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Detection of enteric pathogens by the nodosome

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Abstract

Nucleotide-binding oligomerization domain protein (NOD)1 and NOD2 participate in signaling pathways that detect pathogen-induced processes, such as the presence of peptidoglycan fragments in the host cell cytosol, as danger signals. Recent work suggests that peptidoglycan fragments activate NOD1 indirectly, through activation of the small Rho GTPase Ras-related C3 botulinum toxin substrate 1 (RAC1). Excessive activation of small Rho GTPases by virulence factors of enteric pathogens also triggers the NOD1 signaling pathway. Many enteric pathogens use virulence factors that alter the activation state of small Rho GTPases, thereby manipulating the host cell cytoskeleton of intestinal epithelial cells to promote bacterial attachment or entry. These data suggest that the NOD1 signaling pathway in intestinal epithelial cells provides an important sentinel function for detecting 'breaking and entering' by enteric pathogens.

Keywords

Nod-like receptors; patterns of pathogenesis; pathogen detection; type III secretions system

Distinguishing friend from foe in the gut

The human intestine is host to a large microbial community that provides benefit by conferring niche protection and educating the immune system. An excessive immune response against this beneficial microbial community must be avoided to prevent chronic intestinal inflammation. Mechanisms that restrict immune responses against the resident microbial community include limiting the presence at the luminal surface of innate immune receptors, such as Toll-like receptors (TLRs), which recognize pathogen-associated molecular patterns (PAMPs), effectively distinguishing microbes from self. For example, expression of TLR5, which recognizes bacterial flagellin, is restricted to the basolateral surface of epithelial cells [1]. In addition, resident macrophages in the mucosal tissue lack expression of the lipopolysaccharide (LPS) detector TLR4 and its co-receptor, CD14, thus making them poor inducers of inflammatory cytokine production [2].

At the same time, however, the intestinal mucosa must maintain the ability to mount adequate antimicrobial responses when enteric pathogens enter the resident microbial community. To distinguish pathogens from commensal microbes, the innate immune system detects pathogen-induced processes, such as the presence of microbial products in the cytosol of epithelial cells [3]. These pathogen-induced processes are detected by nucleotide-binding oligomerization domain-like receptors (NLRs); a group of innate immune receptors

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Appendix A. Supplementary data Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.it.2013.10.009.

located in the host cell cytosol [4]. Two of these NLRs, NOD1 and NOD2, are major contributors to responses against bacterial pathogens in the gastrointestinal tract. This article aims to summarize recent advances in our understanding of how NOD proteins enable the host to gauge the pathogenic potential of intestinal microbes by detecting the activation state of small Rho GTPases.

NOD proteins in pathogen detection and maintenance of gut immune homeostasis

NOD1 is expressed constitutively in intestinal epithelial cells [5] where it helps to monitor the integrity of the cytosol and respond to the presence of enteric pathogens [6]. NOD1 contributes to nuclear factor (NF)-κB activation elicited by several enteric bacterial pathogens, including *Shigella flexneri* [7], enteroinvasive *Escherichia coli* [5], *Campylobacter jejuni* [8], *Helicobacter pylori* [9,10], and *Salmonella enterica* serotype Typhimurium (*S.* Typhimurium) [11,12]; a process initiating proinflammatory responses. Furthermore, NOD1 along with a second protein involved in monitoring the integrity of epithelial cytosol, NOD2, is critical for the autophagic response to invasive enteric pathogens [13].

Although NOD2 expression was initially thought to be restricted to monocytes [14], it is now clear that this protein is also expressed in Paneth cells of the intestinal epithelium [15]. A loss-of-function mutation in the human *NOD2* gene is associated with an increased risk of developing Crohn's disease [16] and reduced ileal expression of human defensin (HD)-5 and HD-6, two antimicrobial peptides produced by Paneth cells [17]. HD-5 has bactericidal activity [18] and HD-6 prevents invasion of enteric pathogens through the formation of peptide nanonets, which results from an ordered self-assembly of the defensin into fibrils [19]. Paneth cells of mice produce related antimicrobial peptides, known as cryptins. Interestingly, expression of cryptins is abrogated in NOD2-deficient mice [15]. The opportunistic pathogen *Helicobacter hepaticus* triggers granulomatous inflammation of the ileum of NOD2-deficient mice and inflammatory responses can be reduced by transgenic expression of α-defensin in Paneth cells [20]. These data highlight the importance of NOD2 expression in Paneth cells of the crypt epithelium for maintaining homeostasis of the ileal mucosa.

