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Review

NOD-like receptors: Ancient sentinels of the innate immune system

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Abstract. NOD-like receptors (NLRs) comprise a family of cytosolic proteins that have been implicated as ancient cellular sentinels mediating protective immune responses elicited by intracellular pathogens or endogenous danger signals. Genetic variants in *NLR* genes have been associated with complex chronic inflammatory barrier diseases (e.g. Crohn disease, bronchial asthma). In this review, we focus on the molecular pathophysiology of NLRs in the context of chronic inflammatory diseases and pinpoint recent advances in the evolutionary understanding of NLR biology. We propose that the field of NLRs may serve as a prototype for how a comprehensive understanding of an element of the immunological barrier will eventually lead to the development of targeted diagnostic, therapeutic and/or preventive strategies.

Keywords. Innate immunity, Crohn disease, epithelial barrier, inflammation, NF-κB, caspases.

Introduction

In higher metazoans, two distinct types of immune responses can be functionally defined: (i) a delayed adaptive immune response and (ii) a fast innate immune response. The delayed adaptive immune response is mediated by clonal selection and expansion of T- and B-lymphocytes. It generates an antigen-specific response that matures during the course of antigen exposure. The selection process generates a high level of diversity and specificity and constitutes the individual immunological memory. However, fast-acting pathogens would be able to overwhelm the organism before the immune system would have the chance to mount a sufficient counterattack.

The phylogenetically older innate immune system relies on the fast recognition of invariant molecular patterns by a limited set of non-clonal, germline-encoded receptors. The molecular structures comprise a heterogenous group of molecules from (glyco)lipids and nucleic acids present in pathogens (pathogen-associated molecular patterns, so called PAMPs; e.g. lipopolysaccharides or unmethylated CpG DNA) or endogenous danger signals such as heat shock protein released into the extracellular space by cellular damage. Different sets of pattern-recognition receptors (PRRs), which are often conserved among plants, insects and vertebrates, constitute an integrated cellular process which aims for the effective removal of pathogenic microbiota.

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Different surveillance systems of PRRs are expressed on the cell surface, reside in intracellular compartments or may be released as secreted molecules in the extracellular space. Whereas the Toll-like receptors (TLRs) represent the archetype of the transmembrane PRR with an extracellular ligand binding domain [1], the intracellular NOD-like receptor (NACHT-LRR receptors; NLR) family seems to play a pivotal role for the recognition of intracellular PAMPs [2].

Structure of NLRs

NLR genes encode for a growing family of regulatory cytosolic proteins (overview in Fig. 1). Common to the NLR protein family is a conserved tripartite domain structure. The NLR proteins are characterized by a central nucleotide-binding and oligomerization domain (NOD or NACHT; see below) and C-terminal leucine-rich repeats (LRRs). The third, N-terminal located effector binding domain can be a pyrin domain (PYD), a caspase activation and recruitment domain (CARD) or a BIR (baculovirus inhibitor of apoptosis protein repeat) [2–6]. The presence of the different N-terminal domains, PYD, CARD and BIR, divides these proteins into the subfamilies NALPs, NODs and NAIPs (for neuronal apoptosis inhibitor protein), respectively. The parallel description of the identical genes/proteins in different contexts by different groups has led to a historical co-existence of aliases (e.g. NALP1, also known as NAC, CARD7, SLEV1, DEFCAP, PP1044, VAMAS1, CLR17.1, KIAA0926, DKFZp586O1822). Studies on NLR proteins by different groups using different aliases have led to a confusing number of synonyms which have continuously been used in parallel. Initially, NOD1 and NOD2 proteins were named due to the presence of a NOD domain [7, 8]. The gene symbols (NOD1 = *CARD4*, NOD2 = *CARD15*), which were created simultaneously, were chosen because of the presence of CARD domains, but did not allow further discrimination of the heterogeneous group of CARD-containing genes into functional or structural subgroups. The NLRs containing a pyrin domain as N-terminal effector binding structure were initially termed NALPs [NACHT-, LRR- and PYD-containing proteins, also named as PYPAF (PYRIN-containing APAF-1-like protein)]. The group of Ting named the family as CATERPILLER [CARD, transcription enhancer, R (purine-) binding, pyrin, lots of leucine repeats] [9, 10]. In this review, we used the generally accepted nomenclature from Martinon and Tschopp [11]. The confusing use of historical gene names has stimulated a broad and ongoing discussion on a unifying nomenclature monitored and initiated by the Human Genome Organization (http://www.genenames.org/genefamily/nlr.php). An overview of the family members is depicted in Figure 1.

LRR Domain

The LRR domain was first described in 1985 as a 24-residue repeated sequence with characteristically spaced hydrophobic residues [12]. Since then, thousands of LRR domains (reviewed in [13–16]) have been identified in viruses, bacteria, archaebacteria and eukaryotes and are classified in a variety of subclasses [16, 17], characterized by different lengths and consensus sequences of the variable segments of repeats [14]. LRRs are protein motifs with a length of 20–29 residues present in a number of proteins with diverse functions and may serve as protein interaction platforms or regulatory modules of protein activation [16]. These include plant immune response and the mammalian innate immune response (reviewed in [18]). Repeated LRRs form an arc- or horseshoe-like 3D structure. The conserved hydrophobic residues of the LRR consensus motif point inward and form the core. The LRRs of NLRs have been bioinformatically deduced from the crystal structure of the human and porcine ribonuclease inhibitor protein (RanGAP) [15, 16]. The general structure of LRR-ligand complexes indicates that the ligand-binding site is located on the concave surface of the LRR arc/horseshoe [19]. It is assumed that the identified activators of NLRs, which will be discussed below in detail, bind to the respective LRRs and activate subsequent signalling by inducing conformational changes. However, we want to emphasize that no convincing experimental evidence exists demonstrating the direct interaction of NLR LRRs with their cognate ligands. Thus, we encourage the use of the term 'ligand sensing' rather than 'ligand binding' to reflect this situation. Interestingly, the direct interaction of LRRs with the recognized pathogenic components also lacks a clear structural solution for R proteins. For the extracellular LRR domains of TLR1/2, a co-crystal structure with the lipopeptide Pam(3)CSK(4) was published recently [20] that demonstrates direct binding of the PAMP to the respective receptors mediating heterodimerization and activation via close proximity of the TIR domains.

