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TOLL-LIKE RECEPTORS AND NOD/CARD PROTEINS: PATTERN RECOGNITION RECEPTORS ARE KEY ELEMENTS IN THE REGULATION OF IMMUNE RESPONSE

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Summary

The magnitude of the response to a specific immunogen such as an infectious agent is the result of a complex interaction between genetic and environmental factors. For example, in intestinal inflammation, the inflammatory response appears to be regulated by the indigenous microflora of the gut, by receptors in epithelial cells and antigen-presenting cells in the intestinal mucosa, and by immunologic factors. Recent evidence suggests that genetic variants of human immunomodulating genes influence the susceptibility to and severity of infectious diseases and the subsequent clinical outcome of disease.

This review will focus on recently identified pattern recognition receptors which are located on innate immune and epithelial cells, and recognize pathogen-associated molecular patterns. The binding of specific pathogen-associated molecular patterns to these receptors results in the activation of a signal transduction pathway through nuclear factor (NF)-κB which leads to either enhanced or inhibited immune responses that modify the production of inflammatory effectors, such as cytokines.

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This article reports on the identification and functional characterization including the discovery of mutants which completely abolish NF-κB signal transduction of pattern recognition receptors, such as the extracellular Toll-like receptors and the intracellular nucleotide oligomerization domain/caspase recruitment domain (NOD/CARD) receptors, as well as their role in clinical disease.

Knowledge of pattern recognition receptors such as Toll-like receptors and NOD/CARD intracytoplasmic proteins, including their functions and their downstream signaling pathways, may provide a new molecular basis for preventing or blocking inflammation associated with pathogenic microorganisms. This could direct a new focus for better and more specific therapeutic treatments based on immuno-intervention that can promise a better quality of life for those suffering from chronic disturbances of the immune response.

Introduction

The ability of a multicellular organism to defend itself against microorganisms depends on its ability to mount a proper immune response. The innate immune system is an ancient defense system that provides the first line of host defense (1). This defense system appeared before the separation of vertebrates and invertebrates. The vertebrates have not only innate immunity but are also able to mount defense mechanisms that constitute adaptive immunity as well (2, 3). The innate immune system is composed of humoral factors (such as complement, acute-phase proteins and cytokines) and cellular components (such as macrophages, monocytes, granulocytes, dendritic cells, T cells, B cells and natural killer [NK] cells). Pattern recognition receptors located either on the surface or in the cytoplasm of innate immune cells appear to play a crucial role since they are able to recognize pathogen-associated molecular patterns without the specificity required by the acquired immune system. Upon activation of these receptors, a cascade of intracellular events culminates in the production of inflammatory effectors such as interleukin (IL)-12 and IL-23 (4–6).

Pathogen-associated molecular patterns

In prokaryotes, molecular structures are essentially polysaccharides and polynucleotides that differ little from one pathogen to another but are not found in the host. They are conserved structures within a class of microbes, and in most cases they are expressed constitutively, which is probably the reason why they were selected as targets by the innate immune response during evolution. Examples of pathogen-associated molecular patterns are mannans in the yeast cell wall, flagellin in bacterial flagella, peptidoglycan, lipopolysaccharide, double-stranded ribonucleic acid (dsRNA) and unmethylated cytidine-phosphate-guanosine (CpG) DNA (4, 7–9).

Pattern recognition receptors

This group of proteins is responsible for recognizing pathogen-associated molecular patterns and detecting the presence of infection. The pattern recognition receptors could be classified into three subgroups: 1) secreted pattern recognition receptor molecules such as C4 and C2, which induce destruction of pathogens by the complement system or phagocytosis; 2) surface pattern recognition receptors on phagocytic cells, which bind the pathogen for engulfment; and 3) cell pattern recognition receptors (*i.e.*, Toll-like receptors and NOD/CARDs), which bind the pathogen, and, upon activation, cause the release of effector molecules against the pathogen, thus initiating the inflammatory response (Table I).

Commensal and pathogenic microorganisms have pathogen-associated molecular patterns, but the recognition and development of an immune response depends on where the pathogen-associated molecular patterns can be accessed by the pattern recognition receptors and consequently contribute to inducing an immune response (4, 7–9).

Toll-like receptors

The Toll-like receptors are pattern recognition receptors that activate signaling pathways that sense invasion by microorganisms and induce antimicrobial responses triggering inflammation upon recognition of pathogen-associated molecular patterns. These molecules play a central role in innate immunity (8).

dTOLL

Studies on the innate immunity of *Drosophila melanogaster* identified the first member of the Toll family, *Drosophila* Toll (dToll). This Toll receptor plays a role in dorsal-ventral development during embryogenesis and in the innate immune response in this fly. Lemaitre *et al.* (10–12) reported that the Toll receptor and downstream elements of the Toll signaling pathway were implicated in the control of

Table I. Principal characteristics of Toll-like receptors and NOD/CARD proteins.

	Toll-like receptors (ref.)	NOD/CARD (ref.)
Function	Participate in the innate immune response to microbial agents (8) Act via MyD88 and TRAF6 (15), leading to NF-κB activation, cytokine secretion and inflammatory response (4, 15, 99)	Induces NF-κB and apoptosis (81) Confers responsiveness to intracellular bacterial lipopolysaccharide and peptidoglycan (42, 82, 91, 137)
Domains	1 TIR domain and LRR domains (4, 9, 15)	CARD domain(s), NBS domain and LRR domains (81)
Subunits	Bind MyD88 (and TIRAP for TLR4) via their respective TIR domains (7)	Binds to RIPK-2 (RICK, RIP or CARDIAK) by CARD-CARD interaction (77, 78, 103)
Subcellular localization	Type I membrane protein (8)	Cytoplasmatic (77, 78)

the expression of antifungal peptides (Drosomycin) in *Drosophila*. Spätzle factor is a secreted ligand that stimulates dToll. The activation of this transmembrane receptor culminates in the translocation of a transcription factor, Dorsal, to the nucleus (13). To date, there are nine Toll members described in *Drosophila* (13).

Human Toll-like receptors

There is a close analogy between the signaling pathways of mammals and flies, but with the difference that dToll does not function directly as a pattern recognition receptor. This analogy appears to represent an ancient host pathway conserved during evolution. Members of the Toll family play a key role in innate antibacterial and antifungal immunity in insects as well as in mammals (8, 14). The signaling pathway activated by hToll receptors activates NF-κB, a key molecule in the activation of proinflammatory and immunoregulatory genes (15). Activation of Toll-like receptors by microbial lipoproteins may initiate innate defense mechanisms against infectious agents and trigger inflammation upon recognition of pathogen-associated molecular patterns. Brightbill *et al.* (16) demonstrated that microbial lipoproteins stimulate the Toll-like receptors in human macrophages and the production of IL-12, a special cytokine of the innate immune system which may be the link with the acquired immune response.

In 1997, Medzhitov *et al.* (1) reported on a human homologue of dToll, Toll-like receptor (TLR)4. The mRNA of this human Toll-like receptor was detected in many tissues and human cell lines. All human Toll-like receptors are type I membrane proteins with an extracellular leucine-rich region (LRR) domain and an intracellular domain homologous to Toll-interleukin-1 receptor (4, 9, 15) (Fig. 1). Despite the important role of Toll-like receptors in the recognition of pathogen-associated molecular patterns, it is possible that these proteins cannot identify all microbial antigens because they may have limited functional plasticity. Other molecules probably participate in this process (1, 8, 17).