Collectively, these studies highlight the importance of NOD1 and NOD2 in the detection and control of pathogens at the epithelial surface. To understand immune homeostasis, it is important to be familiar with the signals activating this surveillance system and to know the identities of host proteins participating in the NOD1/NOD2 signaling pathway.

Formation of the nodosome

The NOD1 and NOD2 signaling pathways can be triggered by the presence of peptidoglycan fragments in the host cell cytosol, which might be indicative of bacterial invasion or escape of bacteria from a pathogen-containing vacuole. Diaminopimelic-acid-type peptidoglycan fragments derived from Gram-negative bacteria, such as γ-D-glutamyl-meso-diaminopimelic acid (iE-DAP), lead to the activation of the NOD1 signaling pathway [21,22], whereas muramyl dipeptide (MurNAc-L-Ala-D-isoGln) – a peptidoglycan motif common to all bacteria – activates the NOD2 signaling pathway [23,24].

Upon stimulation with peptidoglycan, NOD1 or NOD2 form a protein complex, termed the nodosome, which contains receptor-interacting protein (RIP)2 [25,26]. Recruitment of RIP2 to the nodosome leads to activation of NF-κB and mitogen-activated protein (MAP) kinases [7,27,28], thereby inducing proinflammatory and antimicrobial responses [5,15,29].

Furthermore, NOD1 and NOD2 interact with BID BH3 interacting-domain death agonist, a BCL2 family protein, thereby providing a link between inflammation and programmed cells death [30]. Several studies have suggested that recruitment of NOD1 and NOD2 to the cell membrane is required for the formation of a functional nodosome. For example, NOD1 is recruited to the membrane at the site of bacterial entry during *S. flexneri* infection, where it colocalizes with NF-κB essential modulator (NEMO); a key component of the NF-κB signaling complex [31]. Similarly, recruitment of NOD2 to the membrane is required for responding to stimulation of intestinal epithelial cells with muramyl dipeptide [32]. NOD2 promotes the membrane recruitment of RIP2 [33], leading to ubiquitinylation of RIP2 and NEMO [34,35]. The nodosome contains the co-chaperone-like, ubiquitin-ligase-associated protein SGT1 suppressor of G2 allele of SKP1, which is required for NF-κB activation [36,37]; presumably because it activates signaling components by assisting in the ubiquitinylation of NOD1, RIP2, and/or NEMO. Additional regulators acting downstream of NOD2 in the signaling cascade have been identified in genome-wide small interfering RNA (siRNA) screens [38,39]. However, the functional mechanism leading to a membrane association of NOD1 and NOD2 has long remained elusive.

Recently, a siRNA screen identified FRMPD2 FERM and PDZ domain containing 2 as a membrane-scaffolding complex that supports a basolateral localization of NOD2 in epithelial cells [38]. Furthermore, small Rho GTPases were identified as components of the NOD1 nodosome, which provides a plausible explanation for its assembly at the membrane [40]. Small Rho GTPases are a family of membrane-bound signaling proteins that transition between an inactive GDP-bound form and an active GTP-bound form. The transition from an inactive to an active form is catalyzed by guanosine nucleotide-exchange factors (GEFs), whereas GTPase-activating proteins (GAPs) accelerate GTP hydrolysis, thereby facilitating transition from an active to an inactive form of small Rho GTPases [41]. The small Rho GTPases studied most extensively include RAC1, cell division control protein 42 homolog (CDC42), and Ras homolog gene family member A (RHOA). Active forms of the small Rho GTPases RAC1, CDC42, and RHOA can activate NF-κB [42]. Recent evidence suggests that NF-κB activation mediated by small Rho GTPases requires functional NOD1, RIP1, and RIP2 proteins [40,43].

