NLR-PROTEIN FUNCTIONS IN IMMUNITY

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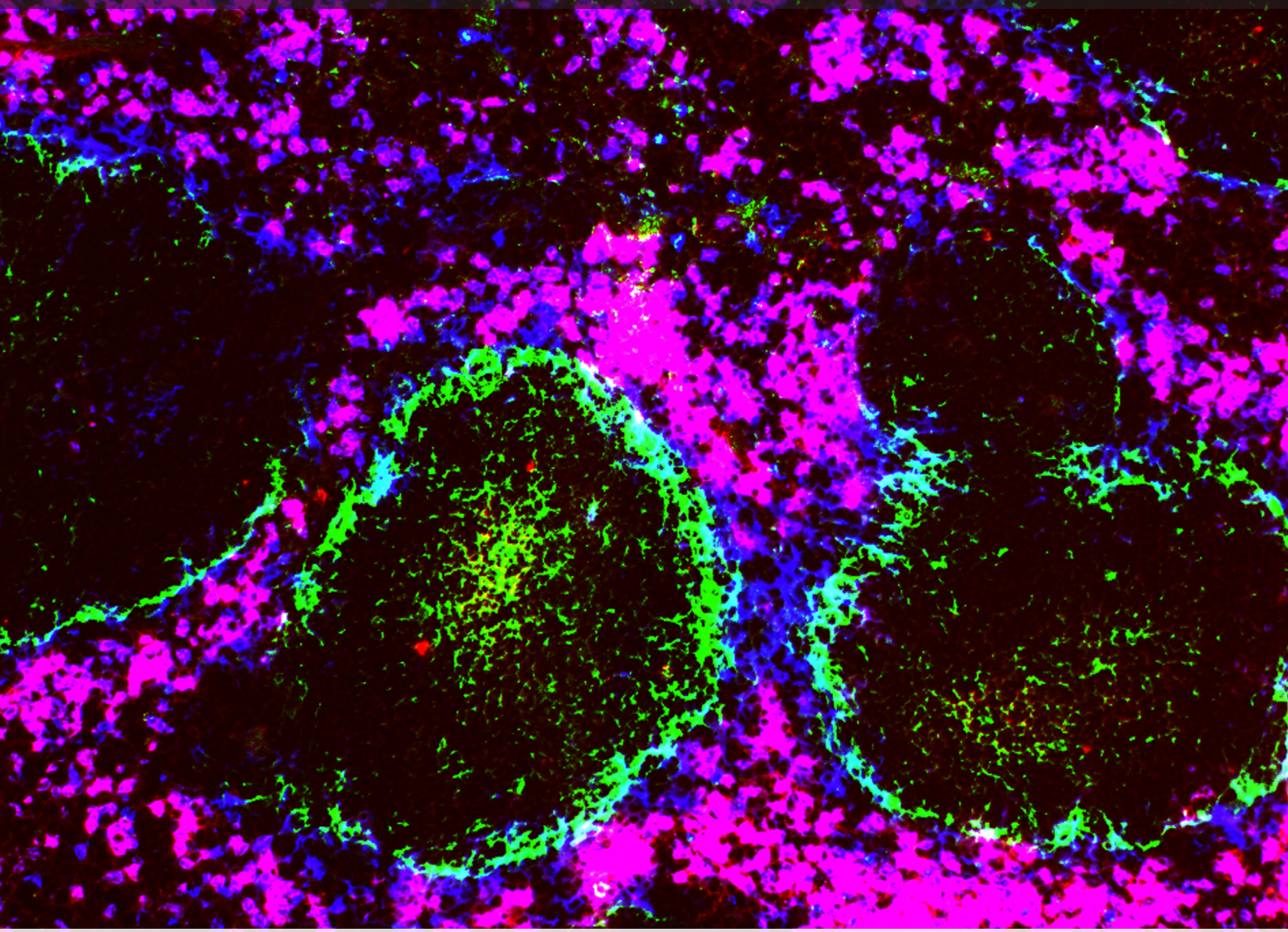

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NLR-PROTEIN FUNCTIONS IN IMMUNITY

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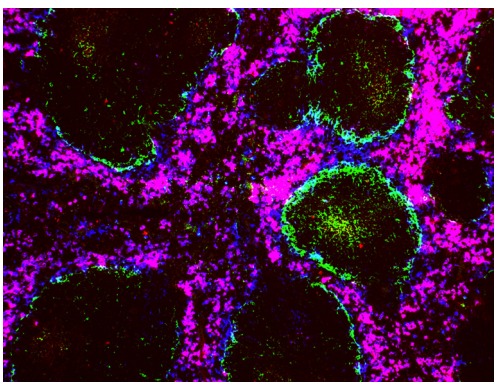

Nod1 stimulation leads to neutrophil recruitment to the splenic red pulp. Sections of spleens from C57BL/6 wild type mice 12 hours post injection with the Nod1 agonist FK156 were stained with specific fluorochrome-tagged antibodies for MadCAM1 (green), CD11b (blue) and Ly6G (red).

Image by Jörg H. Fritz

The Nod-like receptor (NLR) family of proteins are evolutionary conserved molecules that in plants and mammals have been implicated in innate immune sensing of microbes and infection-associated physiological changes, contributing to immune protection of the challenged host organism through the instruction of inflammatory responses, antimicrobial defense and adaptive immunity. Recent data however suggests that the biological roles of NLR go beyond the function of classical pattern recognition molecules (PRM) as they have been implicated in essential cellular processes including autophagy, apoptosis, modification of signal transduction and gene transcription as well as reproductive biology.

In this research topic, we aim to provide a comprehensive state-of the art overview of the emerging functions of NLR in plant and mammalian immunity, cell biology and reproductive biology. Potential topics may include, but are not limited to the following areas:

- Functions of NLRs as PRMs in infection
- Cross-talk of NLRs with other PRMs
- Signal transduction pathways of NLRs
- New functions of NLRs other than pattern recognition

- Structural aspects of NLR activation
- Mechanisms of NLRs in cell biological processes
- Aspects of NLRs in reproductive biology
- Functions of NLRs in plant immune responses

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EDITORIAL
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Editorial: NLR-protein functions in immunity

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Keywords: innate immunity, NLR, protein, microbes, inflammatory responses

Since Janeway (1) and Matzinger (2) put forward two distinct concepts of innate immune recognition, arguing that the driving force that initiates immune reponses is the recognition of microbial patterns or endogenous danger signals, respectively, we have acquired a tremendous wealth of knowledge of the protein families involved. Host-encoded pattern-recognition molecules (PRM) sense conserved microbial structures, referred to as microbe-associated molecular patterns (MAMP) or pathogen-associated molecular patterns (PAMP), as well as endogenous danger-associated molecular patterns (DAMP). Protein families of PRM include the well-described membrane-associated toll-like receptors (TLR) (3–5) and C-type lectins (6). Beside that, the host also evolved intracellular PRM that were identified only more recently (7, 8). One important class of such intracellular PRM is the family of NOD-like receptor (NLR) proteins (9).

In this research topic on "NLR-protein functions in immunity" leading experts in the field discuss various aspects of NLR biology. The proteins of the NLR family are evolutionary conserved molecules that in plants and mammals have been implicated in innate immune sensing of microbes and infection-associated physiological changes, contributing to immune protection of the challenged host organism through the instruction of inflammatory responses, antimicrobial defense, and adaptive immunity.

Plant NLR, in contrast to mammalian NLR, recognize pathogen-derived effector molecules or the activity of these in the cytosol and can act as transcriptional regulators in the nucleus. Notably, the function of most of these proteins is conserved in phylogenetically distant species. Jacob and co-workers present current concepts on the evolution and function of NLR in plants providing an insightful comparison of the repertoire of NLR and NLR-like proteins in different plant species (10). The wiring of plant NLR to signal transduction processes, their molecular activation, and the role of sub-cellular localization are covered by a review by Qi and Innes (11).

To date, our structural understanding of the mechanisms underlying activation and signaling by NLR is hampered by the intrinsic difficulty to obtain recombinant proteins suitable for structural assessment. However, functional studies and in particular evolutionary perspectives allow the acquisition of novel insights into these mechanisms. Monie and colleagues provide new insights by an evolutionary analysis of the NLR proteins Nod1 and Nod2, defining the nature of the interaction surfaces with their ligands and downstream adaptors (12).

Since the first report demonstrating the involvement of NLR in sensing bacterial components (13), their contributions to the control of infection and their impact on immune regulation is becoming increasingly understood. Opitz and co-workers summarize our understanding of the function of NLR in infectious lung diseases (14). In addition, Rosenstiel and Lipinski (15) and Flavell and colleagues (16) detail the roles of NLR in sensing intestinal bacteria in regulating intestinal immune homeostasis at steady state and during infectious challenge. Ferrero and co-workers describe how NLR drive immunity toward extracellular bacteria by recognition of MAMP in released bacterial outer-membrane vesicles (17), while Olivier and colleagues discuss the role of NLR in sensing malarial pigment hemozoin (18). Recently, the role of xenophagy in anti-bacterial host-defense is

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becoming increasingly evident and NLR proteins have been shown to be involved in triggering this cellular event. The molecular details of how NLR target the autophagy pathway are discussed by Carneiro and Travassos (19).

The functional interplay between NLR and regulatory proteins is a research area with many open questions. Le and Harton discuss how a family of Pyrin- and CARD-only proteins interact with NLR to regulate signaling (20).

It is well accepted that activation of PRM is pivotal to trigger adaptive immunity. The review by Eisenbarth (21) details the impact of NLR proteins in shaping antigen-specific immune responses. The role of the NLR member CIITA, acting as a master regulator for MHC expression, is long known. Very recently, several laboratories demonstrated that NLRC5 exerts a key role in MHC expression as well. Kufer and colleagues provide an overview of the current progress in understanding the function of NLRC5 (22).

The important contribution of NLR proteins to immune homeostasis is well underscored by the findings that polymorphisms in their human genes that are linked to disease. Saleh and colleagues provide an overview of known associations of NLR and disease (23), and Kanneganti and Lupfer discuss open questions of NLR biology (24). Moreover, Stehlik and co-workers detail the role of mutations in PYD-containing NLR in disease, which are important for inflammasome formation that drives Il-1β and IL-18 release (25). Finally, the increasingly recognized role of NLR in carcinogenesis is reviewed by Sutterwala and colleagues (26).

Although most mammalian NLR proteins contribute to immunity, some members of this family show restricted expression in the germ line and are associated with developmental processes. This often-neglected role of NLR is illustrated by the consequences of polymorphisms in NLRP7, resulting in embryonic malformations that are reviewed and discussed by Slim and Wallace (27).

The NLR and NLR-like molecules in mammals and plants, respectively, represent a very interesting protein family with diverse functions expanding beyond immune regulation. Particularly in plants, we witnessed many important advances for our understanding of the functions of NLR in cell autonomous pathogen recognition and subsequent signaling. Although first described about 20 years ago, our understanding of the biology of many NLR members in mammals is still fragmentary. Much progress has been made regarding the characterization of the biology of Nod1, Nod2, NLRC4, and NLRP3. In contrast, other NLR have still not been experimentally assessed at all. The collection of articles presented here aims to give an overview of our current understanding of NLR functions and highlight open questions. Utilizing more powerful genetics and advanced cell biology tools will enable us to address controversies in the field and help to further our understanding of the biology of this important protein family.

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Unsolved mysteries in NLR biology

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NOD-like receptors (NLRs) are a class of cytoplasmic pattern-recognition receptors. Although most NLRs play some role in immunity, their functions range from regulating antigen presentation (NLRC5, CIITA) to pathogen/damage sensing (NLRP1, NLRP3, NLRC1/2, NLRC4) to suppression or modulation of inflammation (NLRC3, NLRP6, NLRP12, NLRX1). However, NLRP2, NLRP5, and NLRP7 are also involved in non-immune pathways such as embryonic development. In this review, we highlight some of the least well-understood aspects of NLRs, including the mechanisms by which they sense pathogens or damage. NLRP3 recognizes a diverse range of stimuli and numerous publications have presented potential unifying models for NLRP3 activation, but no single mechanism proposed thus far appears to account for all possible NLRP3 activators. Additionally, NLRC3, NLRP6, and NLRP12 inhibit NF-κB activation, but whether direct ligand sensing is a requirement for this function is not known. Herein, we review the various mechanisms of sensing and activation proposed for NLRP3 and other inflammasome activators. We also discuss the role of NLRC3, NLRP6, NLRP12, and NLRX1 as inhibitors and how they are activated and function in their roles to limit inflammation. Finally, we present an overview of the emerging roles that NLRP2, NLRP5, and NLRP7 play during embryonic development and postulate on the potential pathways involved.

Keywords: inflammasomes, NOD-like receptors, DAMPs, PAMPs, innate immunity, caspase-1, embryonic development

INTRODUCTION

Innate immunity is initiated by germline-encoded pattern-recognition receptors (PRRs). Among these, the nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) comprise a large receptor family of more than 20 members (1–4). Only about half of the NLRs have been characterized in any detail. However, it is well documented that NLRs play a critical role in protection against infectious diseases, including bacteria (5, 6), viruses (7, 8), fungi (9, 10), protists (11, 12), and helminthes (13). Of the NLRs which have been studied, most of them fall into one of four categories: (1) Inflammasome activators, (2) Activators of Nuclear Factor-κB (NF-κB) and mitogen activated protein kinase (MAPK), (3) Inhibitors of inflammatory signaling, (4) and trans-activators of MHC expression. However, several NLRs have definite roles in embryogenesis, uterine implantation, and fetal development (14). Intriguingly, some NLRs appear to play multiple roles within inflammation or development. This suggests alternative functions for some NLRs in different cell types or multiple activation mechanisms with separate downstream effects for other NLRs.

One set of NLRs that regulates NF-κB and MAPK are NLRC1 (NOD1 or CARD4) and NLRC2 (NOD2 or CARD15). NLRC1 recognizes iE-DAP, a subunit of peptidoglycan found in some bacterial cell walls (5, 15). NLRC2 recognizes MDP, another peptidoglycan fragment (16–19). NLRC1 and NLRC2 then act through the adaptor RIPK2 to activate NF-κB and MAPK signaling (20–22). However, there are now multiple reports that demonstrate NLRC2 can respond to cytosolic RNA during viral infection (23–25). Although viral RNA also induces an interaction between NLRC2 and RIPK2, this appears to regulate autophagy mechanisms, instead of NF-κB, and subsequently represses inflammasome activation and prevents immunopathology (25). Furthermore, viral RNA mediated activation causes NLRC2 to interact with the antiviral adaptor protein MAVS. This interaction was shown to be essential for the production of IFN-β during viral infection and for suppressing virus replication (24). Additionally, NLRC2 regulates other antiviral pathways like 2′-5′ oligoadenylate synthease (OAS2), which activates RNAse L and degrades viral RNA, thus potentiating antiviral signaling (23). It is not clear how NLRC2 would bind to both MDP and RNA, but one possibility is that additional upstream adaptor proteins, which have yet to be discovered, actually provide specificity.

In the case of NLRC4, it is activated by bacterial flagellin (26, 27) or the rod complex of bacterial type III secretion systems (T3SS) (28). Once activated, NLRC4 forms a multimeric complex, known as the inflammasome, with the adaptor ASC and caspase-1 (26, 27). Inflammasome formation results in a proinflammatory cell death termed pyroptosis (29) and the release of IL-1β and IL-18 (30–34). Recently, the ability of NLRC4 to recognize flagellin and T3SS components was tagged to an association between NLRC4 and another class of NOD proteins known as NAIPs. NAIP5 and NAIP6 in the mouse recognize flagellin and NAIP2 recognizes T3SS rod complexes respectively and then activate NLRC4 (35–37). Furthermore, NAIP1 in mice activates NLRC4 in response to the needle protein of some T3SS (38). In human cells, only one NAIP exists, and this recognizes the needle protein of T3SS similar to mouse NAIP1 (38). These results demonstrate that one

mechanism for the recognition of multiple ligands by NLRs is the presence of upstream adaptor proteins like NAIPs.

Distinct NLRs recognize microbial or viral components such as peptidoglycan, flagellin, or viral RNA. These pathogen specific molecules are known as pathogen-associated molecular patterns (PAMPs). Alternatively, some NLRs, like NLRP3, detect damage associated molecular patterns (DAMPs). DAMPs consist of byproducts of pathogen invasion or sterile cellular damage such as uric acid crystals, reactive oxygen species (ROS), or extracellular ATP release (39–42). Sensing of DAMPs by NLRP3 is not only critical for detection and clearance of pathogens but also for protection and repair of tissues during inflammation (43, 44). NLRP3 is one of the most ubiquitously important NLRs. Once activated, NLRP3 also forms an inflammasome with the adaptor ASC and caspase-1 (45, 46). NLRP3 responds to an incredibly broad range of pathogens making it unlikely that it senses PAMPs directly. Many lines of evidence support a role for NLRP3 in DAMP sensing, where damage to the host results in the release of certain danger signals not present under homeostatic conditions. ROS, potassium efflux, and release of proteases from endosomes have all been reported to activate NLRP3 (41, 42, 47–51). Although much is know about the range of stimuli that can activate NLRP3, much research remains to be done to understand how NLRP3 becomes activated.

The ability of NLRP3 to respond to multiple PAMPs or DAMPs from such a broad range of pathogens strongly indicates the presence of upstream adaptors, as is the case for NLRC4, or common danger signals which funnel into one pathway. In the case of NLRC2, the different signaling pathways activated by MDP or viral RNA would suggest that different modes of NLRC2 activation lead to different protein conformations or other alterations in NLRC2 activity. This is subsequently responsible for activation of NF-κB, autophagy, or antiviral signaling. Indeed, these are some of the great-unsolved mysteries of NLR biology. In addition, other NLRs, like NLRC3, NLRP6, NLRP12, and NLRX1, play inhibitory roles during inflammation. Yet how these proteins are activated or perform their inhibitory functions is not well understood. Finally, there are numerous NLRs for which there are different reports indicating a multiplicity of potential functions. In this review, we discuss several of these unsolved mysteries and potential future directions in the field of NLR biology.

ACTIVATION MECHANISMS OF NLRP3

NLRP3 was initially described as an activator of caspase-1 in 2002 and was subsequently associated with autoinflammatory periodic fevers like Muckle–Wells syndrome or bacterial infection (45, 46). Since then, there have been many proposed mechanism for how NLRP3 is activated. There is no evidence that NLRP3 interacts directly with any PAMP. Although NLRP3 is activated in response to bacteria and viral RNA (7, 52), lipopolysaccharide, and MDP (53), most PAMPs appear to only be required for the transcriptional up-regulation of NLRP3 and pro-IL-1β (54). Once NLRP3 is upregulated, a second signal, generally a DAMP or a pore forming toxin like nigericin, is required for NLRP3 to interact with ASC and caspase-1 to form an active inflammasome. This second signal is frequently associated with the production of ROS or endosomal rupture (41, 42, 49, 50). Changes in intracellular and extracellular calcium (55–58) and potassium efflux (47, 48, 51) have also been proposed to activate NLRP3, as have changes in cytosolic or extracellular pH (59, 60). This dichotomy of signals for priming and activation is required for NLRP3 inflammasome formation. The big question that remains is how ROS, ion flux, or other DAMPs regulate NLRP3 (**Figure 1**). One possibility is that the structure of NLRP3 is sensitive to changes in ion concentrations, and exposure of the pyrin effector domain occurs when ion concentrations deviate from their homeostatic state (48, 51). Alternatively, protein sensors of cellular redox or ion sensors could regulate NLRP3 activation following their own activation. Studies into the structure of NLRP3 and the effects of different ions on the ATPase activity, pyrin effector domain exposure, and ASC binding affinity of NLRP3 would greatly increase our understanding of how NLRP3 is activated.

Proteomic studies directed at understanding the NLRP3 interactome using different activators of NLRP3 may also provide further insight into potential upstream regulators such as NAIPs or ion/ROS sensors. A recent paper by Mitoma et al. (61) found in human macrophages that NLRP3 is activated in responses to double stranded RNA through an interaction with the RNA helicase DHX33 (61). Protein kinase R (PKR) is an RNA dependent kinase involved in antiviral defenses. Activation of NLRP3 was also proposed to be dependent on PKR, although phosphorylation of NLRP3 was not required (62). However, another group attempted to examine the role of PKR mediated activation of NLRP3 but found no role for PKR (63). Finally, the adaptor protein MAVS, which is required for antiviral signaling downstream of the RNA helicases RIG-I and MDA5, has been shown to interact with NLRP3 and regulate its activation and localization to the mitochondria (64). Although this was shown in the context of RNA transfection, LPS + ATP treatment or nigericin, how LPS or nigericin could activate MAVS remains to be investigated further. In all, there is a significant body of research that would indicate the presence of upstream PRR that tie into the NLRP3 pathway (**Figure 1**).

Although multiple upstream sensors may regulate NLRP3, it is possible that NLRP3 interacting partners regulate its activation through the addition or removal of post-translational modifications. Post-translational modification of NLRs has been reported to regulate their activation. For example, phosphorylation of NLRC4 by PKCδ regulates its activation during *Salmonella typhimurium* infection in macrophages (65). In the case of NLRP3, nitric oxide produced during chronic inflammation *in vivo* during *Mycobacterium tuberculosis* infection results in nitrosylation of NLRP3 and inhibition of inflammasome activation (66). Similarly, the addition of NO donor compounds to macrophages or induction of NO by IFN-γ treatment inhibited NLRP3 activation (66, 67). The role of NO for NLRP3 inhibition during LPS-induced sepsis in mice has also been reported (68). Therefore, proteins that can regulate the nitrosylation status of NLRP3 may be able to regulate its activation. Ubiquitination and deubiquitination were also found to regulate NLRP3 activation (69, 70). Thus far, deubiquitination by the BRCC3 deubiquitinase is the only post-translational modification that is reported to activate NLRP3 (70). It is clear that post-translational modifications can affect NLRP3 activation,

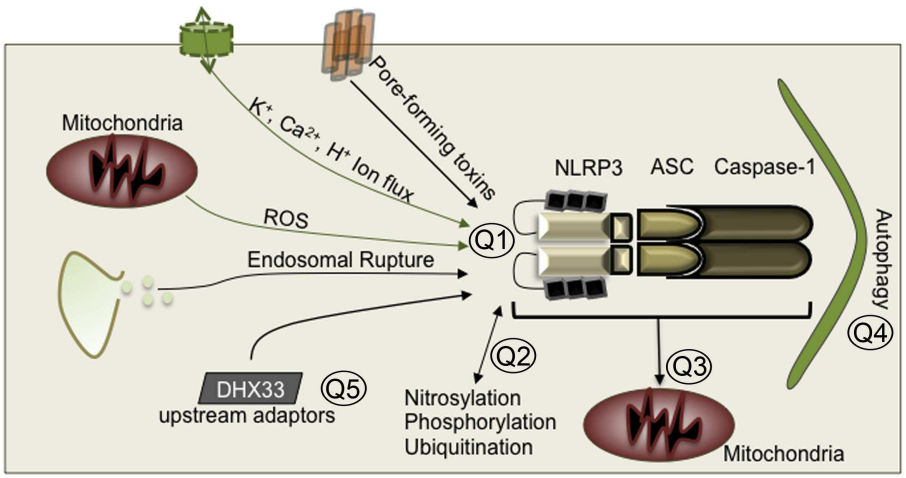

FIGURE 1 | Unsolved mysteries in NLRP3 biology. Q1: Is there a common DAMP that activates NLRP3? Do DAMPs directly activate NLRP3? Do DAMPs induce structural rearrangement of NLRP3? Q2: How do post-translational modifications regulate activation on a structural level? Q3: Is mitochondrial localization essential for NLRP3 inflammasome formation? Q4: How does autophagy inhibit NLRP3? Does autophagy directly engulf NLRP3 inflammasomes? Does it engulf damaged mitochondria where NLRP3 is localized? Does autophagy merely remove the source of DAMPs? Q5: Are there additional upstream sensors or adaptors that facilitate NLRP3 activation?

although how ubiquitination, or nitrosylation affect the function of NLRP3 needs further biochemical examination (**Figure 1**).

The cellular autophagy pathway, which is required for recycling damaged organelles and proteins, has been reported to inhibit NLRP3 activation. Ubiquitinated inflammasomes are degraded through the autophagy pathway (71). This report, in combination with those above, may indicate that deubiquitination of NLRP3 prevents autophagic degradation and allows for inflammasome formation. Alternatively, the removal of damaged mitochondria, which produce NLRP3 activators like ROS or release of mitochondrial DNA into the cytosol, constitutes another mechanism by which autophagy regulates NLRP3 activation (25, 72, 73). It is also possible that autophagosomal degradation of damaged mitochondria simultaneously removes inflammasomes. Several recent publications demonstrate that NLRP3 inflammasome formation is dependent on localization to the mitochondria (64, 74). However, another report demonstrated that inflammasome activation was not associated with any organelle but occurred in the cytosol (75). Why there are conflicting reports regarding the mechanisms that activate NLRP3 are unclear. However, in the case of cellular localization, differences in fixation or staining methodologies may result in aggregation of inflammasomes with mitochondria or their disassociation, respectively. In all, mitochondria appear to play a role in the regulation of NLRP3 inflammasome activation, but whether they serve as an activation platform, a source of stimuli, or both requires further investigation (**Figure 1**).

To more fully understand NLRP3 regulation, the interactome of NLRP3 including kinases and ubiquitin ligases still need to be discovered and the regulation of post-translational pathways examined. Clearly there is need for a concerted effort from biochemists, molecular and structural biologists, and immunologists to collaborate on these issues. As NLRP3 is associated with numerous autoinflammatory and autoimmune diseases, understanding how NLRP3 is regulated will be necessary for understanding and potentially preventing disease development, as well as for the design of inhibitors which are useful under specific inflammatory conditions.

REGULATION OF INHIBITORY NLRs

Intriguingly, all inhibitory NLRs studied thus far have been found to inhibit NF-κB activation. NLRP12 was examined during colon inflammation and colon tumorigenesis and found to negatively regulate NF-κB down stream of Toll-like receptors (TLRs) (76, 77) or to regulate the alternative NF-κB pathway downstream of TNF family receptors (76, 78). NLRP12 appears to interact with NF-κB–inducing kinase (NIK), interleukin-1 receptor-associated kinase 1 (IRAK1), and TNF receptor-associated factor 3 (TRAF3), which are known mediators of NF-κB signaling (78, 79). These interactions appear to regulate the phosphorylation of IRAK1 and the degradation of NIK, thus resulting in inhibition of the alternative NF-κB pathway. However, the mechanism by which NLRP12 inhibits TLR mediated activation of the classical NF-κB pathway is not known (**Figure 2**).

Currently, it is unclear how the inhibitory function of NLRP12 is regulated (**Figure 2**). ATP binding appears to be a requirement for activation (79) but the mechanism by which NLRP12 structural rearrangement occurs to permit ATP binding has not been examined. NLRP12 expression increases following NF-κB activation (80). It is also apparent that NLRP12 interacts with other proteins which regulate its function, including HSP90, which stabilizes NLRP12 and prevents its proteasomal degradation (81). Whether expression alone is sufficient for its inhibitory function, or if NLRP12 is regulated by post-translational modifications of some kind is unclear.

Recently, *Nlrc3*-deficient mice were generated and inflammation examined in response to LPS treatment (82). Sub-lethal LPS

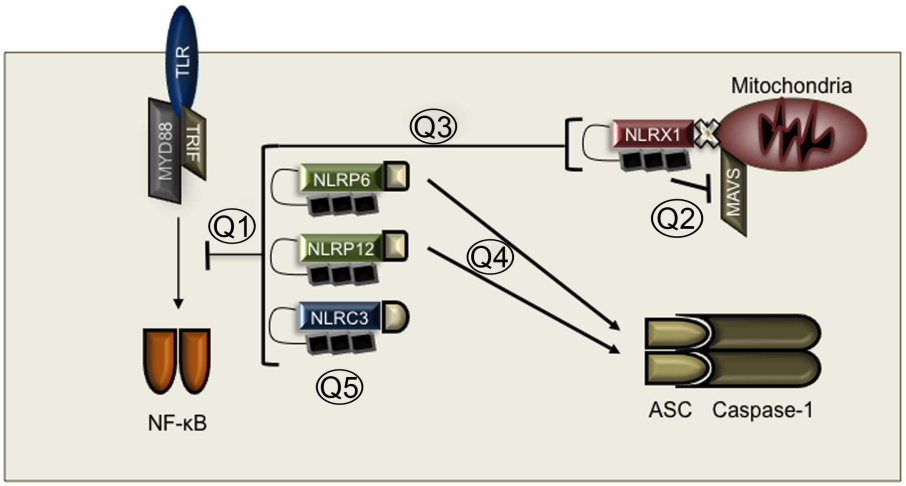

FIGURE 2 | Mechanisms of inhibitory NLRs. Q1: How do inhibitory NLRs function? Is PAMP recognition required for inhibitory NLR function? Is NLR expression sufficient for inhibitory function? Q2: Is NLRX1 an inhibitor of MAVS or a modulator of mitochondrial ROS? Q3: How does NLRX1 inhibit NF-κB if it is localized to the mitochondria? Q4: Do NLRP6 and NLRP12 regulate inflammasome activation and how? Does gut flora play a role in inflammasome activation in the absence of NLRP6 and NLRP12. Q5: Why are there so many inhibitory NLRs? Do inhibitory NLRs play redundant or context specific roles?

administration resulted in increased IL-6, increased macrophage numbers and increased hypothermia in *Nlrc3*$^{-/-}$ mice. Examination of *Nlrc3*$^{-/-}$ macrophages showed that there was enhanced NF-κB activation down stream of TLR signaling (82). Mechanistically, NLRC3 appears to regulate TRAF6 activation by modulating its K^{63}-linked ubiquitination and stability. Once again, the mechanisms that regulate NLRC3 activation remain to be examined (**Figure 2**).

Similar to NLRC3 and NLRP12, NLRP6 also inhibits NF-κB activation down stream of TLR signaling. NLRP6 was shown to suppress NF-κB activation during *Listeria monocytogenes* and *Salmonella typhimurium* infection, and in the absence of NLRP6, bacteria were cleared more rapidly (83). In other studies, *Nlrp6* deficiency predisposes mice to increased inflammation in models of colitis and to increased tumorigenesis in colon cancer models (84, 85). However, the mechanisms proposed for susceptibility to colitis and tumorigenesis are reportedly due to NLRP6 mediated inflammasome activation. *Nlrp6*$^{-/-}$ mice have reduced IL-18 in the colon in these models (84, 85). It should be noted, though, that no biochemical or molecular evidence for an NLRP6 inflammasome has been presented to date. It is therefore possible that NLRP6 regulates inflammasome activation indirectly. In fact, there are significant differences in the gut microbiota in *Nlrp6*$^{-/-}$ mice in the above models, which could result in altered inflammasome activation (**Figure 2**).

NLRP12 has also been proposed to form an inflammasome. During *Yersinia pestis* infection, NLRP12 is reported to recognize acylated lipid A and *Nlrp12*$^{-/-}$ mice were more susceptible to infection and had reduced IL-18 levels (86). In humans, NLRP12 polymorphisms are associated with inflammasome activation during periodic fever syndromes (87, 88). It is possible that both NLRP12 and NLRP6 have regulatory roles during NF-κB activation as well as in inflammasome formation. To verify these functions, however, much needs to be done on the molecular and biochemical level to determine the mechanisms by which these proteins activate the inflammasome and what stimuli activate them to form an inflammasome verses inhibit NF-κB activation. Finally, as discussed above, several NLR deficient mouse strains have been found to harbor altered gut microbiota compared to WT controls. The ability of the microbiome to regulate immunity is clear, but the exact effects of these changes are still not well-understood. Especially during models of colon inflammation, differences in gut flora between mice may be an essential factor in the phenotypes observed. The use of germ free or gnotobiotic mice for studying the roles of NLRs in general, but NLRP12 and NLRP6 in particular, may help resolve their functions as immune activators or repressors and the mechanisms by which they perform these functions.

The last NLR with a proposed inhibitory function is NLRX1. NLRX1 was originally reported to inhibit antiviral signaling through inhibition of the adaptor MAVS (8, 89). Subsequently, NLRX1 was shown to inhibit TLR mediated activation of NF-κB (90). How NLRX1 inhibits NF-κB is not clear though, as NLRX1 is localized to the mitochondria. Furthermore, the role of NLRX1 as an inhibitor is debated. Several groups have found no role for NLRX1 in regulating MAVS but have instead reported NLRX1 as a modulator of mitochondrial ROS (91–93). Recently, the crystal structure of NLRX1 was solved along with biochemical evidence for the binding of NLRX1 to the viral RNA mimic poly(I:C) (94). Although this finding would support a role for NLRX1 in antiviral signaling, the exact function of NLRX1 will require further examination (**Figure 2**).

As discussed above, the *in vivo* importance of inhibitory NLRs has been demonstrated in various models of inflammation. However, why there are so many NLRs that inhibit NF-κB signaling is

a conundrum. If expression of these inhibitory NLRs alone were sufficient to suppress NF-κB activation, then why would there need to be four. One possibility is that they function as a whole to modulate NF-κB activation appropriately. Another possibility is that they are activated only in response to certain infections or stimuli. However, treatment with LPS or poly(I:C) both resulted in increased NF-κB activation in *Nlrp12*$^{-/-}$ macrophages (77), suggesting that ligand recognition is not required for its function. Understanding the individual and combined roles of NLRC3, NLRP6, NLRP12, and NLRX1 during specific infections or models of inflammation will be important as this field moves forward (**Figure 2**).

NLRs AS DOUBLE AGENTS

As discussed in the last section, NLRP12 and NLRP6 have roles in inhibiting inflammation by modulating NF-κB activation (77–79, 83). In addition, both of these NLRs are reported to regulate inflammasome activation (84–86). As discussed in the introduction, NLRC2 is able to respond to both MDP and viral RNA and activates distinct pathways including NF-κB, autophagy, or antiviral signaling (**Table 1**). All of these pathways are important for inflammation and immunity. However, NLRs are also implicated in numerous non-inflammatory roles. NLRC1 and NLRC2 have been shown to regulate the differentiation of human umbilical cord blood-derived mesenchymal stem cells (MSC). Although NLRC1 and NLRC2 had no effect on MSC proliferation, they enhanced their differentiation into chondrocytes and osteocytes and inhibited adipocyte formation *in vitro* (95). The ability of NLRC1 and NLRC2 to regulate MSC differentiation was associated with increased ERK1/2 MAPK signaling; a known function of these NLRs (**Table 1**). The ability of NLRs to affect MSC may play an important part of wound healing and the resolution of inflammation. In fact, NLRP3 was found to play an important function in tissue repair in the lung during influenza A virus infection, although this was likely due to impaired recruitment of macrophages or other cells necessary for wound repair and healing (43).

The role of NLRs in tissue repair or MSC differentiation may be a logical progression following inflammation but several additional NLRs have been reported to regulate seemingly disparate functions. NLRP2 is reported to inhibit NF-κB activation (96, 97) and to enhance caspase-1 activation (96). In addition, siRNA mediated knockdown of NLRP2 in primary human astrocytes was recently reported to impair inflammasome activation (98). How NLRP2 affects inflammasome activation is not entirely clear, as knockdown of NLRP2 resulted in decreased caspase-1 expression as well. Furthermore, the stimulus used for NLRP2 activation was the NLRP3 activator extracellular ATP (98). These findings might indicate that NLRP2 regulates the expression of key NLRP3 inflammasome components as opposed to a novel NLRP2 specific inflammasome. In addition to the role for NLRP2 in inflammasome activation and inhibition of NF-κB signaling, NLRP2 has a definite role in embryonic development (**Table 1**). A truncation mutation of NLRP2 was found in association with Beckwith–Wiedemann Syndrome (BWS) (99). The NLRP2 mutation resulted in developmental defects that stemmed from altered DNA methylation and gene expression initially present in the maternal oocyte (maternal imprinting) and perpetuated in the fertilized embryo and developing fetus (99). Another study found some association between NLRP2 and recurrent miscarriages (100). Finally, siRNA knockdown of NLRP2 in murine oocytes or embryos leads to nearly complete developmental arrest (101).

Other NLRs have also been proposed to regulate inflammasome activation and development. NLRP7 regulates inflammasome activation in response to acylated lipopeptides like FSL-1 or triacylated Pam3CSK4 (102). In addition, NLRP7 is associated with recurrent miscarriages and recurrent hydatidiform molar pregnancies (100, 103–105). The above findings definitely support roles for NLRP2 and NLRP7 in inflammation and development. Interestingly, NLRP7 is not present in the mouse genome and appears

Table 1 | Functionally distinct roles of NLRs in biology.

NLR	Dual roles	References
NLRP12[a]	NF-κB inhibition, caspase-1 activation	Williams et al. (76), Arthur et al. (81), Ye et al. (79), Jeru et al. (87), Jeru et al. (88), Zaki et al. (77), Allen et al. (78), Vladimer et al. (86), Chattoraj et al. (80)
NLRP6[a]	NF-κB inhibition, caspase-1 activation	Chen et al. (84), Elinav et al. (85), Anand et al. (83)
NLRC2[b]	NF-κB and MAPK activation, type-I IFN production, autophagy, MSC differentiation	Bertin et al. (20), Girardin et al. (21), Park et al. (22), Dugan et al. (23), Sabbah et al. (24), Kim et al. (95), Lupfer et al. (25)
NLRP2[c]	Embryonic development, caspase-1 activation	Bruey et al. (96), Fontalba et al. (97), Meyer et al. (99), Peng et al. (101), Huang et al. (100), Minkiewicz et al. (98)
NLRP7[c]	Embryonic development, caspase-1 activation	Murdoch et al. (103), Messaed et al. (104), Khare et al. (102), Huang et al. (100), Ulker et al. (105)

The NLRs listed in this table have been implicated in multiple functional roles. However, the mechanisms by which they perform these distinct roles have not been elucidated. [a]It is unclear how NLRP6 and NLRP12 function under some inflammatory conditions as inhibitors of NF-κB but under other conditions can serve as inflammasome activators. [b]NLRC2 responds to a variety of PAMPs including MDP and viral RNA, but the downstream signaling pathways triggered by NLRC2 are distinct for specific PAMPs suggesting alterative activation mechanisms. [c]Finally, NLRP2 and NLRP7 may serve as inflammasome regulators, but whether their functions in embryonic development are tied to inflammasome activation or are separate functions is unclear.

to have arisen from a gene duplication event from NLRP2 (103). Therefore, it is not surprising that these two NLRs possess similar functions, but how they regulate both inflammasome activation and development is currently unknown (**Table 1**). Indeed, the role of NLRs in development is severely understudied, and many biochemical and cell specific studies on the function of these NLRs are needed to understand their differential roles. One possibility is that inflammasome activation is the mechanism by which NLRP2 and NLRP7 regulate embryonic development. The role of IL-1β in oocyte maturation and development has been appreciated for over a decade and has been reviewed previously (106, 107). Intrafollicular injection of IL-1β in horses induces ovulation but also inhibits embryo development (108), which is similar to the developmental arrest seen with NLRP2 and NLRP7 mutations. Furthermore, treatment of rabbit ovaries *in vitro* with IL-1β also arrests developing embryos (109). However, a lack of IL-1β signaling does not significantly affect fertility and embryo viability as IL-1 receptor deficient mice reproduce normally (110). Therefore, increased levels of IL-1β in patients with NLRP2 and NLRP7 mutations may be the cause of developmental arrest. However, much additional research on the roles of NLRP2 and NLRP7 needs to be performed before any conclusions can be reached regarding their functions in development.

