

Sensor molecules in intestinal innate immunity against bacterial infections

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Purpose of review

Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-containing proteins are innate immune sensors for microbial signature molecules. This review highlights new insights into the functions of these sensors in intestinal physiology.

Recent findings

TLRs are membrane bound and survey the extracellular space for microbe-derived molecules, while NOD-containing proteins are cytoplasmic and detect microbial molecules in the cytoplasm. Most microbial sensors recognize components of the bacterial cell wall and its appendages. For example, TLR4 detects lipopolysaccharide in the Gram-negative bacterial cell wall. TLR5 recognizes flagellin, a component of bacterial flagella required for motility. NOD1 recognizes diaminopimelic acid-containing dipeptide or tripeptide motifs in the Gram-positive bacterial cell wall, while NOD2 detects muramyl dipeptide, a ubiquitous cell wall peptidoglycan motif. These sensors are important for host defense against gastrointestinal pathogens. Thus, TLR4 is required for *Salmonella* eradication, NOD1 contributes to controlling *Helicobacter pylori* infection, and NOD2 is involved in mucosal defense against *Listeria monocytogenes*. These sensors also regulate mucosal inflammation independent of pathogen infections.

Summary

Toll-like receptors and nucleotide-binding oligomerization domain-containing proteins not only play critical roles in host defense against known gastrointestinal bacterial pathogens, but also contribute to mucosal homeostasis in the apparent absence of such pathogens.

Keywords

innate immunity, microbial pathogenesis, mucosal immunology

Abbreviations

CpG	unmethylated cytidine-phosphate-guanosine
IKK	inhibitor κ B kinase
LRR	leucine-rich repeat
MDP	muramyl dipeptide
NF	nuclear factor
NOD	nucleotide-binding oligomerization domain
TLR	toll-like receptor

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Introduction

The gastrointestinal tract, more than any other organ in the human body, is constantly in contact with an abundant commensal bacterial microbiota and is frequently exposed to food and waterborne microbes, both harmless and potentially pathogenic. This poses a great challenge for the intestinal immune system to contain and control enteric bacteria, and prevent their access to the remainder of the body, which is essentially free of microbes under normal conditions. Effective control of intestinal microbes can occur without specific recognition (e.g. killing by gastric acid), but ultimately requires recognition by the host when excessive nonspecific control mechanisms would compromise host functions (e.g. acid hypersecretion). Recognition of enteric bacteria is mediated by several mechanisms, the most important of which rely on host receptors specific for conserved bacterial structures ('molecular patterns') not found in the host. The two major currently recognized groups of such receptors in humans are the toll-like receptors (TLRs) and the nucleotide-binding oligomerization domain (NOD)-containing proteins. This review focuses on recent advances in understanding the functions of these microbial sensors and how they apply to intestinal physiology under normal and disease conditions.

Toll-like receptors

TLRs are single-spanning (type I) integral membrane glycoproteins and members of a receptor superfamily that also includes the receptors for interleukin-1 (IL-1R) and IL-18, as well as several cytoplasmic adaptor molecules important for TLR and IL-1R signaling. The group members share a structurally conserved 'toll/IL-1R' (TIR) motif in the cytoplasmic domain, but they differ markedly in their extracellular domain, since TLRs contain numerous leucine-rich repeat (LRR) motifs, whereas IL-1Rs contain three immunoglobulin-like domains. Humans have 10 TLRs (*TLR1–TLR10*),

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while mice appear to have 11 *Tlr* genes (*Tlr1–Tlr9*, *Tlr11* and *Tlr12*, but not *Tlr10*) [1]. The following discussion highlights recent progress made in defining the functions of selected TLRs, TLR4, TLR5, and TLR9, which have particular relevance to intestinal physiology. Comprehensive accounts of these and other TLRs can be found in excellent recent reviews [1,2].

