

Published in final edited form as:

Curr Opin Gastroenterol. 2008 March ; 24(2): 108–114. doi:10.1097/MOG.0b013e3282f50fdf.

Caspase recruitment domain-containing sensors and adaptors in intestinal innate immunity

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Abstract

Purpose of review—The present review discusses the physiological functions of selected caspase recruitment domain (CARD)-containing sensor and adaptor proteins and their role in the pathogenesis of intestinal diseases.

Recent findings—Myeloid and lymphoid cells as well as intestinal epithelial cells express several intracellular CARD-containing proteins. CARD-containing sensors, particularly NOD1 (CARD4), NOD2 (CARD15) and IPAF (CARD12), have an important role in the detection of conserved microbial structures of invading microbial pathogens. Upon ligand recognition and activation, the sensors interact through CARD domains with downstream CARD-containing adaptors including CARD9, RIP2 (CARD3) and ASC (CARD5). Recent data suggest that multiple signaling pathways from Toll-like receptors and non-Toll-receptor pathways converge on these adaptor proteins and that their functions are crucial for the initiation of innate immune responses to invading microbial pathogens.

Summary—CARD-containing adaptors and sensors represent an important family of molecules involved in innate host defense against gastrointestinal pathogens and in the regulation of inflammatory responses, suggesting that further insights into their physiological functions may yield new pharmacological strategies for treating intestinal inflammatory conditions.

Keywords

caspase recruitment domain; innate immunity; intestinal host defense; microbial sensor

Introduction

A single-cell epithelial layer forms a physical barrier between the body and potentially harmful microbes in the intestinal lumen. If the barrier is penetrated, local immune defenses minimize or prevent systemic spread of invading bacteria. Such defenses are commonly divided into adaptive immune responses, with narrow antigen specificity, and innate immune defenses, capable of rapid, broadly specific responses to conserved microbial molecules known as pathogen-associated molecular patterns. Such ‘patterns’ include molecularly diverse components present in bacteria, viruses, parasites and fungi such as lipopolysaccharide, bacterial lipoproteins, flagellin, zymosan, and certain nucleic acids. In contrast to adaptive immunity, the recognition capacity of which is based on millions of different antigen receptors, microbial sensing by the innate immune system is mediated by a much smaller number of receptors, called pattern recognition receptors (PRRs).

Upon recognition of a pathogenic microbial motif by PRRs, multiple downstream signaling cascades are activated through a limited set of adaptor molecules. The activation of mitogen-activated protein kinases (MAPK) and key transcription factors such as nuclear factor- κ B and activator protein-1 leads to secretion of cytokines with chemotactic and other proinflammatory functions, and thereby the recruitment of inflammatory cells to the site of infection as a first-line defense against further microbial invasion.

In the past decade many PRRs have been discovered and characterized. The first family of PRRs widely studied was the Toll-like receptor (TLR) family. TLRs are membrane-anchored proteins that sense microbial components with their leucine-rich repeats domain exposed to the extracellular space or the lumen of cellular vacuoles that can take up extracellular materials. Upon activation, TLRs stimulate downstream signaling molecules with their cytoplasmic Toll/IL-1 receptor domain.

A second important group of PRRs, termed nucleotide-binding and oligomerization domain (NOD) proteins or NOD-like receptors (NLRs), is located primarily in the cytosol and also functions in the recognition of conserved microbial products. The NLR family, consisting of more than 20 proteins to date, is characterized by three structural domains: a C-terminal leucine-rich repeats domain involved in ligand recognition, a central NOD domain that plays a role in oligomerization and activation, and an N-terminal effector domain. The latter, which can comprise a pyrin domain, a caspase recruitment domain (CARD), or a baculovirus inhibitor-of-apoptosis repeat domain, enables protein-protein interactions and initiates signal transduction cascades that lead to expression of inflammation-associated genes.

The present review focuses on new developments that demonstrate the importance of selected CARD-containing sensors and adaptor proteins in the pathogenesis of intestinal diseases (Fig. 1). The reader is referred to excellent recent review articles [1,2] that provide comprehensive overviews of the biology of NLRs.

