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Innate immune signaling in defense against intestinal microbes

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Summary

The gastrointestinal system is a common entry point for pathogenic microbes to access the inner environment of the body. Antimicrobial factors produced by the intestinal mucosa limit the translocation of both commensal and pathogenic microbes across the intestinal epithelial cell barrier. The regulation of these host defense mechanisms largely depends on the activation of innate immune receptors by microbial molecules. Under steady-state conditions, the microbiota provides constitutive signals to the innate immune system, which helps to maintain a healthy inflammatory tone within the intestinal mucosa and, thus, enhances resistance to infection with enteric pathogens. During an acute infection, the intestinal epithelial cell barrier is breached, and the detection of microbial molecules in the intestinal *lamina propria* rapidly stimulates innate immune signaling pathways that coordinate early defense mechanisms. Herein, we review how microbial molecules shed by both commensal and pathogenic microbes direct host defenses at the intestinal mucosa. We highlight the signaling pathways, effector molecules, and cell populations that are activated by microbial molecule recognition and, thereby, are involved in the maintenance of homeostatic levels of host defense and in the early response to acute enteric infection. Finally, we discuss how manipulation of these host defense pathways by stimulating innate immune receptors is a potential therapeutic strategy to prevent or alleviate intestinal disease.

Keywords

intestine; Toll-like receptors; antimicrobial proteins; innate lymphoid cells; dendritic cells; microbiota

Introduction

The lumen of the intestinal tract is residence to a diverse population of 100 trillion microbes. Microbial molecules, such as β -glucans, flagellin, and polysaccharide A (PSA), are shed by the intestinal microbial flora and detected by the host via innate immune receptors expressed by immune and non-immune cells of the intestinal mucosa. This interaction has been shown to be important for the healthy development of the mucosal immune system and the maintenance of mucosal barrier function (1–5). The interaction is complex and tightly regulated to maintain a healthy inflammatory tone and distinguish commensal microbes

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from pathogenic organisms. Many infectious pathogens gain entry to the body through the gastrointestinal tract; therefore, surveillance and rapid innate immune response mediated by innate immune receptors is critical for early recognition and response to invasion across the intestinal epithelial barrier.

Once the intestinal epithelial barrier is breached, a rapid and appropriate innate immune response followed by a pathogen-specific, individually tailored adaptive response is required to control an enteric infection. However, inappropriately vigorous innate immune responses can result in severe tissue damage, which impairs timely reconstitution of the intestinal epithelial barrier and thus results in increased exposure to products of the microbiota, ultimately prolonging intestinal inflammation. Sensing of microbial molecules by innate immune receptors mediates these processes during periods of homeostasis and inflammation. Herein, we review how detection of microbial molecules positively and negatively influences mucosal defenses and the consequences of alterations in innate immune signaling on susceptibility to enteric infections.

Signaling pathways activated by microbial sensing

Innate immune receptors important for defense against intestinal pathogens include Toll-like receptors (TLRs), Nod-like receptors (NLRs), and C-type lectin receptors (CLRs) (6). Activation of these receptors leads to the upregulation of antimicrobial factors, secretion of cytokines and chemokines, and conditioning of immune cells for direct antimicrobial action or instruction of adaptive immune responses. The response to TLR, NLR, or CLR engagement is dictated in part by the signaling cascades triggered by the binding of a relevant microbial molecule. In this section, we highlight innate immune signaling pathways important for defense in the intestinal mucosa.

TLRs are evolutionarily conserved microbial sensing receptors that are able to detect microbial lipids, proteins, and nucleic acids (6, 7). The murine genome encodes 10 functional TLRs, and the human genome encodes 12. TLRs are either located on the cell surface or within endosomes. The structure of TLRs include N-terminal leucine-rich repeat (LRR) domains, a transmembrane portion, and a cytoplasmic, C-terminal TIR [Toll/interleukin-1 receptor (IL-1R)] homology domain. TLR ligation recruits TIR-containing adapter proteins to initiate the signaling cascade. Adapter proteins specific for certain TLRs roughly dictate the type of response that is generated based on differential transcriptional activation (8, 9). Myeloid differentiation factor 88 (MyD88) is an adapter protein that is utilized by all TLRs except TLR3 (10). TLR2 and TLR4 use TIR domain-containing adapter protein (TI-RAP)/MyD88-adapter-like protein (MAL) as a bridging protein between MyD88 and the TIR domain on the TLR (11). MyD88-independent TLR signaling occurs in TLR3 and TLR4 signaling pathways through TIR domain-containing adapter inducing IFN β (TRIF) (12). In addition, TRIF-related adapter molecule (TRAM) serves as a bridging protein for the interaction of TLR4 and TRIF (13).

In the case of TLRs located on the plasma membrane, which include TLR1, TLR2, TLR4, TLR5, and TLR11, transcriptional activation of pro-inflammatory cytokines, chemokines, and antimicrobial proteins is mediated by nuclear factor- κ B (NF- κ B) and mitogen-activated

protein kinase (MAPK) pathways through initial activation of MyD88 (7). Receptor engagement induces MyD88 to activate IL-1R associated kinase-4 (IRAK-4), which in turn activates IRAK-1 and IRAK-2 (14). These IRAK members activate the E3 ubiquitin protein ligase TNFR-associated factor 6 (TRAF6). TRAF6 associates with other members of an E2 ubiquitin-conjugating enzyme complex. Through a series of ubiquitination steps, transforming growth factor- β (TGF- β)-activated kinase 1 (TAK1) complexed with TAK1-binding proteins activates inhibitor of NF- κ B (I κ B) kinase (IKK) complex and MAPK pathway (15). The IKK complex along with NF- κ B essential modulator (NEMO) phosphorylates and ubiquitinates the NF- κ B inhibitor I κ B α , marking it for degradation. NF- κ B is released from I κ B α and translocates to the nucleus to initiate the expression of pro-inflammatory cytokines (16). The MAPK pathway activates the transcription factor activator protein-1 (AP-1), which is also responsible for the expression of the pro-inflammatory cytokines.

TLR7, TLR8, and TLR9 sense nucleic acids from bacteria and viruses within the endosome. Transcriptional activation of type I interferons is the primary outcome of engagement of these TLRs, although NF- κ B is also activated. This signaling pathway differs from the pathway initiated by MyD88-dependent TLRs located on the cell surface as TLR7, TLR8, and TLR9 activate interferon response element 7 (IRF7) or IRF1 via IRAK1 and TRAF6 (17, 18). In addition, TRAF6 activates NF- κ B and induces the transcription of pro-inflammatory cytokines.

TLR3 and TLR4 also induce a type I interferon response, but in a way that is independent of MyD88 and instead dependent on TRIF (7). Receptor engagement of TLR3 or TLR4 results in the activation of TRIF, which interacts with TRAF3, TRAF6, and receptor-interacting protein 1 (RIP1). TRAF3 activates TRAF-family member associated NF- κ B activator binding kinase 1 (TBK1) leading to the phosphorylation of IRF3 and IRF7, which translocate to the nucleus and mediate the expression of type I interferons (19, 20). NF- κ B is activated in this pathway by RIP1 (21).

Nucleotide-oligomerization domain (NOD)-like receptors (NLRs) are a major class of innate immune receptors demonstrated to have a role in intestinal homeostasis and defense against intestinal pathogens (22). NLRs reside in the cytosol, where they encounter microbial products that have entered the cell cytoplasm by direct infection with intracellular bacteria, such as *Listeria monocytogenes*, or by cytoplasmic injection by extracellular or vacuolar bacteria expressing type III or IV secretion systems. NLR ligands may also enter the cytosol from the extracellular environment through active transport or pathogen-induced pores (23). The general structure of the majority of microbe-sensing NLRs includes a central nucleotide-binding domain (NBD), C-terminal LRR domains, and protein-binding motifs involved in initiating downstream signaling cascades. Among the protein-binding motifs that are present in NLRs, caspase activation and recruitment domains (CARDs) or pyrin domains (PYDs) are important mediators of innate immune defense against pathogens through their ability either to stimulate the expression of NF- κ B- and MAPK-mediated innate immune effectors or to activate inflammasome assembly and IL-1 β or IL-18 production.