Significance of the domains of Toll-like receptors

The LRR domain

The LRR domains are short sequence motifs (20–29 residues) present in tandem arrays and in different proteins of many organisms with diverse biological functions and cellular locations (Fig. 1). Their primary function appears to be to provide a versatile structural framework for the formation of protein-protein interactions and subsequent signal transduction, such as hormone-receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking (18–20). Bacterial LRR-containing proteins are found in *Listeria monocytogenes*: InlA and InlB, internalins with a critical role in the phagocytosis induced by this pathogen in mammalian cells, promote invasion of permissive cells (21). LRR-containing proteins have also been identified in *Yersinia pestis* (YopM, a plague virulence protein), *Shigella flexneri* (IpaH) (22), *Salmonella typhimurium* (SspH1 and SspH2) (23) and *Burkholderia* (*Pseudomonas*) *solanacearum* (Hrp) (24). Among eukaryotic cells, LRR domains are present in proteins involved in early mammalian development, neural development, cell polarization, regulation of gene expression and apoptotic signaling (*i.e.*, the disease-resistant

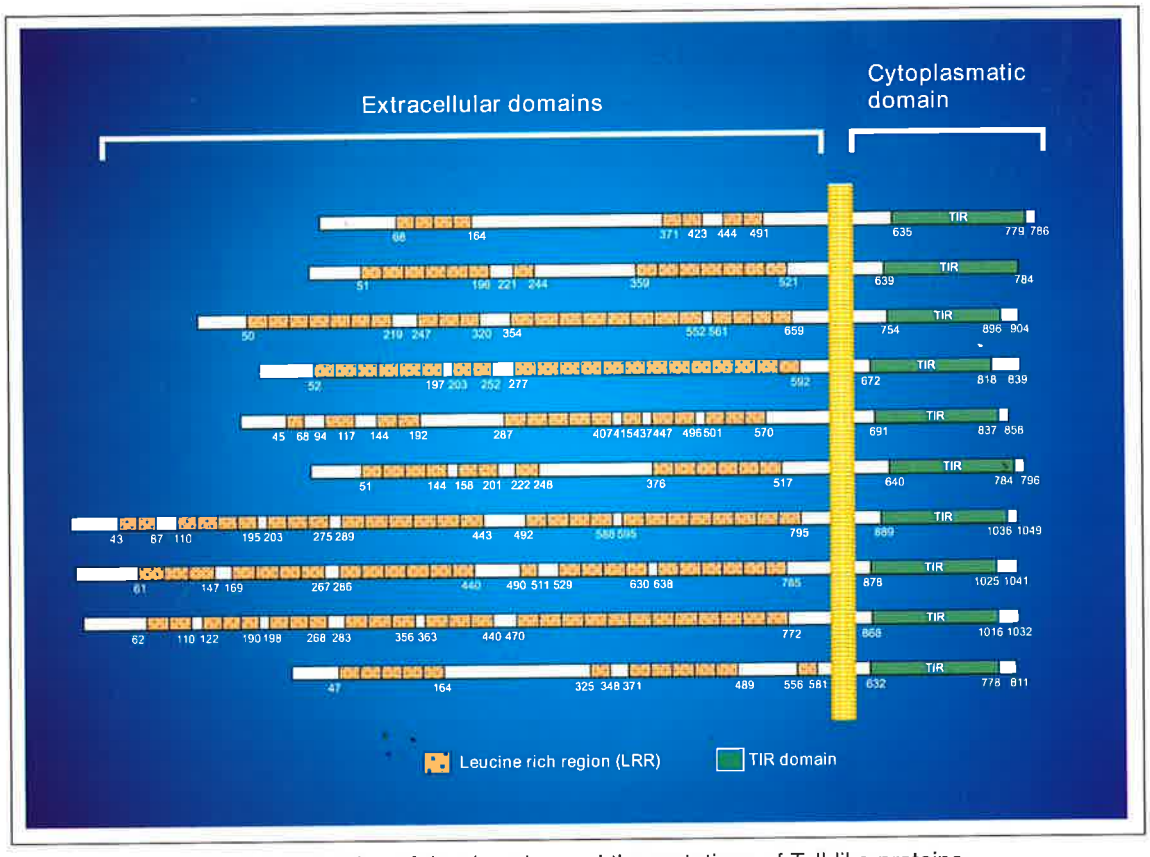

Fig. 1. Localization of the domains and the mutations of Toll-like proteins.

genes of the plant immune system, and the Toll and Toll-like genes of *Drosophila* and mammals, respectively) (18, 19, 25). Sequence analyses of LRR proteins suggested the existence of several different subfamilies of LRRs (26, 27). The most recent classifications reveal at least seven distinct subfamilies characterized by different lengths and consensus sequences of the repeats that have most probably evolved independently. All major classes of LRRs have curved horseshoe structures with a parallel sheet on the concave side and mostly helical elements on the convex side (27, 28). The concave face and the adjacent loops are the most common protein interaction surfaces on LRR proteins. The three-dimensional structure of some LRR protein-ligand complexes show that the concave surface of the LRR domain is ideal for interaction with α-helix, supporting earlier conclusions that the elongated and curved LRR structure provides an outstanding framework for achieving diverse protein-protein interactions. Little is known about the three-dimensional structure of LRRs, although it is believed that they can form amphipathic structures with hydrophobic surfaces capable of interacting with membranes (26–28) (Table II).

Toll-interleukin-1 receptor domain

Toll-interleukin-1 receptor (TIR) domain is a membrane component found in transmembrane and cytoplasmatic proteins (8) (Fig. 1). TIR functions as a transmembrane receptor, a protein-interaction module involved in animal and plant immunity (Table II). This domain is found in a number of plant proteins and is implicated in host defense. In *Drosophila* spp., at least two types of proteins with this domain, myeloid differentiation primary response gene (MyD)88 and Toll-like receptor family, have been described (29). These two proteins are also found in mammals and additional members have been identified: IL-1R family (IL-1R and IL-18R) and TIR domain-containing adaptor protein (TIRAP). MyD88 and TIRAP are signaling receptors that

Table II. Characteristics of the most important domains in Toll-like receptors and NOD/CARDs proteins.

	TIR (ref.)	CARD (ref.)	LRR (ref.)
Process	Host defense Apoptosis (30–32)	Apoptosis (3, 77, 78, 84, 85)	Signal transduction for different mechanisms, such as hormone receptor interactions, enzyme inhibition, cell adhesion and cellular trafficking (18, 19, 104)
Function	Transmembrane receptor (8)	Apoptosis regulator (3, 77, 78, 84, 85)	Protein-protein interaction. In combination with a NBD domain, mediates innate defenses against pathogens in diverse organisms (4, 19)
Component	Membrane (8)	Intracellular (3, 77, 84, 85)	Extracellular or intracellular (4, 19, 77)

participate in Toll-like receptor signal transduction, and the TIR domain of MyD88 is associated with a so-called DEATH domain (DD/DED) (30–32). The defined similarity found between Toll-like receptors and IL-1R is not restricted to sequence homology; these proteins also share a similar signaling pathway with activation of a Rel-type transcription factor via an adaptor protein and a protein kinase (15, 17, 29, 33, 34).

The essential role of TIR in Toll and IL-1R activities has been shown by site-directed mutagenesis and deletion analysis. Using sequence analysis, Slak *et al.* (34) identified three highly conserved regions or boxes among the different members of the family: box 1, box 2 and box 3. Boxes 1 and 2 may be implicated in the binding of proteins involved in signaling, whereas box 3 is primarily involved in directing receptor localization, perhaps through interactions with cytoskeletal elements.

Human Toll-like receptor family: A brief description

At present, ten Toll-like receptor each specialized, often with the aid of accessory molecules, in a subset of pathogen-associated molecular patterns have been identified in mammals (Table III). The ligand identification is still in progress for many of the Toll-like receptors.

TLR1

TLR1 cooperates with TLR2 of other Toll-like receptors and modulates the response to bacterial constituents. Rock *et al.* (35) found that TLR1 is ubiquitously expressed in leukocytes and at higher levels than the other Toll-like receptor genes. TLR1 expression is unchanged in response to lipopolysaccharide (35, 36). TLR1 is supposed to functionally interact with TLR2 in the recognition of bacterial triacyl lipopeptides and lipoproteins from *Mycobacterium* spp. and soluble factors of *Neisseria meningitides* (13, 37).

TLR2

TLR2 plays a pivotal role in the recognition of Gram-positive bacteria (38), mycobacteria (39, 40) and fungi (41). Aderem and Ulevitch (7) demonstrated that Gram-positive cell wall components, as well as mycobacterial cell wall components (lipoarabinomannan and mycolylarabinogalactan) and yeast cell wall zymozan, activate TLR2. Recently, Inohara *et al.* (42) reported that TLR2 in combination with TLR1/TLR6 do not activate NF-κB in response to muramyl dipeptide derivated from peptidoglycan. Atypical lipopolysaccharide present in *Leptospira interrogans* (43) and *Porphyromonas gingivalis* (44–46) is, however, recognized by TLR2. This broad range of ligands recognized by TLR2 can be partially explained by the collaboration between TLR2 and another Toll-like receptor (TLR1 or TLR6) (47). Heterodimeric formation between TLR2 and TLR1 or TLR6 determines the specificity for the ligand to be recognized. TLR2 cooperates with TLR6 in the recognition of mycoplasmal macrophage-activating lipopeptide (MALP)-2, phenol-soluble modulin and *Borrelia burgdorferi* outer surface protein A lipoprotein (OspA-L) (48). TLR2 stimulation resulted in the release of the inhibitory IL-12 p40 homodimer, which favors Th2 development and IL-8 and IL-23 p19 expression (49).