CARD9: a central adaptor of innate immune signaling pathways

CARD9 is an intracellular adaptor molecule that consists of an N-terminal CARD domain and a C-terminal coiled-coil domain [3] (Fig. 1). It is mainly expressed at the organ level in the bone marrow, thymus, spleen, lung, and liver, and at the cellular level in myeloid dendritic cells and macrophages, but only at low levels in normal T lymphocytes and B lymphocytes [3,4^{**}]. CARD9 expression in the small intestine and colon is low under normal conditions [3], but is probably increased in the course of mucosal inflammation due to an influx of myeloid cells. Originally identified as a binding partner of BCL10, a CARD-containing signaling protein involved in the development of B-cell lymphomas of mucosa-associated lymphoid tissues [3], CARD9 was recently shown to be a key integrator of innate and adaptive immune responses [4^{**}-6^{**}]. Most importantly, mice lacking CARD9 exhibit increased susceptibility to the enteric bacterial pathogen *Listeria monocytogenes* [4^{**},5^{**}], as well the fungal pathogen *Candida albicans* [6^{**}], yet have normal T-lymphocyte and B-lymphocyte development [5^{**}]. The exact mechanisms by which CARD9 mediates effective host defense against different microbial threats are not fully understood, but this adaptor is involved in signaling from a number of innate cell surface receptors, including TLRs, NLRs, and immunoreceptor tyrosine-based activation motifs (ITAM)-associated receptors (Fig. 2).

CARD9 is required for signaling from most TLRs in dendritic cells, since activation of nuclear factor- κ B and/or MAPK, and induction of the inflammatory cytokines TNF α and IL-6, in response to different TLR ligands such as lipopolysaccharide (TLR4), Pam₃CSK₄ and zymosan (TLR2), flagellin (TLR5), CpG (TLR9), poly(I:C) (TLR3), and loxoribine (TLR7) was defective in CARD9-deficient bone-marrow-derived dendritic cells [4^{**}-6^{**}].

CARD9 may also be required for TLR2/TLR3/TLR7 signaling in macrophages [4**], although another study found only a limited role for CARD9 in TLR7 signaling [5**]. Furthermore, CARD9 mediates signaling by the intracellular microbial sensor NOD2, since CARD9-deficient macrophages failed to increase IL-6 production and/or MAPK activation in response to the NOD2 activator muramyl dipeptide or to infection with the intracellular bacteria *L. monocytogenes* [4**] (Fig. 2). CARD9 associates with NOD2, as well as the intermediate kinase RIP2 (also termed RICK or CARD3), upon over-expression and/or *L. monocytogenes* infection [4**], further supporting a role of CARD9 in NOD2 signaling.

Beyond TLRs and NLRs, CARD9 has been shown to be a key adaptor in innate immune signaling by dectin-1 [6**], a sensor of fungal cell wall components and a prototype of innate non-TLRs containing ITAMs [7*,8*]. Other prominent ITAM-containing receptors are FcR γ , which associates with and mediates signaling through several Fc receptors, and DAP12, which associates with the triggering receptor expressed on myeloid cells (TREM) family of receptors and others. Activation of such receptors induces phosphorylation of specific tyrosine residues in the ITAMs, which leads to downstream formation of a signaling complex of CARD9 with other adaptor proteins, particularly BCL10 and MALT1, and activation of nuclear factor- κ B and MAPK [5**] (Fig. 2). ITAM-containing receptors are found on lymphocytes, natural killer cells, and myeloid cells, including macrophages, neutrophils, mast cells, and dendritic cells. The importance of CARD9 in signaling through ITAM-containing receptors is demonstrated by the findings that CARD9-deficient macrophages and/or dendritic cells have impaired cytokine production in response to zymosan stimulation of dectin-1, or after crosslinking of FcR γ or of several DAP12-associated receptors [5**]. Consequently, host defense against *C. albicans*, which may partly depend on dectin-1 [7*], is compromised in CARD9-deficient mice [6**]. The underlying mechanism may involve a role of CARD9 in the development of IL-17 producing CD4 T-cell effectors (Th17 cells), which are induced in a CARD9-dependent manner upon *C. albicans* infection [9*].