Mutations of LRR proteins and abnormal LRR protein expression have been linked to a variety of human diseases, including cancer [21], Crohn disease [22–24] and Bernard-Soulier syndrome, a rare inherited form of platelet dysfunction [25–27]. The mutations occur frequently within the LRR domains as well

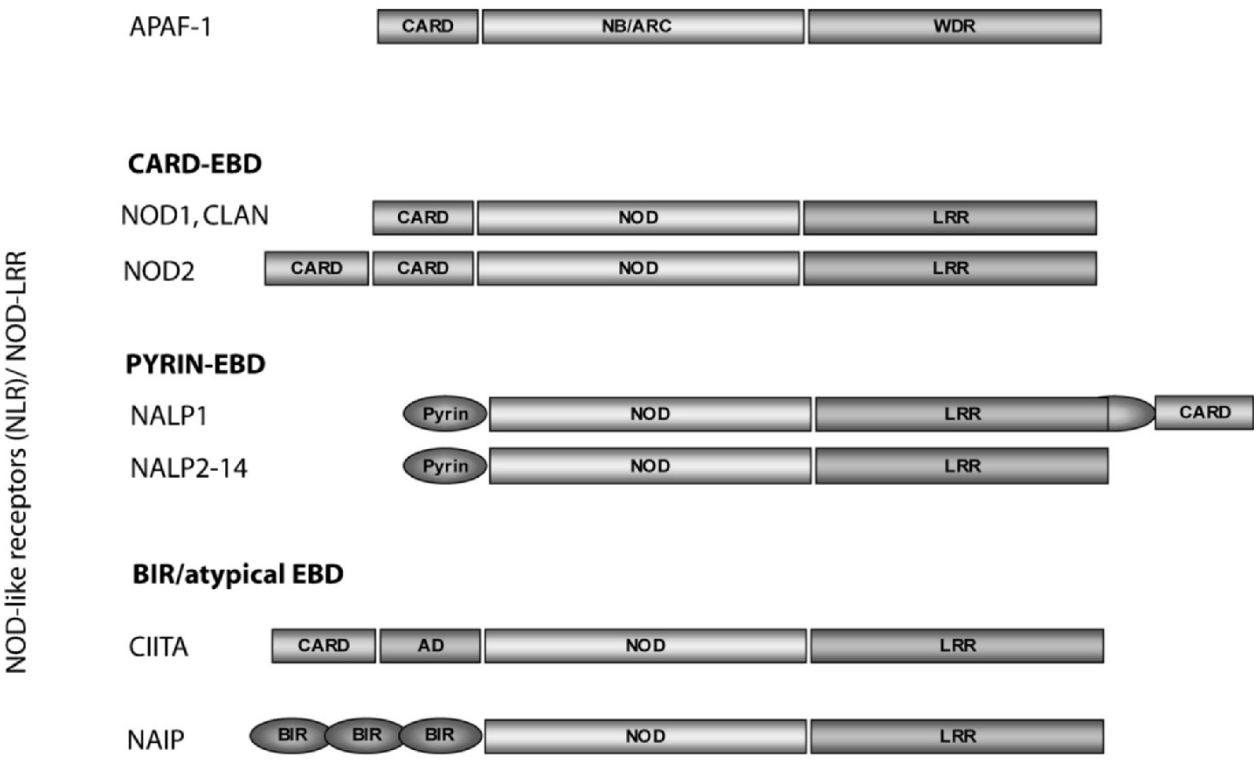

Figure 1. Overview of domain architectures of NLR proteins from different eukaryotes. NLR proteins typically comprise three domains with a C-terminal ligand recognition domain consisting of leucine-rich repeats (LRRs), a central nucleotide binding and oligomerization domain (NOD) and the N-terminal effector-binding domain (EBD), which determines subgroups of the NLR family. CARD (caspase activation and recruitment domain family) can be found in NOD1, NOD2 and CLAN. PYD (pyrin domain) domains characterize the NALP (NACHT-, LRR- and PYD-containing) proteins. The individual number of LRRs varies within the NLR proteins. The other abbreviations are AD, activator domain; BIR, baculovirus inhibitor of apoptosis repeat; FIIND, an interaction domain that is involved in inflammasome formation; NB/ARC, nucleotide binding/found in APAF-1, *R* genes and CED-4; WD40, a domain with a length of ~40 amino acids that typically ends with trytophane ('W') and aspartic acid ('D'), sensor domain for cytochrome c.

as in their neighbouring domains, including cysteine clusters at the N- and C-termini.

Most of the identified elicitors of NLRs are small/low molecular molecules, and the sensing is more or less specific. Whereas first studies identified lipopolysaccharide (LPS) as a NOD2 ligand [23], it is now well established that the NOD1 and NOD2 ligands are the peptides γ-d-glutamyl-meso-diaminopimelic acid (iE-DAP) [28, 29] and muramyl dipeptide (MDP) [30, 31], respectively. These small peptides derived from peptidoglycan (PGN) are thought to interact with NOD1 or NOD2 through the LRR domains. Albeit the evidence for a direct binding of the NOD1 and NOD2 ligands to the LRR domains is still missing, the sensing of MDP and iE-DAP is very specific and can be abolished by either mutations of critical residues within the LRRs [32, 33] or minimal changes of the ligand (e.g. stereoisomers MDP-LD/MDP-DD).

Instead of the highly specific sensing of MDP and iE-DAP by NOD1 and NOD2, NALP3 senses a broader range of ligands. It is reported that NALP3 is involved in the recognition of bacterial PGN (minimal-activating structure: MDP), but surprisingly not LPS [34]. Further reports showed that NALP3 is involved in the sensing of bacterial RNA, viral infection, and TLR ligands and gout/pseudogout crystals [35–37]. The promiscuous liaison of NALP3 to various elicitors is discussed below in detail.

NACHT domain

The NACHT [domain present in neuronal apoptosis inhibitor protein (NAIP), the major histocompatibility complex (MHC) transactivator (CIITA), HET-E and TP1] [6] domain, also designated as NOD (nucleotide binding and oligomerization domain) [6], belongs to the recently defined STAND family of P-loop NTPases. It has a sequence homology with the nucleotide-binding motif of apoptotic protease activating factor-1 (APAF-1), which is responsible for the dATP/ATP-dependent oligomerization of APAF-1 upon cytochrome c sensing during intrinsic apoptotic processes. The oligomerized APAF-1 serves as a molecular platform inducing the recruitment and activation of pro-caspase-9, a process called induced

proximity signalling (reviewed in [38]). It is thus tempting to speculate that the NOD domain of the NLR proteins is pivotally involved in the initiation of a cellular signal upon binding of the respective ligand. An intramolecular complex formation between the LRR and NACHT domain has been proposed to inhibit autoactivation of NLRs [39]. Constructs encoding for forms of NOD2, IPAF and NAIP without LRRs or point mutations of putative interaction sites render the proteins constitutively active, whereas small truncations within the LRR of NOD1 and NOD2 that may interfere with the muropeptide recognition lead to inactive protein species. Disease-associated sequence variants in the NACHT domain of NLRs are in close vicinity to conserved regions of the NACHT domain, e.g. certain NTPase motifs (Walker B Box), which may interfere with the cycle of nucleotide-binding, -hydrolysis and -release and/or conformational changes induced by NTP hydrolysis [39]. Two seminal papers have elucidated the initial molecular requirements of NACHT-mediated NLR activation. Using a reconstituted NALP1 inflammasome, Faustin et al. have shown that a two-step mechanism is required for NALP1-mediated caspase-1 activation [40]. A similar study for NALP3 [41] revealed striking differences in the requirements of nucleotides for this activation process. Whereas NALP1 was rather promiscuous, with ATP, GTP, CTP, UTP and TTP supporting NALP1-dependent caspase-1 activation *in vitro*, only ATP was bound by NALP3 with high affinity. In contrast, modifications of the ribose ring, including deoxy and dideoxy analogs, led to marked reduction of NALP1-mediated caspase-1 activation, whereas dATP and ATP were equally effective in inducing NALP3 activation. For both NLRs, an apparent requirement for the triphosphate form of nucleosides exists which is consistent with a role of ATP hydrolysis in this process. However, the exact function of ATP hydrolysis in NLR assembly or disassembly remains to be characterized.