TLR3

TLR3 is the pattern recognition receptor that recognizes dsRNA, which it is predominantly expressed in dendritic cells but not expressed in monocytes/macrophages (13, 50). Activation of TLR3 induces the production of α- and β-interferons and other multiple mechanisms to enhance and sustain a strongly antiviral response (51). This molecular

Table III. Characteristics of Toll-like receptor (TLR) proteins.

	TLR1	TLR2	TLR3	TLR4	TLR5
Function	Cooperates with TLR2 and modulates the response to microbial constituents (35, 36, 70)	Cooperates with MD-2 to mediate the innate immune response to bacterial lipopolysaccharide and other microbial cell components (156, 157). May also promote apoptosis in response to lipopolysaccharide (156,157). Recognizes MALP-2, PSM and OspA-L cooperatively with TLR6 (48)	May be involved in the recognition of double-stranded RNA (52)	Cooperates with MD-2 and CD14 to mediate the innate immune response to bacterial lipopolysaccharide. Activation of the NF-κB pathway also occurs via TIRAP (8)	Mediates detection of bacterial flagellin (65)
Subunit	Binds TLR2 via their respective extracellular domains (35, 36, 70)	Binds MD-2, TLR1 and TLR6 via extracellular domain (95)		Belongs to the lipopolysaccharide receptor, a multiprotein complex containing at least CD14, MD-2 and TLR4. Binds MD-2 via extracellular domain. Lipopolysaccharide binding protein can form a complex together with lipopolysaccharide and CD14 and activates TLR4 (7, 8, 95)	
Tissue specificity	Ubiquitous. Highly expressed in spleen, ovary, leukocytes, monocytes/macrophages, myeloid dendritic cells, thymus and small intestine (35, 36, 70)	Highly expressed in leukocytes (monocytes/macrophages), myeloid dendritic cells, mast cells, bone marrow, lymph node and spleen. Detected in lung and in fetal liver. Low levels in other tissues (35)	Highly expressed in placenta and pancreas. Predominantly expressed in dendritic cells and not in other leukocytes, including monocytes (36, 70)	Highly expressed in placenta, spleen and leukocytes, monocytes/macrophages, dendritic cells, mast cells and several types of T-cells, corneal epithelial cells, dermal endothelial cells and low expression in intestinal epithelial cells (36, 50, 70). High expression in myocytes (61)	Highly expressed in ovary and leukocytes, monocytes/macrophages, myeloid dendritic cells and in the basolateral surface of intestinal epithelial cells. Detected in prostate, testis (36, 70, 159)
Nº of LRR	8	14	22	21	15
Synonyms	Toll/interleukin-1 receptor-like TIL (35)	Toll/interleukin-1 receptor-like 4; TIL4 (159)	None	hToll, homologue of Toll *Drosophila* (35)	Toll/interleukin-1 receptor-like 3; TIL3 (159)
Gene map	4p14 (162)	4q32 (35)	4q35 (35)	9q32-q33 (35)	1q41-q42 (35)
Length	786 AA (35)	784 AA (159)	904 AA (35)	841 AA (8)	858 AA (159)
Polymorphisms		Allele 2 (677W) is associated with susceptibility to lepromatous leprosy and affects IL-12 production by monocytes in lepromatous leprae (104, 105). Allele 2 (Gln-753) is potentially associated with staphylococcal infection (106)		Allele 2 (299G, 399I) is associated with blunted response to inhaled lipopolysaccharide, lower level of certain cytokines, acute phase proteins, soluble adhesion molecules, more susceptibility to bacterial infection, lower risk of carotid atherosclerosis and a smaller intimae-media thickness in the common carotid artery (58, 107, 108)	

TLR6	TLR7	TLR8	TLR9	TLR10
Participates in the innate immune response to Gram-positive bacteria and fungi. Recognizes MALP-2, soluble tuberculosis factor, phenol-soluble modulin and OspA-L cooperatively with TLR2 (8, 48)	Mediates the immune response to imidazoquinolines: imiquimod and resiquimod-induced response. Deficiency produces undetectable IL-12 and TNF in response to imidazoquinolines (71)		Detects the unmethylated CpG motifs present in bacterial DNA (71, 158)	Closely related to TLR1 and TLR6 (72)
Binds TLR2 via their respective extracellular domains (69)				
Detected in monocytes/macrophages, myeloid dendritic cells, mast cells, plasmacytoid dendritic cells, mast cells and dermal microvessel endothelial cells (8, 36, 70)	Detected in brain, placenta, spleen, stomach, small intestine, lung, plasmacytoid and myeloid dendritic cells and macrophages (73, 160)	Detected in brain, placenta, lung, liver, heart, trachea, monocytes, mast cells and myeloid dendritic cells (73, 160)	Highly expressed in spleen, lymph node, tonsil and peripheral blood leukocytes, especially in plasmacytoid dentritic cells, monocytes. Also detected in lung and liver (160, 161)	Highly expressed in macrophages, spleen, lymph node, thymus, tonsil and a lower levels in lung. Highly expressed in promyelocytic HL-60 cells and in B-cell lines (72, 160)
20 (69)	27 (73, 160)	24 (73, 160)	26	12 (160)
None	None	None	None	None
Chr. 4 (69)	Xp22 (73, 160)	Xp22.3-p22.2 (73, 160)	3p21.3 (73, 160)	–
796 AA (69)	1049 AA (73, 160)	1041 AA (73, 160)	1032AA (71)	811 AA (160)
			The -1237C allele has been associated with asthma among the European-American population	

pattern is associated with viral infection because most viruses produce it at some point during their replication (52). TLR3-deficient mice showed reduced responses to a synthetic dsRNA, polyinosine-polycitidylic acid (polyIC), and reduced production of the inflammatory cytokines IL-6, IL-12 and tumor necrosis factor (TNF)-α. TLR3 induces cytokine production through a signaling pathway dependent on MyD88 (52). In intestinal epithelial cells of active Crohn's disease but not in ulcerative colitis, a down-regulated expression of TLR3 has been related to a selective change in Toll-like receptor expression that may contribute to alteration of the innate immune response and to the pathogenesis associated with inflammatory bowel disease (IBD) (53, 54).

TLR4

TLR4 protein is the first characterized mammal Toll-like receptor expressed in a variety of cell types, (*i.e.*, macrophages and dendritic cells) (1, 9, 12). It functions as a signal-transducting receptor for the lipopolysaccharide of Gram-negative bacteria (55–57) and is involved in the recognition of lipoteichoic acid (38) and mannuronic acid polymers (58). Recognition of viral products has also been proposed to be mediated by TLR4, and studies using animal models point to a major role of TLR4 in the response to F protein of respiratory syncytial virus (9, 59). Heat shock protein (Hsp)60, a molecular region conserved from bacteria to mammals that induces inflammatory responses, is also bound by TLR4 (9). The lipopolysaccharide recognition is complex and requires several accessory molecules, such as membrane or serum monocyte differentiation antigen (mCD14 or sCD14), lipopolysaccharide binding protein and MD-2 (9, 37). This receptor is highly expressed in the cardiac myocytes of normal myocardium and is unregulated in injured myocardium (60, 61). Muzio *et al.* (36, 62) reported an increased expression of this receptor in the presence of bacterial products and proinflammatory cytokines. TLR4 is expressed at low levels in intestinal epithelial cells (63). A strongly upregulated expression of this receptor in patients with IBD has been associated with an inflammatory response to intestinal flora potentially related to the phenotype of IBD (53, 54). C3H/HeJ mice, which have a defective *TLR4* gene, exhibit defective lipopolysaccharide signaling (55, 56) and an increased susceptibility to disseminated candidiasis (64). A remarkable finding is that the *TLR4* promoter gene region is characterized by the absence of TATA and CCAAT boxes (35).