Among the TREM family members, whose signaling depends on CARD9, TREM-1 has garnered recent interest in intestinal immunology as a regulator of mucosal inflammation. TREM-1 is expressed constitutively in neutrophils and monocytes/macrophages, and is further induced in these cells by stimulation with microbial products (e.g. lipopolysaccharide and lipoteichoic acid) and exposure to extracellular bacteria such as *Pseudomonas aeruginosa* and *Staphylococcus aureus* [10,11]. Activation of TREM-1 by crosslinking with monoclonal antibodies leads to secretion of proinflammatory chemokines, especially when TLR or NLR ligands are used as a costimulus, indicating that TREM-1 can amplify proinflammatory responses induced by TLRs or NLRs [12,13**]. It is presently unclear whether TREM-1 recognizes a specific microbial ligand. TREM-1-expressing macrophages are significantly increased in the inflamed mucosa of patients with inflammatory bowel disease and in mouse models of colitis [13**], whereas little TREM-1 expression is found on resident macrophages of the normal human small intestine or colon [11]. Furthermore soluble TREM-1 levels were elevated in the serum of inflammatory bowel disease patients [14]. Importantly, specific blocking of TREM-1 with an antagonistic peptide attenuated inflammation in mouse colitis models [13**]. These data indicate that this ITAM-containing receptor contributes to mucosal inflammation, and suggests more broadly that CARD9-dependent signaling processes promise to be valuable targets in the treatment of inflammatory bowel disease.

CARD4 (NOD1) and CARD15 (NOD2): cytoplasmic sensors of bacterial peptidoglycans

The NLR proteins NOD1 (encoded by *CARD4*) and NOD2 (encoded by *CARD15*) have a tripartite structure with one (NOD1) or two (NOD2) N-terminal CARD domains, a centrally located NOD domain, and a C-terminal set of leucine-rich repeats involved in ligand recognition (Fig. 1). NOD1 is expressed in many cell types, while NOD2 is constitutively expressed predominantly in myeloid cells (neutrophils, macrophages, dendritic cells) and in Paneth cells of the small intestine [15,16]. Both proteins are cytosolic sensors of peptidoglycans, components of bacterial cell walls. NOD1 senses the peptidoglycan-derived peptide γ -D-glutamyl-meso-diaminopimelic acid, which is present mainly in Gram-negative bacteria [17,18]. NOD2 detects muramyl dipeptide, which can be found in a wide range of both Gram-positive and Gram-negative bacteria [19], indicating that its recognition specificity is markedly broader than that of NOD1.

NOD1 contributes to recognition of several enteropathogenic bacteria including *Shigella flexneri* [20], enteroinvasive *Escherichia coli* [21], *Helicobacter pylori* [22], and *Campylobacter jejuni* [23], while NOD2 was shown to be involved in innate detection of *L. monocytogenes* and *Salmonella typhimurium* [24–26]. Several of these pathogens (e.g. *S. flexneri*, *L. monocytogenes*) invade host cells and reside in the cytoplasm, where they may release cell wall components that can make contact with cytoplasmic NOD1 or NOD2. Others (*C. jejuni*, *S. typhimurium*) are invasive but are not known to enter the cytoplasm of host cells, or are only minimally invasive (*H. pylori*), which raises questions about the exact mechanisms by which microbe-derived peptidoglycans are delivered into the cytosol and are sensed by NOD1 or NOD2. Intracellular peptidoglycan delivery can occur via phagolysosomes or via specialized bacterial secretion systems [22,27], or by subversion of peptide transport pathways such as the intestinal peptide transporter hPEPT [28]. Upon activation, NOD1 and NOD2 associate through CARD–CARD interactions with the CARD-containing serine/threonine kinase RIP2 (CARD3), which leads to activation of nuclear factor- κ B and MAPK, and consequently to secretion of proinflammatory cytokines [24,25].