CONCLUSION

The role of NLRs in immune function is unequivocal. However, there is much molecular, biochemical and structural research which remains to be done to better understand how NLRs are activated and regulated. Due to the diversity of functions among NLRs, understanding their activation and regulation should provide a cornucopia of new opportunities to modulate the immune system. The activation of proinflammatory NLRs has already been demonstrated to be important for the function of many adjuvants used in research or in the clinic (111, 112). Targeting NLRs specifically for the generation of novel adjuvants may provide for more effective vaccines. On the other hand, targeting NLRs may provide for new treatments against numerous diseases such as arthritis (40, 113), diabetes (114, 115), colitis (44, 85, 116), multiple sclerosis (117–119), Alzheimer's (49, 120), and many other diseases associated with mutations or disregulation of NLRs.

Several unstudied NLRs have recently been assigned some putative functions. NLRP10 has been reported to play a critical role in the induction of Th1 and Th17 mediated T cell responses through a defect in dendritic cells migration during *Candida albicans* infection (121, 122). As discussed above, NLRP7 was recently reported to assemble an inflammasome in response to bacterial diacylated lipopeptides (102). The fact that after a decade of research, new inflammasome activators are still being discovered may indicate that more NLRs fill this function than those previously described. Furthermore, recent studies have also validated roles for NLRP5 in embryonic development, although the exact mechanisms underlying these observations have not been elucidated (123–125). With more than 10 NLRs unstudied, it will be of interest to determine the function of these remaining NLRs in inflammation and development.

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Recognition of extracellular bacteria by NLRs and its role in the development of adaptive immunity

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Innate immune recognition of bacteria is the first requirement for mounting an effective immune response able to control infection. Over the previous decade, the general paradigm was that extracellular bacteria were only sensed by cell surface-expressed Toll-like receptors (TLRs), whereas cytoplasmic sensors, including members of the Nod-like receptor (NLR) family, were specific to pathogens capable of breaching the host cell membrane. It has become apparent, however, that intracellular innate immune molecules, such as the NLRs, play key roles in the sensing of not only intracellular, but also extracellular bacterial pathogens or their components. In this review, we will discuss the various mechanisms used by bacteria to activate NLR signaling in host cells. These mechanisms include bacterial secretion systems, pore-forming toxins, and outer membrane vesicles. We will then focus on the influence of NLR activation on the development of adaptive immune responses in different cell types.

Keywords: NLRs, extracellular bacteria, OMVs, adaptive immunity, innate immunity

INTRODUCTION

A balanced relationship between humans and their microbiota is required for a variety of biological functions, including optimal protection against invasion by microbial pathogens, development of the mucosal immune system, and control of metabolic processes [reviewed in Ref. (1)]. The ability of the host immune system to distinguish between commensals and pathogens is required to avoid the development of persistent immune responses against the normal microbiota, yet maintain appropriate immune responses to pathogens. However, bacterial pathogens are able to avoid or subvert the host immune system to promote their survival and colonization. To this end, bacteria can either secrete different components into the extracellular medium or inject molecules into the host cell cytoplasm. In parallel, host cells have developed a wide range of pattern-recognition receptors (PRRs), including Toll-like receptors (TLRs) and Nod-like receptors (NLRs), to detect microorganism- and/or danger-associated molecular patterns (MAMPS and DAMPS, respectively) present in the extracellular medium or in their cytoplasm. MAMPS include virulence factors, but also essential components of both commensals and pathogens, e.g., lipopolysaccharide (LPS), peptidoglycan, or nucleic acids. Recent studies have shown that the recognition of the microbiota that takes place in the gut is necessary for the development of a normal epithelium, by controlling the balance of proliferation and differentiation, as well as maintaining a properly functioning immune system (1, 2).

Over the previous decade, the general paradigm was that extracellular bacteria were only sensed by cell surface-expressed TLRs, whereas cytoplasmic sensors, including members of the NLR family, were specific to pathogens capable of breaching the host cell membrane. Structurally, NLRs share a typical tripartite architecture with a conserved central nucleotide-binding domain, which restrains the catalytic activity of NLR family proteins. This central domain is named NACHT after the original proteins which defined the features of this domain: neuronal apoptosis inhibitory protein (NAIP), MHC class II transcription activator (CIITA), incompatibility locus protein from *Podospora anserine* (HET-E), and a telomerase-associated protein (TP1). At the C-terminal region of NLR proteins are a series of leucine-rich repeats (LRRs) that are believed to initiate NLR activation after recognition of the appropriate signal, although this mechanism is still unclear (3). The N-terminal effector domain, which specifies the function of NLRs, is less conserved. Indeed, NLRs may harbor either a pyrin domain (PYD), a caspase-activation and recruitment domain (CARD), a baculovirus inhibitor of apoptosis domain (BIR), or an as yet characterized domain (**Table 1**) (4). To date, 23 NLR family members have been reported, each playing different roles in pathogen recognition, homeostasis, apoptosis, or gut development [reviewed in Ref. (4)]. In the context of host-pathogen responses, NLR activation has been shown to induce the production of pro-inflammatory effectors through either nuclear translocation of Nuclear Factor-κB (NF-κB) or formation of high-molecular-weight platforms, named inflammasomes, which activate caspase-1. The main substrates of caspase-1 are cytokine proforms of IL-1β and IL-18, which are usually expressed in an NF-κB-dependent manner. Hence, to be expressed in a fully mature form, these cytokines require regulation at both transcriptional and post-transcriptional levels. Several distinct inflammasomes have been described in the literature, consisting of different scaffolding proteins of the NLR or the PYHIN (PYRIN and HIN-200) superfamilies [reviewed in Ref. (5)].

Despite their intracellular localization, it has become apparent that NLRs play key roles in the sensing of not only intracellular, but also extracellular bacterial pathogens or their components. In this review, we will summarize the mechanisms used by extracellular pathogens to deliver bacterial components into host cells and how

Table 1 | NLR family members and bacterial recognition.

Effector domain	NLR family member	Bacteria	NLRs ligands	Activation mechanism	Reference
CARD	NOD1	*H. pylori*	Peptidoglycan	T4SS	Viala et al. (6)
		H. pylori	Peptidoglycan	OMVs	Kaparakis et al. (7), Bielig et al. (8),
		P. aeruginosa			Chatterjee and Chaudhuri (9)
		N. gonorrhoeae			
		V. cholerae			
		S. typhimurium		T3SS	Keestra et al. (10)
	NLRC4/NAIP2[a]	*S. typhimurium*	PrgJ	T3SS	Kofoed and Vance (11), Zhao et al. (12)
		B. pseudomallei	BsaK		
		E. coli	EprJ, EscI		
		S. flexneri	MxiI		
		P. aeruginosa	*Psc*I		
	NLRC4/NAIP5[a]	*S. typhimurium*	Flagellin	T3SS	Kofoed and Vance (11), Zhao et al. (12)
	NLRC4/NAIP	*C. violaceum*	CprI		Zhao et al. (12)
	NLRP12	*Y. pestis*	?	T3SS	Vladimer et al. (13)
PYR	NLRP3	*N. gonorrhoeae*	Lipooligosaccharide	OMVs, LOS	Fisseha et al. (14), Duncan et al. (15)
		L. monocytogenes	Listeriolysin O	PFT	Gurcel et al. (16), Mariathasan et al.
		S. aureus	Hemolysins and PVL		(17), Harder et al. (18), Munoz-Planillo
		A. hydrophila	Aerolysin		et al. (19), Dunne et al. (20), McCoy et
		A. veronii	Aerolysin		al. (21), McCoy et al. (22), McNeela et
		B. pertussis	CyaA		al. (23), Kebaier et al. (24), Holzinger et
		S. pneumoniae	Pneumolysin		al. (25)
		S. pyogenes	Streptolysin O		
		V. vulnificus	HlyA		
		V. cholerae	MARTX		
BYR	NLRP1	*B. anthracis*	Anthrax lethal toxin		Boyden and Dietrich (26)

NLR family members are classified depending on the function of the N-terminal effector domain: CARD, caspase-activation and recruitment domain; PYD, pyrin domain; BIR, baculovirus inhibitor domain.

[a] *Indicates that proteins are expressed in mice only. The ligands and the activation mechanisms are detailed in the text.*

infections by these microorganisms are sensed via NLRs. Lastly, we will discuss the importance of the activation of innate immunity receptors, such as NLRs, in tailoring an appropriate adaptive immune response.

MECHANISMS WHEREBY BACTERIAL COMPONENTS GAIN ACCESS TO THE CYTOPLASM

BACTERIAL SECRETION SYSTEMS

Bacteria that need to deliver their effectors across both bacterial and cell membranes have developed highly specialized secretion systems to reach their cytoplasmic targets. Among the six described secretion systems, the injection of bacterial components through either type-3 or type-4 secretion systems (T3SS and T4SS, respectively) has been reported to result in the activation of NLR signaling in host cells (**Figure 1**).

The T3SS, or "injectisome," is a specialized molecular machine which is closely related to the bacterial flagellar apparatus. T3SSs have been identified in numerous Gram-negative bacteria, including pathogens, symbionts, and commensals, suggesting that the T3SS is not a hallmark of pathogenic microorganisms [reviewed in Ref. (27) and (28)]. The global architecture of injectisomes is conserved between bacteria and comprises a needle complex, composed of two pairs of rings that are connected by a rod, spanning the inner and outer bacterial membranes. This structure has at its end a hollow needle, a filament, or a pilus. The main function of T3SSs is to deliver effector proteins across the membranes of host cells, in which these molecules are able to activate cell signaling pathways.

Similarly, T4SSs are specialized macromolecular machines that can deliver DNA and/or proteins to host cells. In contrast to T3SSs, however, those of the type-4 family are believed to be related to bacterial conjugation systems, rather than the flagellar apparatus. T4SSs have been identified in many Gram-negative and -positive bacteria, as the complex can span both types of membrane [reviewed in Ref. (28) and (29)]. T4SSs are classified as Type A or B, depending on structure composition, but both aim to deliver bacterial effectors to host cells. The Type A T4SSs are defined by their homology with the VirB/D4 system of the plant pathogen *Agrobacterium tumefaciens*, whereas the Type B T4SSs are closer to the conjugal transfer systems of the self-transmissible IncI plasmid (29).

The first study to report a mechanism for the recognition of extracellular bacteria by intracellular receptors was described by Viala et al. who showed how virulent *Helicobacter pylori* strains are able to activate the cytosolic NLR family member, nucleotide-binding oligomerization domain-containing protein 1 (NOD1)

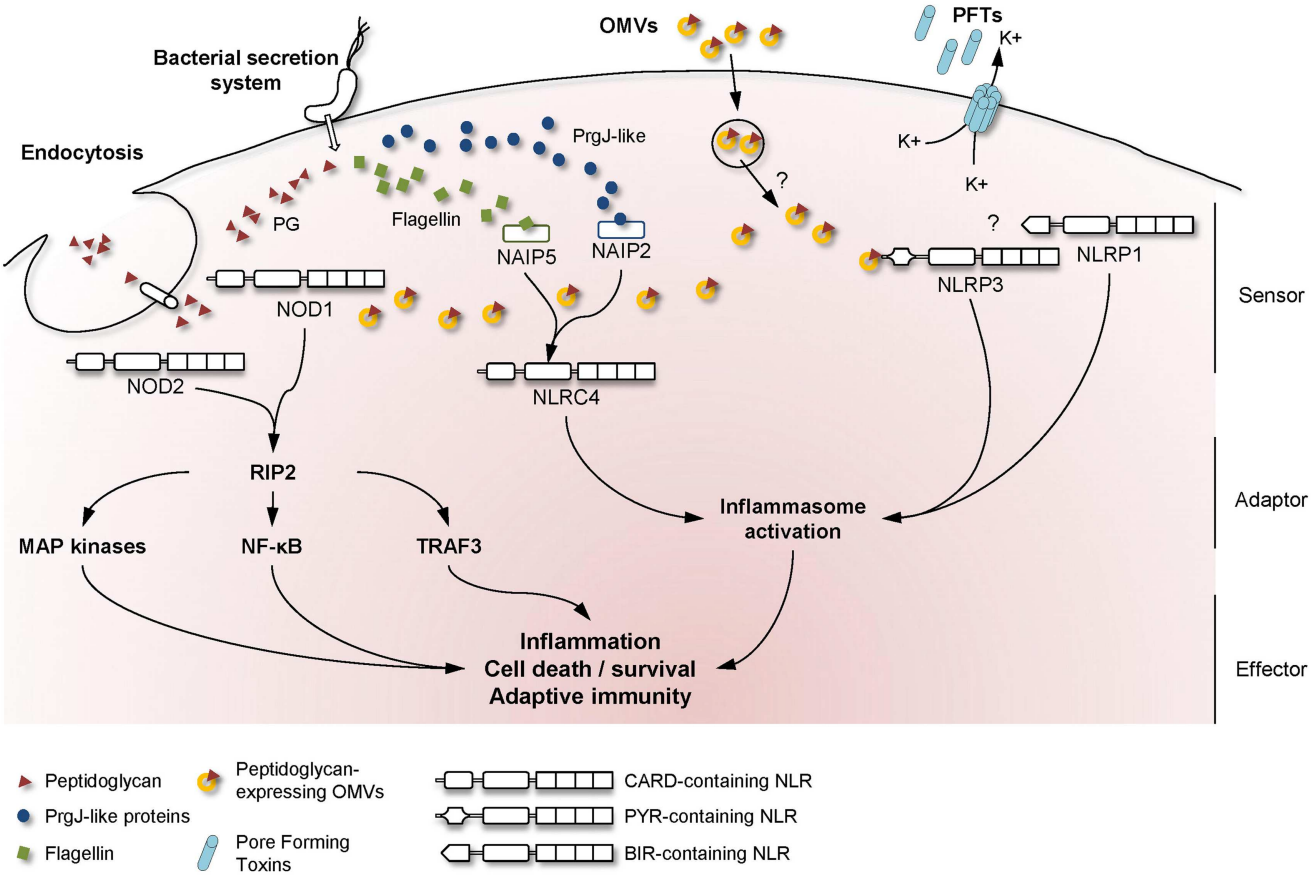

FIGURE 1 | Mechanisms used by bacteria to activate NLR signaling in host cells. A schematic overview of the major NLR signaling pathways activated during bacterial infection, showing the mechanisms whereby extracellular MAMPs are sensed by intracellular NLRs. Upon detection of the appropriate signal, NLRs are believed to oligomerize and recruit adaptor proteins to transduce the signal to downstream effector proteins.

(6). Specifically, the authors showed that *H. pylori* strains with a functional T4SS were able to deliver degradation products of Gram-negative cell wall peptidoglycan, identified as potent activators of NOD1 signaling (30), to epithelial cells (**Table 1**). The mechanism of how *H. pylori* peptidoglycan is delivered to cytosolic NOD1, however, is still unclear, even if it was reported that depletion of cholesterol-rich domains, or lipid rafts, interferes with peptidoglycan delivery into host cells (31). In any case, *H. pylori* T4SS-dependent induction of the NOD1 pathway was shown to result in the downstream activation of NF-κB and MAP kinases, most likely through the recruitment of the serine-threonine kinase adaptor molecule, RIP2 (32, 33). These findings are consistent with the general view that the NOD1 signaling pathway converges on the master transcriptional regulator, NF-κB, leading to pro-inflammatory cytokine production [reviewed in Ref. (34)]. One group, however, suggested that NOD1 signaling is largely independent of NF-κB and MAPK activation (35). Instead, these workers presented data showing that the dominant response mediated by NOD1 activation involves the formation of a complex known as IFN-stimulated gene factor 3 (ISGF3) and production of CXCL10 and type I IFN by epithelial cells (35). The authors demonstrated that stimulation of AGS gastric epithelial cells with either a synthetic NOD1 agonist or live *H. pylori* bacteria alone induced increased CXCL10 production. Nevertheless, the potential link between NOD1 and type I IFN in epithelial cell responses, though interesting, awaits confirmation by other researchers.

Since the work on the role of NOD1 in *H. pylori* sensing, other bacterial pathogens have been identified as also being activators of this pathway, e.g., *Pseudomonas aeruginosa* and *Campylobacter jejuni* (36, 37). These bacteria have essentially extracellular lifestyles, although some data suggest that NOD1 activation in these infection models may be due to the presence of intracellular bacteria. However, even if invasive *P. aeruginosa* and *C. jejuni* have been shown to be present in defined intracellular structures, peptidoglycan delivery, and activation of cytoplasmic sensors, such as NOD1, must still require an efficient secretion system (38, 39). Furthermore, a recent study has shown that *Salmonella typhimurium* activation of NOD1 and NOD2, a related molecule that senses all forms of bacterial peptidoglycan, may be invasion-independent but requires an intact T3SS for the injection into the cytoplasm of the bacterial protein, SipA (10). This work, identifying a novel agonist of NOD1- and NOD2-signaling, suggests that these NLR family members may be activated through a distinct pathway from that induced by bacterial peptidoglycan. The same group suggested

recently that NOD1 could sense other patterns of pathogenesis, such as modification of the actin cytoskeleton. According to this intriguing new model, a T3SS-secreted protein of *S. typhimurium*, SopE, activates the small Rho GTPases, which then triggers the NOD1 signaling pathway (40).

Another major NLR family member that is activated by extracellular pathogens through the action of bacterial secretion systems is the CARD-containing protein, NLRC4 (previously known as Ice Protease-Activating Factor, IPAF) (**Table 1**). The early paradigm for NLRC4 activation was that this NLR senses bacterial flagellin within the cytoplasmic compartment of cells. Specifically, it was shown that T3SS-dependent translocation of *S. typhimurium* and *P. aeruginosa* flagellin into the host cell triggered NLRC4 activation in macrophages (41–44). Interestingly, subsequent reports contradicted this hypothesis as both *Shigella flexneri*, an aflagellated bacterium, and *P. aeruginosa* were also able to activate NLRC4 inflammasome in a flagellin-independent, but T3SS-dependent mechanism, suggesting the existence of one or several other ligands (45, 46). These findings have since been confirmed in various bacterial species and the molecules responsible for NLRC4 activation have now been identified. Thus, it was shown that the basal body rod components of T3SSs (rod protein) are detected during infection with either *S. typhimurium* (PrgJ), *Burkholderia pseudomallei* (BsaK), *Escherichia coli* (EprJ and EscI), *S. flexneri* (MxiI), or *P. aeruginosa* (*Psc*I) (47). Interestingly, all these proteins appear to share a sequence motif present in the carboxy terminal region that is conserved in the flagellar protein, FliC, and detected by NLRC4 (47). The mechanism of how NLRC4 is able to sense and respond to two distinct bacterial products, flagellin or PrgJ-like proteins, has thus been deciphered. Concomitant animal infection studies from two different groups showed that the specificity of the NLRC4 inflammasome is determined by different NAIP paralogs (11, 12). In these new models, both flagellin and rod protein are injected through the T3SS and are specifically recognized by NAIP5/6 or NAIP2 respectively, confirming previous studies showing a physical association between NLRC4 and NAIP5 (**Figure 1**) (11, 12, 48). Interestingly, humans possess only one *Naip* gene compared with the six *Naip* genomic loci in mice, and the specificity of human NAIP appears to be different to the murine NAIPs as it is unresponsive to intracellular delivery of flagellin or PrgJ-like rod proteins (12). Although it has been shown that a PrgJ homolog, from the bacterium *Chromobacterium violaceum*, is specifically recognized by human NAIP (12), further studies are required to determine the importance of human NAIP during infection with common bacterial pathogens.

T4SS and flagellin have also both been suggested to play roles in NLRC4 activation during *Legionella* replication within macrophages, since the presence of all three elements (i.e., flagellin, a functional T4SS and NLRC4) is required to activate caspase-1 in these cells. Consistent with these findings, *in vivo* studies confirmed that clearance of *Legionella pneumophila* required the presence of both flagellin and NLRC4 (49).

Finally, bacterial secretion systems have recently been implicated in the activation of the NLR PYD-containing protein 12 (NLRP12) by the pathogen, *Yersinia pestis* (**Table 1**) (13). The nature of the NLRP12 ligand is still unknown, however, by using a *Y. pestis* strain which lacks the virulence plasmid necessary for the formation of a T3SS, the authors were able to show that this secretion system was required for inflammasome activation and IL-1β release in bone marrow-derived macrophages (BMDMs) (13). The precise mechanism by which the *Y. pestis* T3SS mediates NLRP12 inflammasome activation remains to be elucidated.

OUTER MEMBRANE VESICLES

In addition to bacterial secretion systems, in which individual proteins or macromolecules are secreted, bacteria have developed other mechanisms to transfer a wide variety of components within the host cells. The release of outer membrane vesicles (OMVs) is one such strategy developed by Gram-negative bacteria to secrete toxins, enzymes, DNA, adhesins, or other periplasmic constituents into the extracellular medium [reviewed in Ref. (50)]. It is also noteworthy that the commensal bacterium, *Bacteroides fragilis*, releases a capsular polysaccharide in its OMVs that has immunomodulatory effects and can prevent inflammation in an experimental colitis model (51). OMVs are released by virtually all Gram-negative bacteria, whereas the level of expression differs between bacterial species (50). More recent reports also suggest that Gram-positive bacteria can secrete membrane vesicles, however, these are less well studied than those of Gram-negative organisms (52, 53). OMVs can be released under different conditions *in vitro* and *in vivo* from free-living cells, biofilms, or by internalized bacteria (54–56).

The primary roles of OMVs are believed to be the delivery of toxins or bacterial components into host cells and the evasion of host immune responses (57). On the other hand, several studies have revealed that OMVs are able to induce inflammatory responses that may protect the host from infection. Indeed, OMVs contain different MAMPs, including LPS, flagellin, or DNA that could be recognized by TLR and NLR family members (51, 58, 59). Traditionally, many studies in the literature have focused on OMV-associated LPS, however, it is now becoming apparent that peptidoglycan represents a major promoter of the inflammatory responses induced by OMVs. An initial clue to the potential role of OMV-associated peptidoglycan in innate immunity arose from the observation that *S. flexneri* culture supernatants, when microinjected into epithelial cells, induced the activation of the NF-κB signaling pathway (30, 60). Indeed, it was shown that the OMVs normally present in such supernatants are able to enter non-phagocytic cells and deliver peptidoglycan directly to cytoplasmic NOD1, resulting in the up-regulation of NF-κB and IL-8 responses in cells (**Figure 1**) (7). This OMV-dependent mechanism of NOD1 activation was demonstrated for three extracellular pathogens: *H. pylori*, *P. aeruginosa*, and *Neisseria gonorrhoeae* (**Table 1**) (7). Moreover, it was shown that Nod1-deficient mice intragastrically fed with *H. pylori* OMVs failed to mount local *Cxcl2* and systemic antibody responses, when compared with their wild-type littermates (7).

Subsequent studies reported that *Vibrio cholerae* strains, which also produce large amounts of OMVs, can promote immune responses in the host via a NOD1- and NOD2-dependent mechanism (8, 9). OMVs isolated from *Moraxella catarrhalis* have also been shown to induce IL-8 production through TLR2-initiated NF-κB activation, but a role for NOD1 could not been excluded, as cellular responses to whole bacteria involved both TLR2 and

NOD1 (61, 62). Pro-inflammatory responses to OMVs were also observed for several other bacteria [reviewed in Ref. (50)], however, the role of peptidoglycan in these responses was not assessed.

Besides peptidoglycan, OMVs contain other molecules able to activate the innate immune system. For example, *N. gonorrhoeae* OMVs contain lipooligosaccharide (LOS) which has been shown to activate NLRP3-induced IL-1β secretion and pyronecrosis in monocytes and macrophages (**Figure 1**) (14, 15). This LOS-mediated activation of the NLRP3 inflammasome is believed to be triggered by the release of cathepsin B (15).

Mechanistic data on OMV uptake are scarce, but recent findings suggest that disruption of lipid rafts by treatment with Fumonisin B_1, an inhibitor of sphingomyelin incorporation into lipid rafts, or methyl-β-cyclodextrin, a cholesterol-depleting agent, abrogates both internalization and NOD1-dependent immunostimulatory capacity of OMVs (7). The mechanism and intracellular compartment(s) involved in NOD1 sensing of OMV-associated peptidoglycan, however, have yet to be determined.

The findings concerning the role of peptidoglycan delivery by OMVs provide a new mechanism to understand how extracellular bacteria, which are unable to invade cells or to inject components through a secretion system, may be able to initiate innate immune signaling in non-phagocytic cells, such as epithelial cells.

PORE-FORMING TOXINS

In addition to OMVs, bacteria may secrete toxins to alter host cell integrity distant to the original point of invasion. Among these proteins are the pore-forming toxins (PFTs), which are produced by a wide range of pathogens. PFTs are secreted in a soluble form and are subsequently multimerized into a transmembrane channel that perforates the plasma membrane of host cells. Pore formation may be an entry door for bacterial molecules to penetrate into host cells or lead to cellular ion imbalance (63). Efforts during the last decade have been focused on determining how cells are able to mount a response against pore formation, thereby contrasting with the existing paradigm, which suggested that cells possessed no defenses against these toxins and that the only outcome was cell death (63, 64). Numerous studies, indeed, found that stimulation of immune cells with different PFTs activates pro-inflammatory signaling or vacuolation in response to treatment [(65); and reviewed in Ref. (64)]. It is also noteworthy that the concentrations of PFTs during *in vivo* infection could be sublytic, thus allowing cells to mount an antibacterial response (66), capable of controlling the infection.

Recent intensive studies on PFTs and cellular responses have revealed a major role for NLRP3 in the sensing of pore formation. Mariathasan et al. demonstrated a role for listeriolysin O from *Listeria monocytogenes*, as well as for an unknown *Staphylococcus aureus* toxin, in inflammasome activation and IL-1β production (**Table 1**). Furthermore, this study speculated that the observed effects were dependent on intracellular potassium levels (**Figure 1**) (17). Concerning *S. aureus*, recent studies showed that caspase-1 activation requires the presence of all three of its α-, β-, and γ-hemolysins and the release of bacterial lipoproteins (19, 24). In addition, a small percentage of *S. aureus* isolates also produce another toxin, named Panton–Valentine leukocidin (PVL), which is able to trigger NLRP3 inflammasome activation (25). These results were confirmed using aerolysin from *Aeromonas hydrophila* which was shown to mediate the efflux of intracellular potassium ions and activation of caspase-1 through the assembly of NLRC4 and NLRP3 inflammasomes (16). Subsequent studies suggested that aerolysin from either *A. veronii* or *A. hydrophila* activates only the NLRP3 inflammasome, through potassium efflux, whereas NLRC4 activation was T3SS-dependent but potassium-independent (21, 22). During the last decade, a large number of bacterial PFTs have been shown to be able to activate NLRP3 via the same molecular mechanism of potassium efflux. These PFTs include the adenylate cyclase toxin (CyaA) from *Bordetella pertussis* (20), pneumolysin from *Streptococcus pneumoniae* (23), HlyA hemolysin and MARTX from *Vibrio vulnificus* and *V. cholerae* (23), streptolysin O from *Streptococcus pyogenes* (18). Even if the molecular mechanism is unclear, NLRP3 inflammasome assembly occurs spontaneously at low potassium concentrations and is prevented at higher concentrations, thus confirming its role in the detection of DAMPs (67). In a recent study, potassium efflux was shown to be the minimal membrane permeabilization event triggering NLRP3 inflammasome activation by PFTs and particulate matter (68).

In contrast to our understanding of NLRP3 biology, far less is known about activation of the NLRP1 inflammasome and its activation by PFTs. The human *Nlrp1* gene has three murine paralogs, which encode proteins lacking the N-terminal PYD sequence found in human NLRP1 (69). Sensitivity of mice to the effects of anthrax lethal toxin from *Bacillus anthracis* has been correlated with a polymorphism in the *Nalp1b* gene, encoding Nlrp1 (26). Due to the absence of a PYD domain in the mouse sequence of Nlrp1, it is not clear whether caspase-1 recruitment requires ASC or dimerization with another NLRP. However, recent data suggest that upon stimulation with anthrax lethal toxin, NLRP1 undergoes autoproteolysis to form an inflammasome (70). Interestingly, inflammasome formation and caspase-1 recruitment are inhibited by high levels of potassium (71). Hsu et al. demonstrated a role for NOD2 in lethal toxin-induced IL-1β production and suggested the formation of a complex between NLRP1 and NOD2 (72). This association has since been confirmed by other authors (73). Interestingly, NOD2-recognition of *S. aureus* is facilitated by the presence of its hemolysin, probably by promoting cytoplasmic access of NOD2 ligand (74).

Synergistic responses from the activation of multiple NLR pathways have been observed following co-stimulation with two different pathogens. Indeed, it was shown that *Haemophilus influenzae* peptidoglycan enters epithelial cells more efficiently in the presence of *S. pneumoniae* pneumolysin, suggesting the ability of intracellular NLRs to sense extracellular bacteria that do not encode secretion systems or express OMVs or PFTs (75). One hypothesis from the authors is that host organisms have evolved to detect a combination of pathogens in order to mount optimal responses in ways that are different to the responses induced by a single infection. It is now recognized that co-infection plays a previously unappreciated yet important role in the development of mucosal immunity and disease progression (76).

In conclusion, increasing numbers of studies suggest that osmotic changes induced by pore formation may be sensed by

intracellular NLRs as an early warning system (68). Furthermore, these signals can cooperate with other signaling pathways, thus leading to the generation of antibacterial responses before the concentration of PFTs reaches a lytic concentration.

ENDOCYTOSIS

Asides from the active processes described above, intracellular NLRs may be activated by passive mechanisms. One such mechanism involves cellular entry by the peptidoglycan fragments that are released during bacterial growth or are degraded by host enzymes. Bacteria express peptidoglycan-degrading enzymes, necessary for maintaining functional growth, division, and development [reviewed in Ref. (77, 78) and (79)]. In addition to their role in shaping bacterial membranes, these enzymes are responsible for the release of free peptidoglycan fragments in the extracellular compartment. It is thus possible that these fragments interact with surrounding organisms, mediating pathogenic effects on host cells, as mutations in peptidoglycan-recycling proteins result in decreased pathogenesis. Conversely, released peptidoglycan fragments can also play a role in symbiotic relationships, such as is the case with *Vibrio fischeri*, which induces developmental changes in its squid host [reviewed in Ref. (77)]. Moreover, peptidoglycan can have effects on host cells distant to the point of its release. For example, peptidoglycan released in the gut was shown to circulate and play a major role in the priming of neutrophils in the bone marrow (80). Interestingly, a study by Hasegawa et al. characterizing NOD1- and NOD2-stimulatory activities in different bacterial preparations, showed that the highest levels of NOD1-stimulatory activity were found predominantly in culture supernatants, whereas NOD2 activity was associated with extracts from whole bacterial cells (81). This study further underscores the likely important role of released peptidoglycan during infection with extracellular bacteria.

On the other hand, host cells have also developed some mechanisms to degrade bacterial peptidoglycan in order to kill invading pathogens and provide ligands to host receptors [reviewed in Ref. (82)]. Enzymes such as lysozyme or peptidoglycan recognition protein family members generate fragments small enough to be sensed by NOD1 and NOD2 (82, 83).

In the context of extracellular pathogens, we can then wonder how these peptidoglycan fragments are processed to be presented to intracellular innate immune sensors. It is now established that different internalization mechanisms can be used by the host cell and these are probably cell-type dependent. In the case of epithelial cells, it has been reported that NOD1 and NOD2 ligands are likely to be internalized by clathrin-mediated endocytosis (84, 85). In immune cells, it is more likely that internalization occurs through phagocytosis. Interestingly, it has been observed that Nod1- or Nod2-deficient mice have decreased phagocytic abilities (86, 87).

Once the fragments have been internalized in vesicles by clathrin-mediated endocytosis or phagocytosis, they have to be delivered across membranes and be presented to the cytosolic molecules, NOD1 and NOD2. This mechanism implies the presence of specific transporters, with the transporter family SLC15 having been proposed to play a role. This transporter family comprises membrane proteins controlling the cellular uptake of di/tripeptides and peptide-like drugs [reviewed in Ref. (88)]. Roles for SLC15A4 (PHT1) and SLC15A2 (PepT2) have indeed been identified for the delivery of NOD1-ligands (**Figure 1**) (84, 89, 90). Another member of this family, SLC15A1 (PepT1), is believed to play a role in NOD2 ligand transport (85, 91). Nevertheless, the specificity of each of these transporters is still unclear, as both the minimal motif recognized by NOD1, iE-DAP (γ-D-Glu-mDAP), and the NOD2 agonist, MDP (MurNAc-L-Ala-D-isoGln, also known as muramyl dipeptide) may be delivered through SLC15A2 (89, 92). Interestingly, there are higher expression levels of SLC15A1, SLC15A2, and SLC15A4 in the small intestine, with lower bacterial loads of 10^3 organisms per gram, than in the colon, where microbial densities reach 10^{12} organisms per gram. This may suggest a role for such transporter proteins in MAMP uptake [reviewed in Ref. (93), and (1)].

CONTROL OF ADAPTIVE IMMUNITY

Activation of NLRs has been shown to prime T and B cells, suggesting the contribution of these innate immune molecules in the development of adaptive immune responses. Interestingly, many of the known NLRs activators (e.g., MDP, flagellin, alum) play roles as adjuvants during vaccination, suggesting a role for these molecules in tailoring the adaptive immune response [reviewed in Ref. (94)].

The most striking example is the role played by NOD2 in the mediation of the adjuvant effect of complete Freund's adjuvant, first described in 1937 (95). The adjuvant activity of this compound, which is composed of paraffin oil and *Mycobacterium tuberculosis* extract, is believed to be attributed to its ability to prolong antigen release, to promote the recruitment of immune cells and antigen presentation by inducing expression of cytokines and chemokines [reviewed in Ref. (96)]. The minimal component of complete Freund's adjuvant was subsequently identified to be MDP, as this molecule provided the same level of adjuvant activity as whole killed *M. tuberculosis* (97). The mechanism responsible for the activity of Freund's adjuvant was determined less than 10 years ago by Kobayashi et al. who showed that NOD2 was required for the development of protective immunity mediated by the adjuvant effects of MDP (98). During immunization assays in $Nod2^{-/-}$ animals, a severe deficiency was observed in the production of antigen-specific immunoglobulins, specifically in those of the IgG1 subclass, suggesting that NOD2 is able to activate the adaptive immune system and promote the production of antibodies to T cell-dependent antigens (98). These results were confirmed subsequently, when it was shown that MDP injection in mice triggered Th2 polarized responses in a NOD2-dependent manner (99). Specifically, it was shown that Nod2-deficient mice displayed impaired chemokine and Th2 responses with low numbers of splenic IL-4- and IL-5-producing T cells, as well as the loss of antigen-specific T and B cell responses (99). Interestingly, NOD2 can also cooperate with TLRs to generate Th1-polarized responses to co-stimulation with MDP and TLR2 or TLR4 ligands, suggesting the importance of complementary effects between TLRs and NLRs in the balance of immune effector responses (99). In addition to the recognition of MDP by NOD2, dual recognition of a mycobacterial glycolipid, also known as cord factor, and peptidoglycan is essential for Th17-differentiation in an inflammasome-dependent

manner (100). It was shown that recognition of both the mycobacterial cord factor by the CARD9-dependent C-type lectin receptor mincle and peptidoglycan, via a NOD1- and NOD2-independent mechanism, induces inflammasome activation and IL-1β secretion and thus drives skewed Th17 responses.

NOD1 stimulation has also been shown to be sufficient to drive antigen-specific immunity with a predominant Th2 polarization profile and to play a role in the onset of Th1, Th2, and Th17 immune pathways in conjunction with TLR stimulation (101). Thus, depending on the presence of different MAMPS and the co-stimulation of TLRs, NOD receptors can initiate different arms of the adaptive response. Although data are still scarce concerning the role of NOD1 and NOD2 in the onset of adaptive immunity during microbial infection, *H. pylori*-infected Nod1-deficient mice exhibited reduced Th1 immune responses compared with their WT littermates (101). Similar results were obtained in *M. tuberculosis* or *S. pneumoniae*-infected Nod2-deficient mice, with lower titers of pathogen-specific serum IgG and diminished antigen-specific T cell responses (102, 103). Consistent with these data, *Citrobacter* and *Salmonella* infections triggered Th17 responses that were dependent on NOD1 and NOD2 (104).

Injection of bone marrow reconstituted mice with NOD1 or NOD2 agonists and ovalbumin allowed the group of Philpott and collaborators to determine the importance of stromal factors, versus hematopoietic cells, in the initiation of Th2 immune responses (101, 105). In addition, that group showed that the capacity of NOD1 ligand to cooperate with TLR agonists was completely abolished in Nod1-deficient bone marrow-derived dendritic cells (BMDCs) (101). Similarly, Nod2-deficiency in BMDCs abolished pro-inflammatory cytokine production upon stimulation with MDP alone, whereas synergy between MDP and TLR ligands was lost in Nod2-deficient BMDMs (98). Additionally, full responses required sensing within the hematopoietic compartment, with a major role for dendritic cells, consistent with the well-established role of dendritic cells in the onset of adaptive immunity (105, 106). Furthermore, co-stimulation with NOD1 or NOD2 agonists in combination with TLR agonist induced a synergistic production of Th1-associated cytokines IFN-γ and IL-12 (107).