NOD1 and NOD2 belong to a subfamily of NLRs that have a CARD domain. The LRR region of NOD1 and NOD2 detects peptidoglycan (PGN) components of the bacterial cell wall (24). NOD1, which is primarily expressed by stromal and epithelial cells, senses g-D-glutamyl-mesodiaminopimelic acid (iE-DAP) a component of primarily Gram-negative bacteria (25). In contrast, NOD2 is predominately expressed by myeloid cells, including dendritic cells (DCs), monocytes, and neutrophils and binds muramyl dipeptide (MDP), which is a common component of all PGN. Bacteria release these products during cell wall synthesis, which is a continuous process throughout cell division and growth. Engagement of NOD1 and NOD2 receptors with their cognate ligands results in polyubiquitination of receptor-interacting protein 2 (RIP2) by the E3 ligase activity of cellular inhibitor of apoptosis protein 1 (cIAP1) and cIAP2 (26). The ubiquitinated region serves as a scaffold upon which TAK1 is able to bind and subsequently to activate the downstream proteins leading to the induction of NF- κ B- and MAPK-mediated gene expression.

In contrast to NOD1 and NOD2 proteins, which activate NF- κ B, inflammasome-associated NLRs are responsible for the maturation of IL-1 β and IL-18 to active forms (22, 27, 28). Inflammasome activation requires a signaling pathway distinct from NOD proteins that involves the induction of caspases or cysteine proteases that cleave inactive pro-forms of the IL-1 β and IL-18. Inflammasome activation involves two signals. The first signal induces the expression of inflammasome components, including inactive zymogen forms of the protein-cleaving enzymes, pro-IL1 β , and pro-IL-18. TLR stimulation or engagement of other innate immune receptors that trigger NF- κ B activation often provide signal I. The second signal is provided by the activation of NLRs by interaction with a relevant microbial molecule. NLR activation induces inflammasome assembly through the homophilic interactions of the CARD or PYD domains on NLRs, an adapter protein called apoptosis-associated speck-like protein containing a CARD (ASC), and pro-caspase-1 (29–31). Autoproteolysis results in the formation of active caspase-1, which then enzymatically processes pro-IL-1 β and pro-IL-18 to generate bioactive forms.

How inflammasome-forming NLR proteins sense microbial products remains unclear. For example, NLRP3 triggers inflammasome assembly upon detection of a wide variety of exogenous and endogenous molecules, including MDP, uric acid crystals, and bacterial RNA; however, the structural diversity of these molecules suggests that their interaction with NLRs is not direct but instead involves intermediate steps (27).

C-type lectins receptors (CLRs) are a large family of both soluble and transmembrane proteins that are defined by a highly conserved C-type lectin-like domain (CTLN) (32). The function of these proteins varies widely from microbial sensing to platelet and complement activation. CLRs that function as innate immune receptors primarily recognize carbohydrate motifs of fungal or bacterial cell wall components. However, this interaction is not exclusive, and these receptors recognize endogenous ligands. Although the CLRs that recognize microbial molecules play a role in endocytosis and antigen processing, the focus of this review is acute innate responses, hence we devote our attention to signaling pathways that induce cytokines, chemokines, and antimicrobial protein expression involved in that response.

Dectin-1 and -2 are CLRs that stimulate a robust innate immune inflammatory response to carbohydrate components of fungal and bacterial cell walls. Dectin-1 recognizes β -glucans, whereas Dectin-2 recognizes α -mannans (33, 34). Dectin receptors trigger signaling cascades that lead to gene expression through activation of immunoreceptor tyrosine-based activation motifs (ITAMs) located in the cytoplasmic portion or located on an adapter such as Fc γ R recruited upon receptor engagement (35). Phosphorylation of ITAMs by Src family kinases provides a docking site for spleen tyrosine kinase (Syk), the primary adapter protein for CLRs. Phosphorylation of Syk leads to the recruitment of CARD9, Bcl-10, and Malt-1, which together activate the IKK complex (36). As mentioned elsewhere, the IKK complex phosphorylates I κ B α , thereby, releasing NF- κ B to migrate to the nucleus to stimulate gene expression. Dectin receptors also trigger signaling cascades that activate MAPK and phosphoinositol 3-kinase (PI3K) pathways. Activation of the PI3K pathway, which leads to nuclear factor of activated T cells (NFAT)-mediated gene transcription, is a unique feature of Dectin receptor engagement that is different from other innate immune receptors and is responsible for the distinctive response generated by Dectin stimulation (37, 38). In addition, engagement of Dectin receptors stimulates Syk-dependent production of reactive oxygen species (ROS) (39–41). Because the Dectin receptor activation can induce pro-IL-1 β and pro-IL-18 expression, providing signal I in the activation of inflammasome, as well as stimulate ROS generation, Dectin and NLR pathways synergize to enhance inflammasome activation.

Microbial sensing by intestinal epithelial cells

The organization of intestinal tissue is complex with many layers serving distinct functions. Recognition of microbes has been shown to influence the function of epithelial cells, immune cells, and stromal cells that reside in the intestinal mucosa. Intestinal epithelial cells (IECs) form a single-cell layer where each cell is joined to the adjacent cell by tight junctions. Thus, the intestinal epithelium is a selectively permeable environmental barrier that restricts microbes and microbial molecules in the intestinal lumen from gaining access to the host. Cells specialized in mucus secretion (goblet cells), antimicrobial factor production (Paneth cells), and hormone release (enteroendocrine cells) reside in the intestinal epithelial cell layer. The intestinal *lamina propria* is loose connective tissue located below IECs. Immune cells exist throughout the intestinal mucosal layers. Innate immune receptors are expressed by different cell types throughout the intestine; however, the function of microbial sensing in each cell type is different, and thus distinct cell types, often play differential roles in intestinal homeostasis and defense against pathogens. In the following sections, we discuss how constitutive detection of molecules shed from microbiota influences the status of intestinal innate immune defense and how the sensing of microbial products during intestinal infection directs critical innate immune responses against invading pathogens.

As a result of the redundancy in signaling pathways and induction of downstream effector mechanisms, the loss of one type of microbial sensing receptor may not reveal a phenotype under homeostatic conditions or in the setting of infection. Gene targeting to delete master regulators of innate immune response signaling pathways has yielded important information about the role of microbial sensing in intestinal homeostasis and during infection,

particularly when these genes are conditionally deleted from specific cell types. The inability to stimulate NF- κ B-mediated gene transcription in IECs leads to the development of spontaneous colitis that is associated with increased pro-inflammatory cytokines, increased apoptosis in IECs, and ultimately ulceration of the epithelial layer of the colon (42). Intestinal epithelial cell-specific deletion of TAK1, an adapter protein necessary to activate both MAPK and NF- κ B pathways following TLR engagement, results in inflammation in the small and large intestine (43). These studies show that NF- κ B activation in IECs is beneficial to the health of the intestinal mucosa. In contrast, NF- κ B signaling in immune cells is associated with enhanced susceptibility to colitis (44). In IL-10^{-/-} mice that develop spontaneous colitis, MyD88-dependent sensing of commensal microbes in bone marrow-derived cells drives colitis development (45). Deficient inhibition of myeloid cell NF- κ B activation in mice lacking the regulatory protein A20 also results in microbiota-driven colitis (46). These studies demonstrate that IEC-specific NF- κ B activation regulates barrier function of IECs, whereas uncontrolled NF- κ B activation in immune cells under homeostatic conditions drives intestinal inflammation that damages the epithelium. As the loss of regulation of NF- κ B activation in immune cells drives colitis development, it is likely that immune cells constitutively receive signals from the microbiota that must be balanced by negative regulatory mechanisms.