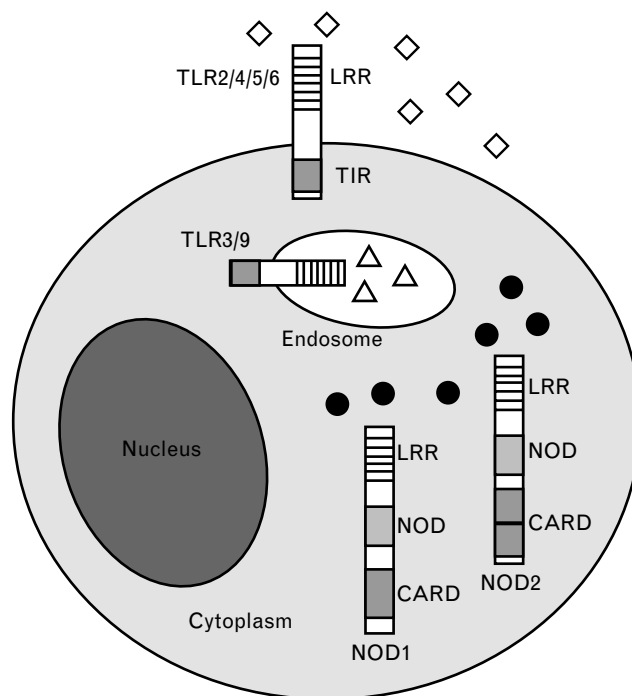
Toll-like receptor-4

The prototypic and best-studied mammalian TLR is TLR4, whose functional importance was first recognized through its identification as the gene responsible for genetic hyporesponsiveness of certain mouse strains to lipopolysaccharide, the major constituent of the outer leaflet of the outer membrane of Gram-negative bacteria. TLR4 is predominantly expressed by hematopoietic cells, particularly macrophages, dendritic cells, and B cells, throughout the body, although intestinal macrophages have only low TLR4 levels normally [3]. Intestinal and gastric epithelial cells have been studied extensively for their TLR4 phenotype, and are generally considered to produce little or no TLR4 under resting conditions, which parallels their reported hyporesponsiveness to lipopolysaccharide [4].

Recognition of lipopolysaccharide depends on formation of a complex of TLR4 with the secreted protein MD-2. Cells that lack either TLR4 or MD-2, or both, are unresponsive to lipopolysaccharide [4]. In addition to lipopolysaccharide, TLR4 was also recently shown to recognize anthrax toxin, a cholesterol-dependent cytotoxin from *Bacillus anthracis*, and other members of this family of cytotoxins in Gram-positive bacteria, indicating that TLR4 can recognize not only products from Gram-negative but also from Gram-positive bacteria [5]. TLR4 may also recognize a variety of host-derived, endogenous ligands, but this contention is controversial as unrecognized contamination with microbial products can be a serious complication in such studies [6].

The lipopolysaccharide receptor, TLR4/MD-2, is membrane bound and expressed at the cell surface of macrophages and other myeloid cells, where it binds lipopolysaccharide (Fig. 1). Although intestinal epithelial cells express low levels of TLR4 under normal conditions, those cells positive for TLR4 appear to express significant quantities of the protein intracellularly in the Golgi apparatus [7]. Accordingly, binding of lipopolysaccharide and activation of cell signaling occurs at the cell surface in macrophages but appears to require internalization in intestinal epithelial cells [7]. Lipopolysaccharide stimulation of TLR4/MD-2 positive cells upregulates expression of a wide range of nuclear factor (NF)- κ B and activating protein (AP)-1 target genes, including multiple proinflammatory and chemotactic cytokines,

Figure 1 Microbial sensors in innate immunity



Toll-like receptors (TLRs) and nucleotide-binding oligomerization domain (NOD)-containing proteins are two major groups of mammalian germline-encoded receptors that can detect microbe-derived molecules. TLRs are membrane bound and are located either on the cell surface (e.g. TLR4), where they sense microbial products in the extracellular space ($\diamond$) or in endosomes (e.g. TLR9), where they recognize microbial products taken up by phagocytosis or generated by partial degradation of internalized, vacuole-bound microbes ($\triangle$). NODs are located in the cytoplasm and detect microbial products present cytoplasmically ($\bullet$), e.g. cell wall components derived from invasive bacteria such as *Shigella flexneri* that enter the cytoplasm. TLRs consist of an extracellular domain of leucine-rich repeats (LRRs) important for ligand binding, and an intracellular 'toll/IL-1 receptor' domain (TIR) involved in signaling. NOD1 and NOD2 are composed of one or two N-terminal protein-protein interaction domains (CARDS), a centrally located NOD, and a C-terminal set of LRRs, which are thought to play a role in ligand recognition, although direct ligand binding has not been demonstrated to date.

such as IL-8, tumor necrosis factor (TNF) α , and IL-6, as well as the type I interferon, IFN- β .

