Events at the Host-Microbial Interface of the Gastrointestinal Tract
II. Role of the intestinal epithelium in pathogen-induced inflammation

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THE HUMAN INTESTINE IS SUBJECT to a vast number of bacteria, including commensal microflora, pathogens, and those that simply pass through following ingestion. Therefore, it is important that adequate systems be in place to accurately identify threats and appropriately react to the given situation. The cells that comprise the intestinal epithelium have evolved sophisticated mechanisms for identification of pathogens and counteraction, when necessary. These mechanisms involve a number of recognition receptors with various locations on and within the cell, including Toll-like (TLR) and Nod receptors that together bind a broad range of ligands. Ligand binding to host cell receptors induces cellular signaling events that lead to the production and secretion of molecules involved directly in defense, in addition to recruitment and activation of the immune cells associated with inflammation. Such molecules vary greatly in composition and activity and include cytokines, chemokines, eicosanoids, and antimicrobial peptides.

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THE INTESTINE UNDER NONINFLAMMATORY CONDITIONS

The state of the intestine lies in a fine balance between health and disease. This proper balance is dependent on the rapid and correct recognition of pathogens in the intestinal lumen and transmission of signals to the immune system to induce an inflammatory response. In contrast to pathogens, the $10^{13}$ microorganisms that comprise the commensal microflora are nonthreatening and beneficial, and unnecessary action taken against microflora would likely lead to a continual state of inflammation. The task of differentiating between pathogens and commensal microflora and eliciting the appropriate action is the responsibility of a single layer of intestinal epithelial cells (IECs) that line the intestinal tract and are in direct contact with luminal contents. IECs are connected by tight junctions at their apical poles to form a monolayer that acts as an impermeable barrier whose proper regulation is critical in restricting the passage of bacteria and their products and maintaining the low reactivity state of the intestine. The intestinal epithelium communicates with immune cells using a suite of signaling molecules and generates a layer of mucus that has many functions, including trapping bacteria within the lumen. Under noninflammatory conditions, the intestinal epithelium not only performs its normal functions (i.e., barrier against bacteria, nutrient absorption), but also continuously “prepares” for an unexpected encounter with a pathogen. Even in the absence of pathogens, IECs are known to continually present antigens to T lymphocytes (11) and express Fc receptors on their apical surface (13). These activities suggest that the underlying immune system is constantly being challenged with luminal antigens and immunoglobulin-bound antigens, even in situations where an offensive immune response is not warranted.

In addition to constitutive antigen presentation, a recent study proposes that TLRs, largely responsible for the recognition of molecular patterns on pathogens, also routinely recognize commensal microflora (22). This recognition evokes cell-signaling events that result in the generation of cytoprotective factors, including IL-6, tumor necrosis factor (TNF), and heat-shock proteins, that are critical for the daily maintenance of the intestinal architecture and protection against future injury (22). Rakoff-Nahoum et al. (22) determined that the recognition of commensal bacterial products by IEC TLRs of intact epithelium resulted in the production of several cellular factors, many of which may be implicated in cytoprotection and tissue repair under normal conditions. These factors were absent in mice following administration of multiple antibiotics to rid the intestine of commensal microflora (22). This suggests that the presence of certain receptors, whose functions were considered to primarily be recognition of pathogens, recognize commensal microflora and induce cell signaling to elicit a form of intestinal homeostasis and protection against injury. Therefore, a basal level of stimulation and continual luminal sampling may aid in the general health and protection of the intestine.

INTESTINAL RECOGNITION OF MICROBIAL THREATS

Multicellular organisms have all developed mechanisms to thwart invasion by pathogens. The primary defense mechanism in most of these potential hosts is the innate immune system that shares many similarities across a wide variety of organisms. Only vertebrates are equipped with the additional layer of defense supplied by the individualized adaptive immune system. In the intestine, innate immunity is dependent on the recognition of microbial patterns found on invading pathogens by pattern-recognition receptors (PRRs) on the surface of the

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IEC. Identified microbial patterns are generally well conserved across many different pathogens, therefore allowing the limited number and variety of receptors on the host cell to recognize and respond to a range of invaders.