NOD2 colocalizes with the activated form of RAC1 in membrane ruffles, indicating that this Rho GTPase might be present in nodosomes [44]. Furthermore, NOD2 colocalizes with GEF-H1; a GEF for RHOA [45]. Evidence for an interaction of NOD1 with the activated forms of RAC1 and CDC42 comes from the observation that these proteins can be coimmunoprecipitated [40]. Similarly, the small Rho GTPase RAC2 colocalizes with RIP2 in membrane ruffles and both proteins are present in a protein complex [43]. In plants, the heat shock protein (HSP)90 chaperone binds RAC1 in innate immune protein complexes [46], and this interaction is thought to function in the recruitment of innate immune receptors to the membrane-bound small Rho GTPase [47]. Interestingly, the nodosome of mammals contains HSP90 [48], the presence of which is required for NF-κB activation mediated by NOD1 [40] and NOD2 [37]. These observations raise the possibility that, similar to its function in plants, HSP90 of mammals associates with NOD1 and NOD2 and functions in recruiting the resulting protein complexes to the activated forms of membrane-bound small Rho GTPases [48]. The membrane localization of small Rho GTPases is essential for their ability to activate the nodosome because a constitutively active form of RAC1 lacking its prenyl-group is unable to activate NOD1 [40]. Thus, the picture emerging from these studies is that excessive activation of the membrane-bound proteins RAC1, CDC42, or RHOA leads to assembly of the nodosome at the plasma membrane; a process that ultimately results in NF-κB activation. The finding that activation of RAC1, CDC42, and RHOA triggers the NOD1 signaling pathway has important ramifications, because small Rho GTPases are well-known targets of bacterial virulence factors [49] (Box 1).

Detection of bacterial virulence factors by the nodosome

Virulence factors activating small Rho GTPases are particularly common among enteric pathogens (Figure 1). For example, many type III secretion systems (T3SSs) of enteric pathogens inject proteins, termed effectors, into host cells that activate small Rho GTPases. The T3SS effectors IpgB1 from *S. flexneri*, EspT from *Citrobacter rodentium* and SopE from *S.* Typhimurium serve as GEFs that activate RAC1 and CDC42 [50–52]. The T3SS effectors Map from enteropathogenic *E. coli* and SopE2 from *S.* Typhimurium serve as GEFs that activate CDC42 [50,53]. Furthermore, EspM2 from enterohemorrhagic *E. coli* and EspM2 from *C. rodentium* serve as GEFs for RHOA [54]. The T3SS effectors IpgB2 and OspB from *S. flexneri* activate GEF-H1, which in turn activates RHOA [50,55]. The *S.* Typhimurium inositol phosphatase SopB alters the inositol phosphate (IP) metabolism of the host cell, thereby indirectly activating CDC42 [56,57]. Recent studies have suggested that VAV2 can activate CDC42 after it senses changes in the IP metabolism [58], which suggest a possible mechanism for SopB-mediated CDC42 activation. Cytotoxic necrotizing factor (CNF)1 – a toxin produced by uropathogenic *E. coli* strains – constitutively activates RAC1 by inactivating its GTPase activity through deamidation of a glutamine residue [59–61]. Finally, *Cam. jejuni* secretes Cia proteins through its flagellar apparatus [62–65], and these proteins are ultimately translocated into the cytosol of host cells [66] where they contribute to activation of RAC1 [67]. A second pathway by which *Cam. jejuni* elicits RAC1 activation is CadF-mediated binding of fibronectin to the bacterial surface, which induces a β1-integrin-dependent signaling pathway leading to the activation of two GEFs termed dedicator of cytokinesis (DOCK)180 and T cell lymphoma invasion and metastasis (TIAM)-1 [68] (Figure 1).

It is well established that activation of small Rho GTPases by bacterial virulence factors leads to induction of MAP kinase signaling and nuclear translocation of NF-κB [52,61,69–74], but evidence that the NOD1/RIP2/RIP1 signaling pathway might be involved in this process has emerged only recently. The first indication that bacterial virulence factors might activate the nodosome was the observation that engagement of GEF-H1 by the *S. flexneri* T3SS effectors IpgB2 and OspB led to NF-κB activation that was NOD1 dependent [55]. The *S.* Typhimurium T3SS effector SipA was subsequently shown to induce NF-κB activation through a NOD1/NOD2-dependent signaling pathway [12]. Work with the *E. coli* CNF-1 toxin established a clear role for RAC2 in inducing RIP1/RIP2-dependent NF-κB activation [43]. Finally, activation of RAC1 by the *S.* Typhimurium T3SS effector SopE was shown to lead to assembly of the NOD1 nodosome at the host cell membrane, thereby inducing NF-κB activation [40]. The guanosine nucleotide-exchange factor activity of SopE is required for NF-κB activation [75] and for the recruitment of NOD1 to membrane ruffles [40]. The observation that SopE can activate inflammasome-dependent cytokine maturation [76,77] might be a consequence of nodosome activation, because NOD1 is known to enhance interleukin (IL)-1β secretion [78]. IL-1β secretion requires two signals. The first signal stimulates TLRs or NOD proteins, thereby driving NF-κB-dependent expression of the *IL1B* gene, which leads to the production of pro-IL-1β. The second signal stimulates NLRs to activate caspase-1, which in turn cleaves pro-IL-1β into its active form. NOD1-dependent IL-1β secretion [78] is likely an indirect consequence of NF-κB activation (signal 1) rather than resulting from a direct activation of caspase-1 (signal 2).