Lipopolysaccharide released by Gram-negative bacteria during the course of an infection, or administered directly, has profound effects on the mammalian host. Most importantly, when given at high doses, it causes endotoxic shock and death due to the release of inflammatory mediators such as TNF α . Recognition of lipopolysaccharide by TLR4/MD-2 is critical for this response, since mice deficient for TLR4 or MD-2 are resistant to the lethal effects of lipopolysaccharide. Furthermore, individuals with a polymorphism of the *TLR4* gene (Asp299Gly), which codes for a receptor with impaired lipopolysaccharide recognition, are more resistant to the airway effects of inhaled endotoxin [8]. Beyond its importance in endotoxic shock, lipopolysaccharide

recognition serves a critical function in defense against Gram-negative bacteria. Mice deficient for TLR4 or MD-2 are highly susceptible to infection with the enteric bacteria, *Salmonella typhimurium* and *Escherichia coli* [9,10], and individuals carrying the *TLR4* Asp299Gly allele are more susceptible to septic shock due to Gram-negative bacterial infections [11].

TLR4 also plays a role in regulating inflammatory responses promoted by poorly defined, possibly multiple, commensal bacteria. Thus, two recent studies have demonstrated that TLR4 deficiency exacerbates experimentally induced colitis in murine models, in which the intestinal microbiota contributes to the development of disease [12^{••},13[•]]. Consistent with a protective function of TLR4, a synthetic agonist of TLR4 was reported to attenuate inflammation in murine colitis [14[•]]. Other studies, in contrast, have found little effect of TLR4 loss on colitis severity, or even improvement [15,16]. The reasons for the apparent discrepancies are not clear presently, but may relate to the varying importance of the microbiota in modulating mucosal inflammation under different, often incompletely understood experimental conditions.

This situation is not unlike that in inflammatory bowel disease patients, with some benefiting from antibiotic therapy while others do not [17]. Individuals with the Asp299Gly allele of *TLR4* exhibit a significantly increased risk of developing Crohn's disease or ulcerative colitis, yet the vast majority of individuals with the mutation do not develop disease [18,19]. Together, these data suggest that recognition of lipopolysaccharide, and perhaps other microbial or endogenous ligands, by TLR4/MD-2 can have a spectrum of functions in intestinal inflammatory disease, ranging from protection to exacerbation, the relative importance of which may be determined by the underlying pathogenetic mechanisms and the involvement of the microbiota in these processes. Any protective function of TLR4 might appear counterintuitive given the strong induction of proinflammatory mediators by lipopolysaccharide in cultured cells, yet is made plausible by the important role of the lipopolysaccharide receptor in host defense against live Gram-negative bacteria, which can contribute to the development of intestinal inflammatory disease in a susceptible host.

Toll-like receptor-5

TLR5, which is expressed by gastric and colonic epithelial cells, microvascular endothelial cells in the colon, as well as dendritic cells and macrophages, recognizes a bacterial protein, flagellin, the major component of bacterial flagella. These complex appendages of the outer bacterial membrane confer motility, which is an important virulence factor in many bacterial pathogens, although some nonpathogenic bacteria are also motile.

As a membrane protein at the cell surface, TLR5 binds directly to a conserved motif present in soluble flagellin monomers but not accessible in polymerized flagella [20] (Fig. 1). Flagellin stimulation of cultured human colon epithelial cells induces the expression of several proinflammatory genes, including the chemokines IL-8 and macrophage inflammatory protein-3 α , which are chemotactic for neutrophils, and immature dendritic cells or T cells, respectively [21,22].

In vivo, flagellin is a potent immunological adjuvant upon systemic administration [23], suggesting that recognition through TLR5 is probably important for mediating this function. Consistent with this notion, flagellin induces maturation and enhanced chemokine production in human dendritic cells, which are key sentinels for capturing antigens and activating T lymphocytes [24]. A role of flagellin, and hence presumably TLR5, in human disease pathogenesis is also suggested by recent studies in Crohn's disease patients, since immunoglobulin (Ig)G and IgA in patient sera were shown to detect bacterial flagellin significantly more frequently than sera from healthy controls or ulcerative colitis patients [25[•]]. Similarly, antibodies in sera from mice with experimentally induced colitis were more reactive to flagellins compared with those from normal mice [26^{••}]. Concomitantly, flagellin-specific CD4 T lymphocytes, but not a polyclonal population of broadly specific CD4 T lymphocytes, induced colitis upon adoptive transfer to immunodeficient recipient mice [26^{••}]. Together, these reports suggest that flagellins can act as immunodominant antigens and stimulate intestinal inflammatory reactions.