Effector domains: CARDs, PYDs and BIRs

Three different types of N-terminal effector binding domains are found in NLRs: CARD, PYD and BIR. PYD and CARD are members of the death domain-fold superfamily that also includes death domains (DDs) and death effector domains (DEDs). Members of this family are involved in apoptosis and/or inflammation [42, 43]. All effector domains are thought to regulate protein function via homotypic and/or heterotypic interactions [8, 44–49].

CARD and NODs

In the NLR family, CARD is the characteristic effector binding domain of the group of NOD proteins. CARD was originally described as a protein-binding motif that interacts with caspase through a CARD-CARD interaction. CARD domains are found in a number of pro-apoptotic proteins, such as caspase-1 and caspase-9. In the case of NOD1 and NOD2, this domain mediates the activation of a pro-inflammatory cascade through its interaction with the CARD domain of receptor-interacting protein 2 (RIP2), a protein capable of activating transcription factor NF-κB [8, 50, 51]. However, CARDs have been found in many adaptor proteins that do not interact with caspases, but instead mediate the assembly of protein complexes in apoptosis and NF-κB signalling and are involved in the regulation of inflammatory processes and cytokine production.

PYD and NALPs

The NLR subclass of NALPs is characterized by the presence of a PYD. This domain was first described in Pyrin (DAPIN, PAAD), a protein whose gene is mutated in patients who suffer from a hereditary disorder called familial Mediterranean fever [42, 52]. The 3D structure of PYD is homologous to the structure of CARD [43, 53, 54]. Similar to the CARD domain, the PYD domain exclusively mediates PYD-PYD homophilic interactions [42, 52], thereby recruiting PYD-containing effector molecules, bringing them into close proximity with each other and leading to their activation. The present structure determination establishes PYD domains unambiguously as a fourth branch in the superfamily of DD-type proteins. CARD and DD domains have been identified in echinoderms, while DED and PYRIN domains seem to be limited to vertebrates [55, 56].

Tschopp et al. identified 14 NALPs in the human genome by database search [6]. NALP2–14 all exhibit the following tripartite structure: PYD-NOD-LRR. Interestingly, an additional CARD and a FIIND (function to find) domain is found at the C-terminal end of NALP1. Another common feature of (at least some) NALPs seems to be the recruitment of the adaptor protein ASC through a homotypic PYD-PYD interaction, which in turn recruits caspase-1 through a CARD-CARD interaction [57].

BIR and NAIPs

Two groups of BIR-containing protein families are known: inhibitor of apoptosis proteins (IAPs) and neuronal apoptosis inhibitor proteins (NAIPs). BIR domains are characterized by a number of invariant amino acids, including three conserved cysteines and one conserved histidine residue within the sequence

$CX_2CX_{16}HX_{6-8}C$. The BIR domains of IAP family members are involved in the function of these proteins as inhibitors of apoptosis [58] by direct inhibition of caspases [59] via the BIR domains and have been identified in several multicellular organisms from *Drosophila* to mammals.

The second group of BIR-containing proteins, the NAIP proteins, belong to the NLR family. Unlike mice that have multiple copies, humans have a single functional *NAIP* gene. NAIP containing three N-terminal BIR domains is an endogenous inhibitor of apoptosis, inactivating caspase-3 and caspase-7 in neuronal tissues [59–65]. Dziargama et al. [66] have shown that NAIP also has an anti-apoptotic function in kidney cells. Molecular genetic analysis of the signal transduction pathway using genetically deficient mouse models has demonstrated that Naip5 in conjunction with other NLRs and the adaptor protein Asc (apoptosis-associated speck-like protein containing a CARD) is critical for the response of mouse macrophages to *Legionella pneumophila*, possibly via the direct detection of cytosolic flagellin [67, 68].

Prominent NLR family members and associated human diseases

In the following paragraphs, expression patterns, signalling pathways and target genes of the archetypical NLR family members NOD2 and NALP3 are discussed. Mutations in NLR genes have been associated with an intriguing number of human diseases. These disorders often affect barrier organs and share several pathophysiological and clinical characteristics. Recent studies have described the genetic association of polymorphisms in NOD-like receptor genes with complex chronic inflammatory barrier diseases, such as Crohn disease and asthma, and with rare autoinflammatory syndromes including familial cold urticaria, Muckle-Wells and Blau syndrome.

NOD2

The *NOD2* gene belongs to the subfamily of NLRs characterized by CARD-containing effector binding domains. NOD2 is comprised of 2 adjacent N-terminal CARDs and 10 C-terminal LRRs [7, 30, 69]. NOD2 is constitutively expressed in monocytic cells, but has recently been shown to be upregulated in intestinal epithelial cells by cytokines TNF-α/IFN-γ *via* an NF-κB-dependent mechanism [70]. NOD2 sensitizes IECs to MDP and enhances the release of the chemotactic cytokine IL-8, defensins and the immune exclusion molecule DMBT1 (deleted in malignant brain tumour 1) [70–73]. An overview of the cellular NOD2 function is presented in Figure 2. In conjunction with other proteins such as GRIM19 (gene associated with retinoid-IFN-induced mortality 19), NOD2 may itself function as an intracellular antibacterial factor [74, 75]. NOD2-deficient mice have an increased susceptibility to oral infection with cytoinvasive *Listeria monocytogenes* [71], which coincides with a decreased expression of the murine α-defensin orthologs. Thus, NOD2 has been implicated as a molecular sentinel in maintaining the integrity of the intestinal barrier against luminal pathogens [76]. The minimal PAMP structure recognized by NOD2 is muramyl dipeptide (MurNAc-L-Ala-D-isoGln, MDP), which is a fragment of peptidoglycan [30, 31]. Upon MDP-stimulation, NOD2 has been shown to interact with the receptor-interacting protein kinase 2 (RIP2) [7]. The complex formation with IKKγ/NEMO leads to an activation of the IκB-kinase/NF-κB pathway via induced proximity signalling. Recent work has shown that NOD2-dependent formation of K63-linked polyubiquitin chains on the NEMO protein is important for temporally and spatially controlled NF-κB activation and target gene expression [77].

A frameshift mutation in the LRR of NOD2 (L1007fsinsC), which leads to a partial truncation of the LRR, and other single nucleotide polymorphisms (SNPs) within the LRR (R702W and G908R) are associated with the manifestation of Crohn disease (CD) [22–24]. CD is a human chronic relapsing-remitting inflammatory bowel disease characterized by transmural and granulomatous inflammation which may affect the whole gastrointestinal tract in a discontinuous manner [78]. The clinical features include abdominal pain, (bloody) diarrhea and complications such as growth retardation in children, anemia, toxic megacolon, stenosis and fistulae. The three main NOD2 variants have been demonstrated to result in a diminished NF-κB activation upon MDP stimulation when transfected into HEK293 cells [79, 80]. Analysis of the translocation of NF-κB subunits to the nucleus confirms the results [30, 81]. A severely defective MDP-induced NF-κB activation in RIP2-deficient macrophages indicates that the activation of NF-κB by NOD2 occurs exclusively through the downstream effector molecule RIP2 [82]. Further, interaction of NOD2 with SGT1 and HSP90 might be required for optimal NF-κB activation [83, 84].