In contrast to the now well-defined role of NOD proteins in tailoring adaptive immune responses, the role of the NOD1/2 adaptor protein, RIP2, is less well-defined. Several early works in *in vivo* or *in vitro* RIP2-deficient models demonstrated impairment in the development of anti-infectious responses, NF-κB signaling, or T cell proliferation and differentiation (108–110). On the other hand, more recent papers, all of them using the same mouse model but different to those used previously, claimed an absence of effect of RIP2 in T cell proliferation and T helper differentiation (111, 112). A comparison of the different RIP2-knockout mouse lines may help to resolve these differences.

Recent studies have shown that non-hematopoietic cells can also be of importance during the development of adaptive immune responses. Indeed, Watanabe et al. proposed that activation of NOD1 and NOD2 in gastrointestinal epithelial cell lines induces production of cytokines associated with a Th1 response (35). They also proposed that NOD1 signaling, through ISGF3 activation and type I IFN responses, may lead to Th1 differentiation and Th1-dependent inflammation (35).

Concerning the other NLR family members, further studies will be needed to help to understand their roles in adaptive immunity. It has been shown using the Listeria infection mouse model that strains that activated the inflammasome generated significantly less protective immunity, a phenotype that correlated with decreased induction of antigen-specific T cells (113). It is noteworthy that IL-1 family cytokines, including IL-1β and IL-18, have adjuvant properties, as they can induce antigen-specific immune responses against infection (114). For example, CyaA, a pore-forming toxin from *B. pertussis*, activates the NLRP3 inflammasome and induces IL-1β expression, thereby playing a critical role in promoting antigen-specific Th17 cells and in generating protective immunity against *B. pertussis* infection (20). Interestingly, a recent report suggested that IL-1β production in trophoblasts after *Chlamydia trachomatis* infection may also be mediated by NOD1, but the signaling pathway involved remains unclear (115). In addition, IL-1β may be secreted after non-canonical inflammasome activation, where an intracellular lipid A moiety of LPS has been showed to play major roles in the induction of TLR4-independent inflammatory responses (116). Although the receptor has as yet to be characterized, these results suggest a new mechanism of intracellular sensing in the mounting of innate immune responses against microbial infection.

CONCLUDING REMARKS

As discussed above, various NLR family members have evolved to detect infection and mount effective immune responses mediated by both innate and adaptive arms of the immune system. Asides from these NLR family members, it is possible that other family members could play roles during infection with extracellular bacteria. Indeed, NLRP6-deficiency in mice was shown to result in increased inflammation, to alter the colonic microbial ecology and was associated with susceptibility to colorectal tumorigenesis (117, 118). More recently, NLRP6 has been shown to inhibit NF-κB translocation and MAPK activation, with NLRP6 activation leading to increased susceptibility to both intracellular and extracellular bacteria (119). Thus, a second subclass of NLR family members, such as NLRP6 or NLRC5, may act as molecular switches to dampen host responses induced by extracellular bacteria. For example, NLRC5 has been suggested to interact with NF-κB regulators, IKKα and IKKβ, and to block their phosphorylation so as to modulate inflammatory signaling to bacterial pathogens (120). Discordant results were, however, obtained in different studies (121–123), suggesting that further investigations are required to fully elucidate the role(s) of NLRC5 in host responses to microbial pathogens. Besides the NLR family members described in this review, other intracellular molecules, such as certain TLRs (i.e., TLR3, TLR9) or Absent In Melanoma 2 (AIM2), are able to detect nucleic acids from extracellular bacteria, allowing a wide range of MAMPs to be sensed.

The different examples of infection sensing described above highlight the existence of dual systems of recognition for MAMPs from extracellular bacteria. First, conserved molecular patterns in bacteria may be recognized by extracellular receptors. For

instance, sensing of bacterial lipoproteins by TLR2, LPS by TLR4 or flagellin by TLR5 have been relatively well described. Activation of these extracellular receptors leads to a transcriptional inflammatory response with production of type I IFNs or pro-inflammatory cytokines, such as TNF-α or IL-12. However, particular cytokines, including IL-1β or IL-18, require an additional post-transcriptional step to be fully functional. A growing amount of evidence suggests that the signaling involved in this post-transcriptional response is due to activation of inflammasome complexes, after sensing of microbes or danger signals by intracellular molecules, including the NLRs. However, some intracellular sensors of extracellular bacteria, such as NOD1 and NOD2, do not induce inflammasome formation and are generally thought to activate NF-κB signaling instead. Nevertheless, we can reasonably hypothesize that host cells are able to distinguish between the signals originating from extracellular and intracellular pathogens, through the intensity, kinetics, or cell-specific nature of the signal.

This dual recognition of the pathogen itself, or of the consequences of the infection, may be of importance to finely tune inflammatory responses in line with the threat. One hypothesis is that non-pathogenic bacteria may be recognized by extracellular receptors only, whereas pathogenic extracellular or invasive bacteria will be sensed by both families of receptors, leading to more intense responses, suggesting that synergy between TLRs and NLRs may be required for optimal responses. As evoked in this review, it was found that NLR family members may synergize with TLR-dependent cytokine expression (124). An interesting example of this possible dual recognition would be the gut, where there is exposure to more than 500 species of commensal microorganisms. It has been shown that TLR agonists induced tolerance to subsequent stimulation with the same agonist (125). This process could thus play a role in the induction of tolerance to commensal bacteria, whereas pathogenic microorganisms could then be sensed by NLR family members.

It is possible that, depending on the cell type, host cells may distinguish between the signal originating from TLRs and NLRs. Gut immunology provides a good example of how this might work. Indeed, the intestinal epithelium is composed of different layers allowing discrimination between commensal and pathogenic bacteria. The outermost of these layers, comprising the mucus, is a barrier surrounding intestinal epithelial cells. Some areas of the intestinal epithelium, such as the Peyer's patches, are devoid of mucus and serve as inductive sites for the mucosal immune system. In addition, dendritic cells can extend pseudopodes through the mucus and reach the lumen [reviewed in Ref. (126)]. This multi-layer system could allow the host to distinguish between a commensal, which should not progress through the mucosa, and a pathogen, which could disseminate beyond this layer and/or present bacterial components to the epithelium (127). Hence, the ability of the host to distinguish between commensals and pathogens and to mount efficient immune responses could be dependent on how and where the MAMPs are sensed (128).

As discussed in this review, activation of NLR-dependent signaling pathways by extracellular bacteria induces the direct production of pro-inflammatory molecules and also tailors and drives adaptive immunity, suggesting that NLR family members are multifaceted proteins. A comprehensive understanding of the functions of NLRs will help decipher their roles in shaping both innate and adaptive immunity during infection with extracellular pathogens.

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The interplay between NLRs and autophagy in immunity and inflammation

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Since they were first described as cytosolic sensors of microbial molecules a decade ago, the Nod-like receptors (NLRs) have been shown to have many different and important roles in various aspects of immune and inflammatory responses, ranging from antimicrobial mechanisms to control of adaptive responses. In this review, we focus on the interplay between NLRs and autophagy, an evolutionarily conserved mechanism that is crucial for homeostasis and has recently been shown to be involved in the protective response against infections. Furthermore, the association between mutations of NLRs as well as proteins that form the autophagic machinery and inflammatory diseases such as Crohn's disease highlight the importance of these proteins and their interactions in the regulation of inflammation.

Keywords: NLR proteins, autophagy, inflammasomes, inflammation, infection, Crohn's disease, innate immunity

INTRODUCTION

Homeostasis in multicellular organisms is dependent on the ability to detect and adapt to a myriad of environmental variations and insults, including exposure to microbes. Early detection of microbes is a crucial step in the defense strategy. Throughout evolution, the continuous interplay between multicellular organisms and microbes has led to the selection of sensors that allow early detection and initiation of the immune response against infections. This detection is based on the recognition of "microbial-associated molecular patterns" (MAMPs), which represent a signature of microbial origin, such as lipopolysaccharide (LPS), peptidoglycan (PG), flagellin, and nucleic acids from bacteria and viruses, or of "danger-associated molecular patterns" (DAMPs), which indicate the existence of cellular damage, such as extracellular ATP and HGMB1 by "pattern recognition molecules"(PRMs). Upon activation, PRMs trigger several protective responses that include the recruitment of phagocytic cells; secretion of chemokines, cytokines, and antimicrobial peptides; and priming of dendritic cells (DCs), engaging the adaptive immune system. Several families of PRMs have been described and include "Toll-like receptors" (TLRs), "RIG-I like receptors" (RLRs), "C-lectin type like receptors" (CLR), and "Nod-like receptors" (NLRs). In addition to their role in innate and adaptive immune responses, all of them have been recently implicated in the control of autophagy, an adaptive cellular response to environmental and microbial-induced stress. In this review, we highlight the role of NLR signaling in the control of autophagy and vice versa, the mechanisms involved and implications for inflammatory diseases such as Crohn's disease (CD) and type 2 diabetes (T2D).

NLR PROTEINS

Soon after the discovery of the transmembrane TLRs, it became evident that additional sensors were necessary for the surveillance of the cytosol. More than a decade ago, the demonstration that a mammalian homolog of plant disease-resistance (*R*) proteins called Nod1 could detect the presence of intracellular *Shigella flexneri* and activate the transcription factor nuclear factor κB (NF-κB) in epithelial cells *in vitro* inaugurated the studies on the role of NLRs as innate immune intracellular sensors (1). Subsequent studies have now set the number of human NLRs at approximately 20 and indicated their involvement in detecting not only microbial components but also DAMPs such as ATP, mitochondrial DNA (mtDNA) and reactive oxygen species (ROS) (2).

Due to the lack of signal peptides or transmembrane domains in their amino acid sequences, NLRs are thought to be exclusively located inside the cell. Both plant and animal NLRs are signal-transduction ATPases with numerous domains (STAND) P-loop ATPases of the AAA^+ superfamily. The typical NLR protein contains the following domains: (a) a C-terminal leucine-rich repeat (LRR) domain, involved in sensing; (b) a central NATCH [Naip, CIITA, HET-E (plant het product involved in vegetative incompatibility)] and TP-1 (telomerase-associated protein 1 that mediates self-oligomerization and is essential for activation of NLRs); and (c) an N-terminal effector domain, responsible for

protein–protein interactions with adapter molecules and signal transduction. Based on the nature of the N-terminal domains, NLRs have been separated into the NLRC subfamily, containing a CARD domain (caspase activation and recruitment domain); the NLRP subfamily, containing a pyrin domain; and the NAIP subfamily, which includes three (BIRs) baculovirus inhibitors of the apoptosis protein repeat domain (3).

Nod1 (NLRC1) AND Nod2 (NLRC2) ARE INTRACELLULAR PEPTIDOGLYCAN SENSORS

Nod1 and Nod2 were the first NLRs identified as MAMP detectors when two concomitant studies demonstrated that Nod2 detects muramyl-dipeptide (MDP), a common motif found in Gram-negative and Gram-positive PG and a major component of adjuvants (1, 4–6). Nod1, in contrast, recognizes PG containing the minimal motif *meso*-diaminopimelic acid (DAP), an amino acid found in Gram-negative and some Gram-positive bacteria, such as *Listeria monocytogenes* and *Bacillus subtilis*. The naturally occurring PG moieties sensed by human and mouse Nod1 are GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-DAP (GM-triDAP) and GlcNAc-MurNAc-L-Ala-D-Glu-*meso*-DAP-D-Ala (GM-tetraDAP), respectively. After additional studies demonstrated that PG recognition by TLR2 was due to contaminants commonly found in PG preparations, it became clear that Nod1 and Nod2 are the only known PG sensors (2, 7–9). The NLR ligands/activators are summarized in **Table 1**.

Nod1 and Nod2 have been implicated in the detection of a vast array of microbial pathogens including bacteria, parasites, and viruses. A key role for Nod1 and Nod2 in the detection of bacterial infection has been demonstrated in *Helicobacter pylori*, *Escherichia coli*, *Chlamydia* spp., *Campylobacter jejuni*, *Salmonella* spp., *Pseudomonas aeruginosa*, *S. flexneri*, and *L. monocytogenes*, *Mycobacterium tuberculosis*, and *Streptococcus pneumoniae* (**Table 1**).

More recent studies have uncovered surprising data regarding microbial recognition by Nod1 and Nod2. Nod1-deficient mice are more susceptible to infection with *Trypanosoma cruzi*, the etiological agent of Chagas disease, apparently due to the lack of a robust nitric oxide production, suggesting that Nod1 may be involved in PG-independent microbial sensing given that *T. cruzi* does not express PG (3, 28).

Supporting a role for Nod2 in the control of infections beyond bacterial/PG detection, Shaw et al. using a *Toxoplasma gondii* infection model, described a T cell intrinsic role in Nod2-deficient mice and a consequent Th1-defective immune response. In their experiments, the authors observed lower amounts of IL-2 not only during infection with *T. gondii* but also following anti-CD28 ligation. Despite the novelty of these results, T cell activation in different models appears to be normal in Nod2-deficient mice (75, 76).

Nod2 has also been implicated in the immune response to viruses. In a recent study, Sabbah et al. demonstrated that Nod2 mediated the *in vitro* production of type I IFN in cells stimulated with single stranded RNA (ssRNA) or infected with various RNA viruses. These results support the observation, made in the same study, that Nod2-deficient mice are more susceptible to respiratory syncytial virus (RSV) (29).

Finally, both Nod1 and Nod2 have been implicated in inflammatory disorders because mutations in the genes that encode these proteins were shown to be related to the establishment of genetic inflammatory diseases. The first piece of evidence of a link between mutations in *NOD2* and CD [an inflammatory bowel disease (IBD)] was provided by Hugot et al. which identified three single nucleotide polymorphisms (SNPs) in the *IBD1* locus associated with increased risk for CD (77). One of these SNPs, *Leu1007fs*, is the most common Nod2 mutation associated with the disease and encodes a protein that is no longer able to sense MDP or localize to the plasma membrane as the normal protein does upon activation (4, 78).

INFLAMMASOMES

By definition, inflammasomes are multimeric protein complexes that comprise a "sensor NLR" and function as platforms for the activation of pro-caspase-1, resulting in the processing of IL-1β and IL-18 and their unconventional secretion (6, 79). Several inflammasomes have been described so far, and among them, the best studied are the ones that contain NLRP3 (formerly known as NALP3) or NLRC4 (formerly known as IPAF). Many NLRs, such as NLRP1, NLRP3, and NLRC4, use the adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) to recruit pro-caspase-1, but this does not apply to all inflammasomes.

NLRP3 INFLAMMASOMES

NLRP3 is mostly expressed in myeloid cells and is activated by a vast array of host-derived and exogenous agonists. One common feature of NLRP3 agonists seems to be a crystalline or polymeric structure associated with danger signals or cell death. For example, monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD) (41), cholesterol crystals (42), amyloid β (80), fatty acids (47), and mtDNA (48) have all been reported to activate NLRP3. Microbial NLRP3 agonists have also been identified. NLRP3 senses bacteria, viruses, fungi, and parasites themselves or virulence factors such as pore-forming toxins. The list of pathogens detected by NLRP3 includes *Staphylococcus aureus*, *L. monocytogenes*, *Klebsiella pneumoniae*, *Neisseria gonorrhoeae*, *E. coli*, *Porphyromonas gingivalis*, *S. flexneri*, *Chlamydia* spp., the influenza A virus, *Aspergillus*, and more recently, *Leishmania* (**Table 1**).

NLRC4 AND Naip5

IPAF (also known as NLRC4) is present in the cytosol of myeloid cells, where it controls the activation of caspase-1 and IL-1β processing in response to the presence of intracellular flagellin. NLRC4 directly binds to cytosolic flagellin, an event that promotes its oligomerization through the nucleotide-binding domain (NBD) and winged-helix domain (WHD) in the presence of adenosine diphosphate (ADP) (81). The importance of IPAF-dependent activation of caspase-1 has been highlighted in infection models *in vitro* using *Salmonella typhimurium*, *S. flexneri*, *Legionella pneumophila*, and *P. aeruginosa* (66, 70, 74). In such experiments, IPAF-deficient macrophages were impaired in their ability to activate caspase-1 and secrete IL-1β and IL-18. Macrophages from IPAF-deficient mice infected with *S. typhimurium* have also been shown to be more resistant to cell death. Indeed, activation of NLRC4 leads to rapid cell death, a feature that differentiates IPAF

Table 1 | NLR proteins involved in autophagy and their activators.

NLR protein	Activator	Reference
Nod1 (NLRC1)	FK156 (d-lactyl-l-Ala-γ-Glu-*meso*-DAP)	Uehara et al. (10) and Magalhaes et al. (11)
	FK565 (Heptanoly)	Uehara et al. (10)
	Meso-lanthionine, *meso*-DAP	Uehara et al. (12)
	iEDAP (γ-d-Glu-*meso*-DAP)	Girardin et al. (159) and Chamaillard et al. (9)
	TriDAP (l-Ala-γ-d-Glu-meso-DAP)	Girardin et al. (4)
	TCT (GlcNAc-(anhydro) MurNAc-l-Ala-γ-d-Glu-mesoDAP-d-Ala)	Magalhaes et al. (11)
	Bacillus species	Hasegawa et al. (13)
	L. pneumophila	Hasegawa et al. (13)
	S. typhimurium	Hasegawa et al. (13) and Le Bourhis et al. (14)
	H. pylori	Viala et al. (15)
	Pseudomonas species	Travassos et al. (7)
	Chlamydia species	Kavathas et al. (16), Buchholz and Stephens (17), Welter-Stahl et al. (18) and Opitz (19)
	L. monocytogenes	Park et al. (20), Kim et al. (21) and Opitz et al. (22)
	E. coli	Kim et al. (23)
	S. flexneri	Girardin et al. (24) and Carneiro et al. (25)
	C. jejuni	Al-Sayeqh et al. (26) and Zilbauer et al. (27)
	T. cruzi	Silva et al. (28)
Nod2 (NLRC2)	Muramyldipeptide (MurNAc-l-Ala-d-isoGln)	Girardin et al. (8) and Inohara et al. (5)
	M-TriLys (Mur-NAc-l-Ala-d-Glu-Lys)	Girardin et al. (4)
	Respiratory syncytial virus (RSV)	Sabbah et al. (29)
	Bacillus species	Hasegawa et al. (13)
	Lactobacillus species	Hasegawa et al. (13)
	Corynebacterium xerosis	Hasegawa et al. (13)
	E. coli	Hasegawa et al. (13)
	Pseudomonas species	Hasegawa et al. (13)
	M. tuberculosis	Juárez et al. (30), Ferwerda et al. (31) and Divangahi et al. (32)
	S. pneumoniae	Travassos et al. (33) and Liu et al. (34)
	C. jejuni	Al-Sayeqh et al. (26)
	S. flexneri	Kufer et al. (35)
	S. typhimurium	Keestra et al. (36) and Hisamatsu et al. (37)
	L. monocytogenes	Kobayashi et al. (38)
Nalp3 (NLRP3)	Muramyldipeptide (MurNAc-l-Ala-d-isoGln)	Martinon et al. (39)
	Bacterial RNA	Kanneganti et al. (40)
	Imidazoquinoline compounds	Kanneganti et al. (40)
	MSU (monosodium urate)	Martinon et al. (41)
	CPPD (calcium pyrophosphate dihydrate)	Martinon et al. (41)
	Cholesterol crystals	Duewell et al. (42)
	Silica	Hornung et al. (43), Kuroda et al. (44) and Cassel et al. (45)
	Aluminum salts	Hornung et al. (43) and Kuroda et al. (44)
	Amyloid-beta	Halle et al. (46)
	Fatty acids	Wen et al. (47)
	Mitochondrial DNA	Nakahira et al. (48) and Shimada et al. (49)
	Aerolysin	Gurcel et al. (50)
	Maitotoxin	Mariathasan et al. (51)
	ATP	Mariathasan et al. (51)
	Nigericin	Mariathasan et al. (51)
	S. aureus	Craven et al. (52)
	L. monocytogenes	Kim et al. (53)
	P. gingivalis	Huang et al. (54)

(Continued)

Table 1 | Continued

NLR protein	Activator	Reference
	Chlamydia species	Abdul-Sater et al. (55) and He et al. (56)
	Influenza A virus	Thomas et al. (57) and Allen et al. (58)
	Aspergillus	Saïd-Sadier et al. (59)
	Leishmania	Lima-Junior et al. (60)
	ROS	Zhou et al. (61)
IPAF (NLRC4)	Cytosolic flagellin	Franchi et al. (62)
	L. pneumophila	Case et al. (63), Vinzing et al. (64) and Coers et al. (65)
	S. typhimurium	Mariathasan et al. (66), Broz et al. (67) and Miao et al. (68)
	S. flexneri	Suzuki et al. (69)
	P. aeruginosa	Cohen and Prince (70), Sutterwala et al. (71) and Franchi et al. (72)
Naip5	Cytosolic flagellin (in cooperation with IPAF)	Zamboni et al. (73)
	L. pneumophila	Lightfield et al. (74) and Zamboni et al. (73)

from the other NLRs (66). Another pathogen whose detection induces cell death through IPAF is *L. pneumophila*. However, in this case, another NLR protein, Naip5 (also known as Birc1e), is required. Both Naip5 and IPAF have been reported to physically interact, but the role of Naip5 in caspase-1 activation remains to be fully elucidated, as A/J mice (mice with a mutation that results in a non-functional Naip5) are able to secrete IL-1β following infection with *S. typhimurium*, *P. aeruginosa*, and *L. monocytogenes* (66, 71, 73) (**Table 1**).

Shigella flexneri also triggers IPAF-dependent activation of caspase-1 and secretion of IL-1β. These data are intriguing considering that *S. flexneri* is a non-flagellated bacterium, suggesting that other factors are able to activate IPAF (69). Indeed, more recent studies revealed that *P. aeruginosa* strains lacking flagellin are still able to induce secretion of IL-1β through NLRC4 (71) (**Table 1**).

Although the importance of IPAF in cytosolic flagella sensing is broadly recognized, it has been demonstrated that flagellin-dependent responses may occur in the absence of IPAF. Recently, a new pathway was reported in which macrophage stimulation with flagellin leads to cell death in a cathepsin B and D-dependent manner even in IPAF-deficient cells. It has yet to be determined whether a new flagellin sensor is involved in such events (82).

NLRX1

In contrast to the huge amount of data regarding other NLR proteins, little is known about the biological function of NLRX1. This protein is highly conserved among species and has sequence homology with Nod3. Unlike other NLRCs, NLRX1 has no CARD in its N-terminal portion but does have a putative mitochondrial-targeting sequence (83). Indeed, what we know about NLRX1 is derived from its mitochondrial localization, even though its precise localization inside this organelle is still a matter of debate. Studies from two independent groups report conflicting results; while Arnoult et al. claimed that NLRX1 is located in the mitochondrial matrix, Moore et al. proposed that the protein localizes to the outer mitochondrial membrane (84, 85). There are also discrepancies concerning the attributed function of NLRX1. Initial results from Tattoli et al. reported that NLRX1 amplifies NF-κB and JNK through the production of ROS. Opposing results from Moore et al. suggest that NLRX1 functions as a brake on innate immune pathways by inhibiting mitochondrial antiviral signaling (MAVS)-dependent NF-κB and IFN-β production upon poly I:C stimulation *in vitro* (85, 86). Further studies are required to clarify of the function of NLRX1.

NLRP4

Very little is known about NLRP4, a 113-kDa protein also known as Nalp4 or PYPAF4. This protein is expressed in tissues as diverse as testis, oocytes, spleen, placenta, thymus, kidney, and lung. NLRP4 has been recently reported as a negative regulator of type I IFN signaling by targeting tank binding kinase-1 (TBK-1) for degradation as well as of TNF-α and IL-1β by inhibiting NF-κB activation by interacting with IKKα (87, 88).

AUTOPHAGY

The term autophagy (meaning "self-eating") was first introduced at the CIBA Foundation Symposium on Lysosomes in 1963 by cell biologist Christian de Duve, who also discovered lysosomes in 1955 (Nobel Prize in Physiology in 1974) and coined several other terms currently used today, such as "endocytosis" and "exocytosis." Autophagy was first characterized by the presence of single- or double-membrane vesicles harboring cytoplasmic content in different stages of degradation – the autophagosomes. At that time, de Duve and others considered autophagy to be a non-selective degradation pathway. However, under specific circumstances, autophagy is highly specific and plays essential roles in maintaining homeostasis. For a complete historical perspective on autophagy and its importance in different pathologies, please refer Ref. (89–91).

Autophagy is a highly conserved cellular homeostatic process in which long-lived proteins, damaged organelles, or parts of the cytosol are delivered to lysosomes for degradation and recycling of functional blocks for anabolic reactions, especially during nutrient shortages. Indeed, for years, autophagy was considered a mere response to nutritional stress given that initial observations demonstrated that glucagon or amino acid deprivation triggered the formation of autophagosomes while exogenous amino acids supplementation inhibited autophagy and protein breakdown (92).

THE MACHINERY OF AUTOPHAGOSOME BIOGENESIS

So far, three types of autophagy have been described, chaperone-mediated autophagy (CMA), microautophagy, and macroautophagy (hereafter, autophagy) (93). The hallmark of autophagy is the generation of autophagosomes. This process occurs in a stepwise manner controlled by over 30 *Atg* genes that were initially identified in yeast species. Interestingly, most of these genes have mammalian orthologs or paralogs with high structural and functional similarities. Briefly, the process starts with the formation of a cup-shaped membrane or phagophore. Once formed, the membrane elongates and selectively and/or non-selectively enwraps the cargo (i.e., the cytosolic target), eventually sealing, completing the formation of the autophagosome. The outer membrane of the autophagosome the fuses with a lysosome membrane, forming the autolysosome, where all the degradation steps of the autophagic response take place (**Figure 1**). The source of the autophagosomal membrane is still a matter of debate. Various studies have proposed the plasma membrane, the endoplasmic reticulum or the outer mitochondrial membrane as the source (92).

THE CORE AUTOPHAGY PATHWAY

At the molecular level, the number of proteins implicated in the control of autophagy is still expanding and linking autophagy with several other pathways. Here, we focus on the core of the autophagic pathway and its links to NLR signaling. For autophagosomes formation, the following two ubiquitin-like (UBL) systems are required: (i) in the Atg12 conjugation system, Atg5 and Atg12 form complexes through the covalent binding of Atg12 to the C-terminal glycine of ATG5 in UBL reactions involving Atg7 and Atg10. Atg16L1, a scaffold protein, is then conjugated to the Atg5-Atg12 complex by binding to the N-terminus of Atg5. The Atg5-Atg12-Atg16L1 complex multimerizes, forming large 800 kDa complexes that are found in the cytosol and in the forming membrane. It has been shown that the Atg16L1 complex acts as a E3-like enzyme, targeting microtubule-associated protein 1 light chain 3 (LC3) to its membrane site of lipid conjugation. (ii) The Atg8 conjugation system is crucial for inducing modifications in LC3. Under normal conditions, LC3 has a diffuse cytosolic distribution pattern. LC3 is cleaved at its C-terminus by Atg4, a cysteine protease, and undergoes UBL modifications by the E1-like enzyme, Atg7, and the E2-like enzyme, Atg3, to form LC3-I (94). During the induction of autophagy, the C-terminal carboxyl group of LC3-I is eventually conjugated with phosphatidylethanolamine to form LC3-II, which is found exclusively on the autophagosomal membrane. For this reason, LC3-II is widely used as an autophagy marker (95).

The Atg proteins, the autophagic machinery, seem to require other proteins to form autophagosomes. Similar to the Atg1-Atg13-Atg17 complex found in yeast, the Unc-51-like kinase (ULK1), focal adhesion kinase family integrating protein (FIP2000), and Atg13 proteins were described to colocalize at the nascent isolation membrane after induction of autophagy in mammalian cells (96, 97). Another protein that was shown to participate in autophagy is Atg9. Studies in mammals have demonstrated that Atg9 is essential for autophagosome formation as it associates with the trans-Golgi, endosomes, LC3, and Rab GTPases (Rab7 and Rab9) and redistributes following the induction of autophagy (92). In **Figure 1**, we summarize the main proteins and all the steps that are part of the formation of autophagosomes.

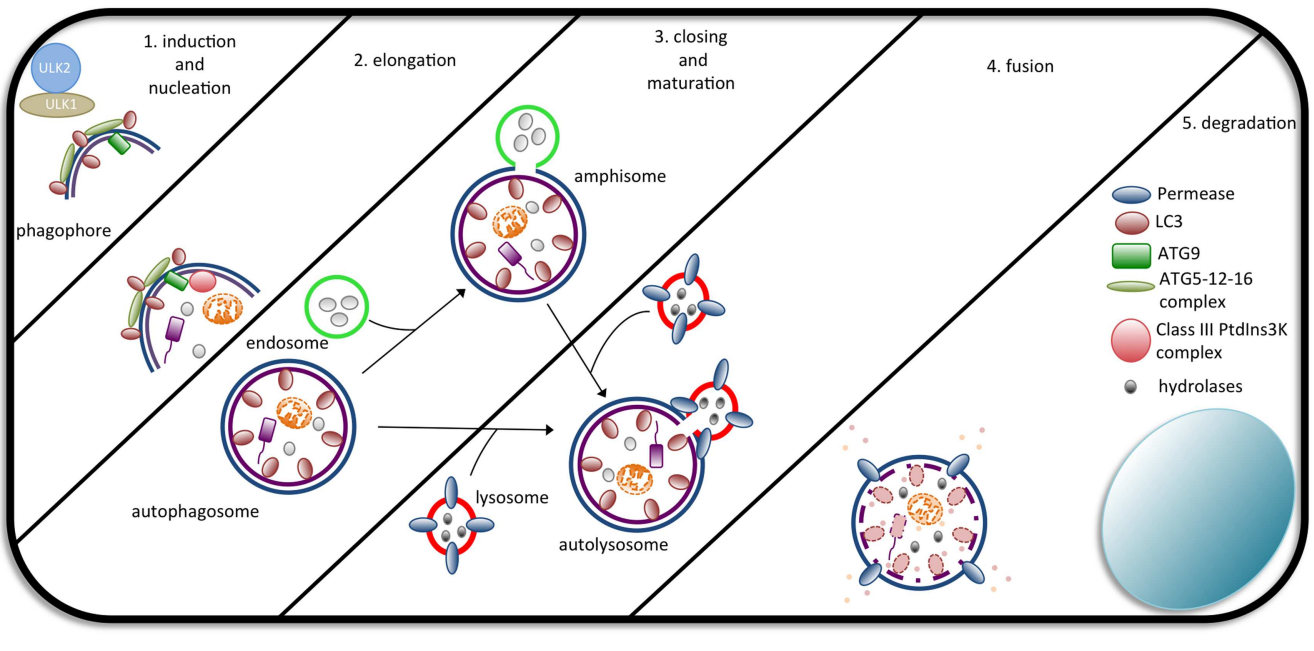

FIGURE 1 | The steps of autophagosome formation. Macroautophagy begins with the formation of a cup-shaped structure called a phagophore as a consequence of the activity of the ULK1/2 complex. In the sequence, the ATG5-ATG12-ATG16L1 complex, Class III PtdIns3K, ATG9, and LC3 assist in the formation of the autophagosome. Autophagosomes completely wrap their cargo and fuse with endosomes (forming an amphisome, which will later fuse with lysosomes) or directly with lysosomes, forming an autolysosome. Upon fusion, the cargo is degraded by lysosomal hydrolases and exported back to the cytosol to be used by the cell.

Interestingly, it has been proposed that autophagy may occur even in the absence of ATG proteins in both insects and mammals. However, the conditions that trigger ATG-independent autophagy and whether this type of autophagy is particular to some cell types remain to be determined (98, 99).

CONTROL OF AUTOPHAGY

The vast number of pathways that connect to autophagy gives rise to an intricate network that makes our understanding of autophagy regulation far from complete. A major breakthrough in understanding autophagy regulation was made when the target of rapamycin (TOR) in yeast and mammals was discovered, implicating the involvement of phosphatidylinositol kinase-related kinases in the process (100–102). Both pathways are linked by the key serine/threonine kinase Akt and are known to participate in several cellular responses such as proliferation and metabolic adaptation (92). Activators of phosphatidylinositol-3 kinase (PI3K) range from cytokines to TLR ligands. After receptor activation, phosphatidylinositol-4,5-biphosphate is phosphorylated by class-I PI3K, activating Akt. mTOR complexes are effectors downstream of Akt and integrate a myriad of cellular signals, especially those related to protein synthesis and translation (103–106). Under optimal nutrient conditions, autophagy is negatively regulated by Akt and mTOR (107). This negative regulation of autophagy by mTOR has been recently shown to require inhibition of ULK kinase-complex activity through its phosphorylation (108). At least in yeast, ULK1 seems to regulate autophagy not only by inhibiting mTOR but also by interacting with Atg8 (109). It remains to be tested whether these findings also apply in mammals. Another mechanism involved in the control of mTOR activation is the recruitment of TNF receptor-associated factor 6 (TRAF6) to the lysosome by p62, where it drives the polyubiquitination of mTOR during optimal nutrient conditions (110).

Upon the induction of autophagy, vacuolar protein sorting 34 (Vps34), a class III phosphatidylinositol-3-phosphate (PtdIns3P) kinase (PI3K) enzyme, specifically phosphorylates phosphatidylinositol and is implicated in trafficking, nutrient sensing, and autophagy (111, 112). In yeast, the role of PtdIns3P seems to go beyond autophagosomal membrane elongation. It has been proposed that the levels of PtdIns3P in the phagophore assembly site (PAS) regulate autophagosome turnover due to the accumulation of ATG proteins in the membrane (113). Other important players in autophagosome formation are Vps15, beclin-1, ultraviolet radiation resistance associated gene (UVRAG), and ambra1, which together form a multiprotein complex with Vps34 that is necessary for the initial steps in autophagosome formation. For a complete view of the autophagy pathway, see the review by Boya et al. (114).

AUTOPHAGY AND IMMUNITY TO INFECTIONS

The contamination of the cytosolic compartment with invasive pathogens is a major step in the activation of innate immune defenses. In this regard, autophagy has emerged in recent years as another component of the innate immune system's arsenal. Several microbial agents are recognized by the autophagic machinery, and their fates can vary from destruction to the creation of a replicative niche. It has been shown that the autophagic machinery can selectively segregate microbes in the cytosol. The mechanisms by which such specificity is achieved are not completely understood but seem to involve both microbial and host factors. Similar to MAMPs, which themselves were shown to induce autophagosome formation, toxins secreted by pathogens also induce autophagy (115). In a landmark paper, Nakagawa et al. demonstrated that group A *Streptococcus* (GAS) lacking streptolysin O (SLO) do not escape from phagocytic vacuoles, and thus, do not activate autophagic sequestration (116).

Gram-negative bacteria are recognized by the autophagic machinery as well. For example, *S. flexneri* uses a type 3 secretion system (T3SS) to deliver effector proteins directly into the host cell. Ogawa et al. showed that, in epithelial cells, the wild-type (WT) *S. flexneri* strain is capable of evading autophagic sequestration. This is dependent on the T3SS effector IcsB, as the mutant strain lacking IcsB is trapped by autophagy. The role of IcsB seems to be to camouflage the bacterial target molecule (VirG) from the autophagy machinery (117, 118). Interestingly, these observations seem to vary depending on cell type, given that in bone marrow-derived macrophages, no difference in bacteria sequestration was observed between the WT and IcsB mutant strains (69). The autophagic machinery apparently relies on redundant strategies to fight *Shigella*; different mechanisms have been described for the induction autophagy by this pathogen. In one mechanism, the phagocytic vacuolar membrane remnants, rather than bacterium itself, trigger autophagy in response to bacterial invasion (119).

Avoidance of autophagic destruction has also been reported for other bacteria. The *Burkholderia pseudomallei* T3SS effector, BopA, which shares some homology with IcsB, contributes to bacterial evasion from autophagosome targeting (120). The Gram-positive bacterium *L. monocytogenes* is able to invade host cells, where it finds a replicative niche within the cytosol following autophagosome escape. These events are dependent on the expression of listeriolysin O (LLO), ActA, and phospholipase C (121).

However, autophagy can serve as a back-up control mechanism for bacteria that are able to escape from other defense mechanisms. For example, after invading the host cell, *S. typhimurium* resides within vacuoles called *Salmonella*-containing vacuoles (SCV). Following SCV damage mediated by its T3SS, *S. typhimurium* gains access to the cytosol where autophagy is immediately activated to confine the bacteria and restrict the infection (122). In the case of *M. tuberculosis*, which is known to subvert host cell phagosomal maturation and survive within macrophages, autophagy induction via rapamycin or IFN-γ circumvents the phagosomal maturation blockade, leading to *M. tuberculosis* elimination (123).

Viral pathogens interact with the autophagic pathway as well. Herpes simplex virus (HSV) ICP34.5 interacts with beclin-1 and blocks autophagosome formation (124). In vesicular stomatitis virus (VSV)-infected DCs, autophagy is essential for the delivery of viral ligands to endosomes to induce type I IFN production (125).

NLR-MEDIATED AUTOPHAGY AND INFECTION

As mentioned before, the first line of host defense against infection relies on various families of PRMs. As it became evident that autophagy is also an innate immune effector mechanism, considerable efforts were made to understand the role of PMRs in the autophagic response to pathogens.

The first study linking MAMP sensing and autophagy induction was the work by Xu et al. who showed a role for TLRs in

autophagy. TLRs are transmembrane proteins that recruit myeloid differentiation primary response protein 88 (MyD88) and Toll-IL-1 receptor (TIR) domain-containing adapter-inducing IFN (Trif) adapter proteins through their TIR domain to initiate downstream signaling. It was demonstrated that LPS induces autophagy in a TLR4-p38-RIP1-Trif-dependent manner (126). Later, a report from Delgado et al. showed that TLR7 could elicit similar responses upon stimulation of macrophages with ssRNA, and this was also dependent on the recruitment of MyD88 (127). The balance between Beclin-1 and Bcl-2 is a major checkpoint in the pathway for autophagy induction. Shi et al. (128) proposed that MyD88 and Trif both target Beclin-1, resulting in decreased binding to Bcl-2 and subsequent autophagy activation upon TLR stimulation. MyD88 and interferon-regulatory factors (IRFs) 5 and 7 are also recruited by mTOR to control cytokine production (128, 129).