A single nucleotide polymorphism has been found in the human *TLR5* gene, *C1174T*, which codes for a truncated, nonfunctional protein, TLR5^{392STOP} [27]. This inactivating mutation, which functions in a dominant-negative fashion, occurs in 5–10% of healthy individuals, indicating that TLR5 is not required for normal development and health, and may even be detrimental under certain conditions. TLR5, however, contributes to host defense against at least some flagellated bacteria, because individuals carrying inactive TLR5 exhibited increased susceptibility to pneumonia caused by the flagellated, Gram-negative bacteria, *Legionella pneumophila* [27]. By contrast, TLR5 deficiency did not increase the risk of developing typhoid fever due to enteric infection with the flagellated, Gram-negative bacteria, *Salmonella typhi* [28], suggesting that TLR5 has no unique role in host defense against these particular bacteria.

Toll-like receptor-9

TLR9 is expressed by epithelial cells in stomach and colon, and follicle-associated epithelial cells (including M cells) in Peyer's patches of the small intestine [29]. Among human myeloid cells, the receptor is found in

B cells and plasmacytoid dendritic cells, but only at low levels or not at all in monocytes/macrophages, T cells, natural killer cells, or myeloid dendritic cells. In comparison with humans, mice express TLR9 on a wider range of myeloid cells which, in addition to B cells and plasmacytoid dendritic cells, includes monocytes/macrophages, natural killer cells, and myeloid dendritic cells.

TLR9 recognizes an unmethylated cytidine-phosphate-guanosine (CpG) motif not present in vertebrate DNA. CpG-containing DNA sequences are taken up by receptor-mediated endocytosis into TLR9-expressing immune cells and bind directly to TLR9 within an endosomal compartment [30] (Fig. 1). Binding is optimal under acidic conditions (pH 6.5 and lower), which explains the requirement for endosomal acidification (also termed endosomal maturation) for TLR9 activation [30]. Stimulation of TLR9 leads to activation of the signaling kinases, inhibitor κ B kinase (IKK) and mitogen-activated protein kinase, and the corresponding transcription factors, NF- κ B and activator protein-1, as well as interferon-responsive factors [31]. Accordingly, CpG-containing DNA induces expression of a wide range of target genes of these signaling pathways, particularly those coding for proinflammatory cytokines and other mediators, as well as type I interferons. In addition to changes in gene expression, activation of TLR9 by CpG-containing oligodeoxynucleotides was recently shown to trigger the degranulation of Paneth cells in the small intestine, a process associated with the release of defensins and other antimicrobial molecules into the lumen [29].

CpG-containing DNA has profound adjuvant effects on the immune system *in vivo*. Systemic administration enhances T and B-cell responses to coadministered protein antigens [32], and protects against systemic infection with several pathogens, including bacteria such as *L. monocytogenes* [33] and fungal pathogens such as *Cryptococcus neoformans* [34]. Common to these events is the fact that CpG DNA promotes the development of T-helper cell 1-type immune responses characterized by enhanced IL-12 and IFN- γ production, as well as the release of type I interferons important for antiviral defenses. Moreover, CpG DNA acts as a mucosal adjuvant, since intranasal immunization of mice with a protein antigen and CpG-containing oligodeoxynucleotides, either as a mixture or as a covalently bonded conjugate, induces antigen-specific IgA production, as well as the generation of specific CD4 and CD8 T cells [35]. Immunomodulation by CpG-containing DNA is also observed in the context of intestinal inflammation, since administration of such DNA protects against experimentally induced murine colitis when administered before, or in the early phase of, induction [36,37], whereas treatment during severe acute colitis exacerbates inflammation [37]. The protective effects of CpG-containing DNA were

recently shown to be mediated by TLR9 through the induction of type I interferons acting as antiinflammatory agents [38^{*}], or the development of regulatory CD4 T cells that can suppress antigen-specific immune responses [39]. Together, these studies indicate that unmethylated CpG-containing DNAs hold promise as immunoregulatory and adjuvant agents for vaccinations and treating immune-mediated diseases in humans, although it must be noted that daily long-term administration was reported to cause immunosuppression and lymphoid follicle destruction in mice, which cautions against potential side effects of drugs based on selective activation of TLR9 [40]. Long-term inactivation of TLR9, however, appears to have no major physiological consequences under normal conditions, since mice deficient for TLR9 are viable, healthy and fertile [38^{*}].