Recent studies have investigated the functional cross-talk between TLRs and NOD2. One of the first studies of this kind demonstrated a marked increase of IL-12 production in NOD2-deficient splenic macrophages that were stimulated *in vitro* upon stimulation with PGN extracts [81]. Further experiments suggested that PGN-mediated activation of TLR2 signalling is negatively regulated by MDP-mediated activation of

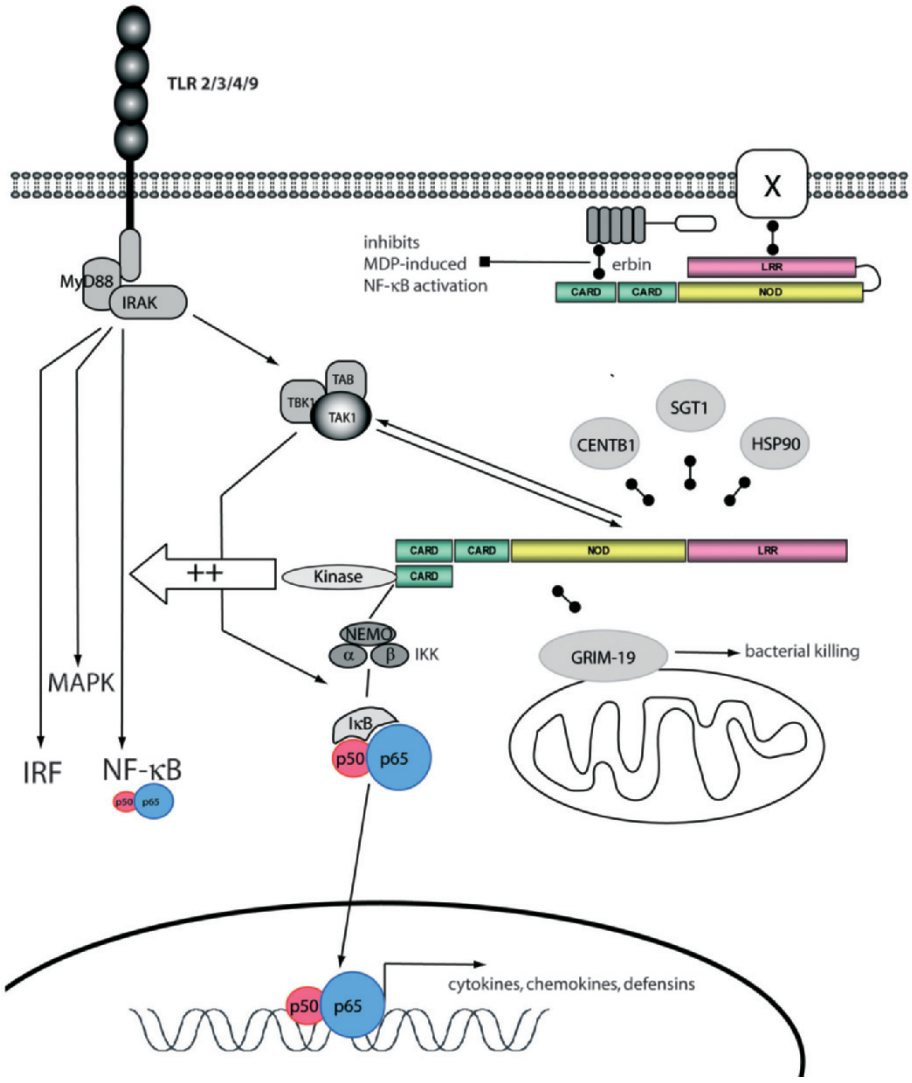

Figure 2. Schematic overview of the cellular signalling pathways of NOD2. Recognition of muramyl dipeptide (MDP) via the LRR domain activates the NOD2 protein, which in turn recruits receptor-interacting serine/threonine kinase 2 (RIP2) through a homophilic interaction of the CARDs. This interaction leads to a canonical activation of the IKK/IκB pathway. Upon phosphorylation of IκBα via IKKα/β, nuclear factor-κB (NF-κB) is released and targeted for translocation to the nucleus. Several other molecules (Erbin, TAK1, SGT1, GRIM-19 and Hsp90) have been described as interactors (halter symbol) and modify NF-κB activation and/or crosstalk with Toll-like receptors (TLRs). 'X' denotes a membrane-associated factor that is responsible for the membrane-association of wild-type NOD2 (see main text for details).

NOD2 signalling and that this negative regulation is abolished in the NOD2-deficient situation [81]. Abbott et al. [77] showed that TLR signalling requires the same K63-linked polyubiquitylation of NEMO as NOD2. This ubiquitination is partially done by TRAF6, which is activated by NOD2.

In contrast, other studies have shown that NOD2 activation via MDP leads to a synergistic enhancement of TLR signals, e.g. as demonstrated by increased production of the cytokines IL-8, TNF-α, IL-1β, IL-6 and IL-10 when APCs were stimulated with ligands for TLR2, TLR3, TLR4, TLR5, TLR7 and TLR9 [75, 85–89]. The mechanisms for this dichotomous response pattern are not clear, but might be related to changes in the expression of key components in the respective signal-transduction pathways. The unresponsiveness of intestinal epithelial cells (IECs) to TLR signals, at least in the absence of inflammation, sets the stage for the function of NLR proteins as important sensors for the detection of bacteria invading the epithelium and upon recognition alter the 'state of alertness' in the now sensitized IECs. This has been demonstrated by a cooperative effect of MDP together with the TLR4 ligand LPS in IECs on the expression of the anti-bacterial factor DMBT1 [90]. Thus, it is tempting to speculate that disturbed additive signals of NOD2 and TLR4 are responsible for decreased mucosal levels of defensins and other protective factors observed in CD patients carrying NOD2 variants. These findings would suggest that NOD2 is at the apex of a protective program which regulates the delicate balance between host intestinal epithelium and the mucosal flora. This may result in the disturbance of a complex defect including facilitated entry of bacteria into epithelial cells through defective regulation of defensin expression, impaired bactericidal capacity and reduced epithelial immune defense ('loss of function').

Subsequently, associations of loss-of-function variants of NOD2 with other chronic inflammatory disorders have been demonstrated. The variants are significantly associated with a heightened risk of atopy-related traits and an increase in serum (immunoglobulin E) IgE levels [91]. Together with asthma and allergic rhinoconjunctivitis, atopic dermatitis (AD) represents an important manifestation of atopy that is characterized by the formation of allergy antibodies (IgE) to environmental allergens. AD is commonly the first clinical manifestation of allergic diseases. The association further corroborates the hypothesis from Crohn disease that NOD2 may play a crucial role as a sentinel for host/bacterial interactions on inner and outer body surfaces. It is tempting to speculate that the precipitation into a given NOD2-associated disease in individual patients depends on the dynamics of the individual immunological interaction with the resident bacterial metagenome. A loss of immunological tolerance to commensal enteric bacteria has been linked not only to inflammatory bowel disease but also to other barrier diseases such as asthma. The findings warrant further studies aiming at the identification of disease-specific bacterial signatures in NOD2-mutated individuals on different body surfaces.