Despite increasing evidence showing a role for TLRs in the induction of autophagy, it remains unclear how the autophagic machinery is directed to trap an entire microorganism during infection, especially considering that they are transmembrane proteins. Several bacteria, such as *Salmonella*, *Mycobacterium*, and *Listeria*, grow within host cells and by doing so can avoid antibody and cellular dependent defenses. Intracellular PRMs, such as the NLR family, are known for their essential role as cytosolic sentinels that can trigger robust cytokine production and inflammation. However, little was known regarding how these sensors contribute to the elimination of intracellular invaders. The first evidence implicating NLRs in autophagy-dependent control of an intracellular infection came from studies using *Drosophila* as a model. Yano et al. (130) reported that, upon infection of hemocytes with *L. monocytogenes*, *Drosophila* PGRP-LE detects diaminopimelic (DAP)-containing PG to trigger autophagy directed against the bacterium. Consistent with these observations, PGRP-LE null mutants were more susceptible to infection (130). The recognition of intracellular PG and subsequent induction of autophagy seem to be conserved features of the innate immune system. In 2010, two independent studies reported Nod1- and Nod2-dependent autophagy upon PG detection. We showed that Nod1 and Nod2 direct autophagy by recruiting ATG16L1 to the plasma membrane during bacterial entry into the host cell (**Figure 1**). Interestingly, the most common mutation in Nod2 associated with CD, Nod21007fs, results in a protein that fails to recruit ATG16L1 to initiate the formation of autophagosomes, although they still interact in the cytosol. In another study, Cooney et al. (131) found that Nod2 induces autophagy in human DCs, increasing bacterial killing, and antigen presentation. DCs expressing CD-associated variants displayed lower autophagy and antigen presentation levels upon MDP stimulation. Interestingly, while we demonstrated that the adaptor protein Rip2 and NF-κB activation is dispensable for autophagy induction because Rip2-deficient fibroblasts displayed similar numbers of *S. flexneri* targeted to autophagosomes, Cooney et al. found that Rip2-deficient DCs had reduced levels of autophagy. The difference in the cell types used could account for such differences (131, 132).

These studies gained additional relevance as a recent link between polymorphisms in ATG16L1 and CD was uncovered. CD and ulcerative colitis (UC) are common presentations of idiopathic IBD. It is estimated that their prevalence in Caucasian individuals reaches 100–150 per 100,000 (133). IBD is the outcome of combined genetic and non-genetic risk factors, and recently genome wide association studies (GWAS) have identified a non-synonymous single nucleotide polymorphism in ATG16L1 (T300A) as one of the most important genetic risk factors for CD. Studies in which the implications of this polymorphism were analyzed show that MDP-induced but not canonical autophagy is impaired in cells of individuals carrying the T300A variant (131, 132). The effect of this polymorphism on the restriction of bacterial growth varies depending on the cellular and bacterial models used. Epithelial cells expressing the T300A variant show decreased bacteria-targeted autophagy during infection with *S. typhimurium* (134). Monocytes from patients with CD and carrying the T300A allele infected with *M. avium paratuberculosis* display no difference in bacterial growth in comparison to patients with the normal allele (135). The impact of this variant on cytokine release has also been evaluated, and again, contradictory findings were observed. Plantinga et al. (136) reported that upon Nod2 stimulation with MDP (but not with TLR ligands), PBMCs from healthy volunteers carrying the T300A variant secreted increased amounts of IL-1β (136). In contrast, in another study, the same group demonstrated that PBMCs with the variant allele do not produce more IL-1β in comparison to the normal allele upon infection with *M. tuberculosis* (137).

Legionella pneumophila, a Gram-negative pathogen of amebae, is also able to replicate within alveolar macrophages and cause the pneumonia known as Legionnaire's disease. Mouse macrophages, in contrast to human cells, restrict *L. pneumophila* replication through the activation of Naip5 and NLRC4 by cytosolic flagellin and activation of caspase-1 resulting in pyroptosis. In a recent study, Byrne et al. demonstrated that flagellin recognition by Naip5 and NLRC4 increases autophagosome turnover (138).

NLRX1 has been shown to enhance autophagy. A recent study demonstrated that NLRX1 enhances autophagy through interaction with the Tu translation elongation factor (TUFM) which, in turn, interacts with the Atg5-Atg12 complex. It is not clear, however, how this NLRX1-TUFM-Atg5-Atg12 interaction leads to increased autophagy. Still, NLRX1 plays an important role as a pro-autophagic factor during vesicular stomatitis (VSV) infection. Lei et al. (139) showed decreased viral replication in NLRX1-deficient fibroblasts, suggesting that autophagy is important for VSV replication, although a previous work demonstrated that VSV succumbs to autophagy in a *Drosophila* model (139, 140). Of note is the fact that the role of NLRX1 in autophagy varies among studies using the different NLRX1 knockout mice available. While Soares et al. and Rebsamen et al. found that the MAVS pathway is fully functional in their NLRX1-deficient mice, Allen et al. reported an enhancement in this signaling pathway using a different NLRX1 knockout mice [the same used by Lei et al. (139, 141–143)]. It remains to be determined whether the role of NLRX1 in autophagy is specific to the knockout animals used by Lei et al. or if it is a general feature of all NLRX1-deficient mice (**Figure 2B**).

Finally, in contrast to what was described above, NLRs can also act as negative regulators of autophagy through mechanisms that are not yet completely elucidated. Suzuki et al. reported that NLRC4- and caspase-1-deficient macrophages display increased targeting of *S. flexneri* to autophagosomes (69). Similar results were found in a study analyzing the impact of NLRC4 and NLRP4

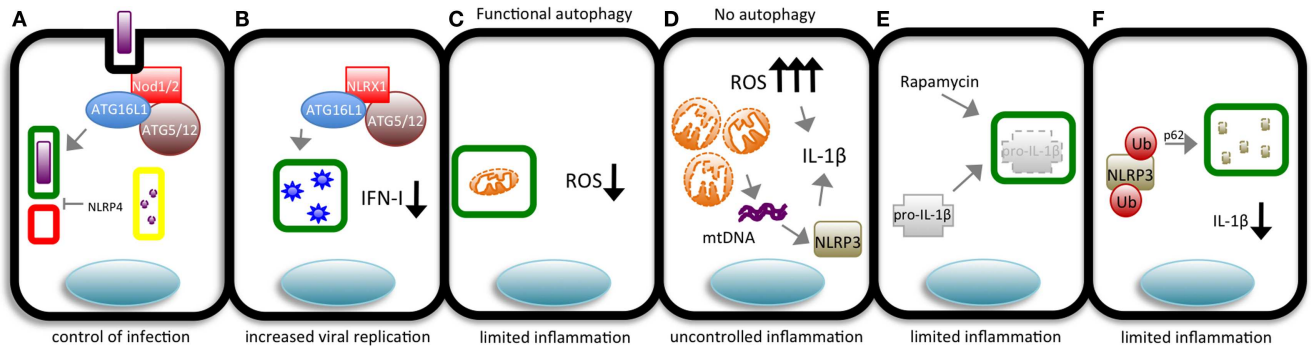

FIGURE 2 | The different means by which pathogens and autophagy interact and their outcomes. **(A)** During bacterial invasion, Nod1/2 recruit ATG16L1 to the bacterial entry site to promote the initiation of autophagosome (green rectangle) formation, targeting the bacterium. After enclosing the pathogen, the autophagosome fuses with lysosomes (red square), giving rise to an autolysosome (yellow rectangle) in which the content is degraded by lysosomal hydrolases to control bacterial replication in the cytosol. NLRP4 can interfere in autolysosome formation. **(B)** In cells with functional autophagic machinery, damaged mitochondria are targeted to autophagosomes (green rectangles) avoiding the accumulation of ROS and limiting inflammation. **(C)** Cells in which autophagy is impaired accumulate swollen mitochondria and high levels of ROS, leading to uncontrolled IL-1β production. Alternatively, damaged mitochondria release mitochondrial DNA (mtDNA), which is sensed by NLRP3 that, together with ROS, induces the production of IL-1β. **(D)** Inflammasome sensors are ubiquitinated and targeted to autophagosomes (green rectangles) for degradation, limiting the production of IL-1β. **(E)** Pro-IL-1β is targeted to autophagosomes (green rectangles) upon autophagy induction (by rapamycin) to limit the magnitude of inflammation. **(F)** NLRX1 associates with ATG16L1 and the ATG5/12 complex to induce the formation of autophagosomes (green rectangles) targeting VSV, which later fuse with lysosomes (red square), forming autolysosomes (yellow rectangles) in which the virus is degraded.

on autophagy, where it was demonstrated that epithelial cells with both proteins silenced displayed enhanced autophagy. A partial explanation for these observations is that NLRP4 is part of the Beclin-1 and C-VPS (a complex consisting of VPS11, VPS16, VPS18, and Rab7 that controls membrane tethering and fusion of vacuolar membranes) complexes, which are essential for the biogenesis and maturation of autophagosomes, respectively (144). The elucidation of the precise mechanisms by which NLRP4 exerts its negative effects on autophagy requires further studies (**Figure 2A**).

AUTOPHAGY-DEPENDENT CONTROL OF NLR-DEPENDENT INFLAMMATION

Another function of autophagy seems to be the control of the magnitude of inflammatory responses (145). In the last few years, several groups have reported that autophagy blockade by pharmacological or genetic means leads to increased production of cytokines by different mechanisms. Two independent studies have shown that ATG5-deficient fibroblasts produce significantly more IFN-I and IL-6 then WT cells during viral infection. The mechanisms likely involve the interaction between ATG12-ATG5 complexes with IPS-1, RIG-I, and MDA-5 and accumulation of damaged mitochondria in the autophagy-deficient cells, as discussed below (146, 147).

AUTOPHAGY AND INFLAMMASOMES

A ROLE FOR ROS

As discussed above, the cells deficient in ATG genes produce increased levels of pro-inflammatory cytokines (146, 147). Tal et al. (147) demonstrated a vital role of autophagy in the removal of damaged mitochondria (mitophagy), and thus, in cell homeostasis. Cells with defects in autophagy, such as Atg5-deficient cells, accumulate damaged mitochondria, and consequently present increased levels of ROS. This in turn results in the enhancement of the levels of IFNα and IL-6 production upon infection with VSV. In addition, the authors show that by using the antioxidants *N*-acetyl-*L*-cysteine (NAC) and propyl-gallate (PG) during VSV infection, they were able to revert the increase in cytokine production. The removal of damaged mitochondria can be regulated by adapter proteins that participate in NLR-activating pathways. For example, besides its well-known and crucial role linking Nod1 and Nod2 sensing to NF-κB activation, Rip2 has also been implicated in mitophagy. In a recent study, Lupfer et al. showed that Rip2 regulates mitophagy through ULK1 to keep ROS at basal levels. Genetic deletion of Rip2 leads to the accumulation of ROS and significantly higher levels of IL-18 and IFN-γ upon influenza infection (148) (**Figure 2C**).

Among all the cytokines that have been studied, IL-1β seems to be the cytokine whose production is most dramatically affected by autophagy. Processing and activation of pro-IL-1β into its active form depends on the assembly of inflammasomes. Increased ROS levels seem to be a prerequisite for inflammasome activation. The manipulation of autophagy by pharmacological or genetic means has a profound impact on IL-1β production and secretion during infection or LPS treatment. Indeed, the use of the autophagy inhibitor 3-methyladenine (3-MA), deletion of LC3B or ATG16L1, silencing of Beclin-1 or dominant negative forms of the cysteine protease ATG4B all lead to remarkably higher amounts of IL-1β (48, 149–151). The first evidence to show that autophagy can modulate IL-1β production surfaced in 2008 with the work of Saitoh et al. These authors reported that macrophages from ATG16L1-deficient mice produced much higher levels of IL-1β after LPS exposure and that this was not due to defects in the generation of pro-IL-1β and pro-caspase-1. These authors proposed a model in which TRIF and loss of K^+ and ROS are required for the activation of inflammasomes, and subsequently, IL-1β processing (150). However, it remains to be shown that these observations were not due to defective mitophagy. Further studies not only

confirmed but expanded the evidence for the requirement of functional autophagy for the maintenance of basal levels of ROS, and subsequently, for the control of inflammasome activation. In an elegant study, Nakahira et al. dissected the role of autophagy in the activation of the NLRP3 inflammasome. In a series of experiments in which autophagy was inhibited either by deletion of LC3B or by heterozygous deletion of Beclin-1, a robust increase in IL-1β processing and release was observed following stimulation with LPS plus ATP. According to the authors, these stimuli led to swollen mitochondria and the release of its DNA (mtDNA). In ρ^0 J774A.1 macrophages, the release of mtDNA into the cytosol is blocked, and IL-1β secretion is impaired. Similar results were obtained when cells were treated with DNAse I. Interestingly, the production of ROS and consequent activation of the inflammasome were dependent on the presence of cytosolic mtDNA. Altogether, the results from this study delineate a model in which LPS plus ATP induce mitochondrial damage, ROS production, and NLRP3-dependent release of mtDNA into the cytosol, resulting in the activation of caspase-1 and release of IL-1β (48). The findings reported by Nakahira et al. were partially contradicted by a more recent study that suggests that ATP actually induces the release of oxidized mtDNA, which in turn binds to NLRP3 to induce IL-1β production. Oxidized mtDNA was not detected in the cytosol of NLPR3-deficient macrophages. According to the authors, this could be explained by the fact that unbound oxidized mtDNA is rapidly degraded, but this later observation still lacks experimental confirmation (49) (**Figure 2D**). It is important to note that the idea of ROS as an activator of NLPR3 inflammasomes was challenged by a study demonstrating that ROS is key for priming of NLRP3 but not for its activation. One way or another, it is clear that ROS is necessary for IL-1β production (152).

The source of ROS required for inflammasome activation is also a matter of debate. Initial reports suggested that nicotinamide dinucleotide phosphate (NADPH) oxidases were the main source for inflammasome activation (153). The NADPH complex comprises the membrane-bound $gp91^{phox}$ and $p22^{phox}$ glycoproteins and the cytosolic components $p47^{phox}$ and $p67^{phox}$. Patients with chronic granulomatous disease (CGD) can have mutations in any of the NADPH oxidases and as a result present defective phagocytes because their cells have impaired capacity to generate superoxide anion and its metabolites, hydrogen peroxide, the hydroxyl anion, and hypohalous acid (154). Meissner et al. reported that in monocytes from CGD patients with mutations in $gp91^{phox}$, $p22^{phox}$, and $p47^{phox}$, stimulation with LPS plus ATP led to the activation of caspase-1 and secretion of mature IL-1β (155). Furthermore, monocytes from CGD patients presented elevated IL-1β levels in comparison to monocytes from healthy controls (154). These findings challenged the notion that NADPH oxidases are the source of ROS necessary to induce inflammasome activation. This question was apparently clarified by the work of Zhou et al. which demonstrated that ROS generated by dysfunctional mitochondria [mtROS, achieved by either by treating cells with the complex I inhibitor, rotenone, or silencing of the voltage-dependent anion channel (VDAC)] activates the Nlrp3 inflammasome. The requirement of mtROS for activation of the inflammasome seems to be specific for Nlrp3 because VDAC1 silencing did not influence activation of NLRC4 of AIM2 inflammasomes. Once again, the crucial role of autophagy in clearing damaged mitochondria was demonstrated in the work of Zhou et al. in which 3-MA treatment or beclin-1 or Atg5 silencing resulted in ROS accumulation and inflammasome activation (61).

As already mentioned, mutations in NLR genes are associated with increased risk for inflammatory diseases. Auto-activation of the NLRP3 inflammasome has been linked to several autosomal dominant cryopyrinopathies or cryopyrin-associated periodic fever syndromes (CAPS), such as familial cold-induced autoinflammatory syndrome (FCAS), Muckle–Wells syndrome (MWS), and neonatal onset multisystem inflammatory disorder or chronic infantile neurologic cutaneous and articular syndrome (NOMID/CINCA) (156). These syndromes, despite their different names, represent a continuum of disease severity where FCAS is the mildest and NOMID/CINCA the most severe. These cryopyrinopathies are associated with periodic fever, rashes, arthralgia, and conjunctivitis, and the aberrant production of IL-1β is the most prominent feature related to all these manifestations (6).

The increasing incidence of T2D has become a global health burden. TD2 has been associated with low-grade inflammation that leads to insulin resistance. In this context, IL-1β is one of the main cytokines implicated in T2D, mediating the destruction of beta cells and resulting in insulin resistance in cells that were initially sensitive to the hormone. A high-fat diet (HFD) is one of the factors associated with T2D. Indeed, T2D patients display augmented levels of free fatty acids in the serum. In a recent study, Wen et al. demonstrated in a bone marrow-derived model that palmitate, an abundant saturated fatty acid in the plasma, inhibits AMP-activated protein kinase (AMPK), leading to defective autophagy, and thus, ROS accumulation. These events contribute to the elevation of IL-1β production and impairment of insulin signaling *in vitro* (47).

A recent report showed that autophagy is involved not only in IL-1β production but also in its secretion. Macrophages from Atg5 conditional knockout mice secreted significantly more IL-1β during the induction of autophagy triggered by starvation. These observations need further confirmation (157).

A ROLE FOR UBIQUITIN

As in many aspects of its biology, autophagy has ambiguous roles in regulating inflammasome activation and acts both as a positive and negative regulator depending on the experimental model.

In a recent study, Harris et al. provided experimental evidence that autophagy controls inflammasomes by targeting its components to autophagosomes. These authors showed that pro-IL-1β is delivered to autophagosomes after TLR stimulation. Upon autophagy induction by rapamycin treatment, pro-IL-1β is degraded, limiting the amount available for the processing and secretion of IL-1β (149) (**Figure 2E**).

One mechanism underlying the targeting of inflammasome proteins to autophagosomes seems to be ubiquitination. In a recent study by Shi et al. it was demonstrated that inflammasomes containing ASC are directed to autophagosomes during NLRP3 or AIM2 activation in primary macrophages. They also provided evidence that beclin-1 and p62 are involved in targeting ASC to autophagosomes after it is K63 ubiquitination. The results presented suggest that by using its separate UBA

and LIR domains, p62/SQTM1 bridges ASC K623 ubiquitination, and autophagy-dependent degradation (151). In addition to ASC, NLRP3 is also ubiquitinated in a mtDNA-, ROS-, and ATP-dependent manner, but its delivery into autophagosomes has not yet been demonstrated (158) (**Figure 2F**).

CONCLUDING REMARKS

The NLR and autophagy fields are two exciting research areas in biology with many unanswered questions related to the precise mechanisms that coordinate the "talk" between NLR proteins and autophagy. It will be interesting to discover in more detail whether autophagy modulation can be used to control NLR-dependent immune pathways to improve therapeutic strategies for inflammatory and infectious diseases. In light of the recent findings that connect autophagy and inflammasome regulation, it remains to be determined whether alterations in autophagy could explain, at least in part, the dysregulated production of IL-1β in NLRP3-associated cryopyrinopathies. We believe that in the near future, some of the findings discussed in the present review have the potential to be translated into new therapeutic strategies that can be applied in daily medical practice.

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Interactions between Nod-like receptors and intestinal bacteria

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Nucleotide oligomerization domain (Nod)-like Receptors (NLRs) are cytosolic sensors that mediate the activation of Caspase-1 and the subsequent processing and secretion of the pro-inflammatory cytokines IL-1β and IL-18, as well as an inflammatory cell death termed pyroptosis. While a multitude of bacteria have been shown to activate one or more NLRs under *in vitro* conditions, the exact impact of NLR activation during the course of colonization, both of pathogenic and commensal nature, is less understood. In this review, we will focus on the role of intestinal NLRs during the various stages of infection with common gastrointestinal bacterial pathogens, as well as NLR function in controlling and shaping the microbiota.

Keywords: nod-like receptors, microbiota, inflammasome, intestine, pathogen

INTRODUCTION

The human body lives in symbiosis with trillions of microbial cells, collectively called the microbiota, with the vast majority of these microbes being bacteria that inhabit the gastrointestinal tract (1). This symbiosis begins with colonization of the gastrointestinal tract at birth and then is sustained throughout life by environmental exposures (2). Occasionally this microbial symbiosis is challenged by invading bacterial pathogens, which perturb the microbial ecosystem and cause disease.

Our ability to harbor trillions of bacteria within our intestines relies on the maintenance of a safe distance between these bacteria and the single layer of intestinal epithelial cells. Crucial protective mechanisms have evolved to help ensure host-bacteria mutualism. A major barrier bacteria encounter in the intestine is formed by the mucus layer, a dense network of glycoproteins that most bacteria are unable to breach (3). To further aid the barrier function of the mucus layer, intestinal cells also secrete an array of antimicrobial proteins, like antimicrobial peptides, lectins, and lysozymes. Furthermore, secreted IgA specifically targets bacteria for immune exclusion (4).

At the cellular level, sensing systems continuously scan for bacteria that are able to actively surpass the mucus layer and attach to and/or invade the epithelium. Two major receptor families that detect microbes are the Toll-like Receptors (TLRs), which control the extracellular compartment, and Nod-like Receptors (NLRs), which sense the presence of intracellular microbes (5). NLRs are crucial for fighting and resolving infections as many pathogenic bacteria (and under certain conditions also members of the commensal microbiota) attempt to exploit and enter the cytosol for nutrients and to escape extracellular threats (6). Here, we provide an overview of the role of NLRs in protection against intestinal pathogenic bacteria and control of the intestinal microbiota.

INTESTINAL NLRs

Nod-like receptors generally consist of a ligand-sensing domain in the form of a Leucine Rich Repeat (LRR) domain, a central ATP binding domain, and a signaling domain (often in the form of a CARD or Pyrin domain) and are categorized by their domain structure. While NLRs are expressed widely in a variety of tissues in humans and mice, we will focus in this review on those that were shown to function in the defense against bacteria in the intestine. While Absent In Melanoma 2 (AIM2) theoretically is not part of the NLR family, we have included it here for completeness.

NOD1 AND NOD2 (NLRC1, NLRC2)

The pattern recognition receptors NOD1 and NOD2 are amongst the best-studied NLRs, and their ligands are well defined. Both NOD1 and NOD2 sense cytosolic bacterial peptidoglycan fragments with high specificity: NOD1 is activated by D-glutamyl-meso-diaminopimelic acid (DAP) containing peptidoglycan fragments, which are mainly found in Gram-negative bacteria (7), whereas NOD2 was shown to bind and responds to muramyl dipeptide (MDP), found in all bacteria (8). Despite the presence of N-terminal CARD domains, NOD1 and NOD2 are non-inflammasome forming NLRs and do not seem to directly activate Caspase-1. Instead, after ligand binding the CARD domain of NOD1 and NOD2 interacts with the signaling kinase RIP2 (RIPK2, RICK) that initiates a signaling cascade resulting in NF-κB activation, as well as the activation of ERK, p38 and mitogen-activated protein kinases (MAPKs) (9, 10). These signaling pathways result in the expression of a variety of pro-inflammatory cytokines and chemokines, as well as the production of reactive oxygen species. While NOD2 expression is more restricted, both NODs are expressed in macrophages, dendritic cells, Paneth cells, and intestinal epithelial cells, making them highly suited to sense infections throughout the intestinal tract (10). In recent years, several

layers of complexity were added onto the basic mechanism of NOD1 and NOD2 sensing and signaling. For instance, NOD2 was shown to interact with NLRP1, NLRP3, and NLRP12 (11), NOD1 and NOD2 were found to play a role in autophagy (12), and NOD1 senses the modification of small rho GTPases injected by *Salmonella* during infection (13).

NLRC4

NLRC4 is an N-terminal CARD domain containing NLR. The elucidation of the NLRC4 crystal structure has revealed that, under resting conditions, NLRC4 resides in a closed monomeric form, kept in place by an ADP-dependent autoinhibitory mechanism involving multiple domains including the LRR (14). Ligand binding is proposed to induce "opening" of the structure, the exchange of ADP for ATP, and subsequent NLRC4 oligomerization. Phosphorylation of a conserved serine residue proximal to the LRR was shown to be required for NLRC4 inflammasome activation in macrophages, although the exact role in this process requires further investigation (15). NLRC4 responds to attaching or invading pathogens by sensing their bacterial secretion systems. So far, two bacterial ligands are well defined: flagellin, which is co-secreted with virulence factors either through type III or type IV secretion systems (T3SS and T4SS, respectively) (16–18), and PrgJ, a structural component of the type III secretion system that leaks or is secreted into the host cytosol (19). Within the cytosol, flagellin and PrgJ bind to the adapter proteins NLR family, apoptosis inhibitory protein (NAIP) 2 and NAIP5, respectively (20, 21), which subsequently bind NLRC4 to initiate its oligomerization into a ring-like inflammasome that recruits the adapter protein apoptosis-associated speck-like protein containing a CARD (ASC) (containing both a Pyrin and CARD domain) and Caspase-1 (22). This complex then processes the pro-inflammatory cytokines pro-IL-1β and pro-IL-18, and induces pyroptosis, an inflammatory form of cell death. Interestingly, unlike mice, humans have only one NAIP protein, which is unresponsive to both flagellin or basal rod protein but instead binds the conserved T3SS needle protein to activate NLRC4 (20).

NLRP3

While NLRP3 is probably the best studied of the NLRs, the mechanism of receptor activation remains relatively unclear. NLRP3, or cryopyrin, was originally shown to play a key role in a collection of autoinflammatory disorders collectively termed cryopyrin-associated periodic syndromes, which all share mutations in NLRP3 that lead to inappropriate IL-1β-mediated inflammatory responses (23). NLRP3 was subsequently found to "sense" a long list of ligands or stimuli, including ATP, pore-forming toxins, particulates like asbestos and silica, bacteria, viral, and fungal infections (24). Initially, three main theories of the activation of NLRP3 were proposed: potassium efflux, lysosomal rupture and subsequent cleavage by released Cathepsin, and ROS production. Several "second generation" unifying NLRP3 ligands were proposed to combine the three, including oxidized mitochondrial DNA released into the cytosol following mitochondrial damage (25); thioredoxin-interacting protein (26), calcium mobilization (27), mitochondrial cardiolipin (28), and changes in cell volume (29). While most of these NLRP3 ligands were recently shown to lead to potassium efflux might, suggesting this to be the common trigger in the end (30), NLRP3 activation remains enigmatic; structural studies similar to those done for NLRC4 might eventually elucidate the elusive NLRP3 ligand.

A new chapter for NLRP3 has been opened through the elucidation of the non-canonical inflammasome pathway. Due to the (re)discovery of the presence of a mutated, non-functional Caspase-11 in the original Caspase-1-deficient mouse, a role for Caspase-11 was found in NLRP3-inflammasome activation by Gram-negative bacteria (31). After prolonged (~17h) stimulation of bone-marrow macrophages with bacteria, Caspase-11 was shown to be activated, leading to cell death and NLRP3/ASC/Caspase-1-dependent IL-1β and IL-18 secretion. It was subsequently shown that the TLR4-TRIF-Type I Interferon pathway was required to induce high levels of Caspase-11 transcription needed for non-canonical inflammasome activation (32). However, it recently was shown that intracellular LPS serves as a ligand able to activate the non-canonical inflammasome pathway, independently of increasing levels of Caspase-11 caused by Type I Interferon (33). Three major questions regarding non-canonical inflammasome activation remain currently unanswered: what is the receptor that senses intracellular LPS or potentially other ligand (presumably a CARD-containing NLR), how does this complex feed into the NLRP3 inflammasome (Caspase-11-dependent pyroptosis resulting in potassium efflux?), and is Caspase-11 activated by any additional receptors?

NLRP6

NLRP6 falls within the group of NLRs that was initially found to induce NF-κB and Caspase-1 activation during overexpression in transfected tissue culture cells (34). In this system, human NLRP6 was also shown to form punctate structures in the cytoplasm, but only in the presence of ASC, suggesting the ability of NLRP6 to form inflammasomes or inflammasome-like structures. Unlike in humans, where NLRP6 is not highly or widely expressed, mice exhibit high NLRP6 expression throughout the intestine, kidneys, and liver (35, 36), which can be regulated by stress factors (37). Mechanistically, NLRP6 was shown to be a negative regulator of NF-κB and MAPK in cultured bone-marrow macrophages from NLRP6-deficient mice (38), which is the opposite of what was initially observed in overexpression studies. NLRP6 function as a negative regulator of NF-κB and MAPK might play a role in the increased intestinal tissue proliferation and inflammation observed in NLRP6-deficient mice (39, 40). Furthermore, NLRP6 was shown to be involved in the production of Caspase-1 dependent IL-18 (36), again suggesting the ability of NLRP6 to form an inflammasome.

NLRP12

Since its identification, NLRP12 has been assigned a number of different functions. Like NLRP6, NLRP12 was originally described to induce NF-κB and Caspase-1 activation when co-expressed with ASC (41). In contrast, without ASC co-expression, NLRP12 overexpression reduced non-canonical NF-κB activation and enhanced the expression of non-classical and classical MHC class I genes (42–44). NLRP12-deficient mice were also reported to have defective dendritic cell and neutrophil responses

to chemokines and subsequent defective dendritic cell migration to draining lymph nodes (45). In addition, NLRP12-deficiency led to enhanced colon inflammation and colorectal cancer development due to increased (non-canonical) NF-κB and ERK activation (46, 47), similar to what was observed previously for NLRP6.

AIM2

AIM2 was originally identified in humans as an interferon-inducible, putative tumor suppressor protein (48), but subsequently found to sense cytoplasmic double stranded (ds) DNA, form an inflammasome complex together with ASC and Caspase-1, and trigger the processing of pro-IL-1β and pro-IL-18 (49–52). Like NLRP receptors, AIM2 contains an N-terminal signaling Pyrin domain; however, the C-terminal consists of a DNA-binding HIN200 domain. AIM2 is able to sense the presence of cytosolic non-sequence-specific dsDNA of both viral and bacterial origin. Bacterial DNA enters the cytosol mainly by a passive process following bacterial lysis, for instance during rapid cytosolic bacterial replication or after intracellular bacteria-containing vesicles are compromised, but appears to be always preceded by bacterial invasion into the host cell, making AIM2 a specific sensor for intracellular bacteria and viruses (53–55). In a mechanism similar to what was observed for NLRC4, the HIN200 domain functions as a negative regulator of the signaling Pyrin domain. Non-sequence-specific binding of dsDNA releases this inhibition, liberating the Pyrin domain to recruit ASC and Caspase-1, and form an inflammasome surrounding the released bacterial or viral DNA (56, 57).

ENTERIC PATHOGENS AND THEIR INTERACTIONS WITH NLRs

Foodborne gastrointestinal pathogens are a major cause of bacterial infections in humans (58). Studies on these pathogens, both in the human host and in various murine models, have provided great insights into microbial virulence mechanisms as well as the immunological defense strategies of the host. For NLRs, pathogen-host interactions have been of great value for the elucidation of the different functions of this family of innate sensors, both in *in vitro* and *in vivo* model systems. Below (and summarized in **Table 1**), we provide an overview of the role of NLRs during infections with the most commonly studied bacterial enteric pathogens.

SALMONELLA

In humans, infections with the Gram-negative bacterium *Salmonella enterica* (*S. enterica*) generally result in one of two distinct clinical phenotypes. *S. enterica* serovars Typhi and Paratyphi are

Table 1 | Role of NLRs in intestinal bacterial infections.

Bacteria	Model	NLR	Mechanism of action	Reference
S. typhimurium	Systemic	NLRC4	Flagellin/T3SS-induced pyroptosis, IL-1β, and IL-18 production	(79–81)
		NLRP3	Caspase-1 activation	(79)
	Systemic, T3SS-1-independent	NOD1	Nitric oxide production in dendritic cells	(69)
	Systemic and colitis in Balb/c	NLRC4	IL-1β-mediated neutrophil recruitment	(84)
	Colitis	NLRC4	IL-1β and IL-18 production	(80)
		NOD1	NOD1-mediated detection of SipA	(70)
	Colitis, T3SS-1-independent	NOD1/2	Innate $CD4^+$ T helper type 17 cell responses in the cecum	(74, 75)
	Systemic (intraperitoneal)	NLRP6	NLRP6-mediated negative regulation of NF-κB and MAPK activation	(38)
C. rodentium	Colitis	NOD2	NOD2-activation in stromal cells, CCL2/CCR2-dependent recruitment of inflammatory monocytes, IL-12-mediated bacterial clearance	(92)
		NOD1/2	IL-6-dependent IL-17 production in the cecum	(75)
		NLRC4	IL-1β and IL-18 production	(94)
		NLRP3	IL-1β and IL-18 production	(94)
H. pylori	Gastritis	NOD1	T4SS-mediated delivery of peptidoglycan, NF-κB-mediated inflammatory responses	(108)
		Unknown	IL-18-dependent IL-17 production, T-cell-mediated antibacterial responses	(110, 112)
		Unknown	IL-1β-dependent impaired bacterial clearance	(111, 113)
Microbiota	Colitis (DSS)	NLRP6	IL-18/CCL5 production, increased intestinal epithelial proliferation and tissue repair	(36, 40)
		NLRP3	Both increased and decreased susceptibility; microbiota-dependent?	(124, 125, 127, 128)
		NOD1/2	Induction of E-cadherin and RegIII-γ expression	(120)
	Colorectal cancer (DSS-AOM)	NLRP6	IL-18/CCL5/IL-6 mediated increased intestinal epithelial proliferation and tissue repair	(39, 40, 116)
		NLRP3	Caspase-1 activation	(126)
	Non-alcoholic fatty liver disease	NLRP3/6	IL-18-mediated control of microbiota	(117)

the causative agents of Typhoid fever, a life-threatening disease characterized by systemic spread of ingested bacteria, high fever, and intestinal bleeding that results in 200,000–600,000 deaths worldwide each year (59). The more common non-typhoidal *S. enterica* serovars, like *S. typhimurium* and *S. enteritidis*, cause a self-limiting gastroenteritis characterized by (bloody) diarrhea, fever, abdominal cramps, and vomiting that usually lasts 4–7 days and affects over 90 million people worldwide each year (60).

Two different disease models in mice represent the two clinical manifestations of *Salmonella* infections. The classical murine model of systemic *S. typhimurium* infection induces a disease similar to Typhoid fever. In this model, orally administered salmonellae reach the distal ileum within hours of ingestion and, aided by flagella-mediated motility and chemotaxis (61), cross the mucus layer toward the epithelium. Here, the bacteria target the follicular-associated epithelium overlying the Peyer's Patches, with a strong preference for the M-cells, as a main port of entry. To gain access into the host cell, *Salmonella* employs the first of two type III secretion systems (T3SS-1), which is only expressed during this initial phase of infection, and injects an array of effector proteins into the host's cytosol that induce cytoskeletal rearrangements leading to bacterial invasion (62). Other effector proteins and the activation of pattern recognition receptors initiate inflammatory responses that attract neutrophils, monocytes, and macrophages. *Salmonella* replicates within the Peyer's Patches and disseminates to mesenteric lymph nodes (MLN), liver, and spleen within infected monocytes and dendritic cells. In a T3SS-1-independent manner, a small proportion of the bacteria is also taken up passively from the lumen by $CD11c^+$ $CX3CR1^+$ dendritic cells and transported directly to the MLN, bypassing the Peyer's Patches (63). To survive and replicate in the host's cells, *Salmonella* resides within a vacuole, the *Salmonella*-containing Vacuole (SCV), whose integrity is maintained by the effector proteins secreted into the cytosol through a second T3SS (T3SS-2). During the later stages of disease, disseminated bacteria in the liver and spleen are found mostly within macrophages, in which they rapidly replicate. Continuous cycles of bacterial replication and dissemination eventually lead to bacteremia from which mice succumb after a week.

In the murine colitis model, infection with *Salmonella* is preceded by a single dose of streptomycin, which is believed to briefly reduce "colonization resistance" provided by the microbiota that occupies the more distal intestinal tract and strongly competes for nutrients. In this short window, *Salmonella* is able to gain a foothold in the cecum and colon, and replicates to high numbers within hours of infection, limiting the need for systemic spread (64). Colonization is accompanied by mucosal penetration of bacteria and the development of colitis, similar to the pathology seen in human infection with non-typhoidal serovars. For efficient colonization salmonellae still require T3SSs, which mediate invasion of enterocytes and induction of (local) inflammation. A reason for this was presented in an elegant study by Winter et al. which revealed that T3SS-induced inflammatory responses are actively exploited by *Salmonella* through the ability to utilize tetrathionate, formed in the intestine under inflammatory conditions, to successfully compete with the microbiota (65). In the colitis mouse model, *Salmonella* also exploits a T3SS-1-independent, "passive" route for uptake through $CD11c^+$ $CX3CR1^+$ dendritic cells. Similar to what is seen in the systemic model, these two invasion pathways act in concert, although T3SS-1-mediated invasion seems much more dominant. While bacteria are able to grow extensively in the distal intestine in the colitis model, substantial numbers of bacteria still continue to disseminate to liver and spleen, and infected mice usually die after 5–6 days.

As several phases of infection largely rely on cellular invasion, NLRs appear to be the ideal sensing mechanism for *Salmonella*. *In vitro*, *Salmonella* is sensed by NOD2 in cultured intestinal epithelial cells, which enables the control of intracellular bacteria though the induction of antimicrobial responses and autophagy (66–68), and by NOD1 in bone-marrow-derived dendritic cells, resulting in nitric oxide production (69). Interestingly, while *Salmonella* peptidoglycan may be involved in the activation of NOD1 and NOD2, it was recently shown that the modification of small Rho GTPases by the T3SS-1 effector SopE, which enables bacterial invasion, is a danger signal sensed by NOD1 (13). Presumably through a similar mechanism, another T3SS-1 effector SipA activated NOD1/NOD2-dependent NF-κB responses both *in vitro* and *in vivo* (70). In addition to NOD1 and NOD2, *Salmonella* is efficiently sensed by NLRC4, both via flagellin and the basal rod protein PrgJ, which leads to rapid pyroptosis and secretion of IL-1β and IL-18 by cultured macrophages, dendritic cells and B-cells (16, 17, 19, 71, 72). These responses are solely dependent on T3SS-1, which is expressed only at the early logarithmic phase of bacterial cultures and is believed to represent the early phase in infection when *Salmonella* needs to invade host cells. The T3SS-2, expressed only at the late logarithmic phase, does not contribute to NLRC4 activation as flagellin expression is now repressed and the T3SS-2 apparatus is not recognized (19). Finally, *Salmonella* has been shown to activate the non-canonical NLRP3 inflammasome through Caspase-11 in macrophages (73).