Mice lacking NOD1 or NOD2 are generally healthy and fertile [17,29,30*], indicating that these sensors have no critical developmental functions. A recent report showed that the Peyer's patches of conventionally reared NOD2-deficient mice had elevated numbers of CD4⁺ T cells and M cells, as well as increased expression of proinflammatory cytokines [30*]. Oral administration of *Saccharomyces cerevisiae* or killed *E. coli* or *S. aureus* led to increased bacterial translocation across Peyer's patches, suggesting that NOD2 deficiency causes an epithelial barrier defect. Furthermore, rectal administration of the colitis-inducing agent trinitrobenzene sulphonic acid caused elevated proinflammatory cytokine production in the colon of NOD2-deficient mice [30*], which suggests that loss of normal NOD2 functions can exacerbate colitis induced by inflammatory agents. This notion is further supported by recent work in NOD2 transgenic mice engineered to overexpress normal murine NOD2 in antigen-presenting and other cells under the control of a major histocompatibility complex class II promoter [31**]. These mice exhibited decreased mucosal inflammation and proinflammatory cytokine production in two different colitis models, consistent with the idea that normal NOD2 suppresses mucosal inflammatory responses. A similar attenuation of colitis was observed when wild-type mice were injected with an encapsulated NOD2 expression plasmid [31**]. Interestingly, injection with a plasmid encoding a mutant form of mouse NOD2 equivalent to NOD2^{1007fs} associated with Crohn's disease caused less attenuation of colitis, suggesting that the mutation interfered with a normal suppressive function of NOD2 [31**]. In this context, NOD2 has been shown to inhibit signaling through the TLR2 pathway [32*], although TLR2 can also protect against colitis under certain conditions [33,34]. The relative roles of TLR2 and NOD2 in regulating

mucosal inflammation of different etiologies, including Crohn's disease, remain to be fully understood [35].

Beyond studies on the role of NOD1/NOD2 in regulating inflammatory processes, several reports have addressed NOD1/NOD2-dependent host defense against specific microbial threats. For example, NOD2-deficient mice infected with invasive *L. monocytogenes* showed increased bacterial numbers in the spleen when challenged orally but not after intraperitoneal injection, indicating that NOD2 had a physiological function in local intestinal defense against the bacteria [24]. Expression of several genes encoding for cryptdins (peptides with antimicrobial activity produced by intestinal Paneth cells) was decreased upon infection of NOD2-deficient mice with *Listeria*, suggesting that NOD2 can regulate innate antibacterial defenses in the intestinal tract. Consistent with this concept, if not the actual mechanisms, intestinal epithelial cells engineered to overexpress NOD2 had reduced numbers of intracellular bacteria upon infection with *S. typhimurium* [26]. In regard to NOD1, mice lacking this sensor were shown to be significantly more susceptible to oral infection with *H. pylori* [22]. A possible underlying mechanism is suggested by the finding that primary gastric epithelial cells of NOD1-deficient mice produced lower levels of the neutrophil chemoattractant MIP-2 (CXCL2) upon stimulation with *H. pylori* [22]. Diminished epithelial chemokine production may compromise effector cell recruitment and thereby host defense against the bacteria.

CARD12 (IPAF/CLAN) and CARD5 (ASC): components of the inflammasome

IPAF is a CARD-containing NLR with a tripartite structure similar to NOD1 (Fig. 1) and serves likewise as a cytoplasmic sensor of microbial components. IPAF appears to be a cytosolic counterpart of TLR5, as it detects intracellular bacterial flagellin, a protein required for bacterial motility, from the enteropathogen *S. typhimurium* and from *Legionella pneumophila*, causative agent of Legionnaire's disease in the lungs [36*]. The dysentery-causing enteropathogen *S. flexneri* is also detected by IPAF, but, unlike *Salmonella* and *Legionella*, flagellin is dispensable in *Shigella* recognition [37]. Upon detection of these invasive pathogens, IPAF forms a complex with the key inflammasome adaptor apoptosis-associated speck-like protein (ASC/CARD5), which leads to caspase-1 activation, and to processing and subsequent release of mature IL-1 β and IL-18. IPAF-deficient macrophages did not process or release IL-1 β upon stimulation with *S. typhimurium*, while they were not defective in IL-1 β processing and release in response to TLR agonists plus ATP [38,39]. Similarly, activation of caspase-1 in response to *S. typhimurium* was abrogated in ASC-deficient macrophages [38]. These data indicate that IPAF (via ASC) mediates the recognition of a narrow spectrum of microbial triggers. Besides its specific role in the activation of caspase-1 and IL-1 β release, IPAF was also reported to inhibit nuclear factor- κ B signaling, since its overexpression in HEK 293 cells suppressed NOD1-dependent and NOD2-dependent nuclear factor- κ B activation [40]. Inhibition was accompanied by association of IPAF with NOD1 and NOD2 upon peptidoglycan stimulation in the human monocytic cell line THP1 [40], suggesting that hetero-oligomerization between different NOD domain-containing signal adaptors modulates innate immune response to bacteria and their products.