Determining precisely how the detection of microbial products in particular cell types influences acute innate immune responses to intestinal pathogens is challenging and not as straightforward as the role of microbial sensing during intestinal homeostasis. The reason for this is that the particular constellation of critical innate immune defense mechanisms activated during infection is pathogen specific. Some pathogens exploit deficiencies in microbial sensing by epithelial cells, whereas others require the induction of a pro-inflammatory program in IECs to initiate invasion of the intestinal mucosa.

Determining the specificity of TLR expression in the intestine has been limited by the techniques used to detect TLRs, which has left the field with an incomplete understanding of which TLRs are functional and expressed by IECs. Current evidence suggests that TLR expression is regionally and spatially separated in the intestinal epithelium and expression can be altered in the setting of inflammation (47–50). Herein, we refer to the preference of TLRs for either apical or basolateral side of an intestinal epithelial cell as spatial segregation. TLR5 has been shown to be expressed at low levels on the basolateral side of the colonic intestinal epithelium (51, 52). The TLR5 ligand flagellin only stimulates a pro-inflammatory response if exposed to the basolateral side of intact human epithelium (53). Similarly, flagellin exacerbates chemical-induced colitis only if administered prior to disruption of the intestinal barrier, which allows flagellin access to the basolateral side of the epithelium. Thus, the spatial segregation of TLR expression helps to distinguish between commensal and pathogenic microbes.

TLR signaling at the apical surface of IECs stimulates a different transcriptional response than stimulation of the basolateral surface (54). Apical stimulation of TLR9 leads to the ubiquitination, but not degradation of I κ B α . Failure to degrade I κ B α prevents the downstream activation of NF- κ B even upon stimulation of TLR9 from the basolateral surface; therefore, TLR activation at the apical side of IECs dampens further activation of

TLRs at the basolateral surface, thus, serving as a innate immune regulatory mechanism. This finding may, in part, explain why TLR9^{-/-} mice are more susceptible to chemical-induced colitis. IECs are simultaneously exposed to two distinct domains: the internal environment of the host and the external environment of the intestinal lumen. These studies demonstrate that determining whether and how IECs respond to microbial sensing depends on where TLRs are expressed and stimulated.

Production of antimicrobial proteins

The primary response to the detection of microbial molecules by cells in the intestinal mucosa includes the upregulation of antimicrobial factors, the induction of epithelial cell repair mechanisms, and the activation of immune cells. Antimicrobial proteins are an evolutionarily ancient form of defense found in organisms across nature from plants to mammals. Paneth cells are specialized secretory cells located at the base of the intestinal crypts whose primary function is to store and release antimicrobial proteins. IECs also secrete antimicrobial proteins in response to stimulation with microbial products. Regulation of antimicrobial factor expression occurs either directly by recognition of microbial molecules by innate immune receptors expressed by IECs or Paneth cells or indirectly through stimulation by cytokines produced by activated immune cells. Under homeostatic conditions, the secretion of antimicrobial proteins into the intestinal mucus layer prevents bacterial overgrowth, limits the translocation of commensal microbes across the epithelial barrier, and prevents enteroinvasive organisms from entering the *lamina propria* (55, 56).

Studies in our laboratory have demonstrated that MyD88-mediated signaling in non-hematopoietic cells is required for clearance of the Gram-positive intracellular pathogen *L. monocytogenes* from the gut (57). The MyD88-dependent effector molecule that mediates *Listeria* clearance in this model is the C-type lectin RegIII γ . By binding PGN on the bacterial cell wall, RegIII γ can disrupt the cell wall structure and induce cell lysis (58). TLR/MyD88-mediated detection of microbial molecules shed from the microbiota leads to the homeostatic expression of RegIII γ (58, 59) (Fig. 1A). Depletion of the microbiota by antibiotics or a deficiency in TLR signaling in non-hematopoietic cells leads to susceptibility to intestinal infection with *L. monocytogenes*, *Salmonella typhimurium*, and vancomycin-resistant *Enterococcus* (VRE) (57, 60, 61). These studies demonstrate that the interaction of the microbiota with the intestinal epithelium plays a critical role in maintaining barrier defense mechanisms.

Many other antimicrobial proteins are regulated by cues from the microbiota. The signals and pathways that induce antimicrobial protein expression in IECs are overlapping and redundant, hence there are few instances where one innate immune receptor is solely responsible for regulating a particular antimicrobial protein. However, numerous examples exist in which colonization with a single microbe is sufficient to induce the expression of an antimicrobial protein. For instance, monocolonization of germ-free mice with the *Bacteroides thetaiotomicron*, a predominate member of the microbiota, induces systemic release of the microbicidal protein angiogenin-4 by IECs and Paneth cells (62). In humans, oral administration of the probiotic strain of *Escherichia coli* increases the level of human β -

defensin-2 in the feces (63). Previous murine studies suggested that flagellin is the primary bacterial ligand responsible for *E. coli*-induced expression of human β -defensin-2 (64, 65).

Microbial molecules also stimulate degranulation of Paneth cells, which results in the release of stored packets of antimicrobial factors into the intestinal lumen. Alpha-defensins are cationic peptides that disrupt microbial cell membranes and are stored in Paneth cell granules. Alpha-defensin activity is regulated by both microbial-induced degranulation as well as activation by proteolysis. Matrilysin-7 (MMP7) is a metalloprotease that is also induced by the detection of microbial products via innate immune signaling (66). The importance of α -defensins was demonstrated by experiments in which human α -defensin 5 (HD5) was overexpressed in mice. These mice were found to be resistant to enteric *Salmonella* infection and colonization with segmented filamentous bacteria (SFB) (67, 68). Although signaling through TLR2, TLR4, and TLR9 has been shown to stimulate α -defensin secretion and activation, NOD2 appears to be necessary for the regulation of α -defensin activity in vivo (69, 70). NOD2^{-/-} mice have defective α -defensin activity in the gut and, thus, are more susceptible to oral infection with *L. monocytogenes* (71). Transgenic expression of HD5 rescues the impaired innate immune defense found in NOD2^{-/-} mice (72). Interestingly, not only do molecules shed from commensal microbes stimulate antimicrobial protein activity through NOD2-dependent signaling, microbial sensing but also induces NOD2 expression (71). These studies demonstrate that one of the most ancient systems of antimicrobial defense is tightly regulated by signals from the microbiota. In the following sections, we consider how further production of antimicrobial factors are induced by signals from activated immune cells during enteric infections.

Restoration of barrier function

NF- κ B-mediated transcriptional activation of IECs prior to and during spontaneous and chemical-induced colitis reduces epithelial cell death and promotes tissue repair mechanisms (73, 74). Products from the microbiota activate NF- κ B through engagement of innate immune receptors expressed by IECs. Thus, the presence of commensal microbes limits damage to the intestinal epithelial barrier and limits the course of colitis (45, 75). NF- κ B plays a positive role in tissue repair by inducing anti-apoptotic factors, stimulating proliferation, and stabilizing tight junctions (73). Although much of the intestinal mucosal biology field is devoted to studying epithelial repair mechanisms in the setting of colitis, NF- κ B-mediated tissue repair mechanisms likely evolved to defend against invasive enteric pathogens that damage the intestinal epithelial cell layer. Herein, we examine examples in which pathogens have taken advantage of repair mechanisms to establish an infection or in which tissue repair is essential for control of an intestinal infection.