TLR5

TLR5 recognizes flagellin from both Gram-positive and Gram-negative bacteria (65). Flagellin is a principal component of bacterial flagella and a virulence factor recognized by the innate immune system in plants, insect and mammals that induces IL-6 production by a MyD88-dependent mechanism in the pathway activation. This monomeric functional unit of the flagella is very important for bacterial invasion and adhesion, and it additionally functions as a modulin (66). Flagellin is important for the activation of epithelial cells and is associated with different clinical symptoms: systemic inflammation, pulmonary inflammation, shock and acute respiratory distress syndrome. Flagellin is critical for bacterial motility and is therefore conserved. Thus, it is a relatively invariant pathogen-associated molecular pattern that serves as a ligand for a pattern recognition receptor. Activation of the receptor stimulates TNF-α production (65, 66). Murine *TLR5* lies within a locus that is associated with susceptibility to *Salmonella* spp. TLR5 is expressed on the basolateral side of the intestinal epithelium, where the flagellin from pathogenic bacteria such as *Salmonella* spp. could be sensed (8, 66–68). The relevance of TLR5 in the development of strategies to counteract flagellin-mediated immune inflammatory responses needs to be studied in greater depth. The development of interferences with the binding of flagellin to TLR5, either by competitive/noncompetitive antagonist molecules or by targeting downstream pathways triggered for TLR5, has been suggested (66).

TLR6

TLR6 is not as well understood as other members of the Toll-like receptor family. It has a 69% similarity to human TLR1, with 90% of the TIR domains of both proteins being conserved (69), suggesting that TLR6 could be the product of an evolutionary duplication (13). TLR6 apparently discriminates between bacterial lipoproteins, which are triacylated at the *N*-terminal cysteine residue, and the diacylated MALP-2 (8, 37). Moreover, TLR6 participates in the innate immune response to Gram-positive bacteria and fungi (69). In addition to recognizing MALP-2, TLR6 also senses soluble tuberculosis factor, phenol-soluble modulin and OspA-L, in cooperation with TLR2. Both *TLR6* and *TLR1* are

expressed constitutively on many cell types, but TLR2 expression is regulated and restricted to antigen-presenting cells and endothelial cells (8, 70).

TLR7

TLR7 appears to be required for imidazoquinoline-induced antiviral and antitumor responses. Macrophages of mice deficient in TLR7 but not other Toll-like receptors did not induce detectable production of the cytokines IL-12 and TNF in response to imidazoquinolines (potent synthetic activators of immune cells with antiviral and antitumor properties) such as imiquimod and resiquimod (71). The activation of TLR7 by endogenous ligands generated during a viral infection or by viral products is suspected (37, 71).

TLR8

The function of TLR8 is still not clearly understood. *TLR8* gene has a 42% similarity to *TLR7* gene and the protein is 73% similar to TLR7. Both genes are located close to each other on chromosome X (72, 73).

TLR9

TLR9 mediates the recognition of bacterial DNA, which has an immunostimulatory effect on the mammalian immune system (*i.e.*, dendritic cell maturation, B-cell proliferation, and production of IL-6 and Il-12 by monocytes) through the presence of unmethylated CpG motifs (37). Human DNA, unlike bacterial DNA, is mostly methylated (37). Hemmi *et al.* (71) showed that TLR9 mediated a cellular response against CpG DNA present in bacteria. It is presumed that TLR9 responsiveness to CpG requires endosome acidification (74–76).

TLR10

TLR10 protein is 50% identical to TLR1 and TLR6. The function of this recently cloned gene is not clearly understood.

NOD/CARD family

NOD/CARDs have striking structural similarity to a class of cytosolic plant disease-resistant gene products and are among the intracellular apoptosis regulators (77, 78). Ced4 is a nematode protein that plays an important role during the execution of programmed cell death during worm development (77). APAF1 is also involved in apoptosis in mammals during the development of different tissues, including brain, limb and eye (79, 80). It has been suggested that NOD/CARDs are involved in the cellular response to pathogen components, as an intracellular bacterial pattern-recognition receptor protein (78, 81) and in the apoptotic process (82, 83). The CED4/APAF1 superfamily is composed of *N*-terminal CARD, a centrally located nucleotide binding domain/nucleotide oligomerization domain (NBD/NOD) and a C-terminal regulatory domain with eight tandem repeats of about 40 residues, each containing a central Trp-Asp motif (WD40 repeats) (82, 83). NOD1/CARD4 and NOD2/CARD15 proteins belong to this family. Both molecules induce activation of the NF-κB pathway. CARD and NBD/NOD domains are found in NOD1/CARD4 and NOD2/CARD15 proteins. LRR domains are located in the carboxy terminal region (81) like the WD40 repeats of the CED4/APAF1 proteins (77, 78, 81, 82).

Significance of the NOD/CARD family domains

Caspase recruitment domain

This domain is an intracellular component widespread among apoptotic signaling molecules and mediates homodimerization. The CARD domain typically associates through homophilic CARD interactions with other proteins, forming either dimers or trimers. It has been predicted that CARD is related in structure and sequence to both DD and DED domains, which activate similar pathways and show similar interactive properties. Its structure consists of six antiparallel helices arranged in a topology homologous to the DEATH and the DED domain (3, 77, 78, 84, 85) (Table II).

Nucleotide-binding oligomerization domain

NBD/NOD domain participates in the process of oligomerization of molecules. The apoptotic signal initiated by ligand-induced oligomerization of death receptors is mediated by a number of adaptor proteins containing specialized interaction domains. The combination of this domain with LRR constitutes the crucial structural domains involved in mediating pathogen recognition and/or inflammation (4, 77).

LRR domains

The carboxy terminal LRR probably plays a negative regulatory role in the NF-κB pathway because the presence of mutations in this region has shown low activation of this pathway. A good example of the role of this domain in *NOD2/CARD15*

Table IV. Characteristics of NOD/CARD proteins.

	NOD1/CARD4	NOD2/CARD15
Function	Mediates responsiveness to lipopolysaccharide and peptidoglycan Induction of apoptosis (86)	Mediates responsiveness to peptidoglycan Induction of apoptosis (81, 121)
Tissue specificity	Broadly expressed	Peripheral blood, leukocytes. Primarily in monocytes (81, 121) and intestinal epithelial cell lines (89, 90)
Domains	1 CARD, 1 NBS, 10 LRR (82, 84)	2 CARDs, 1 NBS, 10 LRR (81, 121)
Gene map	7p15-p14 (82)	16q12 (81, 121)
Protein length	953 AA (84)	1040 AA (81, 121)
Polymorphisms		R702W, G908R and L1007fs are associated with Crohn's disease in Western populations primarily. Ileum-terminal lesions, early onset and less frequency of colonic involvement are the common phenotypes of Crohn's disease in cases with *CARD15* mutations (81, 120, 121)
		R790E associated with Crohn's disease in African-Americans (131, 135)
		Haplotype combination IVS8+158 (C>T) and 268S associated with Crohn's disease in Ashkenazi Jews (139)
		R334Q, R334W and L469F are associated with Blau syndrome (145)
		P268S is inversely associated with ulcerative colitis-spondylitis ankylopoetica and greater disease severity (140)

can be seen in the structurally similar R resistance genes in plants. Each R gene protects plant cell from a specific pathogen. Specificity of R proteins for pathogens is determined by genetic variations in the LRRs of the R proteins (18, 20, 28).

NOD/CARD family: A brief description

To date, there are just two NOD/CARD members in this family described in mammals (Table IV).

Caspase recruitment domain-containing protein 4 (NOD1/CARD4)

The gene map locus of *NOD1/CARD4* is on 7p15-p14. The cytosolic NOD1/CARD4 protein, composed of 53 amino acids, has a CARD domain that along with the NBS/NOD domain induces NF-κB activation (82, 84). The LRR is composed of ten tandem repeats like the LRR of NOD2/CARD15. The CARD domain of NOD1/CARD4 induces activation of NF-κB (through this pathway it promotes apoptosis) and c-JUN *N*-terminal kinase by invasive *Salmonella flexneri* (86). This protein has been found in adult heart, spleen and lungs, as well as in numerous cancer cell lines and fetal tissues (84); it mediates responsiveness to bacterial lipopolysaccharide and peptidoglycan (87). *CARD4/NOD1* gene has not been involved in IBD of its phenotype (88).