There is suggestive evidence that loss-of-function mutations in the *NOD2* gene also predispose for the development of certain types of malignant diseases (colonic adenocarcinoma, breast and lung cancer, and *Helicobacter pylori*-induced MALT lymphoma) [92–97]. Increasing evidence indicates that persistent mucosal or epithelial cell colonization by microorganisms induces carcinogenesis via the concomitant inflammatory response. Indeed, a deficiency in the mounting of NOD2-dependent responses could be involved in the etiopathogenesis of malignant diseases via a sustained inflammation due to impaired bacterial recognition and clearance.

Conversely, other mutations within the NACHT domain of NOD2 have been described to cause rare forms of autosomal-dominant inflammatory syndromes, such as Blau syndrome (BS) and early onset sarcoidosis (EOS). BS is characterized by early-onset granulomatous inflammation (arthritis, uveitis), visceral inflammation and camptodactyly [98]. Miceli-Richard et al. [99] were the first to demonstrate cosegregation of CARD15 missense mutations in families with BS. The identified mutations R334Q, L469F and R334W are in close vicinity to the Mg^{2+} binding sites of the NOD/NACHT domain and cause an increased basal activation of NF-κB ('gain of function') [79]. The closely related EOS shares with BS the distinct triad of skin, joint and eye inflammation. EOS is progressive and in many cases causes severe complications, e.g. destructive arthropathy or blindness [100, 101]. The findings implicate the obvious need for a tight control of NOD2-mediated cellular programs, as uncontrolled overactivation may result in debilitating consequences. One of these negative regulators was identified when a specific interaction of Erbin and NOD2 both *in vitro* and *in vivo* was demonstrated [102, 103]. Both studies showed that NOD2-dependent activation of NF-κB and cytokine secretion is inhibited by Erbin overexpression. Interestingly, the interaction also explains part of the membrane localization of NOD2 described earlier. However, as the interaction between Erbin and NOD2 maps to the CARD domains of NOD2, the observation of a defective membrane recruitment of the L1007fsinsC variant of NOD2 remains unresolved and can only be explained by the presence of an additional factor ('X' in Fig. 2).

NALP3/CIAS1

The *NALP3/CIAS1/Cryopyrin/PYPAF1* gene encodes an NLR protein with a N-terminal pyrin domain and is expressed predominantly in peripheral blood leukocytes. The gene was initially described in a positional cloning effort to identify the gene mutated in familial cold autoinflammatory syndrome (FCAS) and Muckle-Wells syndrome (MWS) on chromosome 1q44 [104]. The gene was termed *CIAS1* as an abbreviation for 'cold-induced autoinflammatory syndrome'. The full-length cDNA corresponds to a nine-exon gene encoding an open reading frame of 3105 bp. The protein sequence of NALP3 displays the classical domain structure of the NLR family, including a N-terminal PYD, a central NACHT domain and seven LRRs at the carboxy terminal end [6, 105]. NALP3 interacts with the apoptosis-associated speck-like protein containing a CARD (ASC) protein, a PYD-CARD-containing protein [105]. There is conflicting data which indicate that NALP3 alone may be inhibitory with regard to NF-κB [106]. A seminal study on NALP protein function in general, however, demonstrated that NALP3 may be of pivotal importance for the processing of pro-IL-1β [34, 107]. Using immunoprecipitation analysis, it was demonstrated that CARD8, which contains a C-terminal FIIND (function to find) and a CARD domain at the amino terminus, associates at the centrally located NOD domain of NALP3 [107]. This interaction together with a recruitment of ASC to the pyrin domain of NALP3 leads to activation of pro-caspase-1. In analogy to NALP1, which possesses an endogenous C-terminal CARD domain and thus does not require CARD8 recruitment, the resulting molecular complex was termed inflammasome (see Fig. 3). A subsequent paper suggested a role for muramyl