TLRs. TLRs are transmembrane receptors that share a high homology to the Toll receptors originally identified in *Drosophila melanogaster*. Toll receptors were found to play key roles in the defense against infection by fungi and Gram-positive bacteria, and TLRs present on the surface of human cells are known to play a similar role. TLRs are classified as membrane PPRs that contain an extracellular domain of leucine-rich repeats (LRR) and a cytoplasmic domain termed the Toll/IL-1 receptor (TIR) domain. After ligand binding to the extracellular domain, the TIR domain recruits a series of TIR-containing adaptor molecules, including MyD88, resulting in the release and activation of NF-$\kappa$B from its inhibitory complex through a complicated sequence of cell signaling. NF-$\kappa$B is then able to move to the nucleus where it induces the expression of a suite of genes, many of which are involved in the proinflammatory response (Fig. 1) (2). Such signaling events do not proceed unregulated; molecules have been identified that interfere with TLR signaling. Such inhibitors, including IL-1 receptor associated kinase (IRAK)-M and Toll-interacting protein, repress IRAK phosphorylation by binding MyD88, thereby dampening or preventing activation of NF-$\kappa$B or MAP kinases (17).

To date, there are 10 identified mammalian TLRs responsible for recognizing conserved bacterial structures termed pathogen-associated molecular patterns (PAMPs). Some PAMPs and their corresponding TLRs include lipoteichoic acid, peptidoglycan, and zymosan of yeast by TLR2, double-stranded RNA by TLR3, LPS, and heat-shock proteins by TLR4, flagellin by TLR5, CpG DNA motifs associated with bacterial DNA by TLR9 (25). Although recognition of the classes of PAMPs by their appropriate TLR is well established, IEC expression of TLRs still remains a matter of debate. Recently, Otte et al. (20) reported that all 10 TLRs are expressed in commonly used colonic cell lines, and all, except TLR10, are expressed in primary IECs, whereas limited expression and/or absence of functional TLRs has been observed by other groups (1). Regardless, expression of certain TLRs within the intestinal epithelium would seemingly offer no selective advantage against bacterial infection and, in fact, would likely contribute to unwarranted inflammation. Of sig-

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**Fig. 1.** Intestinal epithelial cells (IECs) use a number of different receptors to monitor the presence of microbial threats, commensal microflora, or host-generated products. Pathogen-recognition receptors, including Toll-like receptors (TLRs) and Nod, are located on and within the cell where they recognize different threats. Recognition results in NF-$\kappa$B activation leading to the production of cytoprotective factors when stimulated by commensal bacteria and proinflammatory products when stimulated by potential pathogens. Proinflammatory (+) products include various cytokines involved in activating and recruiting macrophages and signaling to dendritic cells and neutrophils. Other non-NF-$\kappa$B-induced products also play key roles in host defense, including the production of antimicrobial peptides (i.e., defensins) and eicosanoids, such as heposphin A$_3$, identified as a neutrophil chemoattractant that signals neutrophils across the intestinal epithelium. Inflammation can be downregulated (−) via a separate eicosanoid, lipoxin A$_3$ (LXA$_3$), and the cytokine CCL20 when bound to the IEC receptors LXA$_3$R and CCR6, respectively. A.A., arachidonic acid; PKC, protein kinase C.
significant interest in terms of intestinal inflammation are TLR4 and TLR5.

