4 activation increases the proportions of goblet cells in vivo[94] and in vitro[86], and that abrogation of epithelial MYD88 or TLR2 signalling leads to a reduction of goblet cells in different mouse models[73,95]. Hypotrophic phenotypes in *Tlr2*$^{-/-}$ mice were associated with a lack of production of the protective trefoil factor 3 (TFF3), which is synthesized by goblet cells in response to TLR2 stimulation[73]. Therefore, most findings suggest that TLR activation is associated with the development and maturation of secretory IECs, goblet cells in particular.

A 2018 study elegantly demonstrated that uniaxial mechanical stimulation of human intestinal organoids engrafted into the mesentery of mice accelerates maturation of the developing miniguts and makes them more similar to adult intestinal tissue[96]. Motility is controlled by enteroendocrine cells via the release of hormones such as cholecystokinin, which acts in a paracrine or endocrine manner on enteric motor neurons or smooth muscle cells[97]. Administration of TLR4, TLR5 and TLR9 ligands induced the release of cholecystokinin into the blood in TLR-sufficient, but not TLR-deficient, mice[77], suggesting that the gut microbiota might also promote IEC differentiation by inducing gastrointestinal motility.

TLR signalling strengthens the epithelial barrier. As previously discussed, the appropriate functioning of the intestinal epithelial barrier depends on the barrier integrity, the density of the mucus layer and the production of antimicrobial peptides and IgA. Early studies suggested an important role for TLRs in the regulation of permeability during infection: MYD88-deficient mice exhibited impaired permeability and increased microbial burden in the mucosa after *Citrobacter rodentium*-induced colitis[98]. Furthermore, Cario et al.[99] demonstrated that *Myd88*$^{-/-}$ and *Tlr2*$^{-/-}$ mice were characterized by early tight junction disruption at day 5 after dextran sulfate sodium exposure that rendered them more susceptible to colitis than wild-type mice. The underlying mechanism involved the TLR2-induced translocation of the proteins zona occludens 1 (ZO1) and occludin to the tight junction, thereby increasing transepithelial resistance in vitro and in vivo[99–101]. Consistent with these findings, later studies reported that *Myd88*$^{-/-}$ and *Tlr1*$^{-/-}$ mice also have increased permeability to small molecules, reduced transmucosal resistance and increased bacterial translocation to the liver, spleen, blood[64] and mesenteric lymph nodes[102], confirming that TLR1–TLR2 signalling sustains epithelial integrity through the tightening of intercellular junctions. Conversely, activation of epithelial TLR4 causes loss of barrier integrity and a leaky gut in both mouse models and human IEC lines[94,103,104] through a mechanism that involves upregulation of myosin light chain kinase (MLCK), a protein that induces the tight junctions to open by promoting the contraction of actin–myosin filaments[104]. Overall, these findings indicate that different TLRs are involved in the control of the intercellular junctions, either enhancing or disrupting intestinal epithelial barrier integrity depending on the bacterial challenge.

The production and density of mucus are largely dependent on the presence of bacteria and the production of MUC2 (REF.[37]). Indeed, the colonic crypts of MUC2-deficient mice are colonized by bacteria, which has been associated with the development of colitis and tumours[105]. Work by Birchenough et al.[75] elegantly identified a subpopulation of colonic sentinel goblet cells in mice that recognize TLR1–TLR2, TLR4 and TLR5 ligands and elicit calcium signals that transmit through gap junctions to other goblet cells to provoke MUC2 secretion. Furthermore, they showed that bacteria reaching the upper parts of the intestinal crypts are expelled from these locations after stimulation of sentinel goblet cells with LPS, demonstrating a dynamic mechanism to restrain bacterial colonization through TLR activation. The disruption of this mechanism might explain why the mucus layers of *Tlr5*$^{-/-}$ and epithelial TLR5-deficient mice are substantially more colonized by commensal microorganisms, leading to the eventual development of spontaneous colitis[106–108]. Similarly, TLR1 is also involved in the production of MUC2, as TLR1-deficient mice are characterized by large areas of the colon with reduced expression of this mucin[64]. Taken together, these studies illustrate how TLRs have important roles in goblet cell physiology and in healthy structural development of the mucus layers (FIG. 2).

Functional development of the mucus layers depends on the production of antimicrobial peptides and IgA. The relevance of TLRs in the synthesis of antimicrobial peptides was demonstrated by Vaishnava et al.[38], who reported that Paneth cells from *Myd88*$^{-/-}$ mice have reduced production of the defensin cryptdin 2 and the lectins RegIIIβ and RegIIIγ. Subsequent studies in diverse mouse models have corroborated the strong dependence of defensins, RegIIIβ and RegIIIγ on TLR activation[39,72,102,109]. Indeed, it has been shown that mice with chronic deficiency of MYD88 develop ileitis with increased bacterial translocation into the lymph nodes owing to the >3-fold depletion in the expression of antimicrobial peptides[95]. Equally importantly, the uptake and transcytosis of secreted IgA from the lamina propria and into the lumen is carried out by enterocytes and relies on pIgR[110], whose expression is MYD88-dependent[102]. Stimulation of primary intestinal epithelial monolayers with LPS or heat-killed *Escherichia coli* in vitro upregulates pIgR and induces transcytosis of IgA in a pIgR-dependent manner[111,112], demonstrating that IgA transcytosis is dependent on TLR-mediated recognition of the gut microbiota.

Findings from the past decade suggest that IECs prevent microbial colonization of the mucosa and reduce bacterial signalling[113], invasiveness[114], motility[115] and virulence[116] through the release of reactive oxygen species (ROS). Indeed, Grasberger et al.[117] have demonstrated that ablation of NADPH oxidase dual oxidase 2 (DUOX2) renders mice more susceptible to colonization of the stomach mucosa in a *Helicobacter felis* infection model. *DUOX2* missense mutations have been associated with an increased risk of Crohn's disease

in two Ashkenazi Jewish families with more than 800 and 200 members[118] and with very early-onset IBD in two cohorts of 59 and 150 patients[114]. In all cases, *DUOX2* variants caused reduced production of ROS[114,118,119], which led to decreased resistance to infection in cells expressing such variants[114]. Given that TLR ligands upregulate the transcription of NADPH oxidase-associated genes[57,120] and induce epithelial release of ROS in a DUOX2-mediated manner[120], it is possible to speculate that IECs might control bacterial invasion of the mucosa through the release of ROS into the lumen upon TLR activation, thereby reinforcing intestinal epithelial barrier function.