In vivo, the role of NLRs during *Salmonella* infection has been rather difficult to define, mainly because of differences in experimental models (systemic versus colitis murine models), variations in growth phase of *Salmonella* at time of infection (T3SS-1-expressing versus T3SS-2-expressing conditions), different mouse intestinal microbiotas, and because of the redundancy in innate receptors. These issues are clearly demonstrated when studying NOD1 and NOD2. Both of these NLRs were shown to be dispensable during systemic infection (69). However, under T3SS-1-independent conditions, during which the bacteria "passively" cross the epithelial barrier through uptake and transport by dendritic cells, NOD1 deficiency led to higher bacterial loads and mortality. The authors show that NOD1-deficient $CD11b^+CD11c^+$ dendritic cells contain higher numbers of *Salmonella*, likely because of a diminished NOD1-mediated nitric oxide response. Similarly, during *Salmonella* colitis, NOD1/NOD2 double knockouts or RIP2 deficient mice exhibited reduced inflammation accompanied by increased mucosal colonization and reduced early IL-17 responses of innate $CD4^+$ T helper type 17 cells in the lamina propria of the cecum, but again only when *Salmonella* was grown under T3SS-2-expressing conditions (74, 75); when T3SS-1 was expressed at the time of oral infection in this model, no differences as compared to wild-type mice were observed (76). These data suggest that only one of the two major entry pathways exploited by *Salmonella* is controlled by NOD1/NOD2 signaling.

During "normal" infections, however, the T3SS-1-mediated invasion seems to outweigh the alternative invasion route, leaving NOD1 and NOD2 to play a non-significant role. Interestingly, NOD1/NOD2-activation by the T3SS-1 effector SipA was shown to lead to a higher gut inflammation score in the colitis model as compared to mice lacking the receptors (70). Combined, the above-mentioned studies suggest that NOD1 and NOD2 mediated detection of *Salmonella* plays a specific but minor role during salmonellosis. As a further complication, differences in microbiota from wild-type and knockout mice dramatically impact *Salmonella* susceptibility, as demonstrated by Kaiser et al.; when "microbiota-matched" littermate controls were used (instead of independently bred or purchased wild-type mice) to test the role of RIP2 during *Salmonella* colitis, the initially observed difference in *Salmonella*-induced pathology was completely lost (64).

Caspase-1 has been shown in several publications to provide moderate protection against *Salmonella* infection. Without Caspase-1, mice succumb to bacteremia sooner and have higher bacterial loads in the MLN, liver and spleen in the typhoid model of infection, and more colitis accompanied by increased bacterial mucosal infiltration (77, 78). While NLRC4 appeared to be the main upstream candidate for caspase-1 activation, NLRC4-deficient mice showed only minor or no defects in bacterial control during infections (77, 79, 80). Two critical findings explained this "lack" of NLRC4 function *in vivo*; first, *Salmonella* actively evades recognition by NLRC4 by downregulating both T3SS-1 and flagellin as soon as the bacteria have invaded the host cell, and T3SS-2 is not recognized by NLRC4 (19, 81). The *in vivo* consequence of NLRC4-evasion, and thereby the role of NLRC4 in protection against invading pathogens, was elegantly shown by Miao et al.: when *Salmonella* was forced to continuously express flagellin, 100 times less bacteria were found in the spleen after 48 h during systemic infection. The increased control of bacterial spreading was attributed to NLRC4-mediated macrophage pyroptosis at peripheral sites, which resulted in release of the intracellular bacteria and subsequent clearance by infiltrating neutrophils (81).

NLR redundancy is a second reason why NLRC4-deficient mice do not phenocopy Caspase-1-deficient mice. Late logarithmic, non-T3SS-1 expressing salmonellae are able to activate the non-canonical Caspase-11/NLRP3 inflammasome (73). Similar to NLRC4, deficiency in only NLRP3 does not lead to differences in *Salmonella* infection. However, deletion of both NLRC4 and NLRP3 recapitulates the Caspase-1 phenotype completely, confirming a role for both NLRC4 and NLRP3 during *Salmonella* infection (79). This also demonstrates that, as was predicted by *in vitro* studies, NLRC4-evasion is not perfect. While pyroptosis has a clear impact on infection, the cytokines IL-1β and IL-18 appear to play minor roles in the control of the bacteria, since IL-1β and IL-18-deficent mice show little delay in bacteremia at 72 h (81, 82).

With the realization that the Caspase-1 KO was in fact a Caspase-1/Caspase-11 double knockout, and the elucidation of the role of Caspase-11 in non-canonical inflammasome activation, Caspase-11-deficient mice were predicted to result in more bacterial spread due to diminished control of infection. However, Caspase-11-deficient mice were indistinguishable from WT mice during *Salmonella* infection (79). Surprisingly, Caspase-1 single deficient mice had even higher numbers of bacteria in liver and spleen than the Caspase-1/Caspase-11-deficient mice, suggesting a protective role for Caspase-11 deficiency, but only in the context of Caspase-1-deficiency. A potential explanation for this may be that, while rapid Caspase-1-mediated pyroptosis clears bacteria, Caspase-11-mediated pyroptosis at later time points is actively used by *Salmonella* to escape the "full" macrophage after extensive replication. Indeed, Caspase-11 senses bacteria escaping from or leaking out of vacuoles into the cytoplasm (83). In the absence of Caspase-1, NLRC4 "evasion" by *Salmonella* is complete, resulting in uncontrolled replication until Caspase-11 is utilized to break out of the macrophage and invade new host cells. Why non-canonical Caspase-11-mediated pyroptosis, like NLRC4-activation accompanied by IL-1β and IL-18 secretion that induces local inflammation and attracts neutrophils, is less potent than Caspase-1-mediated pyroptosis in controlling *Salmonella* infection remains thus far unclear.

Unlike in C57BL/6 mice, in Balb/c mice NLRC4 appears to have a more prominent function in controlling *Salmonella* infection. In these mice, NLRC4-deficiency leads to more systemic bacterial dissemination and mortality, while less inflammation-induced pathology in the cecum was observed (84). It was subsequently shown that *Salmonella* specifically activates intestinal phagocytes that respond by producing IL-1β which triggered the upregulation of endothelial adhesion molecules. The basis of the interesting differential function of NLRC4 between C57BL/6 and Balb/c remains to be determined.

ATTACHING AND EFFACING ENTERIC PATHOGENS: *CITROBACTER*, EPEC AND EHEC

Citrobacter rodentium (*C. rodentium*), Enteropathogenic *Escherichia coli* (EPEC), and Enterohemorrhagic *Escherichia coli* (EHEC) are Gram-negative extracellular enteric pathogens that share a similar virulence strategy, termed attaching and effacing (A/E) (85, 86). EPEC and EHEC are human pathogens; EPEC is a major cause of diarrhea in young children, generally without major complications, while EHEC infections can vary greatly in severity, ranging from mild gastroenteritis to severe hemorrhagic colitis and hemolytic uremic syndrome. *C. rodentium* is a natural mouse pathogen resulting in self-limiting enteritis. While very little is known about activation of NLRs by EPEC and EHEC in humans, several studies have elucidated the role of such responses during infection of their murine counterpart, *C. rodentium*.

Within a couple of hours after oral infection of mice, *C. rodentium* reaches its initial site of infection, the lymphoid tissue in the cecum termed the cecal patch, where it reaches high density over the following 3 days (87). The cecal patch is structurally similar to the Peyer's Patch and, because of their nature as antigen sampling hotspots with decreased mucus layer thickness and absence of microvilli, provide "easy access/entrance" for several intestinal pathogens (including *Salmonella*, as described above). From day 3 to 4, *C. rodentium* starts to spread throughout the distal colon (87). Mouse-adapted strains of *C. rodentium* largely skip the cecal patch phase and colonize the colon readily, suggesting that colonization of the cecal patch also serves as an adaptation phase to the mouse intestinal environment (88). Depending on the strain of *C. rodentium* used, bacterial numbers peak between day 5 and 14 with

limited systemic spread to the MLN, liver, and spleen. The colonization then slowly diminishes until bacterial clearance from the cecum and subsequently the colon after 3–4 weeks post infection.

Upon reaching the cell surface of the cecum and colon, *C. rodentium* employs a T3SS which injects an array of virulence factors into the host's cytosol that result in the attachment of the bacteria to the enterocytes and the accompanying local destruction of the brush border microvilli of the epithelium forming pedestal-like structures termed A/E lesions (86). Two of these virulence proteins are central for this virulence strategy: the adhesin Intimin expressed on the bacterial surface and the T3SS-injected Translocated Intimin Receptor (TIR), which provides a docking ligand for Intimin on the host epithelial surface (89, 90). The attachment of *C. rodentium*, in combination with the secretion of many additional virulence proteins, leads to colonic hyperplasia, observed readily during the peak of infection as larger intestinal crypt length and increased colon weight.

Several reports have shown that NOD1 and NOD2 are able to sense *C. rodentium* both *in vitro* and *in vivo* (75, 91, 92). In the absence of NOD2, *C. rodentium* reaches a higher intestinal abundance as compared to wild-type mice. At the early stages of infection, NOD2 signaling was shown to activate the CCL2/CCR2 axis that resulted in the recruitment of inflammatory monocytes to the site of infection, which initiated IL-12-mediated bacterial clearance. Interestingly, NOD2-activation took place in intestinal stromal cells and not immune cells. NOD2-deficiency led to lower inflammation at the early stages of infection, but more severe colitis later, as a result of reduced clearance and higher bacterial abundance in the intestine (92). In a different study, NOD1 and NOD2 had redundant roles in the protection against *C. rodentium* infection and mediated IL-6-dependent IL-17 production in the cecum at early time point (1–4 days after infection). The observed effect on infection was similar; lower initial inflammatory responses but increased levels of bacterial dissemination to the spleen in the second week of infection (75). NOD1/NOD2 signaling was shown to occur mostly in the radio-resistant compartment, but a role for stromal cells was not further investigated. Although it is expected that peptidoglycan is the major *C. rodentium*-derived ligand of NOD1 and NOD2, T3SS-injected effector proteins may play a role too, as was shown previously for the *Salmonella* effector protein SopE (13). Indeed EspT, which targets small GTPases to induce membrane rearrangement in a similar way as SopE, was shown to induce NF-κB, ERK1/2 and JNK activation, common signaling pathways activated after NOD1/2 signaling (93). Future studies will determine to what extent effector-mediated NLR activation contributes to colonization and bacterial clearance.

Caspase-1/Caspase-11-deficient mice were found to be hypersusceptible for *C. rodentium* infection, as determined by increased intestinal bacterial loads, colitis, and hyperplasia (94). Both NLRP3 and NLRC4-deficient mice, as well as mice lacking IL-1β and IL-18, showed similar phenotypes, suggesting an important role for the NLRP3/NLRC4/IL-1β/IL-18 axis in the control of *C. rodentium*. In a different study, a similar but stronger phenotype was observed in IL-1R-deficient mice, which mostly succumb to infection within 2 weeks (95). In contrast to what was seen in IL-18-deficient mice, neutralizing this cytokine with antibodies had limited to no effect, implicating IL-1β or IL-1α as the critical cytokines that mediated protection against *C. rodentium*. IL-1R signaling during *C. rodentium* infection led to IFN-γ and IL-6 production in the colon, which mediated epithelial repair and maintained barrier function. While bacterial loads remained the same, more bacteria disseminated to the liver in the absence of these cytokines. Like *Salmonella* and most other Gram-negative bacteria, *C. rodentium* is able to activate the non-canonical Caspase-11/NLRP3 inflammasome in cultured bone-marrow macrophages, which occurred in a T3SS-independent (31, 94). The activation of the Caspase-11/NLRP3 non-canonical inflammasome during infection was evident when examining Caspase-11- and TRIF-deficient mice, which were both more susceptible for *C. rodentium* infection (96). Interestingly, while NLRC4 seems to be activated by *C. rodentium in vivo*, bone-marrow macrophages did not sense the T3SS of *C. rodentium* during *in vitro* studies. Whether this is due to tightly regulated T3SS expression or host cell tropism/specificity remains to be determined.

HELICOBACTER PYLORI

The Gram-negative bacterium *Helicobacter pylori* (*H. pylori*) colonizes the gastric mucosa of ~50% of the world's population, although substantial variation exists between countries (97). The majority of people infected by *H. pylori* do not show any symptoms, despite local chronic inflammatory responses induced by the bacterium. However, in a subset of patients, this inflammatory response drives the formation of gastric or duodenal ulcers that can lead to the development of mucosa-associated lymphoid tissue lymphomas and gastric adenocarcinomas (98–100).

In order to survive in the challenging gastric niche and enable persistent colonization, *H. pylori* is highly optimized to evade host antimicrobial strategies. For instance, after ingestion *H. pylori* secretes urease, which increases the gastric pH and reduces mucus viscosity, enabling rapid penetration of the gastric mucus layer and colonization in close proximity to the pH neutral epithelial cells (99, 101). Also, *H. pylori* expresses a modified LPS and flagellin to evade the recognition of TLR4 and TLR5, and has adopted several mechanisms to counteract the effects of host-produced reactive oxygen species (102, 103). Finally, the secreted pore-forming toxin VacA induces epithelial cell apoptosis and inhibits T-cell activation and proliferation (104). In contrast to immune evasion, a subset of *H. pylori* strains also actively induces inflammatory responses by means of the T4SS-mediated delivery of the effector protein CagA. CagA modifies multiple intracellular signaling pathways of host cells and is linked to the development of gastric cancer.

While immune evasion appears to be an important part of the *H. pylori* life cycle, genetic association studies revealed that mutations in NOD1, NOD2, and IL-1β may be associated with increased risk for the development of gastric cancer, suggesting that NLRs play a role in controlling *H. pylori* during human infection (105–107). In addition, several NLR family members have been shown to sense the bacterium and impact on infection or colonization, both in *in vitro* cell culture and *in vivo* murine models. In a manner analogous to the "leakage" of flagellin through the T3SS in *Salmonella*, peptidoglycan fragments were found to enter the host cytosol through the T4SS, where they were subsequently sensed by NOD1 and initiated NF-κB-mediated inflammatory responses (108). NOD1 activation was also observed by

peptidoglycan present in secreted bacterial outer membrane vesicles that were taken up by host cells (109). *H. pylori* was shown to induce the secretion of IL-1β and IL-18 both *in vitro* and *in vivo* (110, 111). As compared to wild-type mice, Caspase-1/Caspase-11-deficient mice showed decreased numbers of *Helicobacter* in the stomach, higher expression of IL-17, and aggravated gastric immunopathology, which was phenocopied by IL-18 and IL-18R, but not IL-1R deficient mice. Loss of IL-18 signaling in dendritic cells was subsequently shown to result in reduced levels of regulatory T-cells and stronger T-cell-mediated antibacterial responses (110, 112). In contrast, different groups reported that Caspase-1/Caspase-11, ASC, IL-1β, and IL-1R-deficient mice were impaired in the clearance of *H. pylori* from the stomach, displayed decreased gastritis and lower levels of IL-1β and IL-18 (111, 113). While the cause of the discrepancies between these different reports is currently unknown, it appears that *H. pylori* strives for the ideal level of inflammasome activation: enough IL-18 and IL-1β to induce regulatory T-cells and decrease gastric acid production, respectively (114), but not so much IL-1β as to lead to T-cell mediated clearance. The nature of the inflammasome NLR that is activated by *Helicobacter* remains unclear. While in cultured dendritic cells NLRP3 was crucial for IL-1β secretion in a T4SS-dependent/CagA-VacA-independent manner, this NLR did not play a role during murine infection.

THE INTESTINAL MICROBIOTA AND NLR-MEDIATED DISORDERS

The intestinal microbiota is predicted to consist of ~100 different bacterial species per person, and displays great variability between individuals (115). Alterations in the composition of the microbiota have been shown to dramatically impact disease susceptibility and progression. Therefore, controlling and (re)shaping the "healthy" microbiota is a crucial function of the intestinal immune system. The role of NLRs in this process is only beginning to be unraveled.

Lack of appropriate immunological control may switch a healthy microbiota into a pathogenic one, as exemplified by mice lacking NLRP6. NLRP6-deficient mice show increased levels of intestinal inflammation during DSS-induced colitis and develop more severe colorectal cancer in a model of colitis-dependent tumorigenesis (36, 39, 40, 116). A potential mechanism was provided by the finding that NLRP6 acts as a negative regulator of NF-κB and MAPK activation, and reduces the levels of cytokines and chemokines during infections with intestinal pathogens or epithelial barrier breach as observed during experimental models of colitis (38). More severe and prolonged inflammation in NLRP6-deficient mice results in increased levels of intestinal epithelial proliferation and increased tissue repair, which was shown to be CCL5 and IL-6 dependent (36, 39, 40, 116). The actions of NLRP6 do not seem limited to infectious or damaging episodes, as NLRP6-deficient mice already display continuous low level inflammation in the steady state, suggesting an interaction with the microbiota (36). 16S rRNA sequencing analysis of the microbiota revealed that NLRP6-deficient mice harbor a dysbiotic, colitogenic microbiota that showed a high relative abundance of *Prevotellaceae* species, that was transmissible to wild-type control mice. Similarly, lack of NLRP6-mediated control of the microbiota induced non-alcoholic fatty liver disease and obesity in mice and increased colorectal cancer, all of which were transmissible through microbiota transfer to wild-type mice (116, 117).

NLRP12 and NLRP6 may play similar roles in the control of intestinal homeostasis. Like NLRP6, NLRP12-deficiency leads to uncontrolled NF-κB signaling and subsequent inflammation and intestinal cell proliferation. Although extensive analysis of the composition of the intestinal microbiota of NLRP12-deficient mice has not been reported, the lack of this NLR might have major effects on the microbiota, either directly through sensing microbial products, or indirectly through the induction of an inflammatory environment via NF-κB dysregulation.

While systemic peptidoglycan from the intestinal microbiota was shown to boost the development of the intestinal immune system and prime immune responses via NOD1 in the bone-marrow in mice (115, 118), NOD1, like NOD2, does not dramatically influence the composition of the microbiota under homeostatic conditions (119). However, during DSS-induced colitis, the murine model of inflammatory bowel disease (IBD) that is driven by the microbiota, NOD1/NOD2-deficiency led to greater susceptibility to colitis (120). Similarly, mutations in NOD2 and NOD1 in humans are associated with susceptibility to Crohn's disease and IBD, respectively (121–123). The role of NLRP3 in controlling the microbiota has been rather controversial. Initially, NLRP3 was reported to have a key role in protecting intestinal homeostasis, as NLRP3-deficient mice were shown to have an altered microbiota and displayed increased susceptibility to DSS-colitis (124, 125) and tumorigenesis (126). However, NLRP3-deficiency led to resistance to DSS-colitis in a different study (127). As the DSS-colitis model is highly dependent on the microbiota, differential compositions of the microbiota may explain the varying outcomes in these studies. Indeed, co-housing NLRP3-deficient mice with wild-type mice, which equalized the intestinal microbiota, also equalized the inflammatory responses and disease in both mice (128). In humans, the role of NLRP3 in Crohn's is equally confusing; polymorphisms associated with NLRP3 were shown to contribute to susceptibility to Crohn's disease (129, 130), but did not replicate in a separate study (131). More detailed investigation of the interactions between specific members of the microbiota and NLRs may provide deeper insights in the function of NLRs in controlling and shaping the microbiota in health and disease.

CONCLUDING REMARKS

Nod-like receptors are crucial components of the intestinal innate immune system, controlling both the commensal microbiota as well as enteropathogenic bacterial infections. While a growing body of scientific evidence now provides clear insight into the role of NLRs in controlling intestinal bacteria, several conflicting reports highlight the importance of precisely controlling experimental conditions like bacterial growth phase and the intestinal microbiota between wild-type and NLR-deficient mice. Several key questions still remain unanswered, suck as the nature of the ligands for NLRP6 and NLRP12, the interplay between NLRs and adaptive immunity in the intestine, the potential role for other NLRs like NLRP7 (which senses bacterial lipopeptides in human cells), NLRP10 (which controls adaptive immune responses), and NLRC3 (which down-regulates NF-κB), and the role of NLRs in

human diseases. Future research will undoubtedly shed more light on these interesting new subjects.

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Debug your bugs – how NLRs shape intestinal host-microbe interactions

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The host's ability to discriminate friend and foe and to establish a precise homeostasis with its associated microbiota is crucial for its survival and fitness. Among the mediators of intestinal host-microbe interactions, NOD-like receptor (NLR) proteins take center stage. They are present in the epithelial lining and innate immune cells that constantly monitor microbial activities at the intestinal barrier. Dysfunctional NLRs predispose to intestinal inflammation as well as sensitization to extra-intestinal immune-mediated diseases and are linked to the alteration of microbial communities. Here, we review advances in our understanding of their reciprocal relationship in the regulation of intestinal homeostasis and implications for intestinal health.

Keywords: NLR, Crohn disease, intestinal mucosa, mucosal immunity, gut microbiota, inflammation, symbiosis

INTRODUCTION

The intestinal epithelium is the largest barrier organ of the human body and the colon harbors the majority of the individual's microbiota (1). It is estimated that more than 1000 different bacterial species colonize the human gut, outnumbering eukaryotic cells at least by an order of magnitude (2). As many of the bacteria represent facultative pathogens (pathobionts), the integrity of the intestinal barrier must be highly secured. This is accomplished by physical and immunological mechanisms formed by cellular (i.e., epithelial- and mesoderm-derived immune cells) and non-cellular components (e.g., antimicrobial peptides, cytokines, and mucus). On the other hand, an extensive crosstalk between host and microbiota contributes to the normal development and maturation of the intestinal epithelium and immune system (3, 4). The recognition of this complex bacterial community is mediated by phylogenetically ancient innate immune receptors, e.g., Toll-like receptors (TLRs) and NOD-like receptors (NLRs). NLR proteins have co-evolved with intestinal microbial communities and are expressed by intestinal epithelial and immune cells. They are characterized by a central nucleotide-binding and oligomerization domain (NOD or NACHT) and C-terminal leucine-rich repeats (LRRs) (5). Upon activation, NLRs initiate assembly of the inflammasome or signaling cascades [e.g., NF-κB, reactive oxygen species (ROS)] leading to a transient pro-inflammatory environment and, ultimately, aim at resolution of inflammation. Dysfunctional NLR signaling is linked to intestinal inflammation and in fact, polymorphisms in NLR genes are associated with complex chronic inflammatory barrier diseases, such as inflammatory bowel disease (IBD) (6). The two major forms of IBD, Crohn's disease (CD) and ulcerative colitis (UC) are chronic relapsing-remittent or progressive inflammatory conditions that affect the gastrointestinal tract.

It has become clear that NLRs play a crucial role for the maintenance of structural and functional composition of the intestinal microbiota. Several lines of evidence have been presented that link dysfunctional NLR signaling to an impaired host-microbiota homeostasis that may predispose to subsequent altered inflammatory responses in animal models. Here, we summarize multiple levels of host-microbe crosstalk in the intestine and review the recent findings and consequences of NLRs in physiological and pathological intestinal host-microbe interactions.

THE ROLE OF NLRs IN THE MULTIPLE LEVELS OF INTESTINAL HOST-MICROBE CROSSTALK – THE NOD2 EXAMPLE

The importance of NOD2 for intestinal homeostasis is emphasized by the finding that genetic variants in NOD2 contribute to dysregulated intestinal inflammatory responses and to manifestation of CD in humans. The three most common single nucleotide polymorphisms (SNPs) are located within the LRR of NOD2 causing either a frameshift mutation (L1007fsinsC), which leads to a truncated LRR or amino acid changes (R702W and G908R) (7–9). Cells that express these variants fail to activate NF-κB upon stimulation with the NOD2 ligand muramyl-dipeptide (MDP) (10, 11). In mouse models of intestinal inflammation, NOD2 has been assigned a protective role, since lack of NOD2 conferred increased susceptibility to DSS and TNBS-induced colitis (12). It must be emphasized that the effects are modest and under regular animal housing conditions no spontaneous inflammatory phenotype has been observed. Although it is still unclear how exactly a loss of NOD2 function predisposes to CD, several mechanisms related to altered host-microbe interactions and consequently increased susceptibility to intestinal inflammation, are currently discussed.

TOLERANCE, POLARITY, AND CONTROL OF PROTECTIVE CELLULAR PROGRAMS IN IECs

An imprinting function of NOD2 on microbial composition and/or active antibacterial responses against pathogens may be explained by its ability to modulate cellular programs in IECs (summarized in **Figure 1**). Furthermore, it was demonstrated that the LRR domain of NOD2 already confers antibacterial properties *per se*. The purified NOD2 LRR domain directly interacted with bacteria leading to bacterial killing, whereas the LRR domains bearing the CD-associated mutation L1007fsinsC lacked antibacterial activity (13). Moreover, NOD2 exhibits additional antibacterial effects by interacting with various proteins, which have been implicated in bacterial clearance. Of these, ATG16L1, a protein involved in antibacterial autophagy ("xenophagy"), was shown to interact with NOD2 and to cooperatively mediate pathogen defense in intestinal epithelial cells (14–16). This is of interest since variants in ATG16L1 are associated with CD (17) and combination of disease-associated alleles of ATG16L1 and NOD2 are assumed to synergistically increase susceptibility for CD (18, 19). Moreover, NOD2 was shown to interact with both components and catalytic proteins of ROS-producing enzymes. ROS production is an integral part of the innate host defense system, and inflammatory responses at mucosal surfaces include moderate (activation of signaling cascades) to excessive (bacterial killing due to oxidative burst) formation of ROS. Intestinal epithelial cells express members of the ROS-generating NADPH-oxidase complex (20) and MDP induces ROS formation (21, 22). NOD2 was shown to interact with the structural NADPH-oxidase component Rac1 (23, 24) and with the DUOX family member DUOX2 (22). Another important facet in the regulation of NOD2 signaling is the specific localization within the intestinal epithelial cell. Despite its intracellular localization, NOD2 can shuttle to the basolateral plasma membrane upon activation (25–27). Moreover, NOD2-mediated cytokine release and defensin production are specifically induced from a membrane complex including Erbin and FRMPD2 from the basolateral side (28).

A link between NOD2 and intestinal mucus production has been established with the discovery that NOD2 interacts with GALNT2 (polypeptide *N*-acetylgalactosaminyltransferase 2), a regulator of mucin biosynthesis. A defect in GALNT2 function due to impaired NOD2 interaction might therefore alter mucin production and hence contribute to CD susceptibility (29).

NOD2 signaling leads to activation of NF-κB and subsequent induction of diverse antimicrobial peptides and proteins like HNP-1 (30), β-defensin-2 (28, 31), and DMBT1 (32), a Scavenger Receptor Cysteine-Rich (SRCR) domain-containing protein, which interacts with and agglutinates several Gram-negative and Gram-positive bacteria [reviewed in Ref. (33)]. Since patients with ileal CD exhibit reduced levels of Paneth cell derived α-defensins HD-5 and -6 (34, 35) and NOD2 is constitutively expressed by Paneth cells (36), several studies investigated an underlying causal role for NOD2. However, contradictory results exist. Whereas

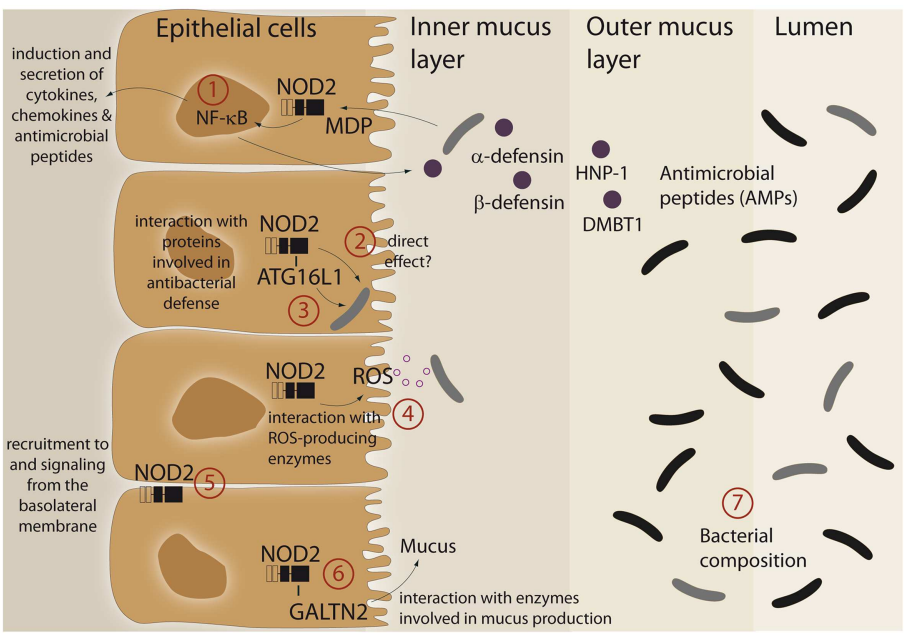

FIGURE 1 | Schematic representation of different functional aspects of the NLR family member NOD2 at the epithelial barrier. (1) Recognition of MDP leads to a complex protective gene expression program including the induction of antimicrobial peptides and cytokines. (2, 3) NOD2 has been shown to interact with the autophagic pathway and may direct xenophagy in a direct manner. A direct antibacterial effect of NOD2 itself has been postulated. (4) There is interaction between NOD2 activation and ROS-generating enzyme complexes that may have an effect on intestinal bacteria. (5) A complex machinery regulates the presence of NOD2 at the plasma membrane (e.g., Erbin and FRMPD2) which may modulate the ability to recognize and act against invasive bacteria. (6) A recent study has shown that NOD2 is involved in mucus generation via modulation of GALNT2. For further details see main text. (7) All aforementioned factors may causally contribute to the reported differences in microbiome composition. For further details see text.

patients carrying NOD2 polymorphism had greater reduction of α-defensins (37, 38), no genotype-dependent correlation was found in another study (39). Similarly, conflicting data exists from transgenic mouse models. Nod2-deficient mice displayed reduced mRNA expression of α-defensins compared to wild-type mice (40). These results were challenged by the recent finding that NOD2 knockout mice that were co-housed with their wild-type littermate had equivalent α-defensin profiles and identical antimicrobial activity against commensal and pathogenic bacterial strains (41). Moreover, NOD2-deficient mice were not impaired in Paneth cell numbers compared to wild-type animals (42). Thus, further work needs to clarify the role of NOD2 in regulating mouse α-defensin status (42).

EFFECTS ON MICROBIAL COMPOSITION

It has been shown that NOD2 is involved in recognition and defense against various intestinal pathogens, including *Helicobacter pylori* (43), *Helicobacter hepaticus* (44), *Citrobacter* (45), *Salmonella typhimurium* (46), *E. coli* (47), and *Listeria monocytogenes* (40, 48). In CD-affected humans, the link between NOD2 status and intestinal dysbiosis has been confirmed in disease patients homozygous for the NOD2 L1007fsinsC mutation. Tissue-attached microbiota from ileal biopsies exhibited higher loads of *Bacteroidetes*, *Firmicutes*, and *Bacteroides* compared to healthy controls. In fecal samples, a similar pattern was observed, however differences were not statistically significant (49). Another study that incorporated CD patients mutated in one of the three major risk alleles (R702W, G908R, and L1007fsinsC) confirmed that genotype and disease phenotype are associated with shifts in their intestinal microbial compositions (50). Nevertheless, NOD2-deficient mice do not develop spontaneous colitis when kept under specific pathogen free (SPF) conditions. With the advent of next-generation sequencing, it has become possible to take an in-depth snapshot of the intestinal bacterial ecosystem and to delineate microbial community structures and composition at the species level. However, considerable differences between published studies exist concerning animal housing and breeding (e.g., hygiene status of animal facility, genetic background, caging effects, use of F2 littermates, or separated WT/knockout strains) study design (age, sex, intestinal sampling location) and sequencing methods (DNA extraction, sequencing, and data analysis). Despite these differences, several independent groups reported that NOD2 status is associated with alterations in the intestinal microbial composition and density (summarized in **Figure 2**) (47, 49, 51, 52). Increased abundance of members of the phylum *Bacteroidetes* was detected in weaning mice and persisted throughout development (49). In line with this, RIPK2-deficient mice displayed increased levels of *Bacteroides* and *Firmicutes* arguing for a RIK2-dependency (47). Greater fecal abundances within the *Alistipes* and *Bacteroides* but an underrepresentation of *Prevotellacea* along with a decreased diversity and richness in the microbiota was found in NOD2$^{-/-}$ compared to WT mice (51). Recently, another aspect of the complex host genotype-microbe interaction was highlighted. Wild-type mice that received disease-predisposing bacterial communities from NOD2 or RIPK2-deficient mice via co-housing or cross-fostering experiments suffered from

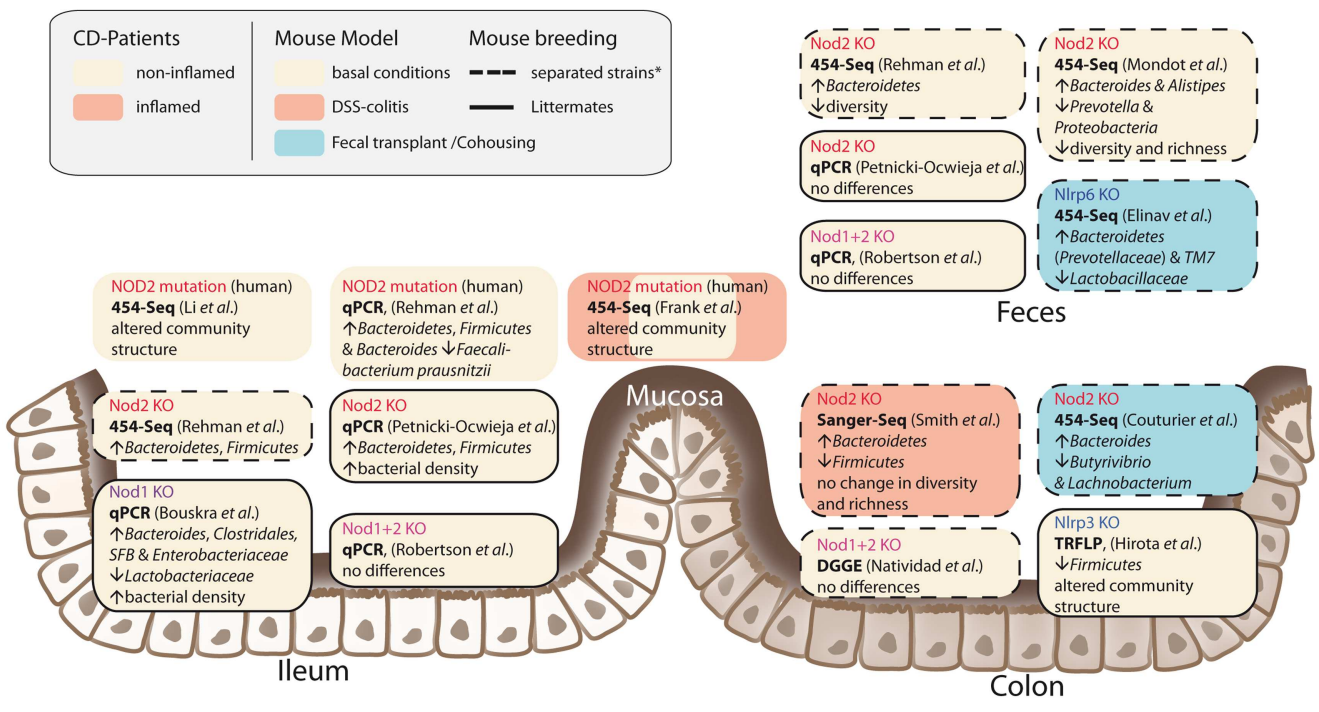

FIGURE 2 | Influences of NLRs on intestinal microbial community structures. The figure summarizes recent studies in humans and mice and depicts different approaches and animal breeding schemes. The asterisk denotes the fact that behind the term separated breeding a variety of strategies is conjoined. For further discussion see main text.

increased susceptibility to DSS-induced colitis and colitis-associated carcinogenesis. Reciprocal microbiota transplantation from wild-type donors reduced disease risk in NOD2-deficient mice (53). However, two recent studies reported only minimal differences in gut microbial composition of co-housed, littermate controlled NOD2-deficient, and wild-type mice (41, 54). The latter one showed that shifts in bacterial communities were independent of genotype and correlated with housing conditions (54). In light of the findings from recent co-housing experiments with NOD2 and other NLRs [e.g., Ref. (53, 55)] this might be partly explained by the restoration of disturbed microbiota due to animal co-housing, however, more studies are needed to fully understand the interference of NOD2 with host-microbe interactions.

NOD ALL NODs ARE CREATED EQUAL – LESSONS FROM NOD1

NOD1 and 2 share similar structural composition, detection of peptidoglycan moieties (iE-DAP/NOD1, MDP/NOD2), and downstream signaling pathways, including RIPK2 and NF-κB activation. In contrast to NOD2, the association between genetic variants in the *NOD1* gene and susceptibility to IBD is less evident. While some studies identified *NOD1* as a risk factor for IBD in some studies (56, 57) this has not been widely replicated (58–60) including a recent meta-analysis (61).

Nevertheless, there is evidence that NOD1-mediated innate immune responses are critically involved in maintaining intestinal homeostasis. Depletion of intestinal microbiota was associated with impaired neutrophil function, which was reversed by administration of NOD1 ligand in the drinking water of mice (62). Moreover, NOD1-deficiency leads to increased susceptibility to *H. pylori* infection (63), impaired clearance of *Clostridium difficile* in the intestine, increased bacterial translocation (64), and enhanced colitis-associated colon tumor formation (65). The NOD1-mediated recognition of peptidoglycan was necessary to induce genesis of isolated lymphoid follicles (ILFs) in the intestine, which in turn influenced the composition of the intestinal bacterial community. In NOD1-deficient mice, the total bacterial population was expanded 100-fold, which was largely due to the groups of *Clostridiales*, *Bacteroides*, and *Enterobacteriaceae* (66). Furthermore, lack of NOD1 led to deficiencies in intestinal barrier integrity reflected by lower expression levels of NOD2, Muc2, α- and β-defensin, and keratinocyte-derived chemokine (KC) as compared to their F2 littermates (54). In line with this, the combined knockout of NOD1 and 2 led to increased paracellular permeability, decreased levels of E-cadherin, and lower colonic antimicrobial RegIII-γ expression in comparison to littermate control mice (67). Nevertheless, both studies did not find genotype-specific differences in the relative abundance of intestinal bacteria (54, 67).Thus, as previously pointed out for NOD2, the impact of breeding strategies and housing conditions may strongly interfere with study results and yet it is still too early to draw final conclusions about the role of NOD1 in physiological and pathological host-microbe interactions in the intestine.