**TLR4.** TLR4 is the most studied member of the family and acts as a homodimer in conjunction with an accessory protein, MD-2, to recognize LPS in the outer cell membrane of all Gram-negative bacteria. Not surprisingly, the intestinal epithelium is generally unresponsive to luminal LPS, an effect that in the past has been attributed to low or nonexistent expression of TLR4 and MD-2, as well as the coreceptor molecule CD14 (1). However, infection by invasive pathogens rapidly induces localized inflammation, an observation that may be explained by the discovery that intestinal crypt epithelial cells express TLR4, MD-2, and CD14 and have been found to display a strong LPS-susceptible phenotype. Interestingly, in these cells, TLR4 was predominantly localized to the Golgi apparatus, and LPS was internalized and found to colocalize with the intra-cellular TLR4 (12). Further studies by Hornef et al. (12) have revealed that disruption of the integrity of the Golgi apparatus reduced LPS-mediated NF-kB activation, thereby dampening the inflammatory response. Placement of TLRs interior to the cell would allow the intestinal epithelium to respond to internalized PAMPs from invasive bacteria, while remaining tolerant of the excessive amounts of LPS or other PAMPs that exist within the intestinal lumen at any given time due to the large commensal bacterial load.

Conflicting evidence suggests that TLR4 trafficking occurs across the intestinal epithelium implying that IECs have the potential to monitor their perimeter using receptors localized to one surface of the polarized cell (4). Otte et al. (20) also contend that TLR4 receptors are expressed in IECs but are functional only during short-term stimulation by PAMPs, whereas long-term stimulation results in hyporesponsiveness with no reactivation upon additional challenge. This perception would be in line with what is observed in a healthy intestine, where constant exposure to certain abundant PAMPs, such as LPS, may result in general state of unresponsiveness.

**TLR5.** TLR5 recognizes flagellin, the major protein that composes bacterial flagella. It is well established that this TLR is constitutively expressed in IECs; however, its expression is predominantly localized to the basolateral surface (10). The cellular placement of TLR5 stipulates that in order for immune activation by flagellin to occur, flagellin must be transported to the basolateral surface of the cell. The mechanism for flagellin transport has newly been demonstrated during infection with *Salmonella* (16). In this situation, flagellin transcytosis was attributed to the vesicular trafficking genes located on *Salmonella* pathogenicity island-2. The requirement for such transcytotic transport may be a regulatory act to avoid unnecessary stimulation by flagellin from commensal bacteria. This would allow IECs to react only when a significant threat is posed, as indicated by the presentation of flagellin on the basolateral surface where commensal bacteria would not be in contact with the IEC. Despite the fact that TLR5 is expressed, recent reports (26) demonstrate that a number of intestinal cell lines respond very poorly, if at all, to flagellin. This may indicate that additional factors are required for efficient signaling in response to recognition of flagellin.

**Nod receptors.** Nucleotide-binding oligomerization proteins (Nods) are a family of cytoplasmic PRRs that reside internally and contain a COOH-terminal series of LRRs capable of detecting PAMPs, in addition to a central nucleotide binding domain. Of most intestinal significance are Nod1 and Nod2 that differ by the number of caspase-activating and recruitment domains (CARDs) located at their NH₂ terminus; Nod1 (CARD4) contains only one CARD, whereas Nod2 (CARD15) contains two. Both Nod1 and Nod2 recognize peptidoglycan in bacterial cell walls; however, they differ in the specific moiety that is recognized. Nod1, expressed ubiquitously in adult tissues, detects a naturally occurring peptidoglycan degradation product (D-Glu-meso-DAP), a signature of Gram-negative bacteria, and may be especially advantageous in situations where Gram-negative bacteria are the largest threat, as in the case of the intestinal epithelium. Nod2 is mainly expressed in cells of the myeloid lineage and detects muramyl dipeptide, the minimal structure found on all bacteria containing peptidoglycans, and is therefore implicated as a broad sensor for both Gram-positive and Gram-negative bacteria (5). Taking into consideration their cytosolic location, Nod1 and Nod2 are thought to be particularly important in detecting invasive enteric pathogens, including *Shigella* and *Salmonella* (5). Detection of commensal microflora is seemingly not an issue for the Nod receptors, because their internal location precludes them from interaction with luminal microflora.