Caspase recruitment domain-containing protein 15

The *NOD2/CARD15* gene located at 16q12 codifies a 1040-amino acid protein. Unlike NOD1/CARD4, NOD2/CARD15 protein possesses two *N*-terminal CARDs. Both CARDs are necessary for the NF-κB pathway activation via the signal transducers IKKs and RICK (81). This cytoplasmatic bacterial pattern recognition receptor protein is expressed in monocytes (78, 81) and intestinal epithelial cells (89, 90), and has been shown to mediate responsiveness to preparations of lipopolysaccharide and peptidoglycan (82). However, recent biochemical and functional analyses identified muramyl dipeptide derived from peptidoglycan as the essential structure in bacteria recognized by NOD2/CARD15. Notably, TLR4, but not NOD2/CARD15, was stimulated by highly purified lipopolysaccharide prepared by gel-filtration chromatography, show-

ing that previous results identifying lipopolysaccharide as the ligand for NOD2 were based on the presence of peptidoglycan in poorly purified lipopolysaccharide preparations. Although NOD2/CARD15 can mediate the recognition of muropeptides, the mechanism involved is unclear and remains to be determined. Because the LRRs are required for recognition, muropeptides could interact directly with NOD2/CARD15 via as yet to be identified cellular factor(s) (42, 91, 92). Because activation of NF-κB in response to bacterial components mediates protection of the host against pathogens, NOD2/CARD15-mediated susceptibility to disease may be caused by a failure to trigger a protective NF-κB pathway in response to muropeptides. Such a defective response against certain bacterial products may secondarily influence the diffuse activation of NF-κB by NOD2/CARD15-independent mechanisms, including those mediated by the Toll-like receptors. Expression of NOD2/CARD15 results in NF-κB activation and mutants lacking the LRRs had reduced NF-κB activation (81).

NF-κB pathway activation

NF-κB is a transcription factor detected in numerous cell types that is involved in the expression of cytokines, chemokines, growth factors, cell adhesion molecules and some acute-phase proteins in health and in various disease states. NF-κB is activated by a wide variety of stimuli such as cytokines, oxidant-free radicals, inhaled particles, ultraviolet irradiation and bacterial or viral products. Inappropriate activation of NF-κB has been linked to inflammatory events associated with autoimmune arthritis, asthma, septic shock, lung fibrosis, glomerulonephritis, atherosclerosis and AIDS (93). In contrast, complete and persistent inhibition of NF-κB has been directly linked to apoptosis, inappropriate immune cell development and delayed cell growth. NF-κB is a heterodimer composed of two subunits: p65 (also called relA) and p50. The inactive form of NF-κB is cytoplasmatic and is bound to Iκ-Kα and Iκ-Kβ, which are phosphorylated by IKKs, causing its degradation and the entry of NF-κB into the nucleus, where it binds to specific promoter regions (93).

In general, binding of the pathogen to the Toll-like receptors or NOD/CARD proteins initiates a signaling pathway leading to the activation of NF-κB. This transcription factor turns on many cytokine genes such as those for TNF-α, IL-1 or chemokines, which attract white blood cells to the site of infection. All of these effector molecules lead to inflammation and can trigger adaptive immunity (93). NF-κB activation by Toll-like receptors or NOD/CARD proteins is described below.

Mechanisms involving Toll-like receptors

The binding of microbial products to pattern recognition receptors such as Toll-like receptors activates intracellular signaling pathways that result in the expression of inflammatory genes through NF-κB and MAPK signaling (94). Figure 2 shows the principal molecules participating in the activation of Toll-like receptors by pathogen-associated molecular patterns. Pathogen-associated molecular patterns such as lipopolysaccharide initiate their biological activities through a heteromeric receptor complex containing CD14, TLR4 (7) and at least one other protein, MD-2 (7, 8). MD-2 is a secreted protein that binds to the extracellular domain of TLR4, where it facilitates lipopolysaccharide responsiveness (8, 95). CD14 works as a receptor and is expressed either anchored to the cell membrane (mCD14) or secreted by myeloid cells (sCD14). Both molecules are critical for the transduction of the signal provoked by lipopolysaccharide, but sCD14 confers sensitivity to cells lacking mCD14. Lipopolysaccharide binds a serum circulating ligand known as lipopolysaccharide-binding protein. This lipopolysaccharide/lipopolysaccharide-binding protein complex is recognized by CD14, which, in the presence of lipopolysaccharide stimulation, become physically closer to TLR4. Lipopolysaccharide-binding protein is an opsonin for mCD14 (96).

TLR4 is activated by the LRR region. The TIR domain of TLR4 interacts with the TIR domain of MyD88 (7). MyD88 is an adaptor protein also involved in TLR2, TLR3, TLR5, TLR6 and TLR9 signaling (97). MyD88 links Toll-like receptors to IRAK, a serine-threonine kinase, by a CARD-CARD interaction. Upon this interaction, IRAK is autophosphorylated and subsequently dissociated from the receptor complex, after which it forms a complex with TRAF6 (7).

TRAF6 is a signal transducer in the NF-κB pathway that activates the I-κB kinase complex. A heterodimeric protein complex, composed of TRAF6-regulated IKK activator (TRIKA)1 and TRIKA2, links TRAF6 to IKK activation. TRIKA1, is composed of the ubiquitin-conjugating enzyme UBC13 and the UBC-like protein UBE2V1. TRIKA2 is composed of TAK1 and its binding partners, TAB1 and TAB2 (98). This process leads to the activation of the

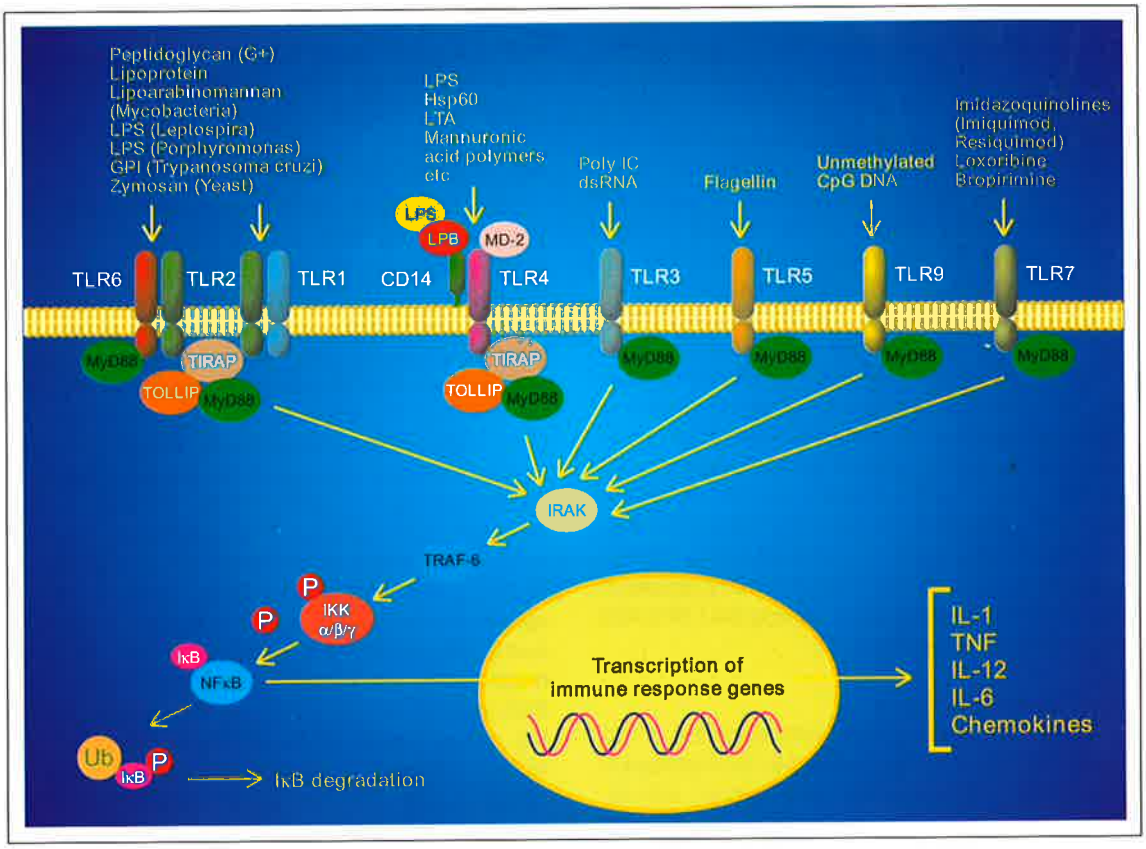

Fig. 2. Activation of the NF-κB pathway through the Toll-like receptors (TLR).