S. typhimurium is a facultative intracellular pathogen that replicates in IECs and macrophages. Interaction of *S. typhimurium* with IECs through a type III secretion system initiates apoptosis in IECs, but anti-apoptotic factors are also induced by flagellin-mediated TLR5 activation and activation of the inflammasome-mediator NLRC4, which ultimately delay cell death by several hours (76). The delay in apoptosis is thought to 'buy time' for the innate immune system to upregulate antimicrobial protein expression and recruit innate immune cells to the site of infection. In fact, TLR5^{-/-} mice develop greater degrees of

intestinal inflammation upon infection with *S. typhimurium* (77). Similarly, *Salmonella* strains in which flagellin has been deleted promote greater intestinal inflammation, and these strains establish a higher burden of infection, which correlates with reduced time to epithelial cell death (76). This correlates with a reduction in time to epithelial cell death. Other pathogens that disrupt the epithelial barrier to establish an infection include *Clostridium difficile*, *Shigella flexneri*, and *Yersinia enterocolitica* (78–80). The anti-apoptotic pathways activated by NF- κ B during these infections serve as a host defense mechanism to restrain invasion of the intestinal mucosa.

Microbial sensing by other cells in the intestinal mucosa helps to regulate tissue repair and restoration. Mesenchymal stem cells (MSCs) located in the *lamina propria* produce high levels of cyclooxygenase-2, an enzyme involved in the production of prostaglandin E2, which promotes IEC survival and proliferation (81). In response to detection of microbial molecules via TLR/MyD88-mediated signaling pathways, MSCs migrate to the base of intestinal crypts where production of prostaglandin E2 adjacent to the IEC stem cell niche leads to resistance of the intestinal epithelial layer to damage during the development of colitis (81, 82). Thus, the microbiota not only influences the maintenance of intestinal barrier function during steady-state conditions but also activates tissue repair programs upon damage to the epithelium that are critical for the resolution of disease.

Microbial sensing by intestinal immune cells

In certain circumstances, TLR signaling in DCs stimulates early activation of the IL-12/IFN γ axis, a requirement for survival from particular infections (83). In other instances, NF- κ B activation of IECs is required to condition DCs to stimulate crucial Th2 responses to other pathogens (84). Much of these requirements depend on the pathogen and the immune responses necessary for control of the infection. The complex network of negative and positive feedback mechanisms coupled with careful segregation and compartmentalization of responses to microbial sensing helps to maintain intestinal homeostasis yet allows the system to remain poised to respond to an invasive pathogen. Herein, we review how sensing of microbial molecules by innate immune cells coordinates effective responses against intestinal pathogens.

DCs

DCs and other mononuclear phagocytes densely populate the intestinal *lamina propria* forming an extensive microbe-sensing network (85, 86). DCs abundantly express a diverse repertoire of TLRs in the intestinal mucosa compared with other cellular residents, particularly in the small intestine (87–91). Activation of innate immune signaling in DCs stimulates the secretion of cytokines and chemokines and induces DC migration to the mesenteric lymph nodes. Local microbe-induced cytokine secretion by DCs serves to stimulate and coordinate the antimicrobial activity of tissue-resident cells and also DCs migrate to the MLNs to initiate adaptive responses. Herein, we focus on the ability of DCs to orchestrating acute innate immune responses, as the role of innate immune signaling in adaptive immunity has been reviewed elsewhere (92, 93).

Diverse populations of DCs and other mononuclear phagocytes with divergent origins and functions reside in the *lamina propria*. These include CD103⁺ DCs, CX3CR1⁺ monocyte-derived DCs, and Ly6C^{hi} monocytes (94, 95). CD103⁺ DCs and CX3CR1⁺ monocyte-derived DCs have distinct functions and arise from two separate myeloid lineages (86, 96). The common DC precursor (cDP) gives rise to CD103⁺ DCs through a Flt3L-dependent process. Circulating Ly6C^{hi} monocytes give rise to CX3CR1⁺ mononuclear phagocytes, which require macrophage colony-stimulating factor (M-CSF) for development. These DC subsets express unique sets of TLRs, which serves to inform their particular function in innate immune defense (88, 89, 97). CD103⁺ DCs are considered tolerogenic and preferentially induce T-regulatory (Treg) cell development by producing high levels of retinoic acid and TGFβ; however, upon sensing of microbes through TLR/MyD88 activation, CD103⁺ DCs migrate to the mesenteric lymph nodes to prime naive T cells (98–100). In contrast, CX3CR1⁺ monocyte-derived DCs have the ability to extend dendrites through IEC tight junctions to sample the luminal contents by a mechanism that requires MyD88 expression in non-hematopoietic cells (101). The purpose of this activity is not entirely clear, but recent evidence suggests that CX3CR1⁺ monocyte-derived DCs are responsible for inducing the proliferation and directing the suppressive activity of *lamina propria* Tregs, which have previously been activated but have migrated to the *lamina propria* (102). In addition, CX3CR1⁺ monocyte-derived DCs are responsible for the production of the colitogenic cytokines IL-23 and tumor necrosis factor-α (TNFα) in the setting of chronic inflammation (86, 103).

Homeostatic sampling of the luminal contents leads to the uptake and transport of commensal bacteria by CD103⁺ DCs to the mesenteric lymph nodes, where they help to generate commensal-specific IgA responses (104–106). This process facilitates the generation and secretion of IgA into the intestinal mucus layer, thereby, fortifying the first layer of defense against bacteria that attempt to cross the epithelial barrier. The absence of commensal bacteria in germ-free mice and antibiotic-treated mice leads to the downregulation of IgA secretion, ultimately contributing to the overall defect in innate immune defense observed under these conditions (5, 107).

Enteroinvasive pathogens undermine the physiologic function of luminal sampling to gain access to the internal environment of the host. *S. typhimurium* has highly evolved mechanisms to subvert the host immune response. Non-virulent strains of *Salmonella* enter the *lamina propria* when transepithelial processes from CX3CR1⁺ monocyte-derived DCs encounter and subsequently phagocytose *Salmonella* during physiologic luminal sampling (101, 108–111). This enables *S. typhimurium* to establish a systemic infection. Virulent strains of *Salmonella* invade IECs and are taken up by CD103⁺ DCs upon crossing into the *lamina propria*. CD103⁺ DCs transport *Salmonella* to the MLNs, also facilitating systemic dissemination. Transport of *Salmonella* to the MLNs by CD103⁺ DCs requires TLR5-mediated detection of flagellin. Therefore, TLR5^{−/−} mice are resistant to disseminated *Salmonella* infection (87).

TLR4- and TLR2-deficient animals are more susceptible to intestinal infection with *Salmonella* (112); however, further deletion of TLR signaling inhibits replication of *Salmonella* within phagocytes (113). TLR signaling stimulates the acidification of bacteria-

containing phagolysosomes, but the *Salmonella* type III secretion systems (TTSS) are activated upon a decrease in pH. The TTSS injects virulence factors into the host cell cytoplasm that inhibit further maturation of the phagolysosome, thus creating a compartment capable of supporting bacterial replication (113). Failure to activate antimicrobial defense by way of TLR signaling leads to susceptibility to *Salmonella* infection; however, in the absence of sufficient TLR activation, *Salmonella* is unable to establish an infection. These findings reveal the intricacies of highly evolved host-pathogen interactions in which innate immune responses, despite being critical for control of a pathogen, are also required for pathogen virulence.

Monocytes

Under normal conditions, Ly6C^{hi} monocytes contribute a fraction of a percent to the total mononuclear phagocyte population residing in the intestinal *lamina propria* (94); however, large numbers of monocytes are recruited to the intestinal mucosa during inflammation. Monocytes serve a crucial role in defense against intestinal pathogens, but can also propagate pro-inflammatory responses that contribute to the pathogenesis of colitis (86, 103). *Citrobacter rodentium* is a murine pathogen similar to enteropathogenic *E. coli*, which infects humans. Both pathogens have the ability to attach to and subsequently damage the IEC layer. A recent study demonstrated that NOD2^{-/-} mice failed to clear enteric *C. rodentium* infection due to the lack of CCR2-dependent recruitment of inflammatory monocytes to the gut (114). Pathogen clearance was restored upon transfer of bone marrow-derived monocytes into susceptible CCR2^{-/-} mice. The authors conclude that the failure to recruit inflammatory monocytes to the gut in NOD2^{-/-} mice is the result of impaired upregulation of CCL2, a CCR2 ligand, by intestinal stromal cells. These intestinal stromal cells, also called mesenchymal stem cells, have previously been shown to direct the proliferation of IECs in response to MyD88-dependent detection of microbial products (82). Furthermore, a recent study has demonstrated that the TLR/MyD88-dependent egress of bone marrow monocytes involves CCL2 expression by mesenchymal stem cells located adjacent to endothelial cells in the bone marrow (115).