CARD8 (TUCAN/CARDINAL): negative regulator of nuclear factor- κ B signaling

At least two CARD-containing adaptors, CARD8 and CARD6, have been identified with regulatory and inhibitory functions in nuclear factor- κ B signaling and/or inhibition of IL-1 β processing. One of the adaptors, CARD8 (TUCAN/CARDINAL), mainly expressed in monocytes/macrophages, is an adaptor protein that inhibits TNF α and RIP2-induced nuclear

factor- κ B activation [41,42]. The adaptor may bind directly to the regulatory subunit of the I κ B kinase complex, I κ B kinase γ (NEMO), and inhibit I κ B kinase activation, although another study did not observe such an association [42]. In addition, CARD8 also interacts with and negatively regulates caspase-1, thereby suppressing release of mature IL-1 β , and inhibits caspase-9 activation and apoptosis [42]. Interestingly, an association between a genetic *CARD8* variant (nonsense mutation rs2043211; stop codon at Cys10) and Crohn's disease has been suggested in one recent report [43], although several other studies in British, German and Norwegian populations [44,45] did not find a significant association between genetic *CARD8* polymorphisms and Crohn's disease, and thus concluded that this gene is not a susceptibility locus for this condition. Beyond its role in inflammation, CARD8 may contribute to colon cancer pathogenesis. Increased CARD8 expression was found by immunostaining in colon cancers compared with adjacent unaffected colonic tissue [46]. Overexpression of CARD8 in epithelial cell lines protected the cells against inducible apoptosis [46], suggesting that this adaptor can contribute to enhanced survival of colon cancer cells by inhibiting normal epithelial cell death.

Conclusion

The mucosal immune system is confronted with an ongoing microbial challenge due to the presence of an abundant commensal microbiota and the intermittent exposure to microbial pathogens in food and water. Intra-cellular CARD-containing sensor and adaptor proteins are able to detect conserved structures present in gastrointestinal and other microbes and to initiate inflammatory responses, making them important players in innate defense against a wide range of invading microbial pathogens. The central immunologic roles of these sensors and adaptors suggest that they will be prime targets in the rational design of novel vaccine-based prevention strategies against gastrointestinal infections, and in the treatment of chronic inflammatory diseases in the intestine. Full exploitation of the CARD-carrying sensors and adaptors as targets of immunomodulatory intervention strategies, however, will require further characterization of their full physiologic functions under normal and disease conditions.

Acknowledgments

The work was supported by a research fellowship from the Swiss National Foundation to P.H. (SSMBS; PASMA 114623), and NIH grants AI56075, DK70867, DK80506, RR17030, and DK35108. The authors thank Gregory Botwin for help with the manuscript preparation.

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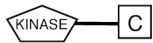
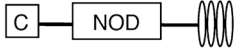
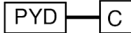



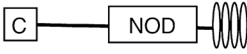
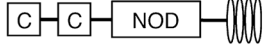
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Name	Alternative Names	Structure	Expression	Function
CARD3	RIP2/RICK/ CARDIAK		T cells, macrophages, dendritic cells, intestinal epithelial cells	Adaptor, activates NF- κ B, MAPK and caspase-1
CARD4	NOD1		T cells, macrophages, dendritic cells, intestinal epithelial cells	Sensor of iEDAP, from Gram-bacteria, activates NF- κ B, and MAPK
CARD5	ASC		Intestinal epithelial cells, macrophages, dendritic cells, hepatocytes	Key adaptor of the inflammasome
CARD8	TUCAN/ CARDINAL		Monocytes, colonic epithelial cells	Inhibitor of NF- κ B
CARD9			Dendritic cells, macrophages, B and T cells, natural killer cells	Key integrator of immune responses through NF- κ B and MAPK activation
CARD11	CARMA1/ BIMP3		B and T cells, macrophages	Adaptor, forms complex with BCL10, MALT1, and activates NF- κ B and MAPK
CARD12	IPAF/CLAN		Granulocytes, macrophages, monocytes, dendritic cells, T cells	Sensor of bacterial flagellin; activation of caspase-1 via CARD5 (inflammasome)
CARD15	NOD2		Granulocytes, macrophages, monocytes, intestinal epithelial cells	Sensor of MDP from Gram +/- bacteria, activates NF- κ B and MAPK