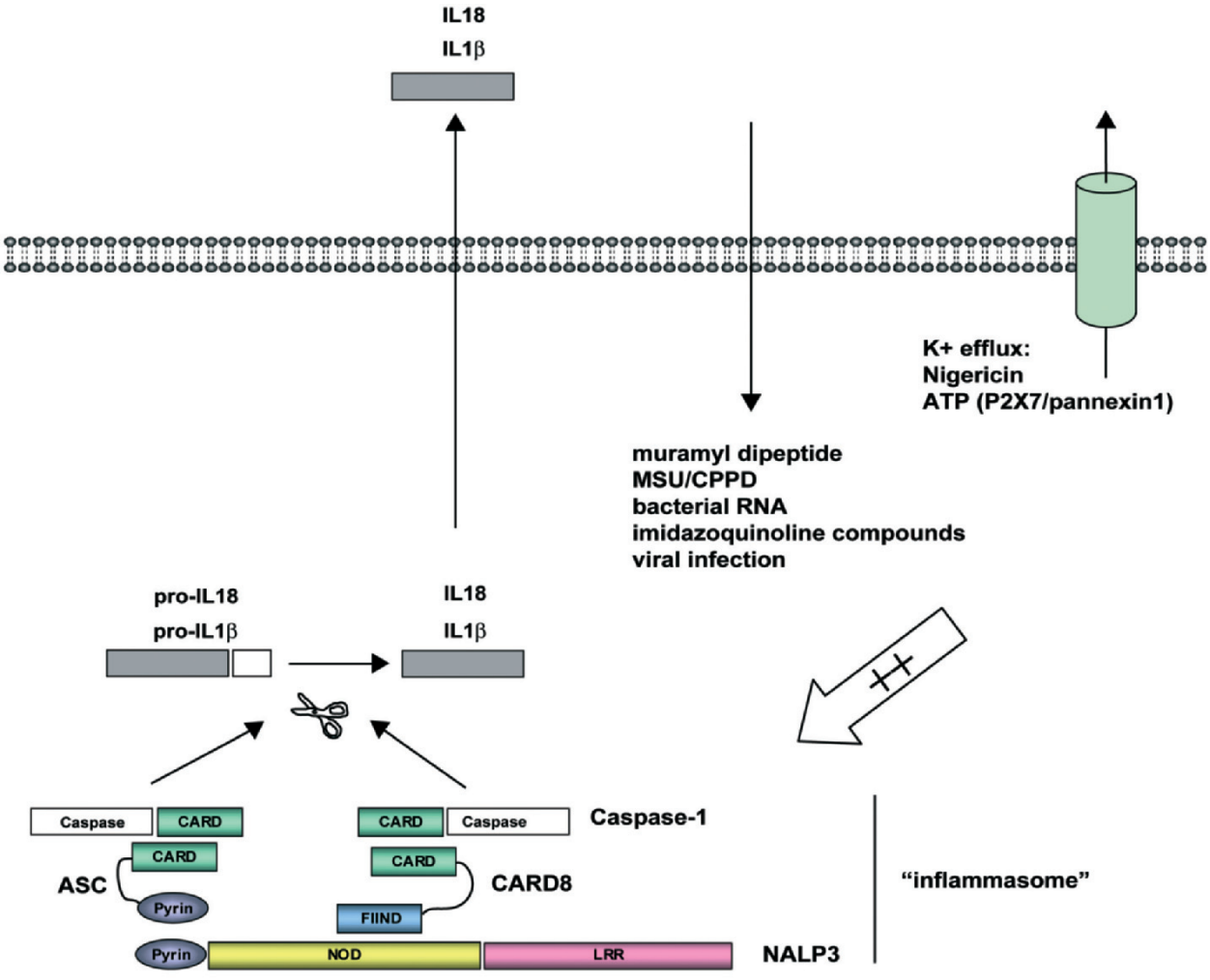

Figure 3. Schematic description of the cellular signalling pathways of NALP3. NALP3 forms a complex with ASC (apoptosis-associated speck-like protein containing a CARD) and CARD8 to activate caspase-1, resulting in the processing of IL-1β. This process is activated by a variety of cellular danger signals/stimuli and is controlled by intracellular levels of potassium. Nigericin is a pore-forming toxin. Pannexin1 is a conducting channel that opens upon P2X7-mediated ATP sensing.

dipeptide, which also constitutes the minimal structure recognized by NOD2, as a minimal structure regulating NALP3-inflammasome formation [34]. Recently, Pan et al. [108] showed that Nod2 and NALP3 have essential, non-redundant roles in MDP-dependent IL-1β production in mice. Three concurrent studies emphasized the promiscuous involvement of NALP3 in the sensing of several cellular danger signals. The first study showed that this process can be induced in response to bacterial RNA and the imidazoquinoline compounds R837 and R848 [35]. A further study clearly demonstrated the involvement of the NALP3 inflammasome in the development of the acute and chronic inflammatory responses known as gout and pseudogout. Monosodium urate (MSU) or calcium pyrophosphate dihydrate (CPPD) crystals lead to NALP3-dependent production of active IL-1β and IL-18 [37].

The third study elegantly unveiled a defective caspase-1 activation in response to Toll-like receptor agonists plus ATP in NALP3-deficient macrophages [36]. The NALP3 mediated release of IL-1β was found to be dependent on intracellular potassium concentrations [109].

Patient-oriented studies revealed that mutations in *NALP3* are not only present in familial cold urticaria (FCU) and MWS, but can also be found in neonatal-onset multi-system inflammatory disease (NOMID), also described as chronic infantile neurologic cutaneous articular (CINCA) syndrome [110]. *NALP3*-associated diseases all manifest in episodes of systemic inflammation associated with arthritis, fever and rash, resulting from perivascular polymorphonuclear infiltrates. Progressive neurological deterioration from chronic meningitis caused by polymorphonuclear cell infiltration can be observed in a subset of patients.

Migrating arthritis may present with or without severe radiologically destruction of the cartilage or epiphyseal structures (reviewed in [111]).

The molecular effects of the mutations in *NALP3* have been extensively studied. All disease-associated missense mutations are located within the central NACHT domain and result in constitutively active protein forms. The most frequent mutation, R260W, is associated with all three diseases, MWS, FCU and CINCA, implicating interaction with other genetic and environmental factors to precipitate into different disease entities. Functional analyses of variant forms of NALP3 have provided strong evidence that the disease-associated forms of the protein are constitutively active and might be analogous to the BS-associated R334Q mutation in the NOD2 gene [41, 112].

It will be interesting to elucidate the crosstalk between NALP3 and other NLRs, as a frequent polymorphism has been reported in the *CARD8* gene which leads to early truncation of the encoded protein. If CARD8 indeed is indispensable for NALP3 activation, it remains unexplained why no obvious immunodeficiency of the affected individuals has been described. The results may point to compensatory mechanisms (such as different protein isoforms of CARD8 [113]) or redundancy of NALP2/3 inflammasome formation for physiological IL-1β processing. Further studies will have to delineate the complex crosstalk between NLRs and their relevance for differential inflammatory responses.

The latter hypothesis of a complex network of NLR interdependencies is corroborated by the ability of heterologous interactions between NLR family members or other proteins that contain CARD or PYD domains. One example is the NLR family member CARD12 (IPAF, CLAN). In CARD12-overexpressing cells, CARD12 binds both NOD1 and NOD2 through NOD-NOD interactions [114], inhibiting NOD1- and NOD2-mediated activation of NF-κB and production of IL-1β. The caspase-recruitment domain-containing adaptor protein CARD9 regulates NOD2-mediated cellular responses by heterologous CARD-CARD interaction. *CARD9*-deficient macrophages display defects in activation of the MAP kinases p38 and JNK, but not of the transcription factor NF-κB after bacterial infection. CARD9-deficient mice failed to clear infection and showed altered cytokine production after challenge with intracellular bacterial infection with *L. monocytogenes*. However, the discovery of more such interactions will result in a mosaic of responses by a network of interacting family members.

Direct recognition of ligands through NLRs? Lessons from plant resistance genes

Despite increasing insights into the molecular function of mammalian NLRs, literature on plant *R* genes, which constitute the group of functional NLR orthologs in plants, still provides a plethora of interesting older findings that may be used to cross-fertilize future NLR research, e.g. in answering the question of a direct or indirect interaction of the receptor and the putative PAMP 'ligand' or in giving ideas on the exact molecular role of the NTPase for activation-induced NLR oligomerization. Resistance (R) proteins are crucial for the immune defense of plants against bacteria, virus, fungi, nematodes, insects, oomycetes and even synthetic products such as insecticides [115]. R proteins mediate – directly or indirectly – the recognition of pathogen-derived molecules (elicitors), which are encoded by pathogen *avr* (avirulence) genes. A resistance to a pathogen is only observed when the pathogen carries a specific avirulence *(avr)* gene and the plant carries a corresponding resistance (*R*) gene [116]. In hosts that express the appropriate R protein, the elicitor leads to an activated defense response, leading to a hypersensitive response (HR). The HR includes programmed cell death of the infected cells, causing a necrotic lesion and the release of antimicrobial products that contribute to the inhibition of pathogen spread [117, 118].

The *R* genes are highly polymorphic but structurally conserved and group in several classes. One R gene subclass contains a nucleotide binding domain called NB-ARC that is closely homologous to the NOD/NACHT domain. The C-terminal-located LRRs from plant *R* genes are homologous to LRRs from NLRs. However, R proteins differ in their N-terminal domain structure and can be split into two related groups that have distinct N-terminal amino acid motif organizations: a Toll/interleukin-1 receptor (TIR) domain or a coiled-coil domain (CC), instead of a CARD, PYD or BIR domain. The CC structure is a repeated heptad sequence with interspersed hydrophobic amino acid residues, of which the leucine zipper is one example. It consists of two or more α-helices that interact to form a supercoil. A CC domain is found in a variety of proteins involved in different biological processes, and is implicated in protein-protein interactions, including oligomerization, and oligomerization-dependent nucleic acid binding. The role(s) of the CC domain in resistance remains unsolved, but results from CC-containing R proteins in *Arabidopsis* on downstream signalling components suggested that this domain may be involved in signalling rather than in recognition [119–121]. The TIR domain is implicated in signalling by its similarity to

the cytoplasmic domain of Toll and IL-1R. In addition to signalling, the TIR domain can play a role in pathogen recognition as well.