Epithelial TLRs shape immune responses. The role of TLRs in mammals was initially described by Medzhitov et al.[49] by cloning a constitutively active TLR4 transgene into a human monocytic cell line. They determined that activation of TLR4 induced IL-1, IL-6 and IL-8 expression, defining the primary functions of the TLR family. Building on this study, different groups addressed epithelial activation of NF-κB, mitogen-activated protein kinase (MAPK) and production of cytokines upon TLR challenge. These initial investigations established that LPS challenge induces the activation of NF-κB and MAPK as well as the release of IL-8 in non-polarized human epithelial cell lines[70,121]. Further reports have corroborated the notion that human cell lines that are stimulated with LPS, lipoteichoic acid, flagellin or more complex structures, such as bacterial outer membrane vesicles, also express IL-6, IL-10, CXC-chemokine ligand 8 (CXCL8), CC-chemokine ligand 2 (CCL2) and cyclooxygenase 2 (COX2)[70,122–126]. A 2018 study has shown that TLR activation also upregulates the expression of TNF, NADPH oxidase family-related genes and inducible nitric oxide synthase (iNOS) in intestinal and colonic organoids prepared from primary mouse IECs[57].

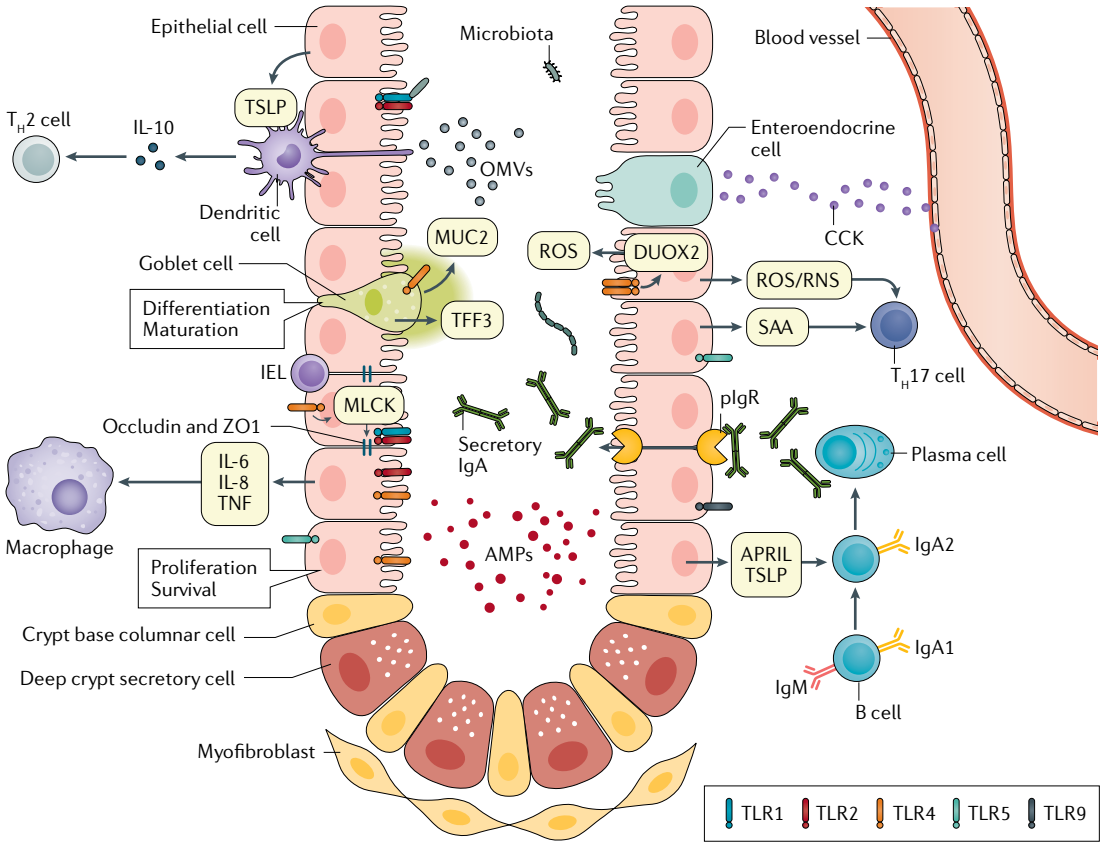

Fig. 2 | **TLRs strengthen the intestinal epithelial barrier.** Activation of Toll-like receptor (TLR) signalling in intestinal epithelial cells (IECs) induces responses that modify intestinal crypt dynamics and IEC functions to increase the integrity of the epithelial barrier. The interactions of crypt base columnar cells and transit amplifying cells with TLR ligands on outer membrane vesicles (OMVs) or live bacteria induce alterations in proliferation, increase apoptosis and modify the lineage fate of daughter cells. To limit such an interaction, TLR activation in differentiated cells promotes secretion of antimicrobial peptides (AMPs), mucus and cytoprotective factors, such as trefoil factor 3 (TFF3); enhances IgA class switching and transcytosis by increasing the expression of a proliferation-inducing ligand (APRIL), thymic stromal lymphopoietin (TSLP) and polymeric immunoglobulin receptor (pIgR); tightens intercellular junctions between IECs by inducing the translocation of zona occludens 1 (ZO1) and occludin to the tight junction; induces the release of hormones such as cholecystokinin (CCK); promotes homing of immune cells to the lamina propria and primes them through the release of IL-1β, IL-6, IL-8, tumour necrosis factor (TNF), TSLP and serum amyloid A (SAA); and upregulates the expression of NADPH oxidases, such as the dual oxidase 2 (DUOX2). Conversely, TLR4 activation induces myosin light chain kinase (MLCK) to cause contraction of actin–myosin filaments, which forces the tight junction to open and promotes epithelial leakiness. IEL, intraepithelial lymphocyte; MUC2, mucin 2; RNS, reactive nitrogen species; ROS, reactive oxygen species; T_H2 cell, T helper 2 cell; T_H17 cell, T helper 17 cell.