I-κB kinase complex, which is finally responsible for the phosphorylation of serine residues on the I-κB proteins, its subsequent degradation via the ubiquitination pathway and translocation of NF-κB to the nucleus (7). The I-κB kinase complex consists of two kinases, IKK-α and IKK-β and a regulatory subunit IKK-γ and is responsible for the serine phosphorylation of the inhibitory molecules of the NF-κB complex, the I-κB that inactivates NF-κB by trapping it in the cytoplasm (4, 99). The activated NF-κB moves into the nucleus, where it acts as a transcription factor, binding to the promoters at κB-binding motifs and/or enhancer of genes of innate host defense, such as encoding IL-1 and other inflammatory cytokines and upregulating co-stimulatory molecules, thus providing clues that are essential to directing adaptive immune responses. The activation of TLR4 also results in the activation of different pathways that involve the c-Jun NH2-terminal kinase (Jnk) and p38 MAPK signaling pathways (8, 15, 94).

In this Toll-like receptor pathway activation, there are other proteins participating, yet not with all Toll-like receptors. Toll-interacting protein interacts with TLR2 and TLR4 and is involved in the recruitment of IRAK (100). TIRAP is involved in TLR4 and TLR2 signaling pathways (101).

MAL (also called TIRAP) is a specific adaptor for TLR4 signaling that selectively interacts with serine/threonine kinase inducers of NF-κB and can compensate for the lack of MyD88 (8).

RP105 is a receptor expressed on the surface of B cells involved in the recognition of lipopolysaccharide and is functionally association with TLR4 (13, 66).

Mechanisms involving NOD/CARDs

The NOD/CARD proteins are able to induce apoptosis (77, 102) and to trigger the NF-κB signaling pathway (81) (Fig. 3). Both processes are initiated respectively through a CARD-CARD inter-

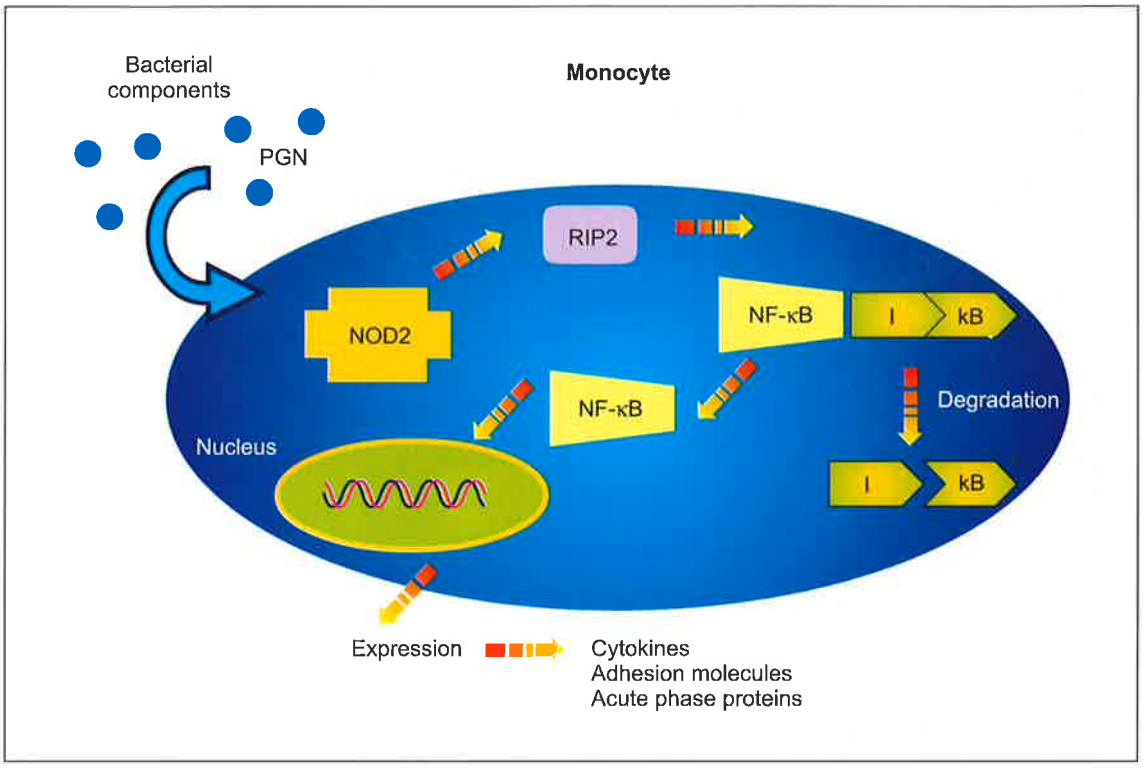

Fig. 3. Mechanism of NF-κB pathway activation by NOD/CARD proteins.

action with caspase-9 (involved in apoptosis) and intracellular signaling mediated by the serine/threonine kinase RIP2 (also named RICK or CARDIAK) (77, 78, 103). This signal transduction pathway is preceded by the interaction of peptidoglycan to the LRR domain of NOD/CARDs (42, 91). Mutation in RIP2 results in reduced IL-2 production, low proliferation of T-cell receptors and low differentiation to the T_H1 cells (103).

Pattern recognition receptor variants associated with disease

A) Allelic variants of Toll-like receptors associated with disease

LEPROMATOUS LEPROSY AND TLR2

Lepra is a chronic disease produced by a defect in cell-mediated immunity in response to the antigens of *Mycobacterium leprae*. Depending on the cell-mediated immune response, it has been divided into lepromatous lepra (with a lack of cell-mediated immunity and therefore a large number of bacilli in the tissue) and tuberculoid lepra (a response reaction to antigens of *M. leprae* resulting in few bacilli present in the lesions). R677W polymorphism of the *TLR2* gene has been associated with susceptibility to lepromatous leprosy in the Korean population (104). This same mutation in the intracellular domain of the *TLR2* gene was later associated with IL-12 production from monocytes in lepromatous leprosy. Patients with this mutation were less responsive to the cell lysate of *M. leprae* and presented lower serum levels of IL-12, in comparison with *TLR2* wild types (105) (Fig. 4).

STAPHYLOCOCCAL INFECTION AND TLR2

R753Q mutation in the *TLR2* gene has been potentially associated with staphylococcal infection (106) in the Western population. Transfection of 293T cells with wild-type and mutant staphylococci showed that the response to lipopolysaccharide was unaffected by the R753Q mutation, although the responses to other bacterial peptides, including Osp-A of *Borrelia burgdoferi* and *Treponema pallidum*-derived 47L, were affected (106) (Fig. 4).