The ability of populations of viral, bacterial and fungal pathogens to shift to virulent biotypes is well known. The ability of plant species to survive over evolutionary time depends on their ability to generate useful diversity at resistance gene loci. Whereas genome-wide duplications are quite rare [122, 123], chromosomal duplications, unequal crossing-over and/or gene conversion are common processes in plant gene evolution [124–126]. It was shown that entirely new clusters of *R* genes are created by these processes [127, 128]. The estimation that more than 1% of the genome of *Arabidopsis* and rice is covered by *R* genes [129, 130], and the organization of *R* genes in clusters highlight the importance of the evolution and diversification of *R* genes.

Database searches and phylogenetic studies identified *R* genes or combination of the domains of the (TIR/CC)-NBS-LRR class of *R* genes in evolutionarily distant branches of flora like monocots, dicots and gymnosperms [131]. The discovery of a large number of *R* genes in the gymnosperm genome of the western white pine and their significant homologies with the angiosperm TIR-NBS-LRR family provides evidence for the hypothesis that the TIR-NBS-LRR subfamily originated before the divergence of angiosperms and gymnosperms [129, 132].

Importantly, the detailed research of R-mediated resistance to phytopathogens has generated valuable hypotheses for putative activation mechanisms of plant R proteins by different pathogenic elicitors. These hypotheses may guide a future approach to a molecular understanding of the recognition of PAMPs or other danger signals by mammalian NLRs.

Receptor-ligand interaction

The most straightforward hypothesis for R protein and Avr interaction (and thus for NLRs and their cognate ligands) is the direct receptor-ligand type interaction. In this model, which is the simplest interpretation of the gene-by-gene hypothesis, R protein and Avr protein interact directly and activate defense, and there are several examples of additional plant proteins required for resistance that may function downstream of recognition [133–135].

Guard hypothesis

The lack of examples for direct interactions has led to several hypotheses evoking indirect interactions in protein complexes, additional cofactors or receptors, or R protein-mediated modification and detection of proteins that interact with Avr or *vice versa* (overview in [136]). One of the most prominent hypotheses that arose from the formation of a protein complex but that does not require direct interaction of R with cognate Avr (or NLR and the corresponding PAMP) is the 'guard hypothesis'. In this hypothesis, the R protein acts as a guard and interacts with a host protein that is directly targeted by the Avr product in its function as a virulence factor. This minimally tripartite interaction causes the generation of an HR, whereas in the absence of the R protein, pathogen virulence occurs. Several modifications have been proposed: (a) In the Bridge model, the effector binds independently to the R protein and to a third protein, recruiting one to the other. The effector-dependent interaction of these two proteins activates downstream signalling for defense. (b) In the Matchmaker model, the effector induces a direct interaction between the R protein and a third protein by causing a conformational change in one or the other, or both. The effector may or may not remain associated with the complex following binding of the two plant proteins. This pattern of interactions could be a guard model if the third protein were a virulence target of the effector. (c) In the Affinity Enhancement hypothesis, the interaction of the effector with the R protein, a third protein, or both, stabilizes a pre-existing, weak interaction between the two plant proteins such that abundance of the complex increases and drives downstream signalling to activate the induced defense response. Steady-state levels of interaction between the two plant proteins may function to maintain basal defense. (d) The De-repression model proposes an effector that de-represses defense responses by disrupting an interaction of the R protein and a third (inhibitory) protein that negatively regulates activity of the R protein. (e) In the Dual Recognition model independent interactions between the effector and the R protein and the effector and a third protein are both required for resistance. This type of mechanism would be costly both in terms of structural evolutionary constraints and physiology, and seems unlikely to be maintained without significant, frequent disease pressure. Figure 4 gives an overview of the described interaction hypothesis.

Further investigations on exemplary R proteins will help to unravel the mechanism of HR activation/initiation. It is plausible that some (or all) above-mentioned mechanisms evolved from an ancient mechanism (gene-to-gene?) due to the need to fine-tune the HR and differentiate between pathogenic and commensal/symbiotic bacteria.

The stunning amount of valid hypotheses but also of unresolved questions in the exact molecular resolution of the interaction of the R proteins and NLRs with their cognate ligands emphasizes the need for an interdisciplinary approach in this area.

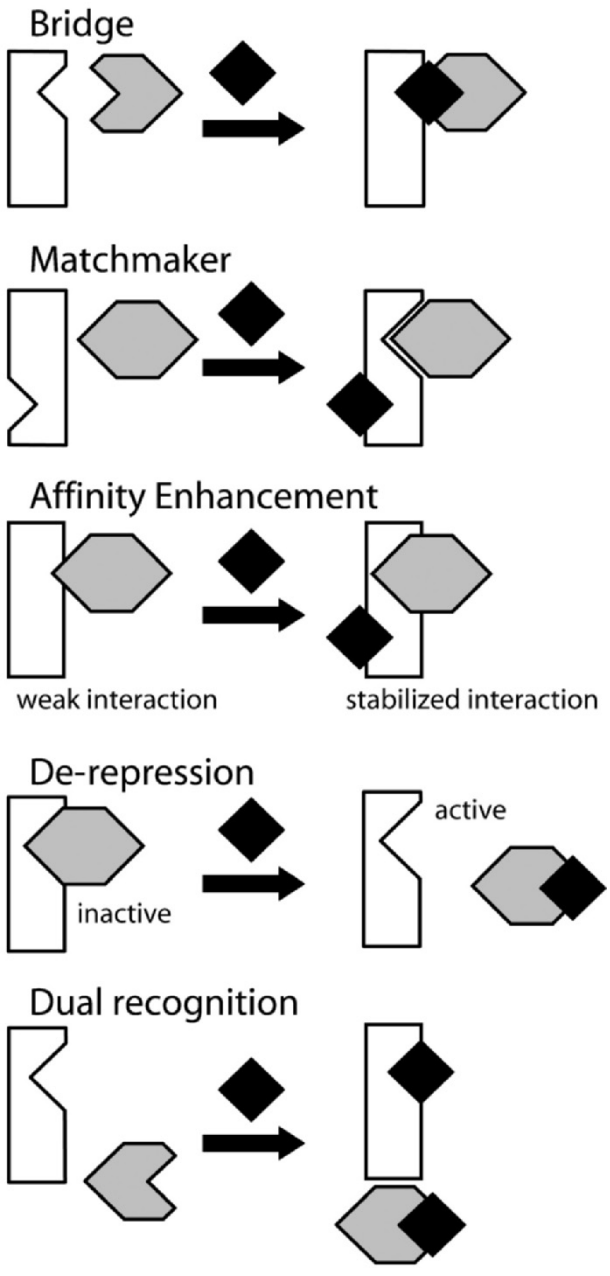

Figure 4. Schematic description of protein-protein interactions of the guard hypothesis. Bridge model: the effector connects the R and a third protein, resulting in a resistance reaction. Matchmaker model: binding of the effector to the R protein results in conformational changes, which allows the binding of a third protein. Affinity Enhancement model: the weak interaction between the R and a third protein (basal defense) is stabilized by the binding of an effector to the R protein and/or the third protein. De-repression model: the R protein is blocked by binding to a third (inhibitory) protein. After binding of the effector to the third protein, the active R protein is released. Dual Recognition model: the independent binding of the effector to both proteins, R and third protein, is necessary for the activation of a HR reaction. All models result in a hypersensitive response. White rectangle, R protein; black diamant, effector (AVR or elicitor); grey hexagon, third protein, unknown.