These in vitro observations suggest that, upon microbial challenge, IECs might induce the chemoattraction of immune cells to the lamina propria and prime their subsequent responses. In support of this hypothesis, in vivo studies in mice have demonstrated that administration of TLR ligands increases the uptake and transportation of 0.2-μm microspheres by the follicle-associated epithelium, which leads to increased homing of DCs to the lamina propria[79,127]. In the small intestine, TLR-induced secretion of mucus could also have a role in antigen uptake by creating antigen-transporting paths through the goblet cells, thereby facilitating the interaction between luminal contents and subepithelial DCs[28,75]. In addition to promoting cell migration, IECs secrete soluble factors and mediators that alter the priming properties of DCs and the differentiation of T cells. Flagellated bacteria increase the epithelial release of thymic stromal lymphopoietin (TSLP), a cytokine that promotes tolerogenic phenotypes in DCs[128]. In co-culture models, mouse DCs conditioned with supernatants of bacterial-challenged human IECs released IL-10, which ultimately led to polarization of naive $CD4^+$ cells into T_H2 effector cells. By contrast, unconditioned DCs directly challenged with flagellated bacteria, even in the presence of IECs, produced higher levels of both IL-10 and IL-12, leading to induction of T_H1 and T_H2 cell responses in naive T cells[128,129].

Similarly, the production of serum amyloid A (SAA) by IECs is MYD88-dependent and can be stimulated by segmented filamentous bacteria[130,131]. As SAA mediates the differentiation of T cells into T_H17 cell phenotypes in vitro, epithelial secretion of this protein could be involved in microbial-induced maturation of the immune system[44,131]. Maturation of T_H17 cells is also regulated by the intestinal redox status[131]. Given that TLR signalling and segmented filamentous bacteria induce the expression of NADPH oxidases and iNOS in vitro and in vivo[57,120,131,132], we speculate that TLRs might be involved in T_H17 cell polarization by inducing ROS and reactive nitrogen species (RNS).

Epithelial TLR activation not only has a role in the transcytosis of IgA from the lamina propria to the intestinal lumen via upregulation of pIgR, but also triggers class switch recombination and IgA secretion by B cells in a T cell-independent fashion. Caco-2 monolayers that were stimulated with LPS and flagellin at their apical pole released a proliferation-inducing ligand (APRIL) and TSLP into the cell culture media[133]. APRIL cooperated with TLR ligands to promote IgA class switching in B cells and the release of IgA, whereas TSLP in turn induced the expression and release of APRIL by DCs[133]. These findings were later confirmed in a mouse model in which constitutively active TLR4 in the epithelium caused increased levels of IgA and APRIL, as well as an increased abundance of IgA-producing B cells, in the lamina propria.

Regulation of TLRs limits immune responses and inflammation. Taken together, these observations suggest that epithelial TLRs control several aspects related to the microbial-induced maturation of host defence, ranging from the conformation and tightening of the epithelial barrier to the appropriate shaping of the adaptive immune responses. Given the importance of these functions, a tight TLR-regulating system is necessary to maintain gut homeostasis and avoid disproportionate reactions of the host towards the gut microbiota. IECs can modulate TLR signalling at different levels, such as by restricting access to ligands or by inactivating downstream signalling cascades[134,135].

IECs reduce the number of interactions between TLRs and their cognate ligands by maintaining a low expression of TLRs and cooperating molecules. For instance, different human cell lines not only express low levels of TLR2, TLR4 and the TLR4-cooperating protein myeloid differentiation factor 2 (MD-2) but, upon apical stimulation, also engulf and relocate the TLRs into cytoplasmic compartments, presumably lysosomes, to limit TLR activation[70,136–138]. Furthermore, TLR signalling in IECs is markedly dependent on cell polarization. Activation of epithelial TLR5 induces the release of IL-8 only when basolateral stimulation occurs, implying that flagellin must translocate to the lamina propria to trigger TLR5-mediated responses[69]. Similarly, apical challenge of TLR9 with CpG oligonucleotides elicits the stabilization of the NF-κB repressor IκB (inhibitor of NF-κB), rendering IECs hyporesponsive to apical interaction with TLR9 ligands[72]. Sequestration and modification of microbial motifs are additional strategies to prevent the recognition of ligands. In the gut, lamina propria mononuclear cells release soluble forms of the TLR2 ectodomain, especially during inflammation[139]. Soluble TLR2 reduces the production of IL-8 in Caco-2 cells challenged with the triacyl lipopeptide Pam3CSK4 and might therefore be involved in modulation of immune responses[140]. Alkaline phosphatase dephosphorylates the immunogenic component of LPS, lipid A, reducing its potency to stimulate TLR4 (REF.[141]). Induction of alkaline phosphatase release in Caco-2 cells reduced LPS-elicited NF-κB activation of reporter cells in co-culture, suggesting that IECs can also use this mechanism to moderate microbial challenge[142].