Intestinal infection of mice with the intracellular protozoan parasite *Toxoplasma gondii* causes inflammation of the ileum (ileitis) that eventually leads to intestinal necrosis and death. Inflammatory monocytes play a role in controlling infection and limiting tissue damage in intestinal infection with *Toxoplasma*. Invasion of the intestinal epithelium by *Toxoplasma* results in the recruitment of inflammatory monocytes to the ileum, where the monocytes produce iNOS, TNF α , and IL-12 (116). A robust IL-12/IFN γ response is required to control systemic Toxoplasmosis (117–120). Currently, it is unknown if microbial products shed from *Toxoplasma* or the microbiota induce iNOS, TNF α , and IL-12 through TLR/MyD88 signaling pathways or other microbial sensing mechanisms. While there is a demonstrated role for inflammatory monocytes in the defense against intestinal pathogens, more research is needed to define their precise contributions to restoration of damaged intestinal epithelium and host defense.

Neutrophils

Neutrophils are a critical component of the early innate immune response to intestinal pathogens. They serve a primary function in innate immune defense by phagocytosing microbes and producing microbicidal factors. Neutrophil depletion or blockade of neutrophil recruitment leads to increased susceptibility to enteric infections (121, 122). Neutrophil-recruiting chemokines, which include CXCL1 and CXCL2, are secreted by both hematopoietic and non-hematopoietic cells in response to the detection of microbial molecules via TLRs or NOD receptors (121, 123, 124). A recent study demonstrated that NOD1 ligands shed from the microbiota influence systemic immunity by positively influencing the activation state of neutrophils located in the bone marrow (125). Under steady-state conditions, neutrophils harvested from mice in which PGN fragments are detectable in the serum have an enhanced ability to kill bacteria. In the setting of antibiotic-induced depletion of the microbial flora, detectable levels of NOD1 ligands in the serum are reduced and, thus, neutrophils exhibit impaired bactericidal activity. Systemic administration of NOD1 ligands restores the activity of neutrophils and enhances resistance to bacterial sepsis. This study demonstrates that commensal microbes influence the innate immune system at distal sites by the detection of microbial molecules released into systemic circulation under physiologic conditions. The role of NOD signaling during acute intestinal infection was recently demonstrated by a study that showed reduced *C. difficile*-induced mortality in mice lacking NOD1 (126). Despite similar levels of epithelial damage compared with wildtype mice, NOD1^{-/-} mice exhibited impaired clearance of *C. difficile*, reduced neutrophil recruitment, and increased dissemination of intestinal bacteria. Impaired neutrophil recruitment is partly attributed to reduced chemokine production during infection. Thus, microbiota-induced NOD signaling regulates the antimicrobial activity of neutrophils under homeostatic conditions; however, NOD signaling is also a critical pathway involved in activating acute neutrophil responses during infection with enteroinvasive pathogens.

T cells

One of the most extensively described relationships between direct TLR signaling and immune cell function is the influence of TLR2 stimulation on T-regulatory cells that reside in the colonic *lamina propria*. Monocolonization with strains of *Bacteroides fragilis* that either express or lack PSA demonstrate that PSA is the critical microbial factor that leads to the direct induction of FoxP3⁺ IL-10-producing Tregs (127, 128). These Tregs play a critical role in limiting inflammation due to infection with colitogenic *Helicobacter hepaticus* (129). The absence of TLR2-induced Tregs in the colonic *lamina propria* leads to an abundance of pro-inflammatory cell populations, including Th1 and Th17 cells. Recently, the Mazmanian laboratory (130) presented evidence that PSA expression by *Bacteroides fragilis* evolved to limit IL-17 levels in the colon, which enables this particular commensal microbe to colonize a niche close to the intestinal epithelium, thereby defining a truly mutualistic relationship with the host. These studies suggest that by altering *lamina propria* T-cell populations, *Bacteroides fragilis* influences the homeostatic inflammatory tone of intestinal mucosal tissue and thus affects susceptibility to enteric infections or colitis.

B cells and IgA production

Because the focus of our review is the regulation of early innate immune responses by detection of microbial products, we limit our discussion here to how the microbiota directly influences IgA production and the impact on susceptibility to intestinal infection.

Remarkably, humans and mice deficient in TLR/MyD88 signaling pathways do not succumb to the frequent translocation of bacteria into the blood stream that occurs when there is diminished antimicrobial protein production, reduced mucus secretion, and an impaired ability to detect enteroinvasive pathogens (61, 131). The reason for this is that the host compensates for deficient TLR signaling by maintaining high antibody titers against commensal bacteria (131). Thus, innate and adaptive responses are finely tuned to tolerate and contain the commensal microbes within the intestinal lumen.

IgA translocates across the IEC layer toward the lumen and is deposited in the intestinal mucus layer. The presence of high concentrations of IgA in the mucus layer restricts commensal microbes from gaining entry to the *lamina propria*. B-cell activation and class-switch recombination occurs in different anatomic sites throughout the small intestine. T cell-dependent class switching involving cognate antigen interactions between B and T cells accompanied by costimulation results in the generation of *lamina propria* B cells that manufacture high-affinity IgA (132). The alternative pathway involves TLR activation of follicular DCs, *lamina propria* DC, epithelial cells, and stromal cells, which secrete soluble IgA-inducing signals (133–136). These factors, include a proliferation-inducing ligand (APRIL), B-cell activating factor belonging to the TNF family (BAFF), and TGF β .

Activation of T-cell-independent class switching also requires direct TLR engagement of B cells in addition to stimulation with BAFF and APRIL (137).

IgA class switching is primarily restricted to the mucosal compartment. The reason for this is that DCs are conditioned by the tissue microenvironment to specifically induce IgA responses (107, 138). Thymic stromal lymphopoietin (TSLP) is an IEC-derived factor that is produced in response to signals from the microbiota. TSLP promotes Th2 responses by preventing DCs from secreting IL-12 and instead inducing IL-10, BAFF, and APRIL expression (139). The importance of NF- κ B-induced TSLP expression was demonstrated in a recent study showing that the inability to activate NF- κ B in IECs leads to reduced secretion of TSLP in response to intestinal infection with the helminth pathogen *Trichuris muris* (84). Reduced TSLP levels in the *lamina propria* result in impaired Th2 responses, which are required to efficiently clear *T. muris* from the gut. Intestinal DCs conditioned by the intestinal microenvironment, in particular CD103⁺ DCs, generate high levels of retinoic acid, which, in turn, induces the expression of the gut-homing receptor CCR9 on B cells, a process that can be induced by flagellin (88). The regulation of IgA class switching in the intestinal mucosa is a prime example of how cues from the microbiota or pathogenic microbes are detected by a diverse repertoire of cell types that respond in a coordinated and specific way to maintain tissue homeostasis.

Innate lymphoid cells

Innate lymphoid cells (ILCs) are a recently described mucosal cell type that share characteristics with lymphoid tissue inducer cells (LTi) and natural killer (NK) cells. Like

LTi cells, ILCs require the transcription factor ROR γ t, the transcriptional repressor Id2, and common γ chain cytokines IL-7 and IL-2 for development. ILCs do not have direct cytotoxic activity like classical NK cells, but do express NK cell markers, most notably in mice NKp46. ILCs reside in the intestinal *lamina propria* primarily in association with DCs within isolated lymphoid follicles or cryptopatches. Research up to this point has revealed that the primary function of ILCs in the mucosa is the secretion cytokines important for host defense and tissue repair.