IL-1β – THE ROLE OF INFLAMMASOME-TYPE NLRs IN THE INTESTINE

Several NLRs form multimeric complexes termed "inflammasomes" that serve as molecular platforms for caspase-1 activation and processing of pro-IL-1-like cytokines into their active forms (68). Until now, this group comprises the NLRPs (NLRs with PYRIN domain) NLRP3, NLRP6, NLRP1, NLRP12, NLRP7, and NLRC4 (69, 70). Although no variants in inflammasome forming NLR genes are among the 163 IBD susceptibility loci (71), their relevance for intestinal health has been shown by various inflammasome-deficient mice in models of intestinal inflammation, as reviewed in Ref. (72). However, in comparison to mice deficient for Nlrp3, Nlrp10, Nlrp12, and Nlrc4, Nlrp6 showed the largest potential to alter microbiota and colitis susceptibility of co-housed mice. In the following paragraph we will therefore focus on the role of Nlrp6 in intestinal host-microbe interactions.

Components of the Nlrp6 inflammasome are expressed in intestinal epithelial cells (73) and throughout the intestinal tract (55), and several studies have demonstrated a protective role of Nlrp6 against colitis and colitis-associated tumor formation (55, 73–75) [reviewed in Ref. (76)]. Importantly, *Nlrp6*-deficiency was demonstrated to significantly alter intestinal microbiota composition (55). On the genus level, *Prevotellaceae* (belonging to the *Bacteroidetes* phylum) were strongly increased, whereas *Lactobacilli* (*Firmicutes* phylum) were decreased. In addition, members of the phylum of TM7, which were highly abundant in *Nlrp6*-deficient mice, have been found to be overrepresented in CD patients (77). Likewise, *Prevotellaceae* were more prominent in the mucosa tissues of patients with UC compared to healthy individuals (78). The distinct bacterial composition of *Nlrp6*-knockout mice was transmissible to co-housed adult mice and cross-fostered litters and resulted in colitis-prone phenotype of recipient wild-type mice. Similarly, mice deficient in the inflammasome adaptor Asc harbored a colitogenic gut microbiota that was transmissible to co-housed WT mice (79). Wild-type mice exhibited increased colonic Il-6 levels compared to single-housed wild-type mice when they were co-housed with either *Nlrp6*- or *Asc*-deficient mice. Of note, the microbiota-mediated transmissible cell proliferation and tumor formation were abrogated when either a neutralizing anti-IL-6 receptor antibody was administered or intestinal IL-6 receptor was conditionally deleted in intestinal epithelial cells. Recently, the role of Nlrp6 for colonic health was extended to the small intestine (80). In a mouse model for small-bowel inflammation, stress-mediated release of corticotropin-releasing hormone (CRH) inhibited intestinal *Nlrp6* (but not *Nlrp3*) expression and altered the composition of the intestinal microbiota, which ultimately led to intestinal inflammation (80). Together, these studies point to a critical role for the NLR-forming inflammasomes, in particular Nlrp6, in modulating intestinal homeostasis via an influence on microbiota composition. It must be emphasized that anti-IL-1 treatment, despite having an effect in DSS colitis, lacks efficacy in IBD. With the above knowledge in mind, this field is now at a point where the translation into the human situation is desperately needed.

CONCLUSION

We are beginning to realize that maintaining the long-term stability of the co-evolved human gut microbe communities is an important mechanism for maintaining human health. The ecology of intestinal microbiota is not only necessary for digestion of nutrients and the delivery of local metabolites (e.g., butyrate)

to intestinal epithelial cells but also critically shapes immune responses of the host. An important fact for future studies will be to delineate how this interaction licenses migratory immune cells for functions in other organ systems such as the brain. It must be emphasized that most of our knowledge of the role of NLRs for this symbiotic relationship originates from animal studies in rodents and that there are also conflicting results in terms of the extent of the influence of single NLRs in this context. Beyond the biology of NLRs, these results teach us two things: (i) we have to reassess how we set up our immunological animal models in the future. From findings that susceptibility to provocation models may be transmissible by genetically determined microbiota to wild-type animals, it is clear that a regular F2 intercross with littermate housing may not be an ideal scenario. On the other hand, drift in microbiota in separated lines over generations may exert bigger effects than the actual genotype. This is a dilemma we have to solve. (ii) We have to be careful how we interpret the findings. In the end only the transfer into the human situation will help us to understand the factual influence of microbiota on traits in health and disease.

Taken together, NLRs represent watchful guardians at the intestinal barrier, which help to maintain immunological homeostasis in an organ system facing strong environmental influences. This environment has changed drastically over the past 100 years and some NLR family members are clearly involved in chronic inflammatory diseases associated with this lifestyle. Decoding the exact molecular signals of NLRs that contribute to the resilience of microbial communities on mucosal surfaces may provide approaches to prevent or ameliorate this range of human diseases. More than 10 years into NLR research we are still far away from understanding how these molecules actually exert their function and how we can target them in therapy.

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Comparative genomic and sequence analysis provides insight into the molecular functionality of NOD1 and NOD2

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Amino acids with functional or key structural roles display higher degrees of conservation through evolution. The comparative analysis of protein sequences from multiple species and/or between homologous proteins can be highly informative in the identification of key structural and functional residues. Residues which in turn provide insight into the molecular mechanisms of protein function. We have explored the genomic and amino acid conservation of the prototypic innate immune genes NOD1 and NOD2. NOD1 orthologs were found in all vertebrate species analyzed, whilst NOD2 was absent from the genomes of avian, reptilian and amphibian species. Evolutionary trace analysis was used to identify highly conserved regions of NOD1 and NOD2 across multiple species. Consistent with the known functions of NOD1 and NOD2 highly conserved patches were identified that matched the Walker A and B motifs and provided interaction surfaces for the adaptor protein RIP2. Other patches of high conservation reflect key structural functions as predicted by homology models. In addition, the pattern of residue conservation within the leucine-rich repeat (LRR) region of NOD1 and NOD2 is indicative of a conserved mechanism of ligand recognition involving the concave surface of the LRRs.

Keywords: NLR, NOD1/NOD2, comparative biology, evolutionary tracing, CARD, innate immunity, LRR

INTRODUCTION

NOD1 and NOD2 are prototypical members of the NLR family of cytosolic pattern recognition receptors and the human and murine proteins have been widely studied. Both receptors respond to different fragments of bacterial peptidoglycan, most likely through direct binding (1–3) although further confirmation of this is required (4). In the absence of ligand the C-terminal leucine-rich repeat (LRR) region contributes to autoinhibition, a state maintained by interaction with chaperone proteins including Hsp90 and SGT1 (5, 6). Exposure to ligand results in conformational rearrangement that permits receptor self-association and nucleotide binding via highly conserved amino acid motifs in the central NOD (or NACHT) region (7). This is coupled with migration to the plasma membrane and caspase activation and recruitment domain (CARD) mediated interaction with the adaptor protein RIP2 and/or CARD9. The net effect is to initiate a pro-inflammatory response mediated by NFκB and stress-kinase activated genes.

The functionality of NOD1 and NOD2 has been well characterized. Despite this we still have a limited understanding of the molecular basis of receptor function. At the amino acid level: changes in NOD2 can lead to an increased susceptibility to inflammatory disorders such as Crohn's disease or cause conditions like Blau Syndrome (8–10); variation in the LRR of NOD1 explains the preferential recognition of tripeptide and tetrapeptide diaminopimelic acid containing peptidoglycan fragments by human and murine NOD1 respectively (11, 12); the C-terminus of NOD2 is important for membrane localization (13); and that specific patches are involved in RIP2 interaction (14, 15), Ubiquitin binding (16), and nucleotide binding and hydrolysis (7).

Amino acids that show high levels of conservation across multiple orthologs or homologs are indicative of residues with important structural or functional roles (17). Consequently comparative sequence analysis can be highly informative in the identification of functionally important residues. We have compared the amino acid sequences of NOD1 and NOD2 across vertebrate species in order to gain a greater understanding of the key functional regions of both proteins. Key functional patches, for example those involved in RIP2 binding and nucleotide binding and hydrolysis show strong, or even complete, conservation across species. Recognition of ligand is likely to be mechanistically conserved between both NOD1 and NOD2 and located on the concave surface of the LRR region and we provide further evidence for the importance of the C-terminus of NOD1 and NOD2 in the function and localization of the receptor.

MATERIALS AND METHODS

BIOINFORMATICS, DATABASE SEARCHING, AND EVOLUTIONARY TRACING

The reference sequences for human NOD1 (NP_006083.1) and human NOD2 (NP_071445.1) were used as search terms to retrieve orthologous protein sequences from the NCBI protein database. Sequences with at least 95% sequence coverage were retained and collated in FASTA format. Sequences were aligned using MUSCLE (18) and then manually refined to remove incomplete and partial sequences. The resulting alignments were subjected to evolutionary tracing using TraceSuite II (19). Consensus sequence images were generated using WebLogo v3.3 (20). NetSurfP was used to predict the surface accessibility of individual amino acids (21). All molecular structure images were

created using the PyMOL Molecular Graphics System, v1.5.0.5 Schrödinger, LLC.

To perform a pairwise comparison between the eight terminal LRRs in NOD1 and NOD2 we manually identified the relevant LRR sequences from the human, chimpanzee, mouse, cow, elephant, platypus, and coelacanth proteins. The number of identical residues between each possible pair of repeats where one repeat is from NOD1 and one repeat is from NOD2 was determined and these values averaged. The average values were tabulated and color coded on a sliding scale from green (most similar) to red (least similar).

HOMOLOGY MODELING

Homology models were built using Modeller v9.8 with the following templates: NOD2 CARD1 – ICEBERG CARD [1DGN; (22)]; NOD2 CARD1– NOD1 CARD (2DBD); NOD1 and NOD2 LRRs – porcine ribonuclease inhibitor LRRs [2BNH; (23)]. Models were refined and the stereochemistry verified using PROCHECK (24).

PLASMIDS

pUNO-NOD1 and pCMV-NOD2 (25) produce full-length untagged NOD1 and an N-terminally FLAG tagged NOD2 respectively; pLuc and phrG (Promega) encode Firefly and Renilla luciferase. Mutant constructs were generated by site-directed mutagenesis.

LUCIFERASE REPORTER ASSAYS

HEK293 cells were maintained in DMEM (Sigma) supplemented with 10% FCS, 100 μg/ml Penicillin/Streptomycin and 2 mM L-glutamine at 37°C and 5% CO_2. Assays were performed in 96-well plates and using jetPEI (Polyplus Transfection) cells were transfected with 0.1 ng of NOD1/2 DNA and 1 ng of pLuc and phrG in each well. Cells were stimulated with specified concentrations of iE-DAP, muramyl dipeptide, or iE-Lys (all Invivogen), concomitant with DNA transfection. Cells were lysed 24 h post transfection with 1 × passive lysis buffer (Promega) and luminescence measured with a LUMIstar Luminometer (BMG Labtech). Protein expression was checked 24 h after transfection of HEK293 cells with 3 μg/DNA per well in a six-well plate without ligand stimulation. Proteins were detected with either monoclonal anti-FLAG (Sigma) or the NOD1 monoclonal 2A10 (26).

SUBCELLULAR FRACTIONATION

Membrane and cytosolic fractionation of transfected HEK293 cells was performed using a Subcellular Fractionation Kit (Pierce) as per the manufacturer's instructions. An antibody against GAPDH (Abcam) was used to characterize cytosolic fractions.

RESULTS

NOD1 AND NOD2 POSSESS DIFFERENT EVOLUTIONARY PATTERNS

Orthologs of human NOD1 and NOD2 were retrieved from the NCBI protein database. NOD1 orthologs were found in a wide range of mammalian species as well as birds, amphibians, and fish. Consistent with previous reports NOD2 was widely present in mammals and fish, but absent from avian and amphibian genomes (27). No reptilian orthologs were recovered for either protein. Given the otherwise ubiquitous pattern of NOD1 possession across vertebrates we examined the genome of the reptilian anole lizard in release 71 of the ENSEMBL genome database. This approach successfully identified NOD1 in the anole lizard, but revealed no evidence of a NOD2 ortholog (**Figure 1**; **Table 1**). Comparing the syntenic positions of NOD1 and NOD2 in a range of vertebrates confirmed the absence of NOD2 from reptiles (**Figure 1**; **Table 1**).

A closer examination of the syntenic position of *Nod1* indicated that for all species investigated, except the frog, *Nod1* was located between *Znrf2* (zinc and ring finger 2) and *Ggct* (gamma-glutamylcyclotransferase). All three genes either side of *Nod1* are strongly conserved, particularly between mammals (**Figure 1A**). The syntenic position of *Nod2* showed even greater conservation across mammals, sharing positions with *Brd7* (bromodomain containing 7), *Nkd1* (naked cuticle homolog 1), *Snx20* (sorting nexin 20), *Cyld* (ubiquitin carboxyl-terminal hydrolase (sometimes referred to as cylindromatosis), and *Sall1* (sal-like 1). The syntenic position is maintained in zebrafish except that *Snx20* has been lost. The chicken and anole lizard retained the whole genomic cluster except for *Nod2*; whilst in the frog only *Sall1* and *Clyd* are located together (**Figure 1B**). Performing a whole genome BLAST search and screening the expression sequence tag database did not detect *Nod2* in any of these organisms, nor in the Zebra Finch or Turkey. This indicates that in birds, reptiles, and amphibians the *Nod2* gene has been specifically lost.

MAPPING KEY RESIDUES IN NOD1 AND NOD2 BY CROSS-SPECIES COMPARISONS

NOD1 and NOD2 amino acid sequences were aligned and evolutionary tracing was used to examine the amino acid conservation at two levels. The first level consisted of residues completely conserved across all vertebrate species. The second level represented residues completely conserved in mammals, but not across all of the non-mammalian sequences. The patterns of conservation are summarized on the human NOD1 (**Figure 2**) and NOD2 (**Figure 3**) amino acid sequences.

Levels of cross-species amino acid conservation were highly similar for NOD1 and NOD2 (**Table 2**). Conserved residues were broadly dispersed across both protein sequences with denser, more focused, patches seen in the CARD, NACHT, and LRR domains (**Figures 2** and **3**). These included motifs of known function such as the RIP2 binding patch in the NOD1 CARD; the Walker A, Walker B, and Sensor 1 motifs crucial for nucleotide binding and hydrolysis in the NACHT; and the LRR consensus repeats (7, 14, 28). In NOD2 the first 27 residues, the C-terminal region of CARD1 and also the linker portion between the end of the winged-helix domain and the start of the regulatory region showed particularly low patterns of conservation (**Figure 3**). The second CARD of NOD2 and three sections of the NOD2 LRRs – A794-Y821, N872-F903, E962-S991 – showed strong conservation across mammals, but not when piscine NOD2 was included.

NOD1 and NOD2 show varying degrees of conservation of the protein interaction motif LxxLL, a motif commonly found in nuclear receptors. Two LxxLL motifs, beginning at L314 and L592, are completely conserved across all species of NOD1 (**Figure 2**).

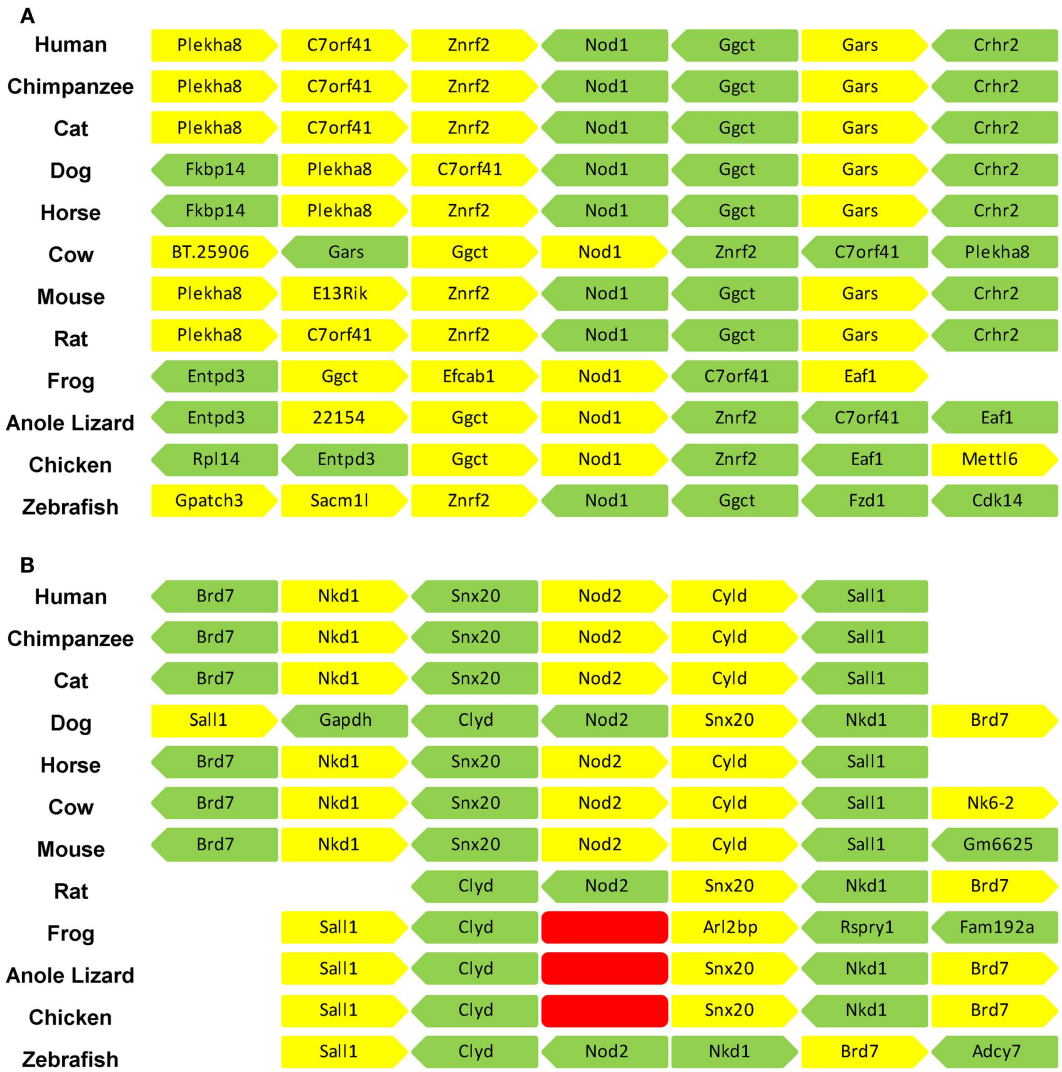

FIGURE 1 | The syntenic positions of *Nod1* and *Nod2* are highly conserved. The syntenic position of **(A)** *Nod1* and **(B)** *Nod2* were compared in 12 different vertebrate species. The three adjacent genes upstream and downstream of *Nod1/2* are displayed. Each gene is represented by an individual block with yellow denoting a position on the forward strand and green a position on the reverse strand. A space indicates insufficient information to definitively identify the gene in that location. The red blocks in **(B)** indicate the absence of the *Nod2* gene from the frog, anole lizard, and chicken genomes. Gene identities are as follows: *Plekha8* – pleckstrin homology domain containing, family A (phosphoinositide binding specific) member 8; *C7orf41* – chromosome seven open reading frame 41; *znrf2* – zinc and ring finger 2; *Nod1* – nucleotide-binding oligomerization domain containing 1; *Ggct* – gamma-glutamylcyclotransferase; *Gars* – glycyl-tRNA synthetase; *Crhr2* – corticotrophin-releasing hormone receptor 2; *Fkbp14* – FK506 binding protein 14; *BT.25096* – corticotrophin-releasing factor receptor 2; *E13Rik* – RIKEN cDNA 241066E13 gene; *Entpd3* – ectonucleoside triphosphate diphosphohydrolase 3; Efcab1 – EF-hand calcium binding protein; *Eaf1* – ELL-associated factor 1-like; *Rpl14* – ribosomal protein L14; *Mettl6* – methyltransferase-like protein 6; *Gpatch3* – G patch domain containing 3; *Sacm1l* – SAC1 suppressor of actin mutations 1-like; *Fzd1* – frizzled homolog 1; *Cdk14* – cyclin-dependent kinase 14; *Brd7* – bromodomain containing 7; *Nkd1* – naked cuticle homolog 1; *Snx20* – sorting nexin 20; *Nod2* – nucleotide-binding oligomerization domain containing 1; *Cyld* – ubiquitin carboxyl-terminal hydrolase; *Sall1* – sal-like 1; *Gapdh* – glyceraldehydes 3-phosphate dehydrogenase; *Nkx6-2* – uncharacterized protein; *Gm6625* – protein Gm6625; *Arl2bp* – ADP-ribosylation factor-like 2 binding protein; *Rspry1* – ring finger and SPRY domain containing 1; *Fam192a* – family with sequence similarity 192, member A; *Adcy7* – adenylate cyclase 7.

Human NOD2 contains four LxxLL motifs starting at residues L57, L407, L554, and L678. The second of these, L_{407}xxLL, is in the NACHT domain and correlates with the NOD1 motif beginning at L314. Unlike NOD1, none of the NOD2 LxxLL motifs are completely conserved across all species. However, L_{407}FNLL is conserved across mammals.

DIFFERENT BIOLOGICAL PROCESSES MERIT DIFFERENT LEVELS OF RESIDUE CONSERVATION

Manon and colleagues (14) previously identified acidic residues in the NOD1 CARD (E53, D54, E56) as crucial for interaction with RIP2. E53 and E54 are completely conserved across all species (**Figures 2** and **4A**), whereas E56 is completely conserved only in

Table 1 | Chromosomal position and ENSEMBL identifier for *Nod1* and *Nod2* across diverse vertebrate species; n.d., not described.

Species	*Nod1*		*Nod2*	
	Chromosome	**ENSEMBL ID**	**Chromosome**	**ENSEMBL ID**
Human	7	ENSG00000106100	16	ENSG00000167207
Chimpanzee	7	ENSPTRG00000019040	16	ENSPTRG00000008106
Cat	A2	ENSFCAG00000012184	E2	ENSFCAG00000008505
Dog	14	ENSCAFG00000003074	2	ENSCAFG00000009818
Horse	4	ENSECAG00000013825	3	ENSECAG00000017005
Cow	4	ENSBTAG00000038235	18	ENSBTAG00000020936
Mouse	6	ENSMUSG00000038058	8	ENSMUSG00000055994
Rat	4	ENSRNOG00000010629	19	ENSRNOG00000014124
Frog	n.d.	ENSXETG00000022012	–	–
Anole lizard	6	ENSACAG00000002919	–	–
Chicken	2	ENSGALG00000011535	–	–
Zebrafish	16	ENSDARG00000036308	7	ENSDARG00000010756

mammals. Closer inspection of the individual sequences shows that only the fish *Takifugu rubripes* differs at this position, possessing a highly conservative aspartic acid substitution. The role of these residues in NOD1 signaling was previously investigated using charge-reversal mutations (14). In order to avoid the potential influence of charge-repulsion effects due to the introduction of a positive charge we instead mutated each residue to alanine and tested their ability to activate NFκB-mediated signaling in response to ligand stimulation. The critical nature of E53 and D54 for NOD1 function was confirmed by the inability of either E53A or D54A to respond to ligand stimulation. E56A activity however did not differ significantly from the wild-type (**Figure 4B**). The slight reduction observed is likely due to the marginally lower expression of E56A compared to wild-type NOD1 (**Figure 4B** inset). Consequently, the impaired signaling of E56K and also its failure to interact with RIP2 (14) may be due to electrostatic repulsion, rather than indicating a critical role for E56 in RIP2 binding and NOD1 signaling.

For NOD2 two arginine residues, R38 and R86 in CARD1, are implicated in the interaction with RIP2 (15). These residues are completely conserved consistent with a crucial role in NOD2 function (**Figure 3**). We mapped R38 and R86, as well as the other completely conserved residues, onto a homology model of NOD2 CARD1 to determine if they associated to the same molecular surface. R38 and R86 were adjacent to each other and clustered with the surface-exposed residues D90 and K95, suggesting the possibility of larger electrostatic interface (**Figure 4C**). L89, which forms part of the hydrophobic core, also clustered to this region. There were fewer conserved residues in NOD2 CARD2 and these predominantly clustered to the helix 2-helix 3 loop, helix 3, and the helix 3-helix 4 loop (**Figure 4D**).

Ubiquitination is important in immune signaling. It regulates RIG-I signaling (29, 30) and is implicated in regulation of RIP2 signaling (31–33). Recently a competitive interaction between RIP2 and ubiquitin for binding to the NOD1 and NOD2 CARDs has been reported (16) with E84 and Y88 in NOD1 and I104 and L200 in NOD2 implicated as important for ubiquitin binding. E84 is completely conserved in mammals (**Figures 2** and **4E**) and only differs in five species of fish in which it is mutated to an alanine. Y88 is less well conserved, although most substitutions are for other bulky residues such as phenylalanine and histidine (**Figure 4E**). I104 and L200 occupy almost identical positions in the first and second CARD of NOD2. However, whilst L200 is completely conserved across mammals, I104 is often substituted for another hydrophobic residue (**Figure 4E**). Mapping these residues on to the structure of the NOD1 CARD and our models of the NOD2 CARDs indicated that neither NOD2 I104 nor L200 are as exposed on the molecular surface as E84 and Y88 are in NOD1 (**Figures 4A–C**). We validated this observation using NetSurfP which predicted that NOD1 E84 and Y88 are surface-exposed, but that NOD2 I104 and L200 are buried.

CONSERVATION IN THE LRRs PROVIDES INSIGHT INTO LIGAND BINDING

Consistent with the repeating modular nature of the LRRs both NOD1 and NOD2 show increased conservation in this region. This is greatest around the consensus LRR motif LxxLxLxxNxL (where L = Leu, Val, Ile, Phe; N = Asn, Cys, Ser, Thr; x = any amino acid; signature residues underlined) (**Figures 2** and **3**). We mapped the completely conserved and mammalian-conserved residues onto homology models of their respective LRR regions but chose not to annotate any of signature residues to allow a focus on functional importance (**Figure 5**). Both NOD1 and NOD2 show molecular surfaces more conserved toward the N-terminus of the LRRs and on a single lateral surface (**Figures 5A,B**).

Mutagenic studies have identified regions of the LRR important for receptor activation (**Table 3**) (12, 34, 35). Five of the seven NOD1 residues are completely conserved across all species (**Figures 2** and **5C**). H788 is predominantly found as a histidine in mammals except for the pig where it is a cysteine and the horse, elephant, West Indian manatee, Northern greater galago, nine-banded armadillo, and white rhinoceros in which it is a tyrosine. In the non-mammalian species this residue is substituted by threonine, arginine, valine, and isoleucine. E816 has previously been implicated in selectivity for preferential activation by ligands with either tripeptide or tetrapeptide stems (11, 12). Consistent with

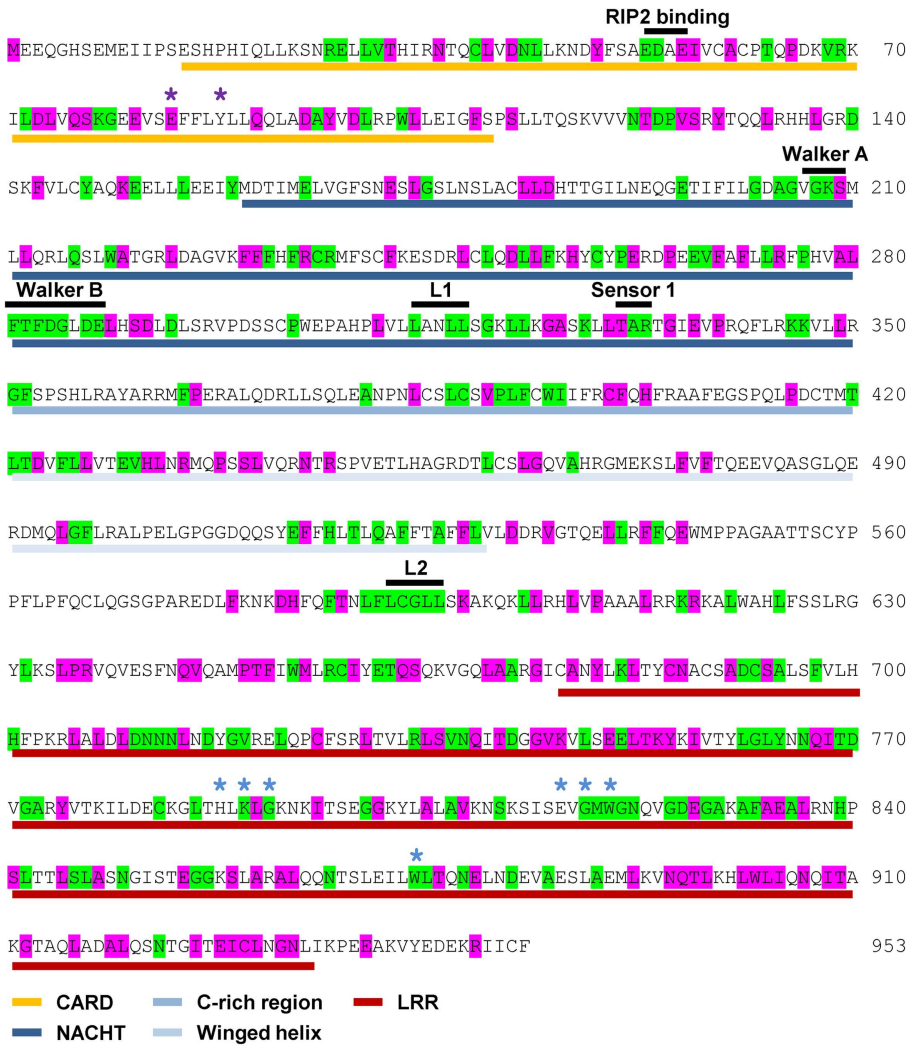

FIGURE 2 | Pattern of cross-species residue conservation in NOD1. Residues conserved across all NOD1 species checked, or just across mammals, are highlighted green and purple respectively. Residues are mapped onto the amino acid sequence for human NOD1. The domain architecture is highlighted underneath the relevant stretch of sequence as follows: CARDs – gold; NACHT – dark blue; C-rich region – blue; Winged-helix – pale blue; LRRs – red. The motifs responsible for RIP2 binding, the Walker A and B motifs and the Sensor 1 region are all labeled in black above the relevant sequence. Also labeled above the sequence are LxxLL motifs [black bars (L1, L2)], residues predicted to be important for ubiquitin binding (purple asterisks), and residues predicted to be involved in ligand recognition (blue asterisks).

a role in selectivity this residue was found as either an aspartic acid (26/53 sequences) or a glutamic acid (27/53 residues). All seven residues previously implicated in NOD2 activation are conserved across mammals, but not other species (**Figures 3** and **5D**). In the case of K989 and S991 this is due to the lack of a single LRR-encoding exon in Actinopterygii orthologs. Mapping these residues to the predicted structures revealed clustering around the edges of the concave surface of the LRR for both NOD1 and NOD2 (**Figures 5C,D**). When all conserved residues are considered the interface extends around the whole concave surface. These residues routinely appear in the second, third, fourth, and to a lesser extent fifth, variable positions in the consensus LRR motif ($Lx_1x_2Lx_3Lx_4x_5Nx_6L$) providing further support for a crucial functional role (**Table 3**).

The similar patterns of residue conservation on the concave surface of NOD1 and NOD2, and the chemical similarities in activatory ligand, led us to ask exactly how alike these regions of the two proteins are. To begin we compared the eight terminal LRRs (LRRs 3–10) from human NOD1 and NOD2 to identify identical residues on the concave surface. Apart from the LxxLxLxxNxL motif, only seven identical residues were found and only three of these – W820, G821, and S846 (NOD1); W907, G908, and S933 (NOD2) – were fully conserved in all examined species of NOD1 and all mammalian NOD2 sequences (**Figures 2** and **3**). Spatially these residues are predicted to be in close proximity and may form a binding site for the shared elements in NOD1 and NOD2 ligands (**Figure 6A**). In support of this possibility, the conserved glycine in NOD2 has been thoroughly investigated as a SNP (G908R)

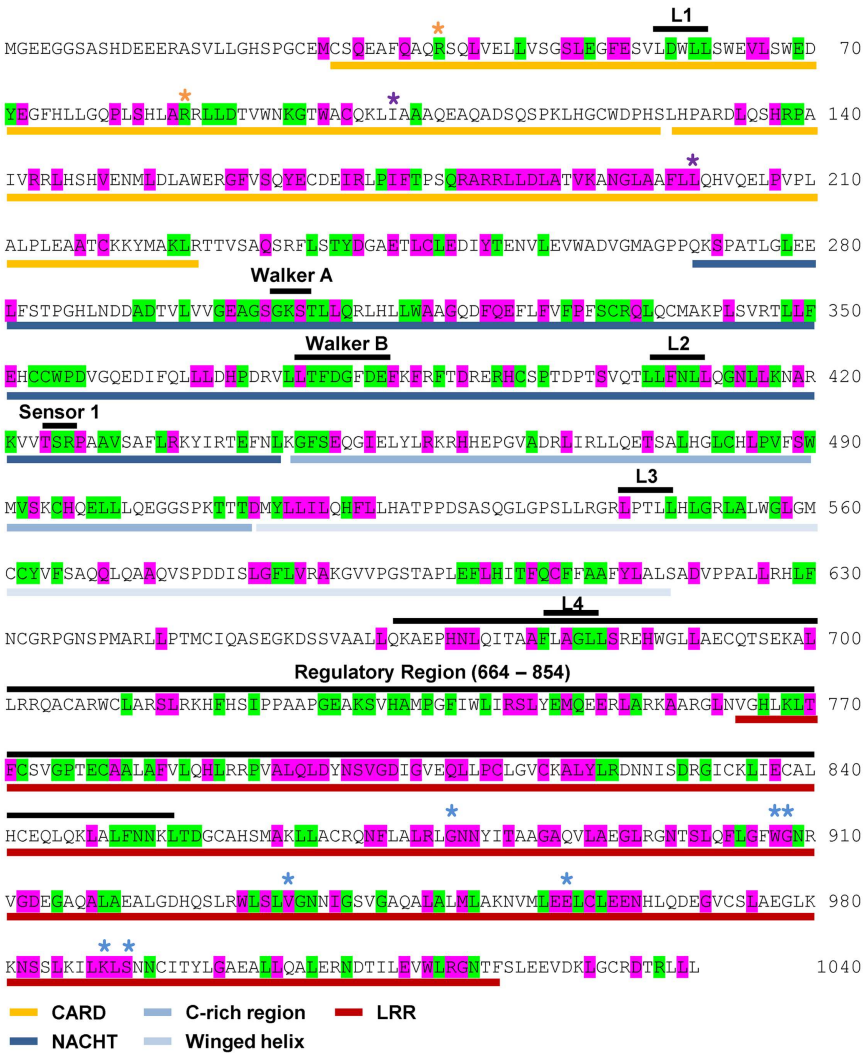

FIGURE 3 | Pattern of cross-species residue conservation in NOD2. Residues conserved across all NOD2 species checked, or just across mammals, are highlighted green and purple respectively. Residues are mapped onto the amino acid sequence for human NOD2. The domain architecture is highlighted underneath the relevant stretch of sequence as follows: CARDs – gold; NACHT – dark blue; C-rich region – blue; Winged-helix – pale blue; LRRs – red. The motifs responsible for RIP2 binding, the Walker A and B motifs, the Sensor 1 region and the regulatory region from residues 664 to 854 are all labeled in black above the relevant sequence. Also labeled above the sequence are the LxxLL motifs [black bars (L1, L2, L3, L4)], residues predicted to be important for interaction with RIP2 (yellow asterisks), ubiquitin binding (purple asterisks), and residues predicted to be involved in ligand recognition (blue asterisks).

which predisposes to Crohn's Disease and reduces the ability of NOD2 to respond to MDP. In addition, a W907L NOD2 mutant was generated by Tanabe et al. and was found to eliminate the response to NOD2 (35). The role of the conserved serine is yet to be investigated.

We accompanied the search for individual residues in the LRRs with a broad examination of repeat similarity. The eight terminal repeats are formed of 28 amino acids each, with the final repeat showing greater sequence divergence and possibly stabilizing the end of the domain in a similar way to LRR capping structures (36). We compared these eight LRRs from the human, chimpanzee, mouse, cow, elephant, platypus, and coelacanth in order to look for identical residues. LRR6 was more similar between NOD1 and NOD2 than any other set of repeats, presumably reflecting a conserved functional role (**Figure 6B**). This repeat contains the WG motif discussed above and the adjacent LRR7 contains the

Table 2 | Levels of cross-species amino acid identity for NOD1 and NOD2.