Following ligand recognition and the production of inflammatory mediators, IECs can also self-limit their responses by upregulating the expression of molecules that inhibit TLR downstream signalling pathways[70,122,126]; TABLE 2 summarizes the molecules known to have such roles in the gastrointestinal epithelium. These modulators can interact with TLRs directly, as is the case with the single immunoglobulin IL-1 receptor-related molecule (SIGIRR), or indirectly, as with signalling complexes that terminate in the activation of NF-κB and AP-1. Engagement of SIGIRR, the Toll-interacting protein (TOLLIP) or peroxisome proliferator-activated receptor-γ (PPARγ) with their target proteins led to attenuated production of IL-8 in response to bacterial components in vitro[70,122]. In vivo, epithelial ablation of TLR-regulating molecules, such as TNFAIP3, PPARγ, TOLLIP or SIGIRR, did not induce disease phenotypes in untreated mice, but instead increased susceptibility to cytokine-induced apoptosis[143,144], dysbiosis[145,146], colitis[147] and tumorigenesis[148] upon induction of experimental models of colitis and infection (TABLE 2). Similarly, total depletion of IL-1 receptor-associated kinase 3 (IRAK3) and MAPK phosphatase 1 (MPK1) in all tissues caused

Table 2 | **Modulators of TLR signalling in the gut**

Molecule	Mechanism of action	Expression and functional evidence in the gut	Refs
SIGIRR	Interacts with TLR4, TLR5 and TLR9 via its TIR domain; hampers subsequent recruitment of downstream adapters MYD88, IRAK and TRAF6	Highly expressed in human colon cell lines; *Sigirr*$^{-/-}$ mice show microbial-dependent hyperproliferation of IECs, increased antimicrobial activity in IECs, hypersensitivity to colitis, increased susceptibility to infection with pathobionts, such as *Citrobacter rodentium* and *Salmonella enterica* serovar Typhimurium, and increased susceptibility to CAC; transgenic expression of SIGIRR in IECs reverts *Sigirr*$^{-/-}$ phenotypes	146,148,219,220
IRAK3	Inhibits phosphorylation of IRAK, preventing its dissociation from MYD88	Expressed in tumorigenic IECs; induced in regular IECs upon stimulation with TLR2/TLR4 ligand and WNT; *Irak3*$^{-/-}$ mice show increased susceptibility to colitis (myeloid compartment) but reduced tumorigenesis (tumour IEC compartment)	149,217,221,222
TOLLIP	Inhibits phosphorylation of IRAK, preventing its dissociation from MYD88	Predominant expression in IECs; downregulated in samples of patients with CD, UC, colon adenoma and carcinoma; overexpression in IECs reduces activation of ERK and secretion of IL-8 in response to TLR2/TLR4 ligands; *Tollip*$^{-/-}$ mice are more susceptible to colitis owing to defects in epithelial compartment	70,147,223–225
TNFAIP3	Disrupts the ubiquitin-dependent interaction between TRAF6 and TAK1	Epithelial *Tnfaip3*$^{-/-}$ mice are more susceptible to experimental colitis and increased cytokine-induced apoptosis of IECs; epithelial and myeloid *Tnfaip3*$^{-/-}$ mice develop spontaneous ileitis and colitis with epithelial apoptosis and hyperproliferation that progresses to CAC	143,144,226
MKP1	Dephosphorylates MAPK p38	Induced in IECs by TLR4, TLR5 and TLR9 ligands; reduces MAPK activation; *Mkp1*$^{-/-}$*Il10*$^{-/-}$ mice show accelerated development of colitis, increased epithelial proliferation and overactivation of MAPK	150,227
PPARγ	Reduces nuclear localization of NF-κB subunits; inhibits phosphorylation of MAPKs ERK and p38	Downregulated in samples of patients with CD, UC and CRC; human IEC lines treated with PPARγ ligands show reduced COX2 and IL-8 expression upon TLR4 stimulation with LPS; epithelial *Pparg*$^{-/-}$ mice show increased expression of iNOS in IECs, production of nitrate, dysbiosis and susceptibility to colitis	126,145,224, 225,228,229

Only molecules with a proven function in intestinal epithelial cells (IECs) were considered for the preparation of this table. CAC, colitis-associated cancer; CD, Crohn's disease; COX2, cyclooxygenase 2; CRC, colorectal cancer; ERK, extracellular-signal-regulated kinase; iNOS, inducible nitric oxide synthase; IRAK, IL-1 receptor-associated kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MKP1, MAPK phosphatase 1; MYD88, myeloid differentiation primary response 88; NF-κB, nuclear factor-κB; PPARγ, peroxisome proliferator-activated receptor-γ; SIGIRR, single immunoglobulin IL-1 receptor-related molecule; TAK1, TGFβ-activated kinase 1; TIR, Toll/IL-1 receptor; TLR, Toll-like receptor; TNFAIP3, TNFα-induced protein 3; TOLLIP, Toll-interacting protein; TRAF6, TNF receptor-associated factor 6; UC, ulcerative colitis.

enhanced immune responses towards the resident microbiota in a spontaneous model of colitis, accelerating the onset of inflammation[149,150].

Overall, the fact that selective deletion of TLR modulators in steady-state conditions does not lead to disease phenotypes suggests that stratification of the gut microbiota and the combination of diverse regulatory mechanisms are efficient at sustaining epithelial immune tolerance towards the resident microbiota. In this state, IECs recognize microbial components and respond by promoting epithelial barrier functions and producing low levels of cytokines, chemokines and inflammatory mediators that maintain immune cells of the lamina propria in a state of surveillance. However, upon colonization of the intestinal epithelium by adherent microorganisms or pathobionts, epithelial and immune TLRs drive robust antimicrobial pro-inflammatory responses to clear the noxious stimuli. In this demanding situation, epithelial expression of TLR regulatory molecules prevents excessive detrimental effects caused by uncontrolled TLR activation, thereby accelerating the resolution of inflammation and a return to homeostasis.