Several studies report that cues from the microbiota are required for development of ILCs, in particular the ability to secrete IL-22 (140, 141). Under steady-state conditions, signals from the microbiota induce IL-7 expression in the small intestine (142). IL-7, in turn, stabilizes the expression of ROR γ t and induces the constitutive expression of IL-22. How commensal microbes stimulate the expression IL-7 is currently unknown. It is also not clear whether IL-7 is required for acute upregulation of IL-22, one of the primary effector functions of ILCs. The role of the microbiota in ILC development may be restricted to the small intestine, as signals from the microbiota have been shown to inhibit ILC development and IL-22 production in the large intestine (143).

While signals from the microbiota regulate constitutive expression of IL-22 in the small intestine, systemic administration of TLR ligands leads to the dramatic upregulation of IL-22 expression by ILCs (144, 145). This process appears to be dependent on TLR signaling in DCs; however, how DCs activate ILCs is currently controversial (145, 146). In humans, IL-23 produced by TLR-activated monocyte-derived DCs triggers IL-22 expression in ILCs (147). In contrast, intrinsic TLR signaling in ILCs was shown to induce the secretion of IL-2, IL-13, and IL-22 via NF- κ B activation (148). The origin, developmental requirements, and regulation of the effector function of ILCs are currently unclear and remain active areas of research. In later sections, we discuss the known cytokine networks involved in the acute expression of IL-22 by ILCs.

Cytokines coordinate antimicrobial activity

An effective host response to an enteroinvasive pathogen requires rapid mobilization of innate immune defenses. This process is typically initiated by the detection of microbe-derived molecules in the context of tissue damage. Resident DCs are likely among the first to detect invasion of intestinal parenchyma due to the vast network of dendrites spread throughout the *lamina propria*. Certain subsets of *lamina propria* DCs respond to microbe-derived molecules by rapidly secreting pro-inflammatory cytokines TNF- α , IL-6, IL-1 β , IL-12, IL-23, and type I interferons. These cytokines direct the first stage of response to intestinal infection, which includes stimulating antimicrobial effector expression and recruiting and activating immune cells. Herein, we review two response axes that have been primarily recognized for their role in inducing polarized adaptive immune responses but are now being appreciated for their critical role in early innate immune defense.

IL-12/IFN- γ axis in innate immunity

The detection of microbial products by DCs is a critical step in the initiation of early IFN- γ responses and is critical for control of the acute phase of infection with intracellular

pathogens (149). The primary effector function of IFN- γ at this early stage is to activate macrophages and inflammatory monocytes to kill phagocytosed microbes. Specific deletion of MyD88 in DCs has demonstrated that TLR/MyD88 signaling is required for the immediate secretion of IL-12 (150). IL-12 directly activates NK cells to secrete IFN- γ . This cytokine response has been shown to be critical for controlling systemic infections with *T. gondii*, *L. monocytogenes*, and *Entamoeba histolytica* (149–151). During natural infection, these pathogens enter the host by ingestion and subsequently invade the intestinal epithelial layer to disseminate to other tissues. Currently, the innate immune role of IL-12/IFN- γ production by DCs has been demonstrated in systemic infections, and few studies on the role of this pathway in limiting pathogen translocation across the intestinal epithelium have been performed. Guo *et al.* (152) have demonstrated that IFN- γ is required for the expulsion *E. histolytica* from the gastrointestinal tract. Others have shown that in the absence of TLR11, the primary innate immune receptor for detection of *T. gondii*, TLR2 and TLR4 activation by the microbiota drives the generation of a protective IL-12/IFN- γ response during enteric infection with *T. gondii* (153). As described previously, pathogens take advantage of the antigen sampling ability of CX3CR1⁺ monocyte-derived DCs and CD103⁺ DCs to gain access to the *lamina propria* (154). It is likely that DCs first encounter pathogens during the physiologic process of antigen sampling. We speculate that this is an important step to initiate an innate immune response early in infection. The type of innate immune response induced depends on the constellation of innate immune receptors that are engaged and the DCs subsets that are activated (91, 97, 155).

IL-23/IL-22 axis in innate immunity

Although the precise role of IFN- γ in innate immune defense against intestinal pathogens remains unclear, numerous studies have demonstrated the protective activity of IL-23 and IL-22 in the intestinal mucosa (156). Primarily expressed by lymphocytes but intended to target epithelial cells, IL-22 serves as a communication line between the immune system and the epithelial cells in contact with the outside world. The expression of the IL-22 receptor is restricted to epithelial and stromal cells (157). Although IL-22 can be expressed by $\gamma\delta$ T cells and Th17 cells, the intestinal source of IL-22 is largely Id2-dependent, ROR γ t-dependent ILCs (140, 141, 158). Activation of the IL-22 signaling cascade occurs through the Janus kinase (JAK)/signal transducer and activator of transcription (STAT) pathway, which leads to the phosphorylation of STAT1, STAT3, and STAT5 (156). The major tissue protective and antimicrobial effector response induced by IL-22 is primarily a result of STAT3-mediated gene transcription (159). STAT3 activation leads to the induction of antimicrobial proteins (RegIII γ , S100 proteins, and β -defensins), anti-apoptotic survival factors, and mitogenic factors which promote tissue repair (156).

The Ouyang laboratory (160) was among the first groups to discover that IL-22 plays a critical role in innate immune defense against intestinal pathogens. They found that mice deficient in IL-22 succumbed to intestinal infection with *C. rodentium*. Compared with wildtype mice, IL-22^{-/-} mice had a higher burden of bacteria in distant organs despite similar bacterial counts in the colon. This correlated with a high degree of inflammatory pathology in the colon, indicating that the inability to express IL-22 during intestinal infection led to the loss of barrier function and subsequently, to the unregulated

translocation of *C. rodentium* into the *lamina propria*. The susceptibility in IL-22^{-/-} mice was attributed to the inability to upregulate antimicrobial proteins RegIII γ and Reg-III β . Exogenous administration of the RegIII γ to IL-22^{-/-} mice restored protection against *C. rodentium*. In this infection model, induction of IL-22 required IL-23 expression but occurred independently of adaptive immune cells (160). The innate immune source of IL-22 was later determined to be IL-23R-expressing ILCs (158). ILCs were found to be essential for protection against *C. rodentium* infection, as specific depletion of IL-22-expressing ILCs cells led to early mortality, whereas transfer of IL-22^{+/+} ILCs but not IL-22^{-/-} ILCs led to protection against death. The source of IL-23 and signals required for IL-23 expression during *C. rodentium* infection are currently unknown.

DCs are widely recognized as primary producers of IL-12 and IL-23. Previous in vitro experiments demonstrated that TLR and CLR ligands stimulate DCs to produce IL-23, which in turn induces IL-22 secretion by ILCs (144, 147). In particular, systemic administration of flagellin induces rapid secretion of IL-22 by ILCs through a mechanism that requires DCs (145, 146). IL-23 also regulates protective IL-22 responses in a variety of other mucosal infections. Oral inoculation with *Candida albicans* leads to colonization of the gastrointestinal tract and infection of the kidneys. IL-23-mediated IL-22 expression protects mice against early dissemination of *Candida* and reduces inflammatory damage to the intestinal wall (161). *Candida*-derived β -glucans interact with dectin-1 expressed by DCs resulting in the activation of Syk-Card9 signaling pathways and preferential secretion of IL-23 (162). IL-23/IL-22-mediated effector responses are also implicated in defense against *S. typhimurium* and *Klebsiella pneumonia* (163–165).