	Percentage of identical amino acids	
	All species	**Mammalian sequences**
NOD1	19.3	39.8
NOD2	18.3	37.0

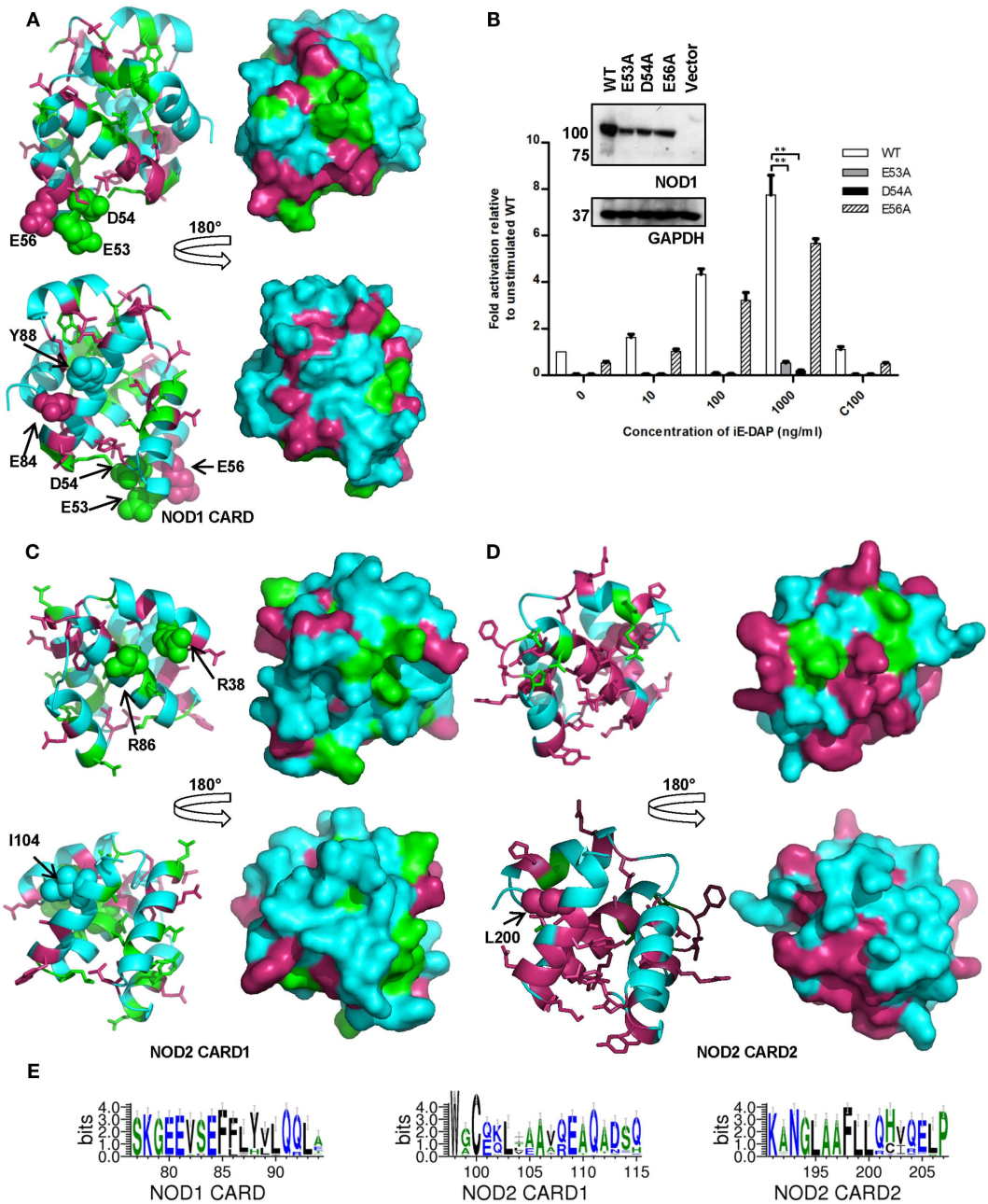

FIGURE 4 | Amino acid conservation in the NOD1 and NOD2 CARDs. Cartoon and surface representations of NOD1 CARD **(A)**, NOD2 CARD1 **(C)**, and NOD2 CARD2 **(D)** showing amino acids conserved across all species (green) and conserved across mammals (pink). In each panel the top and bottom images are related by a 180° rotation around the vertical axis. The left and right images are cartoon and surface representations of the same view respectively. Residues previously implicated in interaction with RIP2 (NOD1 – E53, D54, E56; NOD2 – R38, R36) or in the process of ubiquitination (NOD1 – E84, Y88; NOD2 – I104, L200) are labeled and presented as spheres. Conservation is mapped onto an experimental NOD1 structure (PDB ID: 2DBD) and homology models of the NOD2 CARDs. **(B)** Differential contributions to receptor activation. NFκB luciferase reporter assays were performed in HEK293 cells using wild-type (WT) NOD1, E53A, D54A, and E56A constructs. DNA (0.1 ng/well) and varying concentrations of stimulatory (i.e., DAP) or control (i.e., Lys) ligands were transfected into 96-well plates. After 24 h cells were lysed and NFκB activity determined. Results show the average of four independent experiments and $^{**}p < 0.005$. Error bars indicate SEM. Immunoblots (1.5 μg DNA/well in a six-well plate) were lysed after 24 h and probed with the specified antibodies to determine expression levels of NOD1 WT and mutant constructs. Immunoblots are representative of at least three separate experiments. **(E)** Patterns of conservation in the primary sequence observed around residues implicated in the ubiquitination of the CARDs. Residues are colored according to hydrophobicity (green – hydrophobic; blue – hydrophilic). Sequence images were generated using WebLogo 3.3 (20).

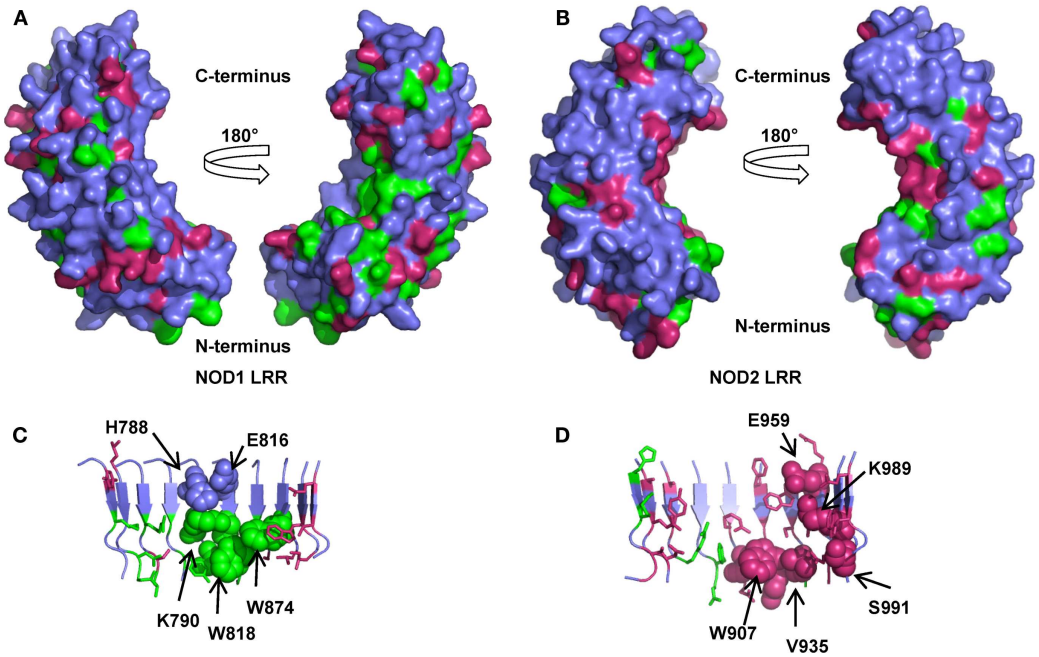

FIGURE 5 | Amino acid conservation in the NOD1 and NOD2 LRRs. Surface representations of homology models of the NOD1 **(A)** and NOD2 LRRs **(B)** showing residues conserved across species (green) or just across mammals (pink). For clarity signature residues conserved in the consensus LRR repeat LxxLxLxxNxL (signature residues L and N) are not represented. The left and right images in **(A,B)** are related by a 180° rotation around the vertical axis. Cartoon representations of the concave surface of the NOD1 **(C)** and NOD2 **(D)** LRRs highlight the spatial relationships of residues likely to be involved in ligand detection. The side chains of residues previously implicated in ligand detection and receptor activation are represented as spheres and labeled appropriately except for G792, G818 (NOD1), and G879, G908 (NOD2). Residues are colored as for **(A,B)**.

Table 3 | Residues contributing to potential ligand binding patches on NOD1 and NOD2.

	Residues previously implicated by mutagenesis[a]	Conserved residues with a potential to form part of a ligand binding interface
NOD1	H788, K790, G792, E816, G818, W820, W874	Y679, L706, D711, N712, R734, S736, V737, I757, G762, Y764, G821, S846, A848, T876, T897, W902, I904, E928, C930, G933
NOD2	G879, W907, G908, V935, E959, K989, S991	H766, K768, T770, A794, Q796, D798, A819, Y821, R823, F851, N852, R877, N880, F903, G905, W931, S933, G936, E958, C960, E962, E963, E1015, W1017

[a] *From (12, 35).*

conserved serine. Comparison across LRRs show that none of these conserved residues are commonly found in this position in multiple repeats and so are unlikely to be structurally important to the domain fold (**Figure 6C**).

DISRUPTION OF CONSERVED C-TERMINAL RESIDUES ALTERS RECEPTOR SIGNALING AND MEMBRANE LOCALIZATION

NOD1 and NOD2 are both targeted to the plasma membrane following activation (13, 26, 37, 38). The NOD2 1007fsincC Crohn's Disease susceptibility polymorphism lacks the last 33 amino acids and doesn't membrane localize (13). In fact the terminal three leucine residues appear important for localization. The final 33 amino acids of mammalian NOD1 and mammalian NOD2 show that the final LRR in both proteins is well conserved (**Figure 7A**). Outside this region residue conservation differs between the two proteins except that both human NOD1 and NOD2 have an EE motif starting 15 residues before the end of the protein, the second residue of which is conserved in mammalian NOD1 sequences. A closer examination of this motif showed that it is in fact highly conserved in NOD1 and NOD2 for most mammals. In NOD1 only the nine-banded armadillo varies in the first position, which is substituted for an aspartic acid. With NOD2 the EE motif is conserved in all mammalian sequences except the star-nosed mole in which the sequence is AD. In light of this degree of conservation we mutated both these residues, and also R1037 (conserved in NOD2 and immediately prior to the terminal LLL motif), in NOD2 to alanine and assessed the impact on receptor activation and protein localization following overexpression in HEK293 cells. All three mutants were significantly impaired in their ability to respond to muramyl dipeptide stimulation in comparison to the WT unstimulated protein (**Figure 7B**; p-values: E1026A = 0.042; E1027A = 0.025; R1037A = 0.029). However, each mutant also

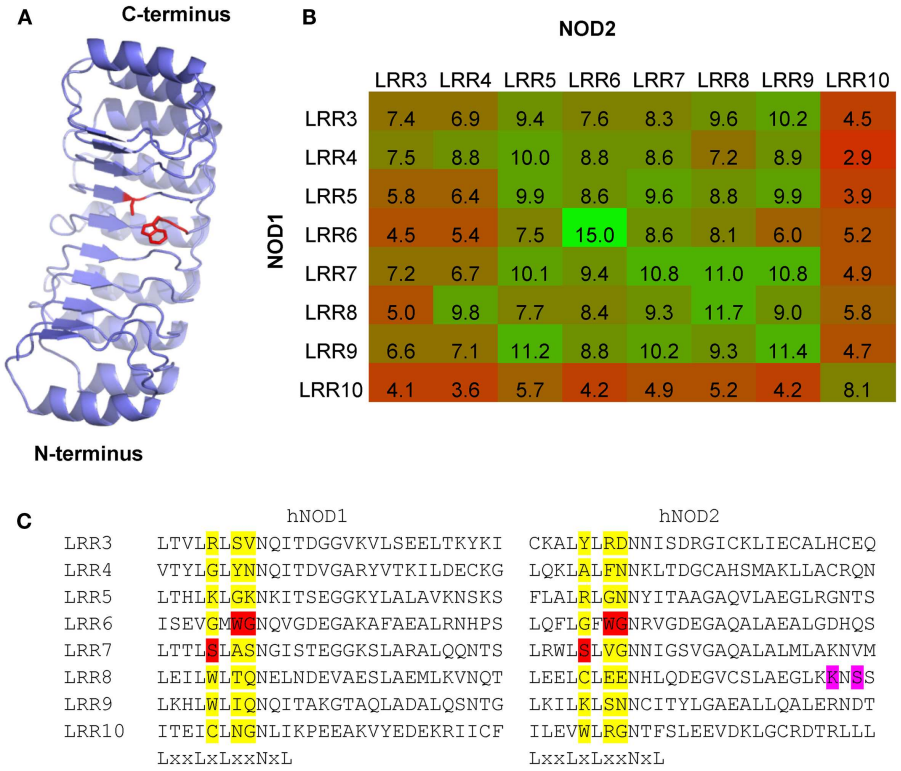

FIGURE 6 | Patterns of LRR conservation between NOD1 and NOD2 support a conserved ligand binding surface. **(A)** Other than the signature residues in the LxxLxLxxNxL motif only three residues – W820, G821, and S846 (NOD1); W907, G908, and S933 (NOD2) – are conserved across all examined species of NOD1 and all mammalian NOD2 sequences. The likely spatial position of these residues on the concave surface of NOD1 is shown. The residue sidechains are represented as red sticks. **(B)** Heat map representation of the relative similarity of the eight terminal LRR repeats in NOD1 and NOD2 from the human, chimpanzee, mouse, cow, elephant, platypus, and coelacanth. The number in each box represents the average number of identical residues in a cross-species pairwise comparison between the relevant LRR motifs. Boxes are colored on a graded scale from green (most similar) down to red (least similar). **(C)** The three residues (highlighted red) are found in the X3, X4, and X5 position of the LRR consensus motif. These positions are populated by a wide range of different amino acids (highlighted yellow). K989 and S991 (highlighted in purple), two residues in human NOD2 implicated in ligand recognition and receptor activation, are located in a region of the protein missing in the Actinopterygii due to an exon deletion.

displayed a reduction in basal signaling in the absence of MDP. This resulted in the following approximate fold-increase in signaling for each construct: WT (threefold), E1026A (fourfold), E1027A (fivefold), and R1037A (threefold). As such, none of the mutants show impairment in their relative responses to ligand stimulation. Despite their ability to still respond to MDP neither E1027A nor R1037A were recruited to the plasma membrane (**Figure 7C**).

DISCUSSION

Comparative biology has the potential to rationalize and explain experimental observations and identify potentially key functional amino acids. We have performed, to our knowledge, the first comprehensive cross-species comparative analysis of the amino acid composition of NOD1 and NOD2. Reassuringly we found that regions of NOD1 and NOD2 already reported to provide essential functional roles showed increased, or even complete, conservation across species. Most notably these related to the Walker A and B motifs in the NACHT domain, the consensus region of the LRR motifs and residues crucial for interaction with the downstream adaptor protein RIP2 in NOD1 and NOD2.

Our analysis identified conserved LxxLL motifs in NOD1 and mammalian NOD2. LxxLL motifs are routinely used in nuclear receptors and form a key part of the nuclear receptor box (39). The precise function of the LxxLL motifs in NOD1 and NOD2 is currently unknown, however, it is highly unlikely that either NOD1 or NOD2 has an as yet unidentified nuclear role. The LxxLL motif has previously been reported in NACHT domains, including those of various plant R proteins which are divergently evolved relatives of the vertebrate NLR family (40, 41). In addition, oligomerization of the NLR protein CIITA utilizes an LxxLL motif in the NACHT domain (42). It is plausible that the conserved LxxLL motifs beginning at L314 (NOD1) and L407 (NOD2) provide a similar functionality.

The pattern of residue conservation in the LRRs of NOD1 and NOD2 is highly similar and points strongly toward a conserved mechanism of ligand binding and/or recognition on the concave surface of the LRRs. The mapping of these key residues to the concave surface is consistent with earlier work (12, 35), however, we have shown here that this interface may be more extensive than previously thought. In both NOD1 and NOD2 highly conserved

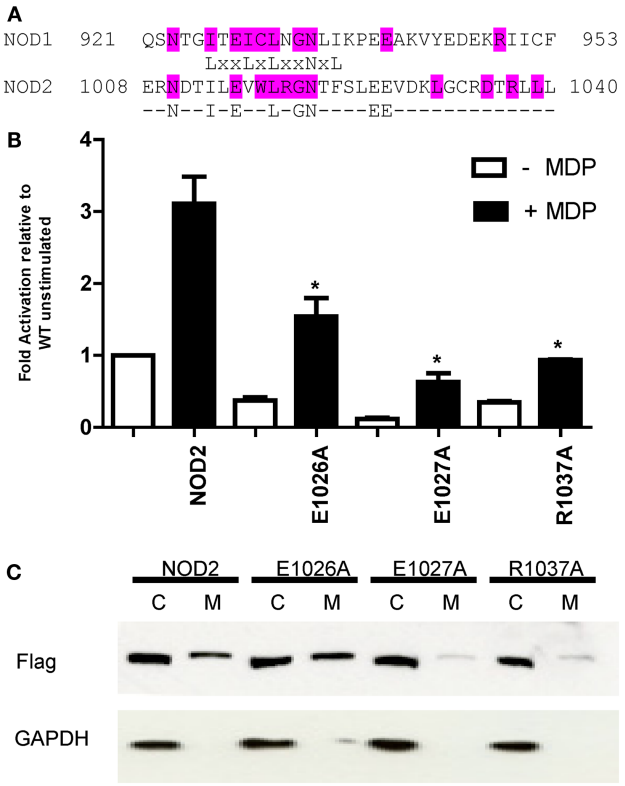

FIGURE 7 | The impact of mutation of conserved residues between the C-terminus of human NOD1 and NOD2 on receptor function. **(A)** Alignment of the terminal 33 amino acids of human NOD1 and NOD2. Residues highlighted in cyan are conserved across mammals in the relevant protein. The consensus sequence highlights residues found in the termini of both human NOD1 and NOD2. **(B)** NFκB luciferase reporter assays were performed in HEK293 cells using wild-type pCMV-NOD2 and the point mutants E1026A, E1027A, and R1037A. DNA (0.1 ng/well) was transfected into 96-well plates with (black bars) and without (white bars) muramyl dipeptide (MDP). After 24 h cells were lysed and NFκB activity determined. Results show the average of three independent experiments and $*p < 0.05$. Error bars indicate SEM. **(C)** Subcellular fractionation was performed with wild-type and mutant NOD2 constructs to separate the cytoplasmic (C) and membrane-bound (M) fractions. Proteins were identified with the specified antibodies. Blots are representative of three independent experiments.

residues increase the potential size of this interface and provide clear candidates for future mutagenesis studies.

Both NOD1 and NOD2 bind peptidoglycan fragments but discriminate between Lys-Type and diaminopimelic acid (DAP)-type muropeptides (34). This binding specificity is also seen in the Peptidoglycan Recognition Proteins (PGRPs), for which structural information has been used to identify the residues responsible for this difference in binding (43, 44). For NOD1 only the D-isoglutamyl-m-DAP moiety is required for signaling, but the presence of the preceding alanine enhances this response (34). In contrast, MDP, which consists of the MurNAc-L-alanine-D-isoglutamine segment, can signal effectively through NOD2. The similar ligands, and the similar patterns of conservation on the concave surface, suggest that the NOD1/2 ancestral gene could bind a muropeptide. Following gene duplication these binding sites evolved to permit the binding of distinct ligands by NOD1 and NOD2. We predict in NOD1 and NOD2 a mechanism similar to that of the PGRPs, where the comparable muropeptide ligands are bound in the same orientation but are told apart by their third peptide. An extra level of subtlety is displayed by the different species sensitivities of NOD1 to tripeptide and tetrapeptide stem lengths (11, 12). We have seen a clear split between the possession of either an aspartic acid or a glutamic acid residue in the equivalent position to human NOD1 E816. Indicating NOD1 has consistently evolved to respond preferentially to either tripeptide stem lengths (glutamic acid) or tetrapeptide stems (aspartic acid). Whether this is driven by exposure to particular microbiota remains unknown.

Unlike NOD1, NOD2 is not ubiquitously present in all species and the specific loss of the gene in birds, reptiles, and amphibians raises many questions about its evolutionary and functional roles. For example, what drives gene loss? Is this due to the absence of specific pathogenic threats in these populations? Interestingly multiple areas of NOD2 show strong conservation across mammals, but differ in the Actinopterygii orthologs. This is particularly noticeable in the LRRs. Actinopterygii orthologs of NOD2 are missing a single LRR-encoding exon which contains two residues which have been reported to contribute to the human MDP response, and which will alter the overall fold of the LRR. While the ability of these orthologs to respond to MDP or other muropeptides has not been investigated, it is possible in light of the NOD2 complete gene loss in birds, the anole lizard, and the frog that this function has also been lost in the actinopterygii.

The patterns of evolutionary conservation observed have increased the clarity of some functions, such as ligand-mediated activation, of NOD1 and NOD2. However, they have also raised questions of other published observations. Previously, Manon et al. reported that NOD1 E56 was essential for signaling as receptor activity was abrogated following mutation to lysine (14). The near-complete conservation of E56 across species supports an important functional role, however, mutating this residue to an alanine retains signaling, suggesting that at least some mutations are tolerated and that E56 is not absolutely critical for NOD1 signaling. Mutation of the acidic residues in the NOD1 EDAE motif will have reduced their spatial occupancy but their predominantly surface-exposed nature makes it unlikely that the native fold of the protein will have been perturbed (45). Our comparative analysis also suggests that the role of ubiquitin in NOD1 and NOD2 signaling may be more complex than previously imagined (16). The observed cross-species variation in NOD1 Y88 and NOD2 I104 suggests that the role of ubiquitin binding might differ between species; or that it is the general surface properties of this region, not the exact residues, that are important. Our homology modeling suggested that NOD2 I104 and L200 may be buried residues, mutation of which could disrupt the overall fold of the CARD. However, in the absence of a structure of the NOD2 CARDs this possibility remains theoretical and awaits experimental confirmation.

We identified a highly conserved di-acidic motif in the C-terminal region of both NOD1 and NOD2. Mutant NOD2 constructs showed reduced signaling compared to unstimulated wild-type NOD2, but also displayed a reduced basal level of activity. This resulted in the relative fold-increase for each construct being

broadly comparable to wild-type NOD2. Despite their ability to still respond to MDP neither E1027A nor the downstream R1037A were efficiently recruited to the plasma membrane. Hence membrane recruitment may not be essential for NOD2 signaling, but might contribute to maximizing the efficiency of signaling. An earlier study by Barnich and colleagues (13) showed that a double EE to GG mutation did not alter membrane localization, or significantly disrupt NFκB signaling. Coupled with our data this raises the question of what the actual role of the EE motif is. Its high level of conservation suggests an important functional role, but this has yet to be experimentally confirmed and merits further investigation.

Overall, our work highlights the applicability of comparative biology and cross-species sequence analysis toward understanding the molecular basis of innate immune receptor function. It is an approach that if more widely used could provide extensive rewards in relation to efficiency savings by readily identifying suitable targets for functional study. Furthermore, it helps to provide a rationalization for the results of mutagenic studies thereby enabling an improved understanding of innate immune function.

ACKNOWLEDGMENTS

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Functions of NOD-like receptors in human diseases

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Nucleotide-binding and oligomerization domain NOD-like receptors (NLRs) are highly conserved cytosolic pattern recognition receptors that perform critical functions in surveying the intracellular environment for the presence of infection, noxious substances, and metabolic perturbations. Sensing of these danger signals by NLRs leads to their oligomerization into large macromolecular scaffolds and the rapid deployment of effector signaling cascades to restore homeostasis. While some NLRs operate by recruiting and activating inflammatory caspases into inflammasomes, others trigger inflammation via alternative routes including the nuclear factor-κB, mitogen-activated protein kinase, and regulatory factor pathways. The critical role of NLRs in development and physiology is demonstrated by their clear implications in human diseases. Mutations in the genes encoding NLRP3 or NLRP12 lead to hereditary periodic fever syndromes, while mutations in *CARD15* that encodes NOD2 are linked to Crohn's disease or Blau's syndrome. Genome-wide association studies (GWASs) have identified a number of risk alleles encompassing NLR genes in a host of diseases including allergic rhinitis, multiple sclerosis, inflammatory bowel disease, asthma, multi-bacillary leprosy, vitiligo, early-onset menopause, and bone density loss in elderly women. Animal models have allowed the characterization of underlying effector mechanisms in a number of cases. In this review, we highlight the functions of NLRs in health and disease and discuss how the characterization of their molecular mechanisms provides new insights into therapeutic strategies for the management of inflammatory pathologies.

Keywords: NLR, inflammation, autoimmunity, IBD, polymorphisms, reproduction, innate immunity, infection

INTRODUCTION

The mammalian immune system encompasses an ancient genome-encoded innate immune system and a more recently acquired adaptive immune system capable of combating pathogens with exquisite specificity and long-term memory. The innate immune system remains a pivotal player in controlling host resistance. This system is equipped with an arsenal of pattern recognition receptors (PRRs) that translate microbial and danger sensing into immediate host defenses as well as provides signals to prime the adaptive immune response for long-lasting protection (1, 2). Nucleotide-binding and oligomerization domain (NOD)-like receptors (NLRs) are a group of evolutionarily conserved intracellular PRRs that play a vital role in innate immunity and host physiology, as reflected by their prevalence among living organisms of both the plant and animal kingdoms (3–9). In humans there are 22 known NLRs, and the association of mutations and single nucleotide polymorphisms (SNPs) in their genes with human diseases reflect their vital role in host defense. The function of NLRs is not restricted to immunity, as they also play important roles in reproduction and embryonic development (10–12).

The characteristic feature of NLRs is a central NOD (or NACHT) domain, required for oligomerization, an N-terminal homotypic protein–protein interaction domain and a C-terminal series of leucine-rich repeats (LRRs) involved in agonist sensing or ligand binding. Mammalian NLRs are sub-divided into four subfamilies based on the variation in their N-terminal domain: NLRA or Class II transactivator (CIITA) contains an acid transactivation domain, NLRBs or neuronal apoptosis inhibitor proteins (NAIPs) possess a baculovirus inhibitor of apoptosis protein repeat (BIR), NLRCs have a caspase-recruitment domain (CARD), and NLRPs a pyrin domain (PYD). NLRX1 contains a CARD-related X effector domain (**Figure 1**). Upon ligand binding, the auto-inhibitory LRR undergoes a conformational change, which exposes the N-terminal domain allowing interaction with downstream signaling adaptors or effectors and formation of an oligomeric complex (13, 14). NLR platforms that recruit and activate the inflammatory protease caspase-1 are referred to as inflammasomes. Caspase-1 is required for the processing and maturation of inflammatory cytokines IL-1β and IL-18 and the induction of an inflammatory form of cell death termed pyroptosis (15, 16). Among the NLRs, NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, and NAIP have been reported to operate via inflammasomes (**Figure 2**). Other NLRs such as NOD1, NOD2, NLRP10, NLRX1, NLRC5, and CIITA do not directly engage the inflammatory caspases, but instead activate nuclear factor-κB (NF-κB), mitogen-activated protein kinases (MAPKs), and interferon (IFN) regulatory factors (IRFs) to stimulate innate immunity. Below, we discuss the different NLRs along with their mechanisms of activation and diseases associated with defects in their activities (**Figure 3**).

INFLAMMASOME-FORMING NLRs

In 2001, the causative mutation of Muckle–Wells Syndrome (MWS), a rare autosomal recessive auto-inflammatory disease, was mapped to *NLRP3* (*CIAS1*) (17). In 2002, Tschopp and colleagues were the first to characterize the inflammasome, defining it biochemically as a complex consisting of an NLR (NLRP1), the bipartite adaptor protein ASC (which contains both a CARD and a PYD), and the two inflammatory caspases, caspases-1 and -5 (18). In 2004, the discovery of the links between the *NLRP3* mutations, NLRP3 inflammasome hyper-activation, and excessive production of IL-1β has set the stage for the use of IL-1 blockade strategies, such as recombinant IL-1 receptor antagonist (anakinra) or anti-IL-1β antibodies (canakinumab), to cure patients inflicted with hereditary periodic fever syndromes [reviewed in Ref. (19)]. Concurrently, Dixit and colleagues reported the generation of the first inflammasome knockouts, namely mice deficient in IPAF (NLRC4) or the adaptor ASC, and showed that macrophages from these mice had a defect in IL-1β production following infection with flagellated bacteria (20). As more inflammasome-forming NLRs are continuously being characterized and studied, their importance in activating immune responses and consequently in conferring host resistance is becoming evident.

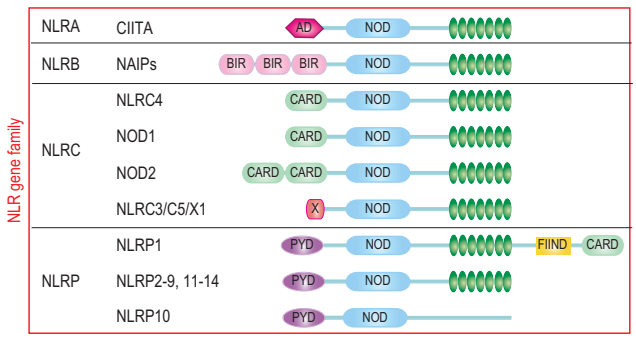


FIGURE 1 | The human NLR gene family. The human NLR gene family consists of 22 members that share a tripartite structure, consisting of an N-terminal signaling domain, a central nucleotide-binding and oligomerization domain, and a C-terminal agonist sensing/ligand-binding domain. The NLR family is sub-divided into four sub-groups NLRA, NLRB, NLRC, and NLRP based on the nature of the N-terminal domain consisting respectively of an acidic transactivation domain, a baculovirus IAP repeat (BIR), a caspase-recruitment and activation domain (CARD), and a Pyrin domain (PYD).

NLRP1

The NLRP1 protein has a unique structure amongst other NLRs. Human NLRP1 contains a PYD on the N-terminus and a CARD on the C-terminus, with ZU5 and UPA domains in the internal region which confers proteolytic activity upon the protein (21). Three murine NLRP1 homologs – Nlrp1a, Nlrp1b, and Nlrp1c – have been identified, although they lack the N-terminal PYD domain present in human NLRP1. Few ligands have been found for NLRP1 to date, and include bacterial products such as lethal toxin (LT) produced by *Bacillus anthracis* which activates murine NLRP1b (22), muramyl dipeptide (MDP), a component of bacterial peptidoglycan that activates human NLRP1; and reduced levels of cytosolic ATP (23–27).

Defects in NLRP1 have been linked to a variety of autoimmune disorders. Candidate gene analysis and Genome-wide association studies (GWAS) have shown a significant association of polymorphic variants in the extended promoter and/or coding regions of *NLRP1* with familial cases of generalized vitiligo (28, 29), celiac disease (30), Addison's disease and type 1 diabetes (31, 32), autoimmune thyroid disorders (AITDs) (33), systemic lupus erythematosus (SLE) (34), systemic sclerosis and giant cell arteritis (35, 36), congenital toxoplasmosis (37), rheumatoid

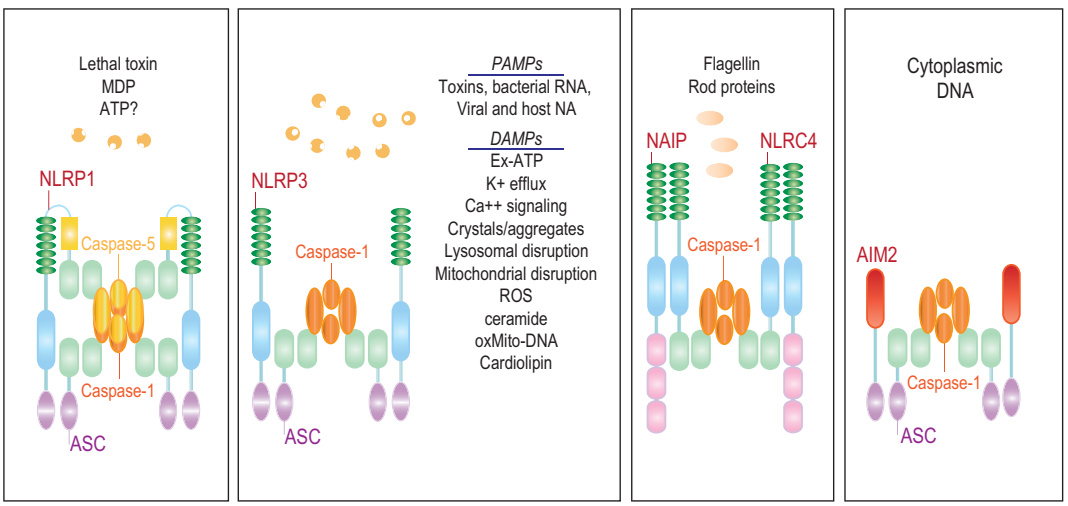


FIGURE 2 | The NLR inflammasomes. The three biochemically characterized inflammasomes are depicted. The NLRP1 inflammasome consists of NLRP1, ASC, and caspases-1 and -5. Little is known about the agonists that activate NLRP1. Anthrax lethal toxin, MDP, and decreased cytosolic ATP have been reported to stimulate this inflammasome. NAIP and NLRC4 form a caspase-1 inflammasome in response to bacterial flagellin and T3SS rod proteins. NLRP3, on the other hand, is activated by a wide range of agonists including a number of MAMPs and DAMPs.

arthritis (38), and Alzheimer's disease (39) (**Figure 3**). A novel missense mutation M77T in *NLRP1*, which destabilizes the protein, has been recently shown to cause corneal intraepithelial dyskeratosis (40).

In a recent analysis of high-risk haplotypes with substitutions in human *NLRP1*, Levandowski et al. demonstrated that peripheral blood monocytes from heterozygous carriers of the haplotype 2A (which contains three non-synonymous substitutions: L155H-V1059M-M1184V) process significantly greater amounts of pro-IL-1β into mature IL-1β under basal conditions. It was thus proposed that the enhanced production of IL-1β predisposes carriers to a wide spectrum of autoimmune diseases (41). Consistently, patients diagnosed with vitiligo commonly suffer from other autoimmune disorders such as SLE (28, 42). However, the molecular mechanisms underlying the link between *NLRP1* genetic variations and these disorders are still unknown. It is plausible that deregulation of an NLRP1 inflammasome effector function is at the basis of the autoimmunity phenotypes. This is consistent with recent results from mice. Masters et al. have recently reported that mice with an activating mutation in *Nlrp1a* exhibited increased T-cell progenitor death (pyroptosis) at the steady state, which rendered them cytopenic (43). In contrast, *Nlrp1a*-deficient mice, which may experience less pyroptosis, develop an over-exuberant immune response (43). However, while Masters et al. demonstrated that the inflammatory disease in *Nlrp1a* mutant mice was dependent on caspase-1, additional proof is needed to show that Nlrp1a formed an inflammasome complex *in vivo* (43). While anakinra has been shown to be successful in treating patients with SLE in preliminary studies, IL-1 blockade strategies have not been tested to date for other autoimmune diseases such as vitiligo or celiac disease (42, 44).

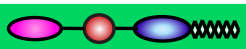
Family	Protein	Structure	Polymorphism	Associated diseases	Mouse phenotypes
NLRA	CIITA		rs3087456(G) allele	Bone density defects Susceptibility to bone fractures	
			CIITA-BX648577 gene fusion product	Hodgkin's lymphoma B-cell lymphona	
				Bare lymphocyte syndrome Celiac disease Susceptibility to myocardial infarction Rheumatoid arthritis Multiple sclerosis Primary adrenal insufficiency Systemic lupus erythematosus Type 1 diabetes	
NLRC	NOD1				
	NOD2		*Mutations in the LRR e.g. 1007fs, R702W, G908R*	Crohn's disease	Mice with mutations in NOD2 did not develop gut inflammation but are more susceptible to bacterial and viral infections
			Mutations in the NOD e.g. L469F, R334Q, R334W	Blau syndrome	
				Asthma Atopic eczema, atopic dermatitis Arthritis Sarcoidosis Prostate and endometrial cancer Gastric lymphoma Leprosy	
	NLRC4			Susceptibility to bacterial infections	
					NLRC4 deficient mice produced decreased levels of inflammatory cytokines and exhibited decreased survival after bacterial infection; impaired expression of adhesion molecules and neutrophil recruitment *Nlrc4*$^{-/-}$ mice do not develop spontaneous colitis
	NLRC5				NLRC5 deficient mice exhibit enhanced immunodeficiency

FIGURE 3 | Continued

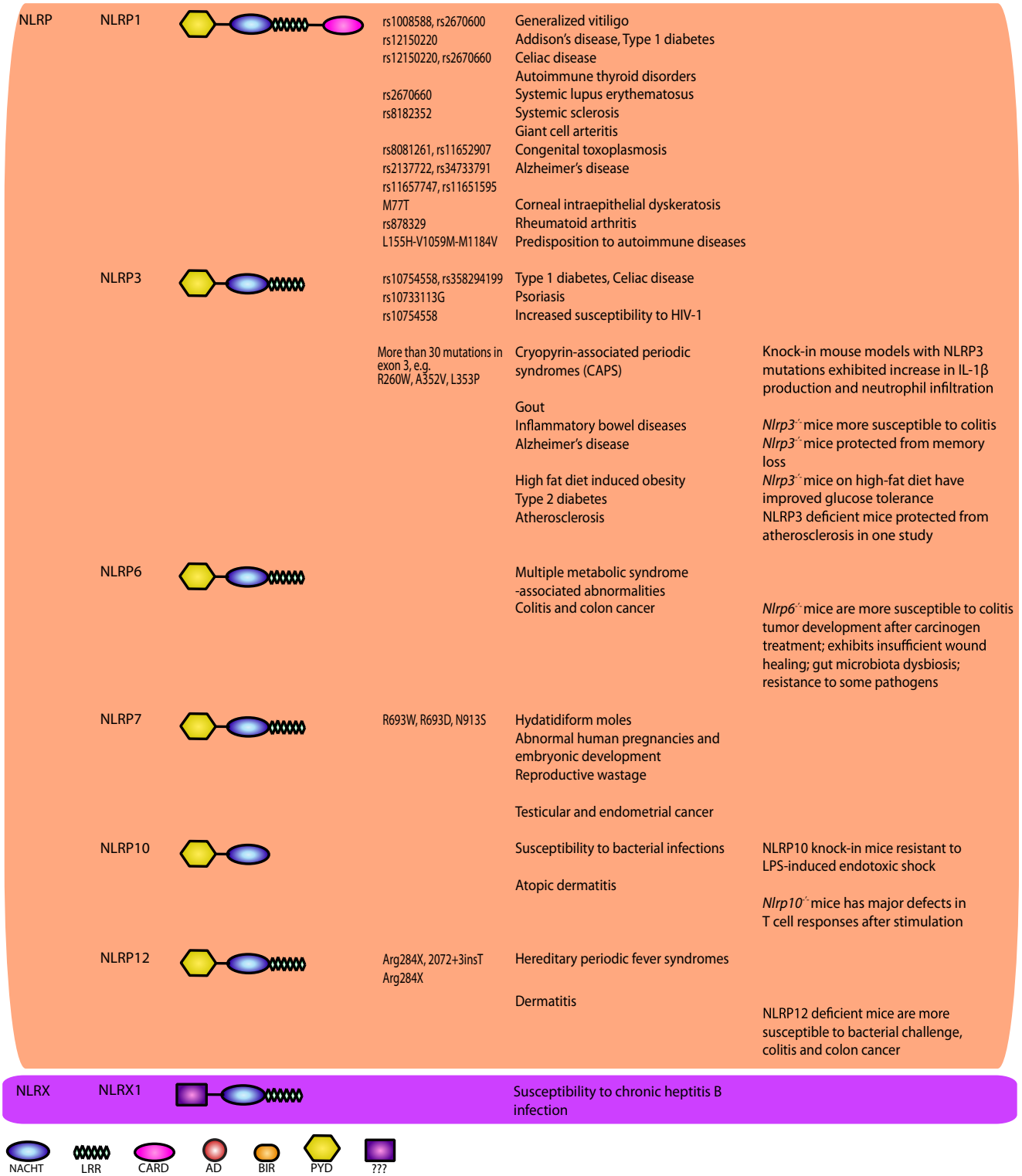


FIGURE 3 | NOD-like receptors and disease. NLRs have been implicated in a plethora of diseases. Genetic studies have uncovered a number of variants in genes encoding NLRs or their signaling mediators associated with human diseases. Animal models have served as a key discovery platform to characterize the underlying functions and molecular mechanisms of NLRs in these diseases and associated pathologies. Together, these efforts have led to therapeutic success in the clinic for a subset of NLR-dependent auto-inflammatory diseases. When available, the mutations and SNPs linked to disease are listed and animal phenotypes are presented.