Not all effector proteins that activate small Rho GTPases do necessarily activate NF-κB. For example, SopE and SopE2 are both GEFs for RAC1 [53]. However, although an *S.* Typhimurium strain translocating only SopE induces proinflammatory responses in host cells [40], a strain translocating only SopE2 does not [73]. Thus, the signal detected by NOD1 might be an excessive or prolonged activation of small Rho GTPases, which is observed for instance with constitutively active forms of RAC1, CDC42, and RHOA [42].

This model suggests that proinflammatory responses are not observed when Rho GTPases are activated during process involved in normal cell physiology.

Collectively, these data suggest that excessive activation of small Rho GTPases by bacterial virulence factors or by the presence of peptidoglycan fragments in the cytosol are pathogen-induced processes that activate the NOD1 signaling pathway. Although the detection of peptidoglycan fragments in the host cell cytosol evidently represents an innate immune surveillance mechanism, the situation is less clear-cut in case of bacterial virulence factors. One benefit to the pathogen of activating small Rho GTPases is that this process leads to cytoskeletal rearrangements (reviewed in [79]) promoting bacterial entry into host cells (reviewed in [80]) or the formation of pedestals on epithelial surfaces (reviewed in [81]). A byproduct of deploying virulence factors to induce these actin rearrangements would in turn be the induction of proinflammatory responses that are aimed at killing microbes [82], which could be viewed as the cost of doing business. By this logic, monitoring the activation state of Rho GTPases could be considered a NOD1-dependent immune surveillance mechanism that sets off an alarm when it detects breaking and entering by enteric pathogens. This is reminiscent of effector-triggered immune responses in plants, which detect bacterial virulence factors, either directly or through their effects on host targets (reviewed in [83,84]).

An alternative, but not mutually exclusive interpretation of the data would be that enteric pathogens deploy virulence factors that activate the NOD1 pathway because they benefit from the ensuing inflammatory host response. This viewpoint is supported by recent data suggesting that intestinal inflammation confers a fitness advantage upon *S.* Typhimurium, *E. coli*, and *C. rodentium* during their competition with the obligate anaerobic microbial community that dominates the healthy gut [85–87]. A bloom of *S.* Typhimurium and *E. coli* in the inflamed gut is supported by host-derived electron acceptors, which are generated as byproducts of the inflammatory host response [87–89]. Interestingly, the SopE protein, which is present in only a subset of *S.* Typhimurium isolates, alters host responses by increasing expression of inducible NO synthase (iNOS); an enzyme that produces NO. Conversion of NO to nitrate increases growth of SopE-producing *S.* Typhimurium strains in the intestinal lumen through nitrate respiration [88]. Enhanced growth of *S.* Typhimurium in the intestinal lumen increases transmission of the pathogen [90], which represents a potent selective force for the evolution of enteric pathogens. In other words, the reason for deploying virulence factors that trigger the NOD1 signaling pathway might be that the ensuing host response selectively feeds the respective enteric pathogens.

In summary, manipulation of small Rho GTPases by bacterial virulence factors is a common theme in microbial pathogenesis (Box 1), and detection of these pathogen-induced processes by the innate immune system enables the host to distinguish pathogens from microbes with lower pathogenic potential. From an evolutionary point of view it is also interesting to note that detection of bacterial effector proteins is a feature of the innate immune system that is conserved from plants to mammals. This observation raises the question whether signaling pathways involved in detecting these patterns of pathogenesis are also conserved between the different phyla.