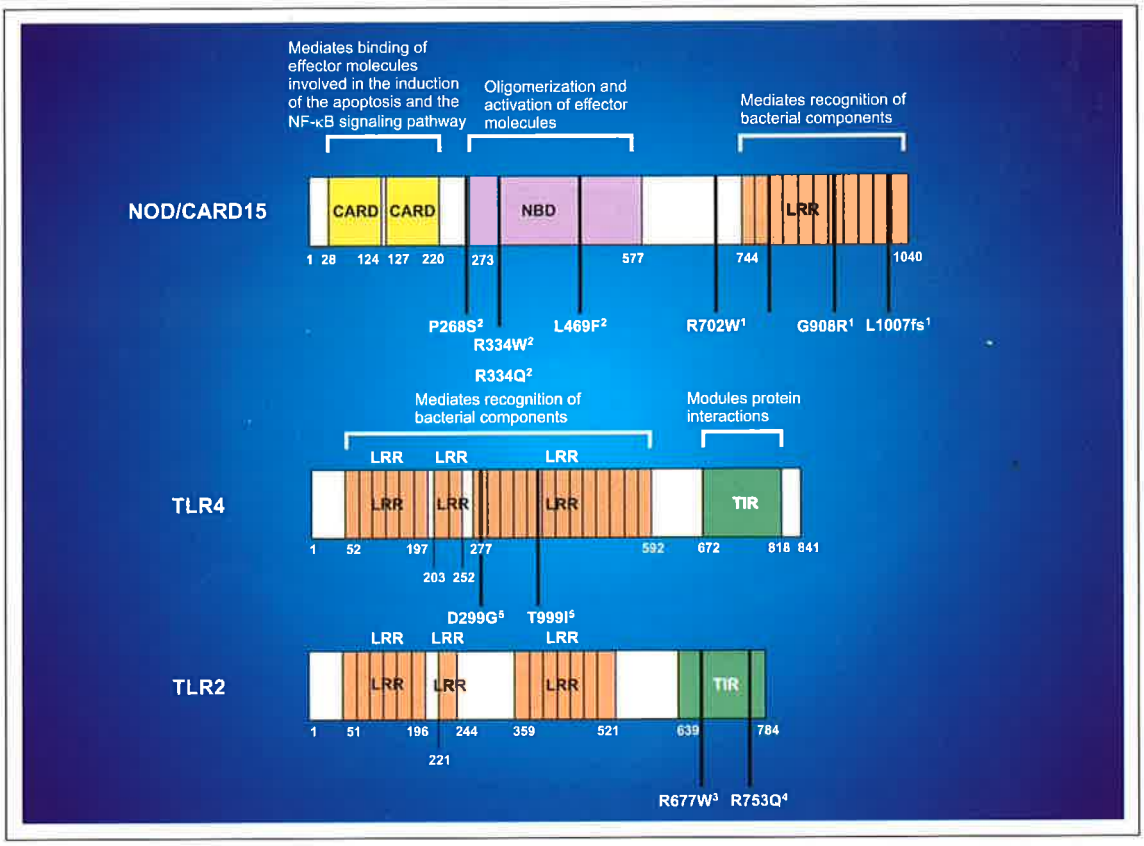

Fig. 4. Localization of the domains and the mutations of NOD2/CARD15, TLR2 and TLR4 proteins. 1: mutations associated with CD; 2: mutations associated with Blau syndrome; 3: associated with lepromatous leprosy; 4: associated with staphylococcal infection; 5: associated with blunted response to inhaled LPS.

Hyporesponsiveness to lipopolysaccharide and TLR4

In humans, the D299G mutation, affecting the LRR domain of TLR4, has been found to be associated with blunted response to inhaled lipopolysaccharide, with a dominant genetic effect in humans (107). A second missense mutation, T399I, which is in tight linkage disequilibrium with D299G, also demonstrated hyporesponsive reaction to lipopolysaccharide (108, 109). The transfection of THP-1 cells demonstrated that only the D299G polymorphism but not the T399I polymorphism interrupts TLR4-mediated lipopolysaccharide signaling (107). The D299G substitution results in reduction in cell surface expression of TLR4 and subsequent disruption of lipopolysaccharide signaling (60, 107–109) (Fig. 4).

Other clinical manifestations and TLR4

Besides the hyporesponsiveness to lipopolysaccharide, the 299Gly allele has been associated with lower levels of certain proinflammatory cytokines, acute-phase proteins and soluble adhesion molecules, with increased susceptibility to severe bacterial infections, with lower risk of carotid atherosclerosis and with a smaller intima-media thickness in the common carotid artery (60). Finally, the D299G polymorphism is associated with an increased incidence of Gram-negative infections in critically ill patients in a surgical setting (110) and may also predispose people to developing septic shock with Gram-negative microorganisms (108). Recently, in a study involving Finnish patients, the 299Gly allele was associated with increased risk of premature births (108).

Other studies failed to show association between the D299G mutation and a variety of clinical manifestations, such as asthma or atopy-related phenotypes (111), or *Candida albicans* (112) and *Chlamydia trachomatis* infections (113).

Other clinical pathologies have been associated with changes in the expression of TLR4. Expression of TLR4 in intestinal epithelial cells is upregulated in Crohn's disease but not in ulcerative colitis (53, 54).

Asthma and TLR9

The T-1237C mutation, affecting the promoter region of the *TLR9* gene, has been associated with asthma. An increased risk of asthma among European-Americans with a C allele at position –1237 was described, but no associations were found with other clinical pathologies, including deep venous thrombosis, myocardial infarction and chronic obstructive pulmonary disease, in this population. Moreover, this polymorphism was not associated with asthma in African-American or Hispanic patients (114).

B) Allelic variants of NOD/CARDs associated with disease

Crohn's disease and NOD2/CARD15

Crohn's disease pathogenesis is the result of enhanced mucosal permeability or absorption of potentially inflammatory bacterial polymers from the small-bowel system (115). Genetics and environmental factors have been implicated in the pathogenesis of this disease (116–118).

In 1996 Hugot (119) reported an association between the IBD1 loci on the pericentromeric region of chromosome 16 and susceptibility to Crohn's disease in French families with multiple members affected. Hugot *et al.* (120) in France and Ogura *et al.* (121) in the United States identified the relevant gene on chromosome 16q12: *CARD15*. Subsequently, an association was demonstrated for Crohn's disease with three of these identified SNPs: a frameshift mutation in nucleotide 3020 in exon 11 produced by a cytosine insertion and two independent missense mutations G908R and R702W, located on exons 4 and 8, respectively (120) (Fig. 4). Ogura *et al.* (121) independently identified the frameshift mutation in association with Crohn's disease. These studies demonstrated that the *CARD15* gene is the first locus causing susceptibility to Crohn's disease in the Western population (81, 119–124).

The frameshift mutation affects the tenth LRR, resulting in a truncated protein with 1007 amino acids instead of 1040 amino acids. This variant has been shown to be defective *in vitro* in the activation of the NF-κB pathway, modifying the response to peptidoglycan. Defects in sensing peptidoglycan might give rise to an abnormal innate and adaptive immune response (77, 125–128). The two nonconservative amino-acid substitutions G908R and R702W affect the LRR and an adjacent region of the LRR respectively (120, 129). P268S, a fourth variant adjacent to the NBD region on exon 4, is described in the literature (130). This variant has strong linkage disequilibrium with the three main variations described above in association with Crohn's disease, forming three independent disease haplotypes (130). Defects in the protein function were shown by Bonen *et al.* in 2003 (131) to result in decreased NF-κB activation for R702W and G908R compared with wild-type *NOD2* and *NOD2 P268S*.

It is now clear that *CARD15* gene variations predispose some but not all populations to Crohn's disease. European populations with at least one of the three frequent variants (R702W, G908R and L1007fs) were more likely to have Crohn's disease. Hugot *et al.* (120, 132) observed a gene dose-effect for susceptibility to Crohn's disease.

In the Japanese population, *NOD2/CARD15* variants were not found in Crohn's disease, ulcerative colitis or controls (133, 134). Bonen *et al.* (135) found lower allele frequencies of the three Crohn's disease-associated variants (R702W, G908R and L1007fs) in African-Americans as compared to Caucasian cohorts, although they identified another variant (R790E) solely present in African-Americans. Adams *et al.* (136) found a similar frequency of the L1007fs in familial Australian Crohn's disease to that previously reported in others populations. In Ashkenazi Jews, a positive linkage was described for the frameshift mutation L1007fs and G908R with Crohn's disease. In this group, no significant relationship between Crohn's disease and the R702W mutation was found (131, 137, 138). Recently, Sugimura *et al.* (139) have reported a haplotype combination IVS8+158 (C>T) and 268S associated with Crohn's disease in Ashkenazi Jews, suggesting a genetic-specific disease-predisposing factor in this population.

A genetic divergence may explain the lack of *CARD15* gene mutations in Eastern populations with Crohn's disease and suggests that these mutations are a relatively late evolutionary development. The same clinical situation may be caused

by different evolutionary responses to the environment. The European stem required a modification in the immune response, allowing changes in the pathogenic mechanisms of response to the ubiquitous bacteria of these populations.

There are different conclusions that can be drawn from the phenotypic effect of these mutations in the clinical subtypes and course of Crohn's disease. This may be the result of ethnic differences in disease classification, particularly when the length of follow-up of the patients is taken into account. In general, the studies have demonstrated an increased risk to ileum-terminal lesions, an early onset and a less frequent colonic involvement in the Western European population with at least one of the mutations. The variability of the phenotype-genotype relations reported does not allow definitive conclusions to be drawn. Efforts should be directed towards building a molecular classification based on genotype and/or haplotype that may help fulfill the need to better predict the prognosis, response to therapy and outcome of Crohn's disease, as well as possibly predicting whether surgery will be needed.