TLRs, dysbiosis and injury

TLR dysregulation promotes dysbiosis and susceptibility to inflammation. The equilibrium established between commensal microorganisms, the epithelial barrier and the immune system can be broken in the presence of pathogenic microorganisms or when microbial recognition and processing defects occur in epithelial or immune cells. Disruption of microbial–host cross-talk precipitates the onset of inflammation, as seen in patients with IBD[151]. IBD involves pathologies that are characterized by relapsing inflammation of the gastrointestinal tract and has been associated with imbalances in the resident bacterial populations, defects in the intestinal epithelial barrier and aberrant immune responses to the gut microbiota[151–154]. Genome-wide association analyses have identified more than 200 loci of susceptibility for Crohn's disease, including the PRR *NOD2* (REFS[155–157]). As NOD2 is necessary to recruit ATG16L1 to the membrane and initiate the formation of the phagosome, NOD2-mediated susceptibility to Crohn's disease is related to a defect in microbial processing[9,158,159]. The resultant impaired autophagy causes inefficient clearance of engulfed bacteria[158,159], antigen presentation[159] and regulatory T cell priming[160] by DCs in patients with Crohn's disease, which translates into a deficient inhibition of the effector immune responses against the gut microbiota and triggers the onset of inflammation. *TLR9* polymorphisms have been associated with mutations in *NOD2* and *IL-23*, both of which are Crohn's disease susceptibility genes, and could therefore be involved in the development of IBD[161]. Conversely, *TLR1*, *TLR2*, *TLR4* and *TLR6* polymorphisms have not been linked to the development of IBD; however, they are more frequent in patients with IBD than in the healthy population[162] and are correlated with more extensive and severe forms of

the disease, such as pancolitis[73,163]. Studies have reported increased epithelial expression of TLR4 in patients with IBD, suggesting that this receptor might be involved in the pathogenesis of these diseases[164,165].

Dysbiosis, a hallmark of patients with IBD, is typically characterized by reduced microbial diversity, increased abundance of facultative anaerobes (such as the phylum Proteobacteria) and reduced abundance of obligate anaerobes (especially of the phylum Firmicutes) when compared with a healthy, balanced microbial community[166–169]. The gut microbiota and dysbiosis are key contributors to the initiation of gastrointestinal inflammation[170]. IL-10-deficient mice develop spontaneous colitis when raised in specific pathogen-free, but not germ-free, conditions[171,172], and humanization of IL-10-deficient gnotobiotic mice with gut microbiota from patients with IBD results in faster development of colitis than humanization with gut microbiota from healthy individuals[173]. Interestingly, IL-10-deficient mice backcrossed to *Myd88*$^{-/-}$ mice do not develop colitis[174], suggesting that TLRs also participate in dysbiosis-mediated onset of inflammation. Although it is still unclear how dysbiosis begins, current hypotheses suggest that atypical conditions, such as metabolic and immune defects in IECs or the presence of pathobionts, increase the availability of oxygen or ROS and RNS in the gut lumen (reviewed previously[175,176]). In this paradigm, facultative anaerobes can outcompete obligate anaerobes by using microbial and epithelial products, such as tetrathionate[177], nitrate[178] or formate[179], to perform anaerobic and aerobic respiration, resulting in the imbalance of microbial populations. In turn, the overgrowth of facultative anaerobes can exacerbate inflammation[180], providing additional ROS and RNS that perpetuate dysbiosis (FIG. 3a). Dysregulated TLR signalling can dampen pathobiont clearance, contributing to dysbiosis. Chassaing et al.[181] demonstrated that monocolonization of germ-free *Tlr5*$^{-/-}$ mice with flagellated or non-flagellated Crohn's disease-associated adherent-invasive *E. coli* followed by conventional housing led to the development of a distinct gut microbiota and subsequent chronic colitis in *Tlr5*$^{-/-}$ mice receiving the flagellated strain, unlike those receiving the non-flagellated strain. Similarly, Kamdar et al.[182] demonstrated that *Tlr1*$^{-/-}$ mice that survive *Yersinia enterocolitica* infection subsequently develop dysbiosis characterized by an increased abundance of δ-Proteobacteria compared with their wild-type littermates. δ-Proteobacteria thrive by taking advantage of the tetrathionate respiration pathway used by *Yersinia* species to outgrow other bacteria. Subsequent transference of the *Tlr1*$^{-/-}$ dysbiotic gut microbiota rendered antibiotic-depleted recipient wild-type mice more susceptible to chemically induced colitis than recipient mice engrafted with the microbiota of wild-type donor mice post infection[182]. In both models, dysbiosis persisted even after elimination of the pathobiont, indicating that a single microorganism can prime a defective intestinal epithelium to modify the microbial ecosystem and perpetuate microbial composition imbalance.

Aberrant TLR activation might also contribute to dysbiosis via the release of antimicrobial peptides, ROS and RNS. TLR4 signalling in IECs increases the expression of iNOS[183] and NADPH oxidases[57], which produce nitric oxide and ROS. The reaction between nitric oxide and superoxide forms peroxynitrite, which can decompose to nitrate, thereby providing facultative anaerobes with terminal electron acceptors for anaerobic respiration[176,178]. Indeed, epithelial TLR4 overactivation not only induced dysbiosis but also increased susceptibility to colitis in transgenic mice that was transmissible by coprophagia to co-housed wild-type mice[94].

TLR-associated dysbiosis has also been associated with metabolic disorders. In mice, deletion of epithelial TLR4 and TLR5 signalling induced microbial alterations that promoted the development of metabolic syndrome, which was transmissible to wild-type germ-free mice by transplanting the gut microbiota[108,184]. Furthermore, the metabolic syndrome in *Tlr4*$^{-/-}$ mice was abolished by broad-spectrum antibiotic treatment and co-housing with conventionally raised wild-type mice[185]. Of note, the specific composition of the gut microbiota in each animal research facility might play a crucial part in the development of disease. Indeed, whereas the same group observed similar disease phenotypes in two strains of mice — total *Tlr5*$^{-/-}$ mice and epithelial-specific *Tlr5*$^{-/-}$ mice — generated using different breeding strategies[108,184], two other groups could not reproduce the development of colitis and metabolic syndrome in total *Tlr5*$^{-/-}$ mice[186]. These controversial results raise additional concerns about the need for standardization of the housing conditions in murine cohorts when studying the interactions between the microbiome and the host[187]. However, all of these models exemplify how excessive or defective epithelial TLR signalling promotes the expansion of the gut microbiota, which can in turn transmit disease phenotypes, highlighting the role of the intestinal epithelium in generating dysbiosis even in the absence of pathobionts.