In addition to mediating the effects of exogenously administered CpG-DNA, TLR9 has critical functions in immune responses against microbes. For example, mice lacking functional TLR9 are highly susceptible to infection with cytomegalovirus, a double-stranded DNA virus that causes acute systemic infection often followed by prolonged persistence in the host, and show impaired infection-induced secretion of IFN- α/β and natural killer cell activation [41^{*},42^{*}]. Thus, TLR9 can play an important role in viral sensing, possibly through recognition of viral DNA intermediates within infected cells. In addition, TLR9 deficiency was recently reported to attenuate chronic colitis induced by multiple cycles of feeding the irritant dextran sulfate sodium, suggesting that recognition of commensal bacterial DNA can promote chronic inflammatory responses in the colon [43].

Nucleotide-binding oligomerization domain proteins

NOD proteins possess a tripartite domain architecture, consisting of an N-terminal effector domain involved in protein/protein interactions, a centrally located nucleotide-binding oligomerization domain, and a C-terminal series of LRRs implicated in ligand recognition and autoregulation. NOD proteins, like TLRs, are involved in recognition of microbial signature structures, but as cytoplasmic proteins differ in their cellular localization from the membrane-bound TLRs (Fig. 1). This localization makes them part of a cytoplasmic surveillance system of innate immunity in the mammalian host. The NOD proteins are expressed in many organs including the gastrointestinal tract, although their physiological functions in different sites are only beginning to be understood. Two of the best characterized NOD proteins relevant to intestinal physiology are NOD1/CARD4 and NOD2/CARD15.

Nucleotide-binding oligomerization domain-1

NOD1 is expressed in epithelial cells of stomach and colon, and macrophages and dendritic cells in multiple

tissues (e.g. pancreas, lungs, kidney, and spleen). NOD1 mediates recognition of a specific diaminopimelic acid-containing di-peptide or tri-peptide motif present in the cell wall of Gram-negative bacteria, and a few Gram-positive bacteria, but absent in eukaryotes [44]. NOD1 activation causes activation of IKK and NF- κ B and subsequent induction of proinflammatory cytokines and other mediators. In addition, NOD1 associates with, and activates, caspase 9, a proteolytic enzyme involved in mediating apoptotic responses to diverse stimuli [45], and caspase 1, the critical protease required for cleavage of pro-IL-1 β into the mature, biologically active form of IL-1 β [46].

NOD1 can act as a sensor or 'trip wire' of intestinal epithelial cells for several important gastrointestinal bacterial pathogens. Thus, infection of cultured epithelial cells with *Shigella flexneri* or enteroinvasive *Escherichia coli*, invasive Gram-negative bacteria that enter the host cell cytoplasm, activates IKK/NF- κ B and expression of proinflammatory cytokines in a manner dependent on intact NOD1 function [47,48]. Similarly, activation of NF- κ B and its target genes in response to infection of gastric epithelial cells with *Helicobacter pylori* is partly dependent on NOD1 [49^{••}]. Although *H. pylori* is not invasive, it can deliver bacterial virulence factors and cell wall components into the host cell cytoplasm through a specialized type IV secretion system [49^{••}]. NOD1-dependent recognition of *H. pylori* products, and the subsequent triggering of signaling pathways, appears to contribute to effective host defense against certain strains of *H. pylori*, since mice deficient for *Nod1*, which are normally healthy and fertile with no gross abnormalities, exhibit greater gastric colonization upon oral inoculation [49^{••}].

Nucleotide-binding oligomerization domain-2

Human NOD2 is expressed predominantly in myeloid cells, particularly macrophages, neutrophils, and dendritic cells, as well as in Paneth cells in the small intestine [50], which makes its distribution more restricted than that of NOD1. Expression of NOD2 can be induced by the proinflammatory cytokines, TNF α and IFN- γ , in cultured intestinal epithelial cells [51]. Accordingly, its expression in enterocytes is low under normal, but elevated under inflamed conditions [51].

NOD2, like NOD1, is localized in the host cell cytoplasm and serves as a microbial sensor (Fig. 1). It differs from NOD1 in its recognition specificity, since NOD2 is involved in recognition of muramyl dipeptide (MDP), a ubiquitous peptidoglycan motif present in the cell wall of Gram-positive and Gram-negative bacteria [52,53]. Thus, NOD2 senses a structural motif found in a wider range of bacteria than NOD1. Although NOD2 is commonly referred to as the MDP receptor, direct and specific binding of MDP to NOD2 has not been demon-

strated. Activation of NOD2 stimulates IKK/NF- κ B activity and expression of multiple proinflammatory cytokines and other mediators.