Nod1 and Nod2 are expressed by IECs and interact with the CARD containing adaptor protein Rip2 to activate the same signaling cascade for both Nod1 and Nod2. Detection of bacterial peptidoglycan is followed by oligomerization of Nod, which recruits and associates with Rip2 by physical association via CARD-CARD interactions. Rip2 then interacts with the IkB kinase complex and activates NF-kB in a similar manner to TLR signaling, although independent of MyD88.

**SYSTEMIC SIGNALING MOLECULES AND THE INFLAMMATORY RESPONSE**

After recognition of potential danger, a threatened IEC is stimulated to produce and release a number of signaling molecules that will lead to the activation and recruitment of various immune cells that play key roles in the inflammatory response. Many secreted signaling molecules act as chemoattractants that signal cells to the site of infection. Others aid in the proliferation, differentiation, and/or activation of immune cells.

**Cytokines and chemokines.** The detection of pathogens by the PRRs and the cell-signaling events that ensue lead to the translocation of NF-kB to the nucleus where it activates the expression of a number of proinflammatory proteins. These proinflammatory cytokines and chemokines aid in the orchestration of the immune response by signaling to the underlying immune system. IECs produce a variety of chemotraactants responsible for recruiting macrophages, dendritic cells, and neutrophils to the site of infection. These chemotraactants have been shown to increase upon infection with *Salmonella* or treatment with bacterial PAMPs, including flagellin from *Salmonella* (23).

Intestinal macrophages are responsible for engulfing and destroying pathogens in addition to producing cytokines, including IL-1 and TNF (7). IECs secrete a number of macrophage-specific chemokines in response to infection that signal macrophages to the site of infection (i.e., MCP-1, MIP-1β) and halt them once they have arrived (i.e., MIF), as demonstrated by *Salmonella* infection. In addition to chemotraaction, cytokines such as GM-CSF, IL-6, and TNF-α stimulate macro-
phage activation, proliferation, and additional cytokine secretion (7), which only further perpetuate the inflammatory response. Another chemokine, CCL20 (MIP-3α), is secreted basolaterally by polarized IECs and is only recognized by the receptor CCR6 expressed on immature dendritic cells, circulating T cells expressing the α4β7-integrin characteristic of mucosal homing lymphocytes, and IECs (Fig. 1) (28). In response to CCL20, dendritic cells migrate to the subepithelial region of the mucosal surface where they slip in between IECs and function by processing and presenting antigens obtained directly from the lumen to T lymphocytes (19). Interestingly, CCR6 on IECs is located on the apical membrane, whereas CCL20 is secreted basolaterally. The only circumstance under which CCL20 would reach its receptor on the apical surface would be if the inflammatory response or infection were able to modulate the integrity of the intestinal epithelial barrier, allowing CCL20 access to the apical membrane. In this situation, CCL20 may signal through CCR6, leading to the down-regulation of epithelial secretory processes, aiding in the restoration of the epithelial barrier (28).

Neutrophils are the most abundant class of white blood cells in the blood and are responsible for the destruction of bacterial pathogens by releasing proteases and reactive oxygen intermediates. Although effective at killing the bacteria, excessive release of such substances can cause damage to the intestinal epithelium. Many cytokine neutrophil chemoattractants are known to be produced at the onset of bacterial infection, including IL-8, GROα/β/γ, and ENA-78 (7, 29). Among these, IL-8 is secreted from the basolateral surface of IECs and associates with the extracellular matrix, creating a notable gradient allowing the initial recruitment of neutrophils from the underlying circulation into the subepithelial region (Fig. 1) (18). This process is dependent on the recognition of flagellin or peptidoglycan in infected IECs. Although proficient at initially luring neutrophils to the subepithelial region, the basolateral gradient of IL-8 is not necessary or sufficient to guide neutrophils across the intestinal epithelium into the lumen where they can destroy pathogens. Additional factors are necessary for such events to occur.