Epithelial repair after inflammation. Even though the gut microbiota precipitates the onset of colitis in spontaneous models, chemically induced models of inflammation have demonstrated that it also has essential roles in the wound healing process. Indeed, both germ-free and antibiotic-treated mice are highly susceptible to chemically induced colitis and show high mortality rates when compared with conventionally raised mice[84,188]. Similarly, MYD88, TLR2, TLR4, TLR5 and TLR9-deficient mice are more susceptible to chemically induced colitis owing to defects in intestinal epithelial permeability, reduced proliferation, increased apoptosis and delayed differentiation of IECs, which ultimately cause inefficient epithelial restitution[84,85,99,108]. On the basis of such observations, several studies evaluated the use of TLR ligands as therapeutic tools to ameliorate colitis and accelerate the recovery process in experimental models (reviewed previously[189]). Most of these studies concluded that TLR activation ameliorates colitis by promoting the production of cytoprotective factors and modulatory cytokines in mesenchymal stem cells and immune cells that migrate to subepithelial locations adjacent to the wound[84,99,190–193].

Intestinal epithelial restitution occurs in three phases: barrier re-establishment, wound channel formation and

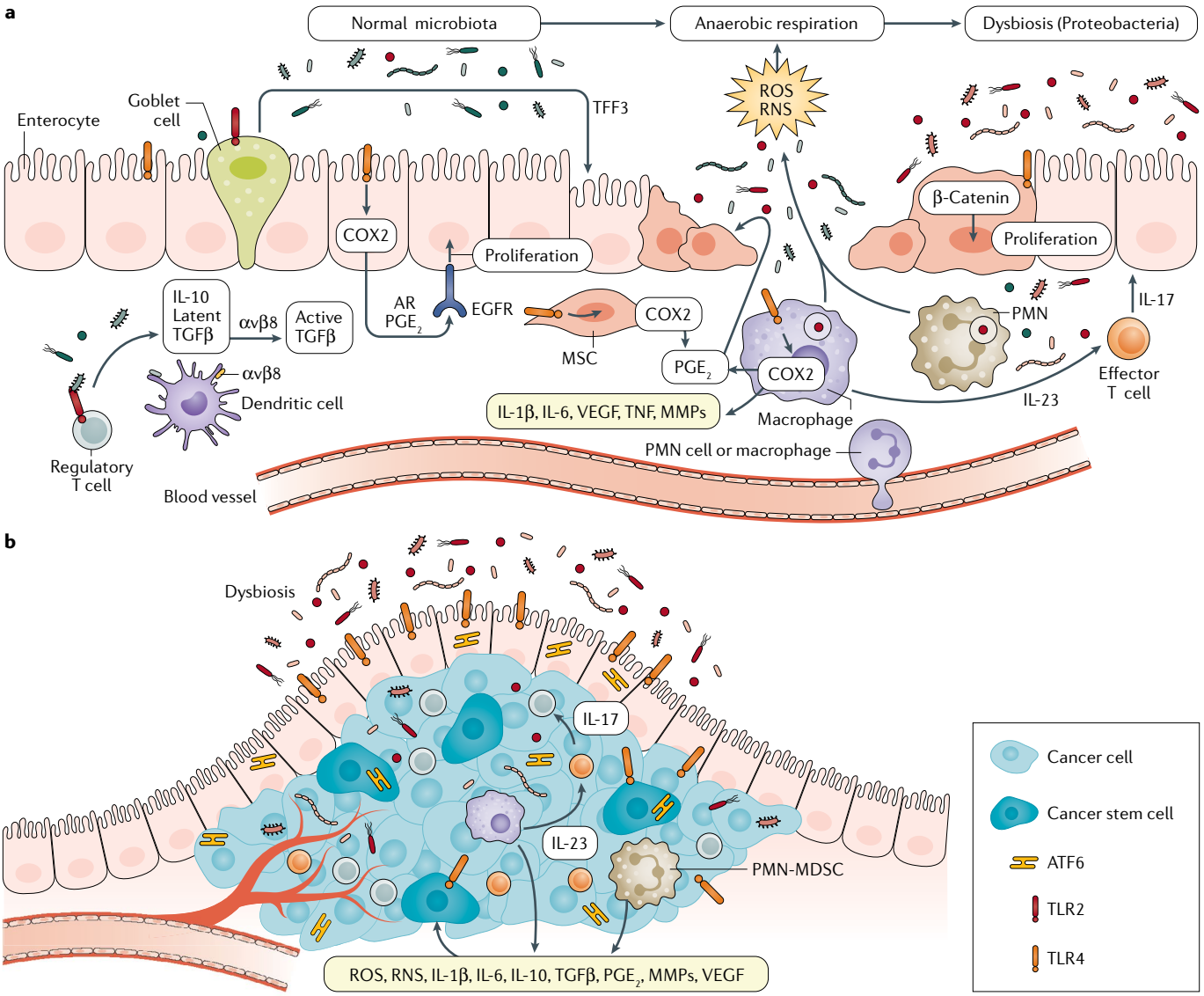

Fig. 3 | **TLRs participate in the generation of dysbiosis, epithelial restitution, and initiation and progression of tumorigenesis.** **a** | Activation of Toll-like receptors (TLRs) promotes dysbiosis and epithelial repair after injury. During inflammation, facultative anaerobes can outcompete obligate anaerobes by using derivatives of reactive oxygen species (ROS) and reactive nitrogen species (RNS) released by host cells to perform anaerobic respiration, leading to dysbiosis. Restitution of the intestinal epithelial barrier requires re-epithelialization of injured areas, which encompasses epithelial proliferation and migration over the wound bed. Activation of TLRs in enterocytes, goblet cells, macrophages and mesenchymal stem cells (MSCs) induces the expression of trefoil factor 3 (TFF3), amphiregulin (AR) and cyclooxygenase 2 (COX2), which in turn synthesizes prostaglandin E_2 (PGE_2). These factors activate the epidermal growth factor receptor (EGFR) and WNT–β-catenin signalling pathways, promoting proliferation of enterocytes in the crypts adjacent to the wound. In later phases of repair, the release and subsequent activation of latent transforming growth factor-β (TGFβ) by the αvβ8 integrin in dendritic cells promotes crypt fission and regeneration of the epithelial architecture. Macrophages and polymorphonuclear (PMN) cells migrating to the wound bed produce cytokines, vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMPs) that participate in neovascularization and stromal remodelling. **b** | Dysregulated TLR signalling is involved in initiation and progression of tumorigenesis. Epithelial activating transcription factor 6 (ATF6) or TLR4 can initiate tumorigenesis by inducing defects in mucus production or activating proliferative pathways in stem cells (FIG. 4). Subsequent microbial colonization of the lamina propria promotes infiltration by myeloid-derived suppressor cells (MDSCs) and lymphoid cells. MDSCs and effector T cells produce ROS, RNS, MMPs, VEGF, PGE_2, IL-23 and IL-17, which enhance tumour progression. TNF, tumour necrosis factor.