Together these studies suggest the following sequence of events during the first moments that the epithelial barrier is breached. Upon damage to the intestinal epithelium, DCs immediately sense microbial products and respond by secreting IL-23. ILCs produce IL-22 in response to activation by IL-23. IL-22 signals the epithelium to secrete antimicrobial factors and induce tissue repair mechanisms. Although extensive research has been carried out to elucidate the downstream effects of IL-22, direct evidence for MyD88-mediated IL-23 expression by DCs that drives rapid IL-22 secretion is currently lacking. In addition, the identity of the intestinal DC population that detects enteroinvasive pathogens and responds by inducing the expression of IL-22 is not known.

IL-23 was initially recognized as a maturation factor for Th17 cells. With the discovery of ILCs as critical mediators of innate immune defense and their functional requirement for IL-23, it is now apparent that IL-23 serves an important role directing a more evolutionarily ancient form of host defense than adaptive immunity. In fact, the ability of IL-23 to influence Th17 development may be a recycling of IL-23 signaling originally evolved to create specialized innate immune responses. While IL-22, for the most part, is protective during colitis, IL-23 has been shown to have a key role in driving chronic inflammation of the gut by inducing both IFN- γ -producing ILCs and T cells (166, 167). Thus, IL-23 is an important cytokine in innate immune defense of the gut, but dysregulation of IL-23 expression results in uncontrolled inflammation. In the future, careful study of IL-23-dependent factors involved in protection against intestinal infections and how these factors

are regulated to avoid the development of colitis are needed to determine how to therapeutically manipulate this pathway to prevent infection or inflammation.

Innate immune signaling shapes the microbiome

The intestinal microbiota has been shown to influence immune responses and lead to the development of disease (168–171). Conversely, innate immune signaling has recently been shown to alter the composition of the microbiota, which consequently influences the development of disease (172–174). Herein, we review the innate immune signaling pathways that have a role shaping the microbiome, and how in the absence of these pathways, colitogenic changes to the microbiome occur.

Although T-bet has been shown to be a master regulator of Th1 development and essential factor for IFN- γ expression (175, 176), Garrett *et al.* (173, 177) discovered an important regulatory role for T-bet in limiting intestinal inflammation by activating negative feedback pathways in DCs. This study demonstrated that in the absence of both T-bet and adaptive immunity, *lamina propria* DCs secreted high levels of TNF- α in response to cues from the microbiota. In this mouse model, designated the TRUC mouse (T-bet^{-/-}RAG2^{-/-}), TNF- α drives epithelial barrier dysfunction and tissue inflammation resulting in spontaneous colitis. Interestingly, the colitis phenotype observed in TRUC mice can be transmitted to non-TRUC mice by exchange of the microbial flora. However, high levels of TNF- α were only found in TRUC mice and not cross-fostered colitic mice of various genotypes. Members of the Enterobacteriaceae family, specifically *K. pneumoniae* and *Proteus mirabilis*, were discovered as necessary but not sufficient components of the colitogenic microflora (178). In TRUC mice, blockade of TNF- α signaling inhibits colitis development and leads to a reduction intestinal colonization of *K. pneumoniae* and *P. mirabilis*; however, Treg transfer to TRUC mice alleviates colitis but induces no change in colonization levels of *K. pneumoniae* and *P. mirabilis*. Thus, a central unanswered question in the TRUC mouse model is how aberrant TNF- α expression changes the intestinal microenvironment in way that supports colonization with colitogenic microbes.

Recently, the inflammasome was shown to play a role in shaping the microbiota (172). The role of the inflammasome in protection against colitis had been previously described for the cytosolic sensor NLRP3 and was attributed to the ability of the inflammasome to activate repair of the intestinal epithelial cell barrier. Although the precise role of the inflammasome has been difficult to sort out, because, like MyD88-mediated signaling, IL-1 β and IL-18, which are products of the inflammasome, induce opposing responses in epithelial cells compared with immune cells. Elinav *et al.* (172) were the first to show that impaired inflammasome activation via NLRP6 leads to the development of a transmittable colitogenic microbial flora. Mice with different genetic deletions in aspects of NLRP6-mediated inflammasome pathway independently developed a transferable colitogenic microbial flora, which was primarily composed of bacteria of the Prevotellaceae family. In the absence of IL-18, these microbes are better able to colonize the gut and initiate intestinal inflammation, which required CCR5 for the recruitment of immune cells.

These studies suggest that the intestinal immune system suppresses or promotes the growth of colitogenic organisms. Certain enteroinvasive pathogens exploit this relationship and, as a mechanism of pathogenesis, these pathogens promote intestinal inflammation to create a more favorable environment for growth. For example, *S. typhimurium* destroys IECs and thus causes severe inflammation. It is thought that inflammation works in favor of *Salmonella* by leading to the upregulation of antimicrobial factors that eliminates certain microbes from the intestinal lumen leaving less microbial competitors (179–181). *Salmonella* has evolved mechanisms to resist host defenses that are activated during inflammation, whereas the commensal microbes are more likely to succumb to the onslaught of antimicrobial processes.

With the advent of genome-wide association studies, hundreds of genes associated with IBD have been identified, but heritability can only account for 20–60% IBD cases (182). Because IBD has a significant environmental component, it is possible that changes to the activity of intestinal innate immune defense mechanisms in IBD patients alter the microbiome to one that is more colitogenic. It is also possible that pathogenic microbes initiate inflammation in the gut and that reaction is propagated by signals from the microbial flora in the setting of impaired negative regulatory mechanisms.

Manipulating innate immune responses to prevent infections with intestinal microbes

At the mucosal interface where the host and intestinal microbiome interact, a constant struggle occurs to maintain tolerance to commensal microbes yet also maintain a state of readiness in case of breakdown of the intestinal epithelial barrier. Under normal conditions, signaling pathways activated by molecular products shed by commensal microbes helps to establish a balance between these competing functions by providing negative and positive regulatory signals to the intestinal innate immune system. Inflammatory bowel disease and increased susceptibility to enteric infections occurs when this balance is disrupted. Herein, we review therapeutic strategies that may be used to manipulate innate immune signaling to restore intestinal homeostasis and mucosal defenses.

Several enteroinvasive pathogens cause damage to the intestinal mucosa through mechanisms that have been found to be involved in the pathogenesis of inflammatory bowel disease (183). For this reason, colitogenic bacteria such as *Helicobacter hepaticus* are often used as experimental colitis models. Infections that occur in the gut are unique from those that occur at other sites because of the effects of the microbiota on the immune response (184). In fact, for many pathogens, the host defense mechanisms important for control of the infection are different depending on whether the infection site is the intestinal mucosa or the bloodstream. In some cases, detection of products shed from commensal microbes via innate immune signaling may act as an adjuvant to promote the development of pathogen-specific responses (153). However, stimulation of pro-inflammatory responses by microbial products from the microbiota may also drive the destruction of the intestinal mucosa. A potential therapeutic strategy to prevent inflammatory disease is to stimulate negative regulatory pathways to keep inflammation in check. As described in a previous section in this review, the common commensal microbe *Bacteroides fragilis* produces a symbiotic factor that

stimulates IL-10 production in Tregs (128, 129). This molecule is the TLR2 ligand PSA, a component of the *B. fragilis* capsule. Prophylactic oral administration of PSA or colonization with PSA⁺ *B. fragilis* prevents gut pathology associated with *H. hepaticus*-induced experimental colitis.

Alternatively, antibiotic treatment disrupts the balance of Treg and effector T-cell (Teff) populations in the intestinal *lamina propria*, leading to susceptibility to certain enteric infections (185). Treg/Teff equilibrium is restored by activation of DCs upon oral administration of TLR9 ligands to antibiotic-treated mice. Thus, innate immune signaling regulates the balance of pro-inflammatory and anti-inflammatory responses in the gut, and disruption of this process by colitogenic bacteria or depletion of commensal bacteria leads to disease. Future study should focus on the mechanism by which Tregs influence mucosal defenses and, in particular, on their interaction with DCs and the expression of the key colitogenic cytokine IL-23. The use of anti-inflammatory microbial molecules such as PSA to downmodulate inflammatory responses in IBD is a promising therapeutic approach.