	Kinase domain		Card domain		Nucleotide-binding oligomerization domain		LRR; Leucine rich repeats region		Pyrin domain
	Function to find		Coiled-coil domain		PSD95/Dlg/ZO-1 homologous domain		Src homology 3 domain		Guanylate kinase like domain

Figure 1. Caspase recruitment domain (CARD)-containing intracellular sensor and adaptor proteins

The scheme depicts the different CARD-containing adaptors and sensors discussed in the review. CARD6/CARD7/CARD10/CARD14 and other CARD-containing molecules (e.g. caspases, APAF1, CIITA, Arc, RAIDD, ICEBERG) are not included here, as little is known about their intestinal functions. iEDAP, γ -D-glutamyl-meso-diaminopimelic acid; MAPK, mitogen-activated protein kinases; MDP, muramyl dipeptide; NF, nuclear factor.

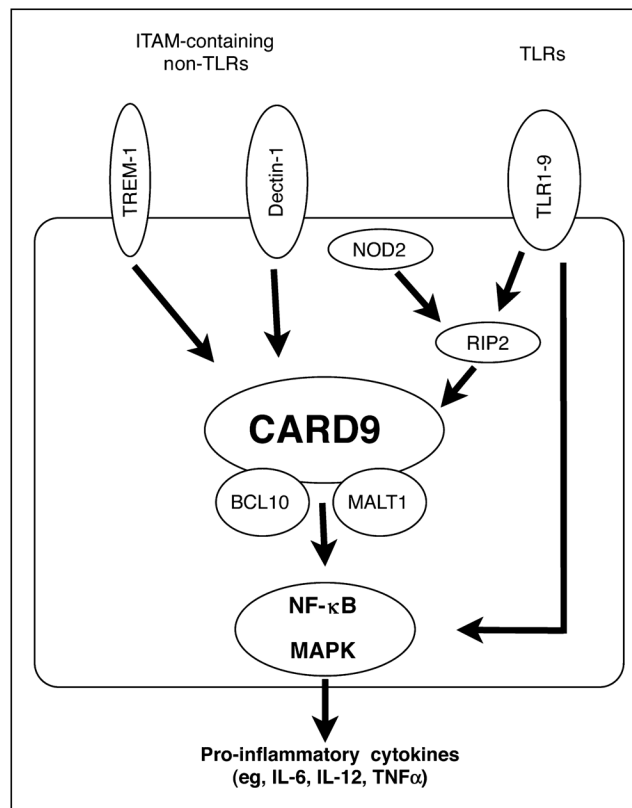


Figure 2. CARD9 functions in myeloid cells

After activation of membrane-bound microbial sensors such as Toll-like receptors (TLRs) and immunoreceptor tyrosine-based activation motif (ITAM)-containing non-TLRs, and the cytoplasmic sensor NOD2 (CARD15), signaling pathways converge on CARD9. Stimulation of dectin-1 with the fungus-derived ligand zymosan or of TREM-1 (unknown specific ligand) induces phosphorylation of specific tyrosine residues in the ITAMs, leading to downstream formation of a signaling complex of CARD9 with other adaptor proteins, particularly BCL10 and MALT1. TLRs can activate RIP2 (CARD3) and CARD9 in response to a broad range of microbial ligands, while NOD2 interacts with RIP2 and CARD9 via CARD–CARD interactions upon intracellular recognition of muramyl dipeptide. Formation of the signaling complex with CARD9, BCL-10 and MALT1 leads to activation of nuclear factor (NF)- κ B and mitogen-activated protein kinases (MAPK), and subsequently secretion of proinflammatory cytokines. TLRs can also activate NF- κ B through a CARD9-independent pathway.