crypt regeneration[194]. Barrier re-establishment consists of an initial re-epithelization of the wound by an IEC monolayer. This phase depends on the production of proliferative, anti-apoptotic and pro-migratory factors, such as prostaglandin E_2 (PGE_2) and TFF3 (FIG. 3a). Mesenchymal stem cells, IECs and macrophages can produce PGE_2 and TFF3 in vitro upon TLR2 or TLR4 stimulation[73,194,195]. Moreover, studies demonstrated in vivo that PGE_2 and TFF3 are necessary to rescue the hypoproliferative and pro-apoptotic phenotypes of *Tlr4*$^{-/-}$ and *Tlr2*$^{-/-}$ mice, respectively, after chemical colitis[73,195]. PGE_2 can signal through its receptor, EP4, to induce non-canonical activation of the WNT–β-catenin[196] and epidermal growth factor receptor (EGFR)[165,195]signalling pathways, whereas TFF3 acts preferentially via the MAPK–EGFR pathway[197].

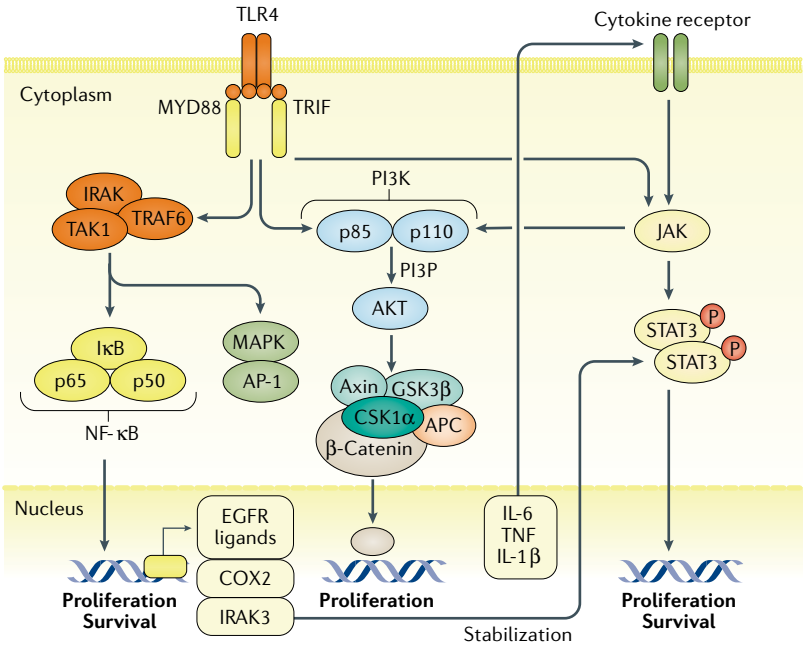

Fig. 4 | **TLR activation elicits anti-apoptotic and proliferative pathways in cancer stem cells.** Activation of Toll-like receptors (TLRs) induces translocation of nuclear factor-κB (NF-κB) and activator protein 1 (AP-1) into the nucleus, promoting the transcription of cytokines, cyclooxygenase 2 (COX2) and epidermal growth factor receptor (EGFR) ligands, and enhancing anti-apoptotic survival responses. TLR4 activation can inhibit the glycogen synthase kinase 3β (GSK3β) in a phosphoinositide 3-kinase (PI3K)-mediated manner, stabilizing β-catenin and promoting its translocation into the nucleus to activate proliferative pathways. Last, TLR signalling in tumours has also been associated with increased phosphorylation (P) of signal transducer and activator of transcription 3 (STAT3), which elicits proliferation and survival responses. Induction of this pathway could occur via activation of the Janus kinase (JAK), stabilization of STAT3 by inhibitor IL-1 receptor-associated kinase 3 (IRAK3) or direct signalling by pro-inflammatory cytokines. APC, adenomatous polyposis coli; CSK1α, casein kinase 1α; IκB, inhibitor of NF-κB; MAPK, mitogen-associated protein kinase; MYD88, myeloid differentiation primary response 88; PI3P, phosphatidylinositol 3-phosphate; TAK1, TGFβ-activated kinase 1; TNF, tumour necrosis factor; TRAF6, TNF receptor-associated factor 6; TRIF, TIR-domain-containing adapter-inducing IFNβ (also known as TICAM1).

The wound channel formation phase is characterized by the activation of the two main proliferative pathways in the gastrointestinal tract, the WNT–β-catenin and EGFR pathways, in the stem cells of the intestinal crypts flanking the wound to completely cover the exposed mucosal surface. This phase can also be potentiated by TLR-mediated recognition of microorganisms: studies have shown that TLR4 can trigger the induction of non-canonical phosphoinositide 3-phosphate (PI3K)–AKT–β-catenin signalling[87] and the transcription of the EGFR ligands epiregulin and amphiregulin[165,195,198,199]. Ungaro et al.[198] also reported that chemical blockade of TLR4 during the recovery phase of experimental colitis in mice reduced the induction of COX2 and the production of PGE_2 and amphiregulin, leading to defective epithelial restitution due to diminished proliferation. Last, the crypt regeneration phase is defined by the division of the proliferating wound channels through crypt fission to restore the number of intestinal crypts. Major drivers of differentiation, such as transforming growth factor-β (TGFβ), control the formation of clefts and the downregulation of mitotic phenotypes during this stage[200]. Work from different groups in mouse DCs has demonstrated that TLR2-mediated recognition of polysaccharide A from *Bacteroides fragilis* not only induces the production of TGFβ by regulatory T cells[201] but also induces the expression of αvβ8 integrin, which converts latent TGFβ into its active form[202].