Evolution of the NOD1 signaling pathway

NOD1 and NOD2 of mammals are thought to have evolved independently from plant NLRs, suggesting that functional similarities and the presence of similar protein domains (i.e., the presence of similar C-terminal leucine-rich repeats and similar central nucleotide-binding oligomerization motifs) are features that developed independently in different lineages. Furthermore, the *Drosophila melanogaster* genome does not encode homologs of the

mammalian NOD1 or NOD2 proteins (reviewed in [91,92]). Instead, diaminopimelic acid-type peptidoglycan from Gram-negative bacteria is detected in *D. melanogaster* through the immune deficiency (IMD) pathway, which shares homology to the tumor necrosis factor (TNF)-α signaling pathway in mammals [93]. In the IMD pathway, peptidoglycan is detected by the transmembrane receptor peptidoglycan-recognition protein (PGRP)-LC [94] or the cytoplasmic receptor PGRP-LE [95]. Upon binding of peptidoglycan, PGRP-LC and PGRP-LE activate IMD, a death domain protein with homology to RIP1, the RIP kinase associated with TNF-α-dependent NF-κB activation [96]. In turn, IMD activates the transcription factor Relish, which is a member of the NF-κB family [97].

Interestingly, recent studies raise the possibility that the IMD and NOD1 signaling pathways represent evolutionary conserved signaling cascades for the detection of diaminopimelic acid-type peptidoglycan (reviewed in [98]). First, the *E. coli* CNF-1 toxin constitutively activates RAC2 in *Drosophila* cells, thereby inducing the IMD pathway [43] and diaminopimelic acid-type peptidoglycan activates the NOD1-signalling pathway in human cells through a RAC1-dependent mechanism [40]. Thus, a requirement for small Rho GTPases is a feature shared between the IMD and NOD1 pathways, but absent from the TNF-α signaling pathway. Second, human RAC2 induces NF-κB activation through RIP1 and RIP2 [43]. These data suggest that the IMD homolog RIP1 is not only involved in TNF-α signaling, but also participates in detecting the activation state of small Rho GTPases, which confirms the similarities between the IMD and NOD1 signaling pathways. There are however some notable differences between the IMD and NOD1 pathways, including the absence of a NOD1 homolog in insects and the fact that mammalian PGRPs do not function in the NOD1 signaling pathway [99].

In summary, recent evidence provides some support for the idea that the signaling pathways detecting diaminopimelic acid-type peptidoglycan in mammals and insects share a common ancestry (reviewed in [98]). This observation along with the finding that the *Drosophila* Toll protein and the homologous mammalian TLRs both function as cellular sensors of microbes highlights the conservation of innate immune surveillance mechanism within the animal kingdom.

Concluding remarks

The picture emerging from these studies is that NOD proteins provide epithelial cells with important sentinel functions for detecting pathogen-induced violations of the integrity of the host cell cytoplasm. Many enteric pathogens produce virulence factors that manipulate the activity of small Rho GTPases to mediate attachment or entry, or to inhibit phagocytosis (Figure 1 and Box 1). As a result, the host can detect the presence of pathogens by monitoring the activation state of small Rho GTPases. The downstream signaling pathways enable the intestinal epithelium to mount responses that are appropriate to the threat, including NF-κB activation, activation of the autophagy pathway, and the release of antimicrobial peptides. The ability of the epithelium to react appropriately is important for balancing host responses against microbial communities in the gastrointestinal tract. When these mechanisms for maintaining immune homeostasis are disrupted by mutations that impair NOD2 signaling or the autophagy pathway, the host has an increasing risk of developing intestinal inflammation.

Direct evidence for the binding of NOD1 to peptidoglycan is lacking, therefore, it has been speculated that this protein might function in signal transduction rather than serving as an innate immune receptor for peptidoglycan [100]. Interestingly, NOD1-dependent NF-κB activation elicited by diaminopimelic acid-type peptidoglycan requires the presence of active RAC1 [40], thereby raising the possibility that peptidoglycan is sensed in mammals

by an innate immune receptor acting upstream of this small Rho GTPase (Figure 1), which represents an exciting area for future research.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Box 1

Small Rho GTPases are common targets for bacterial virulence factors

Over the past two decades, manipulation of small Rho GTPases has emerged as a conserved pattern in bacterial pathogenesis (reviewed in [49]). For example, the T3SS encoded by *Salmonella* pathogenicity island 1 triggers actin rearrangements and NF-κB activation in host cells [69,70] by injecting effector proteins into the cytosol that activate Rac1 and CDC42 [52]. In addition to the many effector proteins and toxins from a variety of enteric pathogens, which activate small Rho GTPases (see Figure 1 in main text), there are multiple examples of virulence factors that inactivate these molecular switches. For instance, *Clostridium difficile* toxin A and B glycosylate Rho GTPases, thereby inhibiting their function [101,102]. The T3SS effector proteins ExoS of *Pseudomonas aeruginosa*, YopE of *Yersinia pseudotuberculosis* and SptP of *S.* Typhimurium inhibit the function of small Rho GTPases by acting as GAPs [103–106].