Eicosanoids. Aside from cytokines and chemokines, IECs have more recently been found to produce eicosanoids that play key roles in host defense. Eicosanoids are a group of lipid molecules, including prostaglandins, leukotrienes, lipoxins (LXA), and hepxoxins, with inflammatory and anti-inflammatory properties. Eicosanoids are produced from the release of arachidonic acid from the cell membrane and its enzymatic conversion by various cyclooxygenases (COX) and lipoxigenases. For example, IEC exposure to many intestinal pathogens (i.e., Salmonella, Shigella, Yersinia, and enteroinvasive E. coli) has been shown to upregulate the expression of PGH synthase-2 and COX-2, both resulting in increased production of PGE2 (3). PGE2 stimulates electrolyte transport and chloride secretion in epithelial cells, ultimately leading to diarrhea. Leukotriene production also results from exposure to pathogens and is a promoter of inflammation.

Of great significance is the novel work regarding a neutrophil chemoattractant previously termed pathogen-elicited epithelial chemoattractant recently identified as the eicosanoid hepxxin A3 (HXA3) (18, 21). HXA3 is generated in response to pathogenic infection when arachidonic acid is converted into 12-HpETE by 12-lipoxygenase (LO), followed by transformation into HXA3 by the putative enzyme hepoxilin synthase. 12-LO inhibitors impede the ability of both IECs to generate HXA3 and neutrophils to migrate across model intestinal epithelia (18). Release of this molecule has been demonstrated in IECs infected with Salmonella when the type III secretion system effector protein SipA is present as the bacterial molecule that seemingly promotes the response (24). As previously mentioned, IL-8 and other CXC chemokines act primarily basolaterally and are capable of recruiting neutrophils from the bloodstream to the subepithelium; however, they are inefficient at inducing neutrophil migration across the intestinal epithelium. On the contrary, HXA3 is released from the apical surface of Salmonella-infected IECs and successfully generates a gradient progressing from the luminal side of the epithelium and strongly attracting neutrophils into the lumen (18). Therefore, IL-8 and other neutrophil chemoattractants released basolaterally may work in concert with the apically released HXA3, the former function to attract neutrophils to the subepithelium, and HXA3 functions to signal neutrophils to cross the intestinal epithelium into the lumen where they can destroy invading microbes (Fig. 1).

In contrast to other neutrophil recruitment molecules, HXA3 is generated independent of NF-κB, because inhibitors of this pathway have no effect on Salmonella-induced neutrophil migration. Instead, generation of HXA3 involves a signaling pathway that is reliant on the activation of protein kinase C-α (PKC-α) (24). SipA of Salmonella activates a novel signaling-transduction pathway involving an ADP-riboseylation factor 6- and phospholipase D-dependent lipid signaling cascade that activates many forms of PKC, of which only PKC-α appears to be involved in trans epithelial signaling of neutrophils (24). Although SipA appears to be specific to Salmonella, other intestinal pathogens also induce neutrophil transmigration, but it remains to be determined whether these pathogens stimulate the generation of HXA3.

Unlike prostaglandins, leukotrienes, and hepxoxins, other eicosanoid mediators such as 15-epimeric aspirin-triggered LXAs and LXA4 are primarily anti-inflammatory in function, offering vasoregulatory and immunoregulatory activity. Lipoxins are generated at the site of inflammation via conversion of arachidonic acid by 5-LO and 12-LO or 15-LO and are likely produced as a result of neutrophil-epithelial interaction. LXAs appear to exert anti-inflammatory effects on both neutrophils and IECs and are capable of inhibiting neutrophil migration across the epithelium (14). Specifically, LXA4 has been shown to downregulate NF-κB-mediated IL-8 production and secretion in polarized epithelia. This eicosanoid is recognized by a G protein-coupled receptor, LXA4R, preferentially expressed on the basolateral surface. This suggests a scenario in which LXA4 is generated in or near the paracellular space as a result of interactions between neutrophils and IECs and is rapidly recognized by LXA4R. This leads to the downregulation of IEC-associated inflammatory events (Fig. 1), including the production of the neutrophil chemoattractants IL-8 and HXA3, and perhaps aids in the prevention of excessive host damage (9).