At the other end of the spectrum of intestinal immune dysregulation is the loss of mucosal defense mechanisms brought about by antibiotic-induced depletion of the microbiota. As described in previous sections of this review, products shed by the intestinal microbiota play a critical role in stimulating innate immune responses important for maintaining a healthy level of inflammatory tone in the intestinal mucosa. The effects of antibiotic treatment include reduced antimicrobial protein secretion, downregulation of mucosal T-cell responses, and disruption of the mucus layer (186). Thus, deficiencies in innate immune defense contribute to susceptibility to certain infections during and after treatment with broad-spectrum antibiotics. Immunosuppressed patients who chronically receive antibiotics to prevent or treat infections are particularly at risk for colonization with antibiotic-resistant organisms. A previous study in our laboratory has demonstrated that the nosocomial pathogen VRE becomes the dominant microbe colonizing the gut prior to the development of a blood-stream infection in highly immunocompromised hematopoietic stem cell transplant (HSCT) patients (187). Thus, the antibiotic-conditioned intestine is a reservoir for multi-drug resistant organisms (188, 189). The use of antibiotics cannot be avoided in HSCT patients and other patients with severe immunosuppression; therefore, we propose that administration of microbial molecules can be used to stimulate the innate immune system and restore mucosal defenses to replace signals normally provided by the microbiota.

Defense against VRE infection depends, in part, on the antimicrobial protein RegIII γ , which has bactericidal activity against Gram-positive organisms (58, 60). RegIII γ expression by IECs and Paneth cells is regulated by TLR/MyD88-mediated detection of microbial molecules. Direct TLR stimulation of IECs and Paneth cells induces RegIII γ expression as shown in Fig. 1A (60, 61), but RegIII γ expression can be further induced by IL-22 produced by radiosensitive bone marrow-derived cells, which are activated by TLR signaling in the hematopoietic compartment as shown in Fig. 1B (140, 141, 145, 160, 190). The dramatic increase in RegIII γ expression that occurs during intestinal infections is primarily mediated by IL-22. Antibiotic treatment reduces RegIII γ expression, thereby, leaving the intestinal mucosa in a state of vulnerability to infections. Thus, antibiotic-treated mice are susceptible to VRE intestinal colonization and dissemination (60). However, the consequences of

antibiotic-mediated depletion of the microflora are reversible. Innate immune defenses, most importantly RegIII γ expression, are restored upon administration of oral lipopolysaccharide or systemic flagellin, thereby leading to enhanced resistance to VRE colonization in antibiotic-treated mice (60, 190) (Fig. 2). Flagellin-mediated RegIII γ expression requires TLR5 signaling in hematopoietic cells and IL-22 secretion by innate lymphocytes, but precisely how TLR signaling in the hematopoietic compartment induces IECs to secrete antimicrobial peptides remains unclear (145, 190).

Bloodstream infections are a leading cause of mortality in severely immunocompromised patients. Many of the organisms that cause bloodstream infections colonize the gut, and it is thought that translocation of these microbes across a weakened intestinal epithelial barrier is the initial event that precipitates bacteremia. Therefore, microbial products may be used as therapeutic agents that fortify the intestinal epithelium by inducing antimicrobial protein secretion and ultimately preventing microbes from entering the systemic circulation.

Flagellin, a TLR5 ligand, has been shown to have protective effects against trauma to the intestinal mucosa caused by other bacterial and viral infections, chemical-induced colitis, and lethal radiation (191–193). Unlike lipopolysaccharide, flagellin does not precipitate a sepsis-like response and is well tolerated by mice and humans (191, 194, 195). Infection with the Gram-positive organism *C. difficile* causes antibiotic-associated diarrhea and, in some cases, toxic megacolon. Many patients acquire the infection in the hospital due to the communicability of the *C. difficile* spores and the prevalence of antibiotic use in hospitalized patients. *C. difficile* destroys the intestinal epithelial barrier by secreting toxins that cause disruption of the tight junctions and stimulate a highly proinflammatory response. Antibiotic-treated mice are protected from death if flagellin is systemically administered prior to inoculation with *C. difficile* spores (192). Prophylactic administration of flagellin delays early *C. difficile* expansion in the colon, reduces IEC apoptosis, and preserves intestinal barrier function; however, the mechanism by which flagellin stimulates these protective effector functions is not known. It is possible that STAT3 signaling in IECs mediates the protective response via flagellin-induced IL-22 as wound healing and restitution of intestinal barrier function after chemical-induced colitis largely involves the IL-22/STAT3 signaling pathway (159). Thus, exogenous administration of microbial products stimulates the innate immune system leading to the upregulation of host defense mechanisms. This is a promising therapeutic strategy to supplement antibiotic treatment in the prevention and treatment of infections caused by multi-drug resistant pathogens that colonize the gut in hospitalized, immunocompromised patients.

Conclusion

Healthy functioning of the gastrointestinal system requires complex interactions between commensal microbes and the intestinal mucosa. Detection of microbial products via innate immune signaling is the central mode by which the microbiota regulates the mucosal immune system. However, this relationship has two sides. Antimicrobial factors produced by cells of intestinal mucosa influence the types of microbes that can be found colonizing the intestinal mucus layer. Antibiotic treatment or impairment of regulatory responses disrupts these finely tuned interactions and leads to susceptibility to infections or to

inflammatory bowel disease. How the intestinal immune system maintains both tolerance and a healthy inflammatory tone yet is able to immediately respond to an enteroinvasive pathogen with a robust, but tightly controlled innate immune response is not completely understood. Elucidating the mechanisms by which a healthy gut is maintained will direct the development of therapeutic agents that manipulate this pathway in patients susceptible to disease.

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Fig. 1. Mechanisms of RegIII γ expression

(A) Under steady-state conditions, microbial products shed by the microbiota induce MyD88-mediated signaling in intestinal epithelial cells (IECs), resulting in the upregulation of RegIII γ . The expression of RegIII γ in the setting of homeostasis serves to limit the translocation of commensal microbes and defend against the establishment of an intestinal infection. (B) Infection with enteroinvasive pathogens requires a rapid and robust innate immune response. The presence of microbial products in the *lamina propria* leads to activation of Toll-like receptor (TLR)-expressing dendritic cells. These activated dendritic cells stimulate innate lymphoid cells to produce IL-22 by a mechanism that is not entirely clear. Signal transducer and activator of transcription 3 (STAT3)-mediated activation of IECs by IL-22 results in the upregulation of RegIII γ . Thus, in contrast to intrinsic TLR/myeloid differentiation factor 88 (MyD88)-signaling in IECs, RegIII γ expression during infection or therapeutic administration of TLR ligands is mediated by immune cells activated directly via TLR/MyD88-mediated signaling pathways. It is not known if these pathways are mutually exclusive or whether the mechanisms of RegIII γ induction overlap under the described conditions.

Fig. 2. Therapeutic strategy for the use of TLR ligands in restoring innate immune defenses impaired by treatment with broad-spectrum antibiotics

A healthy microbiota is composed of a diverse population of microbes that stimulate the expression of homeostatic levels of RegIII γ . Treatment with broad-spectrum antibiotics not only reduces the overall number of commensal microbes but also reduces the diversity of the microbial population in the intestine, leading to the downregulation of RegIII γ expression. Vancomycin-resistant *Enterococcus* (VRE) is able to colonize the intestine under these conditions and become the predominate organism residing in the gut lumen. Prophylactic administration of TLR ligands stimulates RegIII γ expression in intestinal epithelial cells despite antibiotic-mediated depletion of the microbiota. Because RegIII γ has the ability to kill VRE, exogenous stimulation of TLRs is a potential therapeutic strategy to limit intestinal colonization and infection with VRE.