Gene-targeted mice deficient for *Nod2* were reported to be healthy and fertile, indicating that the protein has no critical developmental functions [54,55^{••}]. Very young *Nod2* knockout mice were more resistant to systemic challenge with a high dose of lipopolysaccharide [54], whereas adult knockout mice showed a smaller difference in lipopolysaccharide susceptibility [54,55^{••}]. These mice survived systemic challenge, however, with a combination of lipopolysaccharide and MDP significantly better than wild-type mice [55^{••}]. Importantly, a recent report showed that adult *Nod2* knockout mice were more susceptible to oral, but not systemic, infection with the enteric bacterial pathogen, *L. monocytogenes* [55^{••}]. This was paralleled by decreased expression of specific Paneth cell α -defensins, antimicrobial peptides that can kill *Listeria* and other enteric bacteria [55^{••}]. Together, these data suggest that normal NOD2 mediates systemic responses to the bacterial cell wall component, MDP, and can play a role in innate intestinal defense against at least one enteric bacterial pathogen, *Listeria*. In support of the latter notion, transfection of normal NOD2 into cultured intestinal epithelial cells was shown to limit uptake or growth of another invasive enteric bacteria, *Salmonella*, suggesting that NOD2 can exert, directly or indirectly, antimicrobial functions in epithelial cells [56].

NOD2 has gained prominence over the last few years through its association with increased susceptibility to several clinically important human inflammatory diseases. In particular, mutations in *NOD2* are strongly associated with an increased risk of developing Crohn's disease in North American and European populations [57]. The Crohn's disease-associated mutations are confined to a region within or near to the LRR domain of the NOD2 protein and were reported to abolish the ability to sense MDP and activate NF- κ B in a transient transfection experiment [58[•]]. Furthermore, monocytes isolated from Crohn's disease patients homozygous for mutant *NOD2* were reported to exhibit defects in production of proinflammatory cytokines, TNF α , IL-6, and IL-8, as well as the antiinflammatory cytokine IL-10 [59,60]. Crohn's disease, however, is associated with the presence of epithelial cells and lamina propria macrophages with activated NF- κ B in their nuclei, and these cells overexpress products of NF- κ B target genes including the proinflammatory cytokines TNF α , IL-1 β , and IL-6 under inflamed conditions [61]. These findings appear to be inconsistent with the presumed loss of NF- κ B inducibility by the NOD2 variants.

Several, nonmutually exclusive hypotheses have been advanced to reconcile these observations and provide a

mechanistic explanation for the apparent inflammation-promoting functions of the Crohn's disease-associated NOD2 variants [62]. They can be divided conceptually into those advocating that NOD2 variants are defective in performing critical functions required for limiting inflammation ('loss of function'), and those proposing that the mutant protein directly activates proinflammatory signaling pathways ('gain of function'). For example, lack of normal NOD2 appears to promote the production of IL-12 in macrophages stimulated with bacterial peptidoglycan [63•]. This cytokine is an important regulator of T-cell proliferation and promotes intestinal inflammation. By contrast, a Crohn's disease-associated NOD2 variant was shown in a murine model to stimulate release of mature IL-1 β in macrophages, which can contribute to mucosal inflammation [64•]. The pathophysiological importance of these different hypotheses is an area of active investigation.

Conclusion

TLR and NOD proteins constitute the major known sensors of innate immunity, which detect with great sensitivity and specificity a range of molecules characteristic for whole classes of microbes. It is now clear that these genome-encoded sensor molecules are indispensable for rapid activation of host defenses against microbes that threaten the health and survival of the host. Furthermore, their function is critical for activation of adaptive immunity, a phylogenetically younger system of immune defense that is controlled and assisted by innate immunity. Although the gross outline of the functions of innate immune sensors is now established, many questions remain on the functional details and physiological relevance of these sensors. For example, what is the full spectrum of microbial molecules recognized by each of the microbial sensors? Is recognition of all of these molecules relevant under physiological conditions? Which TLR or NOD proteins are important in immune defense against specific pathogens? What is the role of mutations and defects in microbial sensors in the pathogenesis of inflammatory diseases? Can selective activation of specific TLR or NOD proteins be exploited reliably for purposes of vaccination or immunomodulation? Answers to these questions will not only deepen our fundamental understanding of the immune system, but also have the potential to provide a crucial basis for developing new strategies to prevent and treat infectious diseases and immune disorders.

References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

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Additional references related to this topic can also be found in the Current World Literature section in this issue (pp. 176–177).

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