Evolution of NLRs: lessons from lower phyla of the animal kingdom

Ting and Davis [137] compared R and NLR protein sequences from across kingdom boundaries and could demonstrate that NLRs and R proteins form distinct clades or groups.

Nevertheless, phylogenetic analysis demonstrates that the R proteins are clearly related to the NLR family, and it appears that NLR proteins have their evolutionary roots in plants and are likely an ancient family of genes. Due to the absence/missing of *NLR* genes in different phylogenetic branches of the animal kingdom, it is still unclear whether *R* and *NLR* genes have a common ancestor or whether they developed independently of each other twice during the evolution of the plant and animal kingdom. The discrete number of *NLR* and *R* gene homologs might reflect different evolutionary histories (owing to putative genome-wide or segmental duplications) or different immunological strategies between phyla [138, 139].

The fact that the link between the *NLR* gene family of vertebrates and the *R* genes in plants is still unclear is also due to rather limited information on immune phenomena in the ~30 bilaterian phyla known today. So far extensive molecular information exists on the immune reactions in chordates, molluscs, nematodes, arthropods and echinoderms, with an overwhelming majority of functional and genetic data derived from just two animal phyla: Arthropoda (mostly from *Drosophila melanogaster*) and Chordata (with a special emphasis on mammals and teleost fish). A few other invertebrate species, such as the nematode worm (*Caenorhabditis elegans*) and the sea squirt (*Ciona intestinalis*), have been the focus of functional genomic studies of immunity.

Further investigations to identify *NLR* gene homologs/orthologs in different phylogenetic branches of the animal kingdom will help to solve the question of homologous or paralogous evolution of *R* and *NLR* genes. First hints of an old common ancestor are given by database searches of *Drosophila* and *Caenorhabditis*, where sequences with weak similarities to known NLRs can be found. These sequences contain individual domains of NLR proteins, such as the NACHT and LRR domains, but do not show the NLR characteristic tripartite domain architecture [9]. The NACHT domain is distantly related to AAA+ ATPases, which are proposed to work as switches, regulating signal transduction by conformational changes. AAA+ ATPases have been identified in most prokaryotic and eukaryotic organisms. They are involved in a wide range of cellular regulatory mechanisms. In bacteria, representatives of this superfamily are involved in functions as diverse as tran-

scription and protein folding (e.g. *clpB*) and play an important role in the protein quality control network [140]. Often they employ a common mechanism to mediate an ATP-dependent unfolding/disassembly of protein-protein or DNA-protein complexes. However, involvement in defense mechanisms so far has only been assigned to the tripartite structure of *R* and *NLR* genes. The identified N-terminal effector domains are less common and less well conserved. Examples of invertebrate CARD domains in *Drosophila* and *Caenorhabditis* genomes are Dronc and DARK, and CED-3 and CED-4, respectively, whereas the pyrin domain appears to be vertebrate-specific. A breakthrough with respect to the evolutionary roots of NLRs has been made by investigating the sea urchin genome, where ~200 NLRs have been demonstrated [55, 56, 141]. The number of identified NLRs in *Strongylocentrotus purpuratus* (sea urchin) is in conspicuous contrast to the only ~30 NLR proteins in vertebrates, but resembles the plant situation where more than 600 *R* genes have been identified in the rice genome. Most of the sea urchin NLRs contain one or two DEATH-folds as N-terminal effector-binding domains instead of a single CARD, PYD or BIR domain. This principle is also realized in putative orthologous interacting proteins, e.g. a RIP2-like protein that also carries a C-terminal DEATH domain instead of a CARD.

Unveiling the elicitors and exact signal transduction pathways of the sea urchin, NLRs will shed new light on primary cellular programs, which are just beginning to emerge. The main localization of NLR expression appears to be in intestinal tissue, where they could play a crucial role in maintaining a balance with gut symbionts. This is reflected by the finding that DMBT1 has been described as a target gene of NOD2 in human intestinal epithelial cells, where it serves as a secreted immune exclusion by aggregating bacterial factors [90]. DMBT1 belongs to the family of scavenger receptor cysteine-rich (SRCR) genes, which was first described as dynamically regulated proteins in the immune-challenged sea urchin [142, 143]. However, by analogy to the role of TLRs in developmental processes in lower animal phyla, it is tempting to speculate that NLRs may also play a pivotal role in differentiation and regeneration processes of epithelia. An interesting observation in this respect is the requirement of tracheal cytotoxin (TCT), a peptidoglycan fragment, which is recognized by murine NLR Nod1, as a necessary morphogen to induce normal morphogenesis in the epithelial light organ of the squid [144, 145].

It will be important to build comparative SNP maps of NLRs in different lower species to understand the extent of sequence variability or conservation, respectively. The analysis of the diversification of this class of genes in organisms subjected to high selective pressure and short generation times (e.g. marine animals) will lead to a better understanding of the interaction between barrier and environment and help to understand evolutionary pressures that have shaped human diversity profiles resulting in disease under today's living conditions.

Conclusion

Signals elicited by NLRs are important elements in providing effective pathogen recognition and host defense. The population spectrum of genetic variants in *NLR* genes in humans presumably has been shaped by the major pathogenic challenges of mankind (e.g. bacterial or viral infections); by the same token; precisely these variants may predispose to chronic diseases under changed environmental conditions. Thus, the genetics and functional genomics of NLRs have prototypically demonstrated that the host's interpretation of pathogen-associated molecules is context-dependent. Differences in cellular interpretation can lead to a mutually beneficial animal-microbe association, a protective inflammatory response or chronic, debilitating inflammation.

Several unresolved issues remain, which include the determination of the structural nature of NLR-ligand interactions and the mechanisms by which NLR-eliciting molecules are channelled into the cytosol.

The use of innovative animal models of lower phyla (e.g. sea urchins) will be needed to understand the conserved cellular programs (the 'Ur'-NLR defensome) elicited by host-pathogen interactions and will allow generating model systems where the therapeutic augmentation of barrier function and epithelial drug delivery can be assessed in a simple fashion. An interdisciplinary understanding of the biology of NLRs in the context of barrier disorders and inflammation will drive translation of basic science into patient-oriented fields and eventually allow the development of novel targeted strategies using pharmacological manipulation of this important class of innate immune receptors.

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