Antimicrobial peptides. Antimicrobial peptides are positively charged polypeptides less than 100 amino acids in length that are implicated in the antimicrobial activity associated with phagocytes, inflammatory body fluids, and epithelial secretions. These peptides achieve their microbicidal function by
disrupting the bacterial cell membrane through interactions between the peptide and negatively charged elements of the bacterial membrane (i.e., LPS, teichoic acid, phosphotidylglycerol). This leads to the insertion of these small polypeptides into the bacterial membrane, creating membrane pores that disrupt energy and ionic gradients and subsequently lead to bacterial cell lysis (8).

There are many examples of antimicrobial peptides, including histatins, dermcidins, and anionic peptides, although these particular peptides are restricted to few animal species and tissues. There are, however, two main families widely distributed in human and other mammalian epithelial cells and phagocytes: cathelicidins and defensins. Aside from their antimicrobial capability, cathelicidins have other properties that allow communication with the host immune system, such as promoting neutrophil chemotaxis and recruiting mast cells. The diverse properties of cathelicidins suggest that they play an important role in mediating innate immune defenses and influencing adaptive immune responses (6).

Of documented intestinal significance, defensins are peptides 3–4 kDa in size, further classified into the α- and β-defensin subfamilies based on length, with the highest concentrations found within the granules of leukocytes. In this case, bacteria are subject to the microbicidal activity of the defensin upon ingestion into phagocytic vacuoles. In the intestine, elevated concentrations of various α-defensins termed cryptidins are found within the secretory granules of Paneth cells and are discharged into the intestinal crypts in response to microbial pathogens. The α-defensins of neutrophils are generated only in promyelocytes of bone marrow and, once mature, circulating or inflammation-associated neutrophils also contain high levels of defensins, although they no longer generate defensin mRNA or the peptides themselves. Additionally, barrier and secretory epithelial cells constitutively produce β-defensins and increase expression in response to infection, yielding localized regions of high defensin concentration (8). Nonpathogenic, probiotic bacteria have also been found to strongly induce the expression of certain β-defensins (27).

Similar to cathelicidins, defensins play immune roles beyond that of their direct antimicrobial activity. Various defensins have also been reported to have chemotactic activity directing the movement of monocytes, T cells, and dendritic cells (8). Defensins have also been implicated as signaling molecules where cells exposed to certain defensins generate proinflammatory signals, leading to the onset of additional responses including the induction of cytokines and chemokines, including IL-8 (15). Although in general, this activity is targeted at the defense and protection of the host cell and tissue, certain defensins are cytotoxic at elevated levels and likely contribute to tissue injury on excessive release (Fig. 1).

In conclusion, in this review, we have highlighted some of the approaches used by the intestinal epithelium to recognize and deal with potential microbial threats that arise within the intestine. IECs employ various strategies, namely PRRs, aimed at identifying a range of different PAMPs located on or within bacterial pathogens. Microbial recognition stimulates a cascade of signaling events leading to the generation of multiple molecules, including NF-κB-induced products (i.e., IL-8), eicosanoids (i.e., HXAs), and antimicrobial peptides (i.e., defensins) that drive inflammation. Although antimicrobial peptides are directly involved in damaging bacteria, many of these effector molecules aid in the recruitment and activation of macrophages, dendritic cells, and neutrophils to the site of infection where they can perform their immune functions to eliminate the pathogen. Unfortunately, the same mechanisms that lead to the destruction of bacterial threats are also capable of damaging the host tissue if the products are generated unnecessarily or in excess. Thus the proper regulation of such strategies is of great importance.

REFERENCES