Recently, Hisamatsu *et al.* (89) studied the expression of the wild-type *CARD15/NOD2* gene in a variety of human cell culture lines and primary epithelial cells and compared its function to the 3020insC mutant counterpart in response to challenge by *Salmonella typhimurium*. Several intestinal epithelial cell lines and primary intestinal epithelial cells expressed CARD15/NOD2 and expression could be increased with TNF-stimulation. When expressed in human colonic epithelial Caco2 cells, wild-type CARD15/NOD2 limited the number of viable internalized *S. typhimurium* compared with untransfected or transfected cells with the wild-type (89). Another study reported that TNF-α and IFN-γ upregulate the CARD15/NOD2 expression synergistically on intestinal epithelial cells lines (90). The proinflammatory cytokine TNF-α is involved in the regulation of NF-κB by inducing upregulation of CARD15/NOD2 protein and mRNA levels in epithelial cells lines and primary intestinal epithelial cells (89, 90). This upregulation is dependent on NF-κB activation and on the binding of NF-κB to two different sites of the *CARD15/NOD2* promoter. In Crohn's disease patients, an upregulation of CARD15/ NOD2 expression in colonic epithelial cells, as well as high levels of NF-κB, have been described. This overexpression makes intestinal epithelial cells more sensitive to bacterial components and points to an increased secretion of the chemotactic cytokine IL-8 under peptidoglycan stimulation (90). This antibacterial function of CARD15/NOD2 in intestinal epithelial cells is genetically affected in the presence of defective *CARD15/NOD2* variants that may enhance the inflammatory process by disturbing the epithelial barrier functions (89, 90).

Spondyloarthropathy and CARD15

The term spondyloarthropathy covers spondylitis ankylopoetica, undifferentiated spondyloarthropathy, psoriatic arthritis and reactive arthritis. The pathogenesis is thought to be a combination of endogenic/genetic factors (HLA-B27 and others) and exogenic factors (microorganisms such as *Chlamydia, Yersinia, Shigella* and *Salmonella* spp.). Spondyloarthropathies are a group of rheumatic inflammatory diseases with recognized extra-articular symptoms. They are associated with IBD and Crohn's disease, as well as with ulcerative colitis, suggesting a common pathogenic-genetic background among these clinical entities. Approximately 10–15% of IBD patients have complications such as spondylitis ankylopoetica or other forms of spondyloarthropathy. Interestingly, Crane *et al.* (140) demonstrated that the *P268S CARD15/NOD2* variant was inversely associated with ulcerative colitis-spondylitis ankylopoetica, and the carriership of this SNP was associated with a greater disease severity as assessed by the Bath Ankylosing Spondylitis Disease Activity Index. However, no association has been found between the other described *CARD15/NOD2* variants and primary spondylitis ankylopoetica or between ulcerative colitis and Crohn's disease spondylitis ankylopoetica (140–144).

Chronic granulomatous disease and *CARD15/NOD2*

In granulomatous disease, several mutations in the *CARD15* gene have been found in association with Blau syndrome, a rare genetic autosomal-dominant disorder characterized by granulomatous arthritis, uveitis and skin lesions. This disease is clearly different from Crohn's disease, but both are characterized by granuloma formation. A granuloma is the product of giant and epitheloid cells aggregating; these cells are differentiated forms of monocytes. Three missense mutations (R334Q, R334W and L469F) located in the NBD region of the *CARD15/NOD2* gene were found in families with this syndrome (Fig. 4). The heterozygosity pattern that was found in the affected subjects was consistent with the autosomal-dominant inheritance characteristic of this disorder (145).

PPROM and CARD15

Infectious causes such as chorioamnionitis and uterine causes such as overdistention are known causes of preterm, premature rupture of membranes (PPROM). African-Americans are an ethnic population that presents a high incidence of PPROM. In this population, Ferrand *et al.* (146) did not find mutations of *CARD15/NOD2* in PPROM cases compared to healthy controls. The authors concluded that *CARD15/NOD2* probably does not contribute to the ethnic variation in the incidence of PPROM.

Psoriasis and CARD15

An Italian group (147) studied the relationship between the *CARD15/NOD2* gene and the susceptibility to psoriasis, a relationship that appears to be possible based on the localization of the susceptibility locus on chromosome 16 for this disease. In spite of this hypothesis, the evidence did not support a strong genetic association between *CARD15* gene polymorphisms and psoriasis, at least in the Italian population.

Wegener's granulomatosis and *CARD15/NOD2*

Wegener's granulomatosis is another disorder characterized by granuloma. Given the association between Crohn's disease and Blau syndrome two granulomatous diseases and the *CARD15* gene, an influence for the gene in this third granulomatous disease are possible. However, Newman *et al.* (148) did not find a relationship between the *CARD15* gene and susceptibility to Wegener's granulomatosis.

Clinical implications

The identification and functional characterization of pattern recognition receptors such as Toll-like receptors and NOD/CARDs in mammals have expanded our understanding of the interaction between the innate immune system and pathogens. An understanding of the immune response to this new level and how pattern recognition receptor function affects most aspects of the adaptive immune response could be crucial in identifying targets for immune intervention. Until now, bacterial enzymes that participate in the synthesis of bacterial components have been flanked as targets for the development of new antibiotics. Antibiotics have proven to be a powerful tool against infectious diseases, but with the increased emergence of multidrug-resistant pathogens and, on the other hand, the often undesirable effects of some vaccines, the development of novel therapeutic agents is necessary.

The identification of mutations in these genes with respect to inflammatory or infectious disease could potentially aid in the diagnosis, prognosis and treatment of clinical pathologies in a noninvasive manner. Recent investigations show different associations between the carriership of a specific mutation and certain clinical parameters (*i.e.*, carriership of *CARD15 L1007fs* with an early onset of Crohn's disease, ileum-terminal lesions and a less frequent colonic involvement). These conflicting data may be true for particular ethnic populations. Identification and study of the genes show that the genesis of inflammatory and infectious diseases is the result of complex genetic disorders and that specific risk factors are neither necessary nor sufficient for developing certain diseases.

Evidence of lack of tolerance of the indigenous microflora in chronic IBD

Chronic IBD pathogenesis is the result of a complex interaction between environmental factors (*i.e.*, microflora in the gut), immunologic factors and genetic predisposition (126, 149). The bacterial pathogenesis hypothesis is based on experimental data and clinical observations. The first studies in rodent models of Crohn's disease, such as the transgenic HLA-B27 rats and many others in mice, demonstrated differences in disease symptoms in animals in germ-free environments as compared to experimental animals with normal enteric commensal microorganisms (126, 127, 150, 151). Enhanced mucosal permeability or absorption of potential inflammatory bacterial polymers from the small-bowel system has been documented based on the overgrowth of lumenal bacteria (115). These pathogenic or normal lumenal bacteria constantly stimulate the mucosal and systemic immune systems and perpetuate the inflammatory cascade (152, 153). Pioneering studies in Oxford by the late Sidney Truelove in the 1960s demonstrated the beneficial effect of diverting the lumenal flow in severe colonic disease. Interestingly, the first studies showed better results in Crohn's disease than in ulcerative colitis. In 1983, Harper *et al.* (154) reported an immediate but temporary clinical improvement in colonic Crohn's disease patients who had a split or double-barreled ileostomy. Rutgeerts *et al.* (155) investigated the influence of the fecal stream in the pathogenesis of Crohn's lesions in patients with an ileal resection. They described important differences between Crohn's disease patients with or without a temporally diverting terminal ileostomy. Patients who underwent reanastomosis after six months did not present characteristic inflammatory

lesions of Crohn's disease in the isolated neoterminal ileum. However, recurrence of disease was observed six months after reanastomosis in these patients as well as in patients with one-step surgery. Moreover, the functional responses of intestinal epithelial cells to lumenal bacterial flora and the role of Toll-like receptor expression in these cells have been associated with alterations in the innate response to lumenal bacterial flora in the pathogenesis of IBD (53, 54).

Conclusions

As is known, Toll-like receptors and NOD/CARDs belong to the family of innate immune receptors that respond against different pathogen-associated molecular patterns. This stimulatory response can produce either enhancing or inhibiting immune signaling pathways. Therefore, the expanding knowledge of Toll-like receptor and NOD/CARD specificity and their downstream signaling pathways should provide a new molecular basis for preventing or blocking inflammation associated with infection or other pathological conditions. A better understanding of pathogenic mechanisms and phenotypic classification could provide a new focus for better and more specific therapeutic treatments that can promise a better quality of life for those suffering disturbances of the inflammatory immune response.

Since strictures appear to be more frequent in patients carrying *CARD15/NOD2* mutations, *CARD15/NOD2* genotyping may be proposed to establish prognosis in patients with an established diagnosis. However, the relationship between *CARD15/NOD2* mutations and specific phenotypic clinical disease is still controversial. In addition, although genotyping could theoretically be an ideal method for predicting response to drug therapy, no evidence has emerged on this topic. To elucidate the potential value of *CARD15/NOD2* genotyping in clinical practice, long follow-up studies in large groups of patients are needed.

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