Notably, the epithelial and vascular growth factor-enriched microenvironment that is generated during epithelial restitution is similar to that occurring during tumorigenesis. Therefore, in the context of relapsing inflammation, inefficient wound healing could lead to the accumulation of mutations in mitotic cells and the formation of tumours. It has been reported that *Myd88*$^{-/-}$ mice with CAC showed an early response to inflammation characterized by enhanced induction of the β-catenin, EGFR and signal transducer and activator of transcription 3 (STAT3) signalling pathways and increased DNA damage, leading to the premature formation of neoplastic lesions[203]. Similarly, in germ-free mice, the induction of CAC caused delayed inflammation and epithelial repair, leading to the early development of colorectal adenomas. Such a phenotype could be rescued by LPS administration or bacterial colonization[204].

Microbial signalling in tumorigenesis. Sporadic colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States[205] and is usually caused by mutations in oncogenes, such as the gene that encodes adenomatous polyposis coli (APC), that control major proliferative pathways[206]. Rakoff-Nahoum and Medzhitov[207] demonstrated a major role for TLR signalling in CRC progression by crossing *Apc*$^{Min/+}$ to MYD88-deficient mice. These mice had reduced numbers of tumours when compared with *Apc*$^{Min/+}$ mice, which was associated with a downregulation of genes involved in inflammation and epithelial repair, such as those encoding IL-1β, IL-6, COX2, matrix metalloproteinase 7 (MMP7) and MMP10, and insulin-like growth factor 1 (IGF1). In addition, subsequent studies reported that abrogation of MYD88, TLR2, TLR4 and TLR9 signalling in myeloid cells in mice reduced the number of tumours owing to a decreased activation of STAT3 and downregulation of IL-23 and IL-17 (REFS[208,209]) (FIG. 3b).

Evidence of a role for epithelial TLR signalling in the initiation of CRC came from mice with constitutively active epithelial TLR4, which developed carcinomas in the distal colon upon administration of the mutagenic agent azoxymethane, unlike their wild-type littermates. The mechanism proposed involved TLR4-mediated non-canonical activation of β-catenin[87]. Furthermore, these mice also have spontaneous duodenal adenomas that are completely abrogated in germ-free conditions (J.F.B. and M.T.A., unpublished observations), suggesting that TLR4-mediated alteration of bacterial populations might be an additional driver of intestinal tumorigenesis. A 2018 study also highlighted the involvement of TLR signalling in the formation of spontaneous adenomas in the colons of transgenic nATF6^{IEC} mice[210]. Of note, the development of adenomas depended on the dysbiosis generated by epithelial activation of activating transcription factor 6 (ATF6), which was transmissible to germ-free mice. Almost 80% of nATF6^{IEC} mice backcrossed to *Myd88*$^{-/-}$*Ticam1*$^{-/-}$ double

knockout mice did not develop tumours and displayed reduced activation of epithelial STAT3 when compared with their nATF6^{IEC} littermates[210], indicating that TLR-regulated activation of the Janus kinase (JAK)–STAT pathway might be another mechanism that initiates CRC (FIG. 4).

Patients with IBD are at increased risk of developing CRC compared with the general population, with increased incidence rates ranging from 0.8-fold to 3.2-fold[211–213]. CAC is thought to occur after recurrent episodes of inflammation and repair in proliferating IECs that are continuously exposed to cytokines and ROS[206]. Patients with ulcerative colitis and CRC have increased expression of TLR4 in IECs[87,214]. Epithelial TLR4 expression is associated with the initiation of CAC: whereas TLR4-deficient mice are protected from CAC[165], epithelial overactivation of TLR4 increases susceptibility to CAC[215]. RNA sequencing data from cancer stem cells of mice undergoing a CAC model suggest that the mechanisms underlying TLR4-driven tumorigenesis involve not only direct non-canonical β-catenin signalling[87] but also the activation of the JAK–STAT pathway and increased chemoattraction of tumour-supportive cells, such as myeloid-derived suppressor cells (J.F.B. and M.T.A., unpublished observations). Additional mechanisms that have been proposed for epithelial TLR-dependent promotion of tumorigenesis include the ROS-mediated activation of NF-κB in gastric cancer[216] and the induction of the TLR inhibitor IRAK3, which has been shown to interact with and stabilize STAT3 to avoid proteasomal degradation[217] (FIG. 4).

Conclusions

A growing amount of evidence demonstrates that the gut microbiota are not passive bystanders in the gastrointestinal tract but instead actively participate in establishing homeostasis and in precipitating disease. TLRs regulate host–microbiota interactions and have fundamental roles in maintaining a healthy epithelial barrier at different levels, ranging from the genesis and differentiation of the epithelial lineages to the control of permeability, antimicrobial peptide production and mucus secretion into the lumen. In the presence of pathobionts, epithelial TLRs trigger antimicrobial responses that prevent subsequent microbial imbalances and induce the expression of regulatory molecules that prevent uncontrolled inflammation. Once inflammation occurs, TLRs accelerate epithelial restitution. However, prolonged dysregulation in TLR signalling, especially during relapsing inflammation, can lead to the induction of dysbiosis and the activation of proliferative and anti-apoptotic signalling pathways, which might be usurped by mutated cancer stem cells and grow out of control. The potential use of therapeutic strategies that target epithelial TLRs to prevent or minimize dysbiosis and inflammation is still attractive. However, strengthening our knowledge of the crosstalk between epithelial TLRs, commensal microbiota and opportunist pathobionts will be essential to define the mechanisms causing microbial imbalances that participate in triggering and perpetuating inflammation, as well as in initiating and inducing the progression of tumorigenesis.

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