Small Rho GTPases mediate actin polymerization and NF-κB activation through two distinct pathways [107]. A model for the pathway leading to NF-κB activation is shown in Figure 1, main text. Interestingly, some virulence factors of enteric pathogens target components of the signaling pathway that are located downstream of small Rho GTPases. For example, the *S.* Typhimurium T3SS effector protein SspH2 *Salmonella* secreted protein H2 cooperates with the NLR co-chaperone SGT1 to ubiquitinate NOD1, thereby inducing IL-8 secretion [108] (see Figure 1 in main text). In case of enteropathogenic *Escherichia coli*, the T3SS effector proteins NleD non-LEE-encoded effector D and NleC function in silencing this signaling pathway by cleaving C-Jun N-terminal kinase (JNK) and the NF-κB p65 subunit, respectively [109,110].

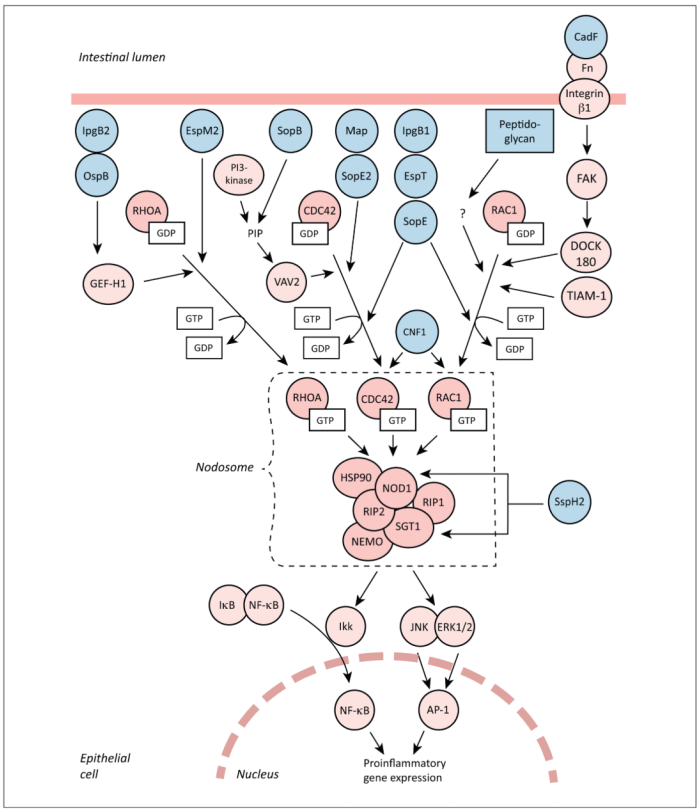

Figure 1.

Model for activation of the Nucleotide-binding oligomerization domain protein (NOD)1 nodosome in epithelial cells by diaminopimelic acid-type peptidoglycan or bacterial virulence factors, which requires excessive or prolonged activation of small Rho GTPases. Peptidoglycan (blue box) or bacterial virulence factors (blue circles) engage host proteins (red circles) to activate (arrows) the nodosome, thereby inducing the expression of proinflammatory genes. AP-1, activator protein-1; CDC42, cell division control protein 42 homolog; CNF1, cytotoxic necrotizing factor 1; DOCK, dedicator of cytokinesis; ERK, extracellular signal-regulated kinase; FAK, focal adhesion kinase; Fn, fibronectin; GEF, guanosine nucleotide-exchange factor; HSP90, heat shock protein 90; IκB, nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor; Iκk, I kB kinase; JNK, C-Jun N-terminal kinase; NEMO, NF-κB essential modulator; NF-κB, nuclear factor-κB; PI3, phosphoinositide 3; PIP, phosphatidylinositol phosphate; RAC1, Ras-related C3 botulinum toxin substrate 1; RHOA, Ras homolog gene family member A; RIP, receptor-interacting protein; SGT1, suppressor of G-two allele of Skp1; SspH2, Salmonella secreted protein H2; TIAM-1, T cell lymphoma invasion and metastasis-1.