NLRP3

The NLRP3 inflammasome is arguably the most studied inflammasome to date. NLRP3 is predominantly expressed in splenic neutrophils, macrophages, monocytes, and conventional dendritic cells, and its expression is inducible in response to inflammatory stimuli (45). There is evidence suggesting that a two-step process is required for NLRP3 activation. The first, or priming signal, converges on the activation of NF-κB and transcriptional induction of inflammasome components including NLRP3 itself and pro-IL-1β. The second, or activating signal, in the form of a microbial or danger signal, is then able to directly activate inflammasome assembly (46). NLRP3 is able to recognize a wide variety of exogenous and endogenous stimuli such as microbial agonists, ATP, and particulate matters (47, 48). There is, however, scarce evidence that NLRP3 binds directly to its activators. Instead its activation is thought to be triggered by signaling intermediates (46). For instance, Shenoy et al. proposed that guanylate binding protein 5 (GBP5) may play a vital role in activating inflammasome assembly and promoting caspase-1 processing in response to live bacteria and bacterial cell wall components (49). A recent study by Zhong et al. suggested that particulate stimuli might induce mitochondrial production of reactive oxygen species (ROS), which triggers a calcium influx mediated by transient receptor potential melastatin 2 (TRPM2) to activate NLRP3 (50). The role of ROS in NLRP3 activation is consistent with earlier results by Zhou et al. who showed that ROS also leads to the dissociation of thioredoxin-interacting protein (TXNIP) from thioredoxin, freeing it to interact with and activate NLRP3 (51). In addition, it has been reported that NLRP3 activators are able to disrupt the mitochondria, resulting in the release of oxidized mitochondrial DNA and/or cardiolipin, which can bind to and activate NLRP3 (52, 53). Alternatively, it was argued that mitochondrial disruption is not required for NLRP3 activation; instead K^+ efflux is sufficient to stimulate this NLR (54). NLRP3 inflammasome activation may involve at least two adaptors, ASC and the mitochondria-associated adaptor MAVS. It was recently shown that MAVS recruits NLRP3 to the mitochondria for activation in response to non-crystalline activators (55) and that microtubule-driven trafficking of the mitochondria is necessary for NLRP3-ASC complex assembly and activation (56).

Gain-of-function mutations in the *NLRP3* gene were first associated with cryopyrin-associated periodic fever syndromes (CAPS), which are a group of rare hereditary auto-inflammatory diseases including familial cold urticaria, MWS, and neonatal onset multisystem inflammatory disease [reviewed in Ref. (19)]. Mutations in *NLRP3* were reported to induce an overproduction of IL-1β that triggers the subsequent development of severe inflammation (57, 58). A knock-in mouse model of MWS have validated the observations made in human patients, and showed that equivalent mutations in murine *Nlrp3* lead to the production of massive amounts of IL-1β, which mediates the disease (59, 60). IL-1 blockade therapies are frequently used to treat auto-inflammatory diseases. Anakinra and canakinumab, for example, have been used to treat CAPS patients with great success, as several groups have reported long-lasting clinical responses as well as the restoration of IL-1β production levels to normal amounts in patients after treatment (61, 62).

NLRP3 was also linked to gout, which is a result of uric acid crystal deposition in the joints as a consequence of a rich diet high in purines (63). The exact mechanism of NLRP3 activation by uric acid crystals is still unknown, but monosodium urate and calcium pyrophosphate dihydrate crystals were found to induce NLRP3 and caspase-1 activation and the subsequent processing of IL-1β and IL-18 (64). Since uric acid can also be released from dying cells as a DAMP (65), there has been speculation that NLRP3 may also detect danger signals released from dying cells (66).

Single nucleotide polymorphisms in the NLRP3 locus have been associated with a wide range of disorders, including type 1 diabetes (67), celiac disease (67), psoriasis (68), and increased susceptibility to HIV-1 infections (69). While no SNP in the NLRP3 region is directly associated with inflammatory bowel disease (IBD), a SNP downstream of *NLRP3* has been previously identified as a risk allele in Crohn's disease (70). Lewis and colleagues, however, were unable to reproduce this result, as they found no significant association between *NLRP3* SNPs and Crohn's disease (71). A recent GWAS meta-analysis has shown that SNPs that affect receptors downstream of NLRP3 such as IL18R1, IL1R1, IL1RL1, IL1RL2, and IL1R2, are associated with susceptibility to IBD (72). Thus, although there is conflicting data regarding the effects of *NLRP3* variants in IBD, defects in inflammasome signaling likely play a role in IBD pathogenesis. Consistently, $Nlrp3^{-/-}$ mice or mice deficient in inflammasome components were found to be significantly more susceptible to experimental models of colitis compared to wild-type mice (73–76). Together, these studies indicate that NLRP3 may be involved in intestinal tissue repair mechanisms following injury.

The NLRP3 inflammasome has also been implicated in different metabolic pathologies. For instance, the NLRP3 inflammasome has been linked to obesity, insulin resistance, atherosclerosis, and Alzheimer's disease. It has been shown that activation of caspase-1 and IL-1β processing downstream of NLRP3 lead to inhibition of adipocyte differentiation and contributes to high fat diet-induced obesity (77). Several studies have also shown that the NLRP3 inflammasome may play a crucial role in insulin resistance and the potential development of type 2 diabetes (51, 77, 78). Consistently, $Nlrp3^{-/-}$ or $Asc^{-/-}$ mice were reported to have improved glucose tolerance and insulin sensitivity when fed a high fat diet. Ceramide, a specific product from the metabolism of long-chain saturated fatty acids, and the saturated free fatty acid, palmitate, have been shown to induce IL-1β in an NLRP3-dependent fashion [Ref. (78) and reviewed in Ref. (63)]. IL-1β produced downstream of the NLRP3 inflammasome, which is also stimulated by islet amyloid polypeptide (79), promotes beta-cell dysfunction, and cell death (80), linking NLRP3 activation to insulin resistance. Crystalline cholesterol was proposed to cause atherosclerosis by acting as a danger signal and initiating inflammation through the NLRP3 inflammasome. Consistently, Duewell et al. observed that mice deficient in components of the NLRP3 inflammasome did not undergo acute inflammation after the injection of cholesterol crystals, and had markedly decreased atherosclerosis compared to wild-type animals (81). There is, however, some controversy in this area, as Menu et al. reported no differences in disease progression in $Nlrp3^{-/-}$ mice compared to wild-type animals (82). In Alzheimer's disease, amyloid-β aggregates were shown to activate

NLRP3 *ex vivo* in primary macrophages and microglia (83). This was supported by *in vivo* results by Heneka et al. who demonstrated that NLRP3 deficiency protected mice with familial Alzheimer's disease mutations from memory loss (84).

NLRP6

Preliminary immunofluorescence data has proposed that the formation of the NLRP6 inflammasome is dependent on the recruitment of NLRP6 to ASC specks in the cytosol via its N-terminal PYD (85, 86). While much of its functions still remain unknown, recent studies have demonstrated that NLRP6 is important in the self-renewal and integrity of the intestinal epithelium, as *Nlrp6*$^{-/-}$ mice exhibited insufficient wound healing after injury (87, 88) and were more susceptible to carcinogen-induced tumor development compared with wild-type mice (89, 90). While the precise mechanisms by which NLRP6 protects against tumorigenesis is not clear. It is known that *Nlrp6*$^{-/-}$ mice are able to sustain increases in intestinal epithelial proliferative activity over longer periods of time along with the observed lower efficiency in wound repair – in other words, the repair mechanism in *Nlrp6*$^{-/-}$ mice fails to promote wound healing but is still able to promote general cell proliferation, leading to higher incidents of dysplasia and tumorigenesis (91).

In a study by Elinav et al., *Nlrp6*$^{-/-}$ mice showed an altered gut microbiota with an increase in colitogenic bacterial strains such as *Prevotellaceae* and TM7, which are also found in increased numbers in IBD patients, indicating a role for NLRP6 in the regulatory sensing system in the gut as well (92). The authors speculated that NLRP6 may act as a "gate keeper" by sensing bacterial products or cell damage and promoting the production of IL-18 during homeostasis which in turn supports the normal microbial flora in the gut to prevent dysbiosis (92). While this proposal raised some interesting points as to the functions of NLRP6 in the gut, a conclusion cannot be drawn as to whether the altered microbiota in *Nlrp6*$^{-/-}$ animals is due to the absence of the protein, as no littermate analysis or maternal microbiota transfer experiments were conducted by the authors to further their hypothesis. A study by Henao-Mejia et al. later linked changes in *Nlrp6*$^{-/-}$ mice microbiota to metabolic diseases (93). Namely, NLRP6 seems to negatively regulate the progression from non-alcoholic fatty liver disease to non-alcoholic steatohepatitis by preventing the increase in colitogenic bacteria (93). Additionally, Anand et al. observed that *Nlrp6*$^{-/-}$ mice were highly resistant to a variety of pathogens including *Listeria monocytogenes* and *Escherichia coli* (94). *Nlrp6*$^{-/-}$ mice had increased numbers of immune cells in their circulation, as well as enhanced activation of MAPK and NF-κB signaling, though Toll-like receptor (TLR) activation, suggesting that NLRP6 may suppress TLR pathways after the recognition of pathogens to prevent amplified inflammatory pathology (94). The exact mechanisms of how NLRP6 functions, however, still remain to be studied.

NLRP7

NLRP7, a human NLR with no murine orthologs, is characterized by an N-terminal PYD along with a NACHT domain and a C-terminal LRR region. Mutations in the *NLRP7* gene are associated with recurrent hydatidiform moles and reproductive wastage (substitutions R693W, R693P, and N913S) (11, 95–97). Furthermore, NLRP7 expression is increased in certain type of cancers such as testicular (98) and endometrial (99) cancers. However, the mechanisms underlying these phenotypes are not clear. Messaed et al. showed that PBMCs from patients with *NLRP7* mutations (at G118X, G380R, C399Y, R693W, A719V) secreted significantly lower levels of IL-1β and TNF in response to LPS despite high intracellular levels of pro-IL-1β and unimpaired pro-IL-1β processing. The authors concluded that NLRP7 might play a role in cytokine trafficking and secretion from the cell (100). Conversely, others have shown that overexpression of NLRP7 inhibited pro-IL-1β synthesis and secretion (88, 101). Moreover, it was recently reported that bacterial acylated lipopeptides (acLP) activated NLRP7 and stimulated formation of an NLRP7-ASC-caspase-1 inflammasome (102). Thus, further studies are needed to clarify NLRP7 mechanisms of actions and functions in reproduction and immunity.

NLRP12

NLRP12 was previously reported to form an inflammasome as well as function in modulating NF-κB signaling (see below). A recent study by Vladimer et al. has shown that the NLRP12 inflammasome has a key role in controlling IL-1β and IL-18 production after *Yersinia pestis* infection, where NLRP12-deficient mice were more susceptible to infection compared to the controls (103). Other pathogens such as *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* and bacterial components such as LPS do not seem to depend on NLRP12 for infection or pathology (104).

While many of the functions and activators of NLRP12 remain unknown, mutations in the NLRP12 gene have been associated with auto-inflammatory diseases such as atopic dermatitis (105) and hereditary periodic fever syndromes (106, 107). Anti-IL-1 therapies, similar to those administered to patients with NLRP3 mutations, have been conducted on patients with NLRP12 mutations with limited success. Patients treated with anakinra showed improvements early on during the treatment process, but developed resistance to the drug within months and suffered from severe myalgia as a side effect (107). Similarly, levels of IL-1β in these patients returned to pre-treatment levels after 14 months of treatment (107). Further clinical studies are needed before conclusions are drawn regarding the efficacy of anti-IL-1 agents in the treatment of diseases associated with NLRP12.

There is currently much debate as to the role of NLRP12 in inflammation, and both stimulatory and inhibitory functions have been proposed. Some studies have suggested that NLRP12 may negatively regulate the NF-κB pathway (86, 108, 109). *Nlrp12*$^{-/-}$ mice were found to be more susceptible to colitis and colon cancer, and polyps isolated from these mice showed significantly higher non-canonical activation of NF-κB with an increased expression of inflammation and cancer-related genes (109, 110). Conversely, Arthur et al. demonstrated in a murine model of allergic dermatitis that proinflammatory cytokine production was unaffected in *Nlrp12*$^{-/-}$ mice (111). Instead, dendritic cells in *Nlrp12*$^{-/-}$ mice exhibited a much-reduced migratory capacity, and neither peripheral dendritic cells nor neutrophils in *Nlrp12*$^{-/-}$ mice responded to chemotaxic signals or chemokines in *in vitro* experiments (111). Yet another function for NLRP12 was proposed by Jéru et al.,

who discovered that mutations in *NLRP12* did not affect NF-κB activation, but rather increased ASC speck formation and caspase-1 activation (112). Altogether, these results suggest that NLRP12 plays a role in suppressing NF-κB while stimulating the inflammasome and assisting in the migration of immune cells.

NLRC4 AND NAIPs

NLRC4 possesses an N-terminal CARD that allows direct interaction with caspase-1 independently of ASC (113, 114). A recent study by Qu et al., showed that the phosphorylation of Ser533 in NLRC4 by PCKδ was crucial for the activation of the NLRC4 inflammasome (115). NAIPs, members of the NLRB sub-family, have been identified as critical components of the NLRC4 inflammasome. They are required for the recognition of bacterial components, as well as the scaffolding of the NAIP-NLRC4 inflammasome. Activators of this inflammasome include bacterial flagellin and components of the bacterial type III secretion system (T3SS) (113, 116, 117). Notably, murine Naip5 and Naip6 were shown to recognize bacterial flagellin and subsequently bind to NLRC4 to trigger the formation of the inflammasome, whereas Naip2 and human NAIP serve as receptors for the rod and the needle components, of the bacterial T3SS (118, 119).

NLRC4 plays an essential role in host survival and pathogen clearance following host infection with pathogens such as *Legionella pneumophila* (120, 121), *Candida albicans* (122), and *Burkholderia pseudomallei* (123). More recently, Cai et al. showed that, upon *K. pneumoniae* infections, *Nlrc4*$^{-/-}$ mice exhibited decreased survival compared to wild-type animals (124). Similarly, Franchi et al. reported that *Nlrc4*$^{-/-}$ mice were highly susceptible to orogastric *Salmonella* infections (125). Interestingly, *Nlrc4*$^{-/-}$ mice do not develop spontaneous colitis in response to the commensal microbiota (126), likely due to low soluble flagellin levels in the gut and a primary role of TLR5 in dealing with gut flagellated bacteria. NLRC4 thus serves as an additional sentinel against pathogenic enteric infections (126, 127). NLRC4 has been previously been shown to act in sync with NLRP3 during *Salmonella* infection (114). More recently, it was demonstrated that both NLRs play non-redundant roles in *B. pseudomallei* infection and melioidosis, where NLRC4 is critical for pyroptosis and NLRP3 for the production of IL-1β and IL-18 (123). Ceballos-Olvera et al. demonstrated that while IL-18 and pyroptosis are both essential for host resistance, the production of IL-1β by NLRP3 was deleterious, as it triggered excessive neutrophil recruitment and exacerbated the disease (123). Thus NLRC4 seems to act synergistically with both TLR5 and NLRP3, but its contributions to their functions seems to be secondary.

NON-INFLAMMASOME-FORMING NLRs

NOD1/2

NOD1 and 2, have been studied primarily in the context of their signaling activity following recognition of the peptidoglycan components diaminopimelic acid (DAP) and MDP from Gram-negative and Gram-positive bacteria (128–132). Despite this focus, much of the nature of the NOD1 and 2 interaction with these structures remains unknown, although recent findings suggest that NOD2 directly binds MDP with high affinity (133), with the N-glycosylated form specific to the mycobacterial cell wall triggering an exceptionally strong immunogenic response compared to N-acetyl MDP (134). The possibility of a role for NOD2 in non-bacterial infections has also been suggested, with NOD2 having been shown to induce an IFNβ-driven antiviral response following recognition of single-stranded viral RNA (135). Indeed, viral ssRNA from respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), and influenza virus has been shown to trigger a non-canonical NOD2-directed signaling pathway that requires mitochondrial antiviral signaling protein (MAVS) and induces IRF3 activity, leading to the production of type I IFNs (135) (**Figure 4**). However, it is still unclear whether lack of NOD2 results in susceptibility to viral infection in humans.

NOD1 and 2 are encoded by the *CARD4* and *CARD15* genes respectively, and as NLRCs, both contain the shared NOD and LRR domains in addition to an amino-terminal CARD. Despite the strong similarities between the two receptors, differences exist; NOD1 contains one CARD domain, while NOD2 contains two (136) and expression of NOD1 is detected in a wide variety of cell types, whereas NOD2 expression is restricted to myeloid cells (136–138), keratinocytes (139) and intestinal, lung, and oral epithelial cells (140–142).

Activation of NOD1 and 2 follows the cytosolic recognition of peptidoglycan ligands that triggers oligomerization of the receptors via their NOD domain and the recruitment of mediators needed to form a signaling complex referred to as the nodosome (143). The nodosome is directed to the point of bacterial entry on the plasma membrane of polarized epithelial cells by the regulatory protein FRMBP2 (144). NOD1 and 2 both interact with RIPK2, via a CARD–CARD homotypic interaction (145–148). This association results in the recruitment of a number of E3 ubiquitin ligases, including TNF receptor-associated factors (TRAFs) (149), cellular inhibitor of apoptosis (cIAP)1 and cIAP2 (150), X-linked inhibitor of apoptosis (XIAP) (151, 152), and ITCH (153). K63-linked ubiquitination of RIPK2 has been established as a means to construct protein scaffolds that transduce downstream signaling. In a step-wise fashion, ubiquitination of RIPK2 leads to activation and recruitment of the TAK1 complex, consisting of TAK1 in association with TAK1-binding protein (TAB)2 and 3. The kinase activity of TAK1 leads to phosphorylation events that activate AP-1 and NF-κB. In parallel to cIAP-induced ubiquitination of RIPK2, XIAP's enzymatic activity results in the formation of polyubiquitin chains on RIPK2, serving as a platform to engage another E3 ligase complex known as the Linear Ubiquitin Assembly Complex (LUBAC) (152, 154). LUBAC attaches linear ubiquitin chains to the regulatory protein NEMO, allowing for activation of the IKK complex. The kinase activity of IKKβ results in the phosphorylation and degradation of the inhibitor of NF-κB (IκB), allowing for NF-κB dimers to translocate to the nucleus and induce proinflammatory gene expression (155). Besides activating NF-κB, NOD1 and NOD2 have also been shown to activate the p38, JNK, and ERK MAPK pathways (147, 156, 157) and to interact with other NLRs such NLRP1 and NLRP12 (158, 159) (**Figure 4**).

NOD1 and 2 have been implicated in a number of chronic inflammatory diseases. Mutations and SNPs in *CARD15* in particular, have been linked to a multitude of inflammatory diseases including Crohn's disease (160, 161), Blau Syndrome (162), asthma (163, 164), atopic eczema (165), atopic dermatitis (105), arthritis

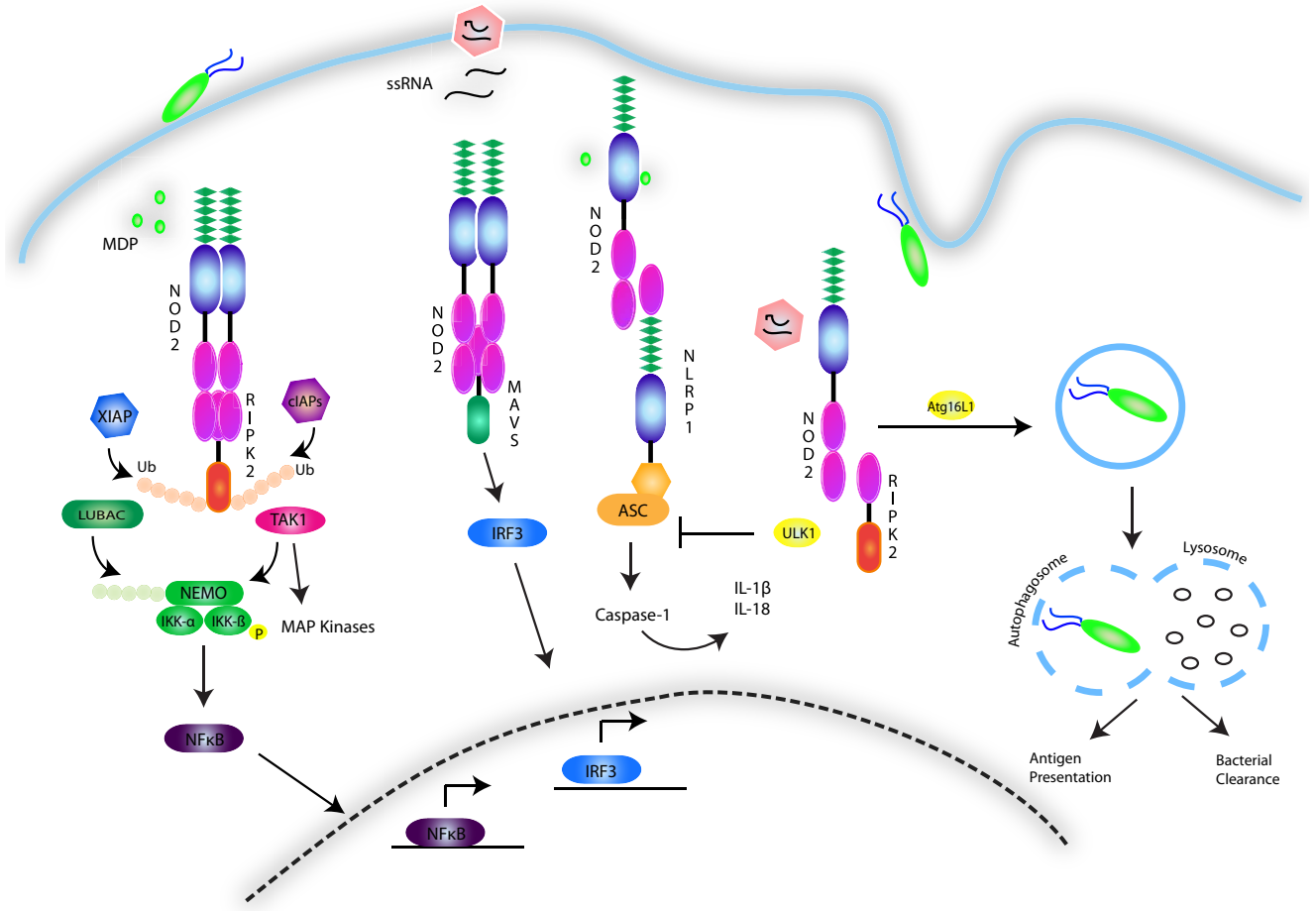


FIGURE 4 | NOD1/2 signaling pathways. The NOD1 and 2 receptors recognize the bacterial peptidoglycan derivatives DAP and MDP. The events involved in signal transduction are depicted and involve the formation of a nodosome complex that is stabilized through a series of ubiquitin scaffolds mediated by a number of E3 ligases including cIAP1/2, XIAP, LUBAC, and ITCH. These scaffolds serve to engage effector kinases, including TAK1 and the IKK complex to activate NF-κB and MAPK pathways. NOD2 is additionally activated by single-stranded RNA viruses and stimulates an antiviral innate immune response by engaging MAVS and activating IRF3. The NOD receptors have also been shown to synergize with NLRP sensors to activate the inflammasome. Conversely, they have also been implicated in triggering autophagy though association with ATG16L1, and in response to viral infection, to inhibit the inflammasome by upregulating ULK1-dependent mitophagy.

(166, 167), and sarcoidosis (168). In the context of Crohn's disease, the most common mutation that confers susceptibility is a frameshift mutation in the LRR region of the receptor (160), while the mutations conferring susceptibility to Blau syndrome occur in the NOD region (162). While the contribution of these mutations to disease is unknown, further work on understanding NOD2 function could unveil the link between the gene and the disease, as well as allow for the creation of new therapies for these chronic and often devastating diseases. Several NOD2 loss-of-function mouse lines have been generated in an attempt to elucidate its role in Crohn's disease. Pauleau and Murray generated the first *NOD2* knockout mice (156). Surprisingly, these mice lacked symptoms associated with spontaneous intestinal inflammation, although stimulation of primary macrophages from these animals with MDP failed to trigger inflammatory responses, confirming loss of NOD2 activity (156). Other NOD2 mouse mutants were later generated to express common Crohn's disease susceptibility mutations (157, 169). While these mice did not develop any gut inflammation resembling that of Crohn's disease patients, they did display increased susceptibility to bacterial infection, and were shown to produce decreased amounts of β-defensins when challenged with *L. monocytogenes* (157, 170). Similarly, mice deficient in NOD1, NOD2, or RIPK2 also exhibited enhanced susceptibility to bacteria including *Helicobacter pylori* (171–173), *Chlamydophila pneumoniae* (174), *L. pneumophila* (172, 175, 176), and *B. anthracis* (177). This susceptibility often resulted from an inability to control bacterial burden, possibly due to a reduced ability to recruit neutrophils as well as a decrease in the production of proinflammatory and antimicrobial molecules (176, 177). Despite the prevalence of NOD2-deficient models, there remains controversy as to whether Crohn's disease-linked mutations in NOD2 diminish or enhance its activity in the context of the disease. Common

Crohn's disease-associated NOD2 variants expressed in HEK293T kidney cells are unable to detect MDP and activate NF-κB (178) and monocytes from Crohn's disease patients with the *1007fs* variant displayed defects in the secretion of TNFα, IL-6, IL-8, and IL-10 (179, 180) and many of these NOD2 variants seem to act recessively (181). While these findings point to a loss-of-function effect of the mutations, Karin and colleagues have argued that the models used in these studies lack resemblance to the natural course of the disease in humans and that the Crohn's disease-associated NOD2 mutations may in fact result in a gain-of-function. Indeed, Crohn's disease has been associated with the presence of activated NF-κB and inflammatory NF-κB target gene products in epithelial cells and lamina propria macrophages (182, 183) rather than in circulating blood monocytes used in studies with cultured cells, and results from experiments using tissue samples have differed from those using monocytes (179).

The study of NOD2 linkage to Crohn's disease has been extended to encompass a key role of NOD1 and 2 in the regulation of autophagy. Autophagy is a housekeeping process in which organelles or other cellular components are degraded and recycled into nutrients during times of starvation or stress. This process results in the formation of a double membrane vacuole known as the autophagosome, which fuses with lysosomes to degrade its contents (184). The role of NOD1 and 2 in this process was initially proposed following GWAS findings of an association between a key component of the autophagy process, ATG16L1, and susceptibility to Crohn's disease (185, 186). Not only have NOD1 and 2 been shown to interact with ATG16L1 (187), but murine Paneth cells expressing the ATG16L1 mutation associated with Crohn's disease were unable to produce antimicrobial peptides despite NOD2 stimulation (188). Additionally, the autophagic machinery is involved in loading antigen onto MHC Class II, a process that has been observed to be defective in Crohn's disease (189). Recently, Lupfer et al. further substantiated the link between NOD2 and autophagy by demonstrating a role for NOD2-RIPK2 signaling in the regulation of the NLRP3 inflammasome following infection with influenza A virus. By triggering the phosphorylation of the autophagy inducer ULK1, RIPK2 induces autophagy of disrupted mitochondria (mitophagy), preventing the accumulation of ROS and NLRP3 inflammasome activation. Mice lacking Nod2, Ripk2, or Ulk1 were hypersusceptible to influenza A infection due to a hyperactive NLRP3 inflammasome and excessive IL-18 levels (190) (**Figure 4**). Collectively, these studies provide evidence for a key role of NOD2 in autophagy-associated processes such as xenophagy, antigen presentation, antimicrobial peptide secretion, and mitophagy.

Other diseases have also been associated with genetic variants in loci encompassing the genes encoding NOD1 and/or 2. GWAS have linked SNPs in *CARD15* to prostate (191) and endometrial (192) cancer, as well as to gastric lymphoma induced by *H. pylori* infection (173). Similarly, SNPs in *CARD15* were linked to susceptibility to leprosy (193, 194) and tuberculosis (195, 196). The observation that *CARD15*, *RIPK2*, and *NF-κB* have been linked to leprosy (193), tuberculosis (195, 197, 198), and IBD (150, 199) by GWAS and other genetic studies in humans and mice, has led to speculation of a common etiology between mycobacterial diseases and Crohn's disease (197, 200, 201).

NLRP10

NLRP10 was discovered based on its homology to NLRP3 and APAF1 (202). Lack of LRRs in NLRP10 may indicate a role for this protein as a signaling adaptor rather than an NLR sensor. NLRP10 has been found in human and murine skin (203), colon, kidney, and testis (204), with mRNA and protein expressed in epithelial cells (202, 205) and hematopoietic cells (206). NLRP10 was previously proposed as a negative regulator of NF-κB, cell death, and IL-1β release (202). These results have been supported by NLRP10 over-expression studies in *Nlrp10* knock-in mice and in *in vitro* studies. In the murine model, *Nlrp10* knock-in mice were found to be resistant to LPS-induced endotoxic shock, due to a decreased release of inflammatory cytokines (203). This was consistent with the observation that cells from these animals secreted reduced amounts of IL-1β following infection with *Salmonella* or TLR7 stimulation (203). However, another group proposed a role for NLRP10 in augmenting the NOD1 immune response to *Shigella flexneri*, indicating the possibility of an inflammasome-independent function for NLRP10 (205). While this mechanism is still poorly understood, the ability of NLRP10 to interact with NOD1 as well as its signaling targets RIPK2, TAK1, and NEMO, suggests that NLRP10 may be involved in optimizing cytokine release following bacterial infections. Furthermore, Flavell and colleagues reported a role of NLRP10 in adaptive immunity. Using NLRP10 knockout mice, this group examined T-cell responses to ovalbumin and aluminum hydroxide, complete Freund's adjuvant with myelin oligodendrocyte glycoprotein, and LPS. Interestingly, $Nlrp10^{-/-}$ mice displayed major defects in TH2, TH17, and TH1 responses, potentially due to a defect in the ability of dendritic cells to transport antigen to draining lymph nodes (207). These findings, as well as those of another group that reported hematopoietic compartment-dependent susceptibility of $Nlrp10^{-/-}$ mice to *C. albicans* (206), highlight a role for NLRP10 in bridging innate and adaptive immunity. Despite these findings, understanding the role of NLRP10 in immunity is still in its infancy, and applications of this protein to human diseases are limited while the function of NLRP10 remains largely uncharacterized. However, GWAS have linked *NLRP10* to atopic dermatitis (105, 165, 208), an interesting find considering the abundant expression of NLRP10 in the skin.

NLRX1

NLRX1 is unique among NLRs in that it contains an N-terminal mitochondrial targeting sequence (209, 210). The protein is broadly expressed in the mitochondria, although it is yet unclear whether it is localized to the matrix or to the outer membrane (209, 211). NLRX1 has been shown to enhance ROS production when it is overexpressed (212), following *Chlamydia* (213) and *Shigella* infection, as well as in response to TNFα and poly(I:C) (212). Like NOD2, NLRX1 has been implicated in host antiviral responses following viral RNA detection (135, 212) and has been shown to directly bind both single and double stranded viral RNA via its LRRs (210). Moore and colleagues initially characterized NLRX1 as a negative regulator of MAVS and antiviral signaling (211). Recently, Lei et al. demonstrated a role for NLRX1 and the mitochondrial protein TUFM in enhancing autophagy and reducing type I IFNs following viral infection (214, 215). However, these findings have been contested and the generation of

NLRX1-deficient and *Nlrx1* knockdown mice by several groups has produced conflicting results. In some laboratories, the NLRX1 mutant mice did not display any differences in MAVS antiviral signaling compared to wild-type controls (216–219). In contrast, another group's findings supported their original claim of a role for NLRX1 in inhibiting MAVS signaling pathways (216, 218). Zhao et al. recently associated a missense mutation in the LRR of NLRX1 with susceptibility to chronic hepatitis B infection in human patients. The replacement of the highly conserved Arg707 with a cysteine between a α helix and a β strand was hypothesized to interfere with the electrostatic potential of the region and consequently modulate the activity of the protein (220). Lastly, a group characterizing the molecular signature of SIV-induced gastrointestinal dysfunction found an increase in NLRX1 expression in rhesus macaques 90 days following SIV infection (221). These findings highlight the role of NLRX1 in antiviral defense, but more research is needed to elucidate the precise mechanism.

NLRC5

One of the newest additions to the NLR family, NLRC5 has been shown to have a similar structure to other NLRs, although the CARD domain has been found to be structurally distinct from CARD domains expressed in other NLRs. The protein is most similar to CIITA, both in structure and activity. NLRC5 has been shown to be able to enter the nucleus, and its main function is believed to be as a MHC Class I transactivator, forming the basis of an enhanceosome for MHC Class I transcription (222). Accordingly, NLRC5 is expressed constitutively in both humans and mice, unlike the more restricted expression of CIITA (223, 224) and knockdown of NLRC5 in cells using siRNA and in knockout mice resulted in a decrease in MHC Class I expression, without significantly affecting expression of MHC Class II (222, 225, 226). Despite the widespread expression of NLRC5, however, there is a distinct upregulation of NLRC5 in lymphocytes compared to other hematopoietic and somatic cells (227, 228). As a key regulator of MHC Class I transcription, NLRC5 expression has been shown to be induced by a number of signals, including IFNβ, poly(I:C), VSV, and LPS (222–225, 227, 228). However, the most efficient activator of NLRC5 known is IFNγ, which several of the aforementioned signals are known to induce. IFNγ functions via signal transducer and activator of transcription 1 (STAT1) and cannot induce NLRC5 expression in the absence of STAT1 (225, 228).

The effect of NLRC5 on human health and disease has yet to be extensively studied. However, inferences can be made based on NLRC5's role in MHC Class I presentation and phenotypes observed in NLRC5-deficient mice. Yao et al. observed extreme immunodeficiency in these mice, with the animals unable to mount an effective CD8+ T-cell response when challenged with *L. monocytogenes*. Interestingly, NLRC5 deficiency also seemed to result in a decrease in NLRP3 inflammasome activation, suggesting that NLRC5 may play a role in the regulation of this pathway (226). Murine and cellular models of NLRC5 deficiency have also implicated NLRC5 in the negative regulation of TLR signaling (223, 229, 230), as well as in RIG-I-like receptor signaling(229). However, other groups have disputed these findings (222, 230), and more research needs to be done in order to gain a more comprehensive understanding of the functions of NLRC5.

CLASS II TRANSACTIVATOR (CIITA)

MHC CIITA was discovered in 1993 as the genetic basis of hereditary major histocompatibility complex Class II deficiency, or bare lymphocyte syndrome (BLS), a disease characterized by severe immunodeficiency due to a lack of MHC Class II expression (231). Its detection via complementation cloning marked the discovery of the first NLR family member, although the classification of NLRs was only later introduced, following the discovery of NOD1. Although CIITA retains the tripartite structure consistent across the NLR family, it contains an additional acidic domain and a proline/serine/threonine (PST)-rich domain at its N-terminus. Unlike other NLRs, the function of CIITA lies in transcriptional regulation of MHC Class II. The previously mentioned additions to the structure of CIITA do not allow it to bind DNA, but provide a platform for the recruitment and interaction of proteins required for the transcription of MHC Class II in leukocytes or other cells following IFNγ stimulation (232–234). Accordingly, CIITA contains nuclear localization signals and nuclear export signals (235–237). As its role suggests, CIITA is expressed in cells that express MHC Class II, mainly lymphocytes, dendritic cells, macrophages, and other professional antigen presenting cells.

In addition to BLS, CIITA has been linked to a number of other human diseases. GWAS and patient exome sequencing studies have linked SNPs in CIITA to celiac disease (238, 239), susceptibility to myocardial infarction (240), rheumatoid arthritis (240–242), multiple sclerosis (240, 242), primary adrenal insufficiency (243), SLE (244), and type 1 diabetes (245, 246), although these results have not always been replicated in subsequent studies (247–249). Gyllenberg et al. suggested that age-dependent variation in the gene encoding CIITA could be responsible for false associations in GWAS (250). Interestingly, women over 75 years of age expressing the rs3087456(G) allele were found to have a higher average bone mineral density and a decrease in bone fractures compared to controls, although the association was not observed in women aged 25 years (251). Ulrich Streidl and colleagues recently used RNA sequencing to identify a novel and frequently expressed *CIITA-BX648577* gene fusion product in the KM-H2 Hodgkin lymphoma cell-line which was associated with decreased HLA Class II expression and increased programed cell death 1 (PDL1) on the surface of affected cells (252). Genomic *CIITA* breaks were found to occur frequently in B-cell lymphoma patients; 38% of primary mediastinal B-cell lymphoma patients and 15% of classical Hodgkin's lymphoma patients displayed them. The group also observed a decrease in survival in B-cell lymphoma patients expressing genomic *CIITA* breaks compared to control. The role of *CIITA* gene fusion products in B-cell lymphomas remains a field of considerable interest. Understanding the effect of these genomic breaks could lead to novel therapies for a highly treatment-evasive cancer. At the very least, the discovery of these abnormal gene products could lead to the discovery of new biomarkers, which aid clinicians in stratifying patients according to prognosis and predicted therapeutic response.

CONCLUSION

NOD-like receptors have been described as master regulators of innate immunity, and research performed on the functions and signaling pathways of these proteins continues to support this claim. NLRs are essential in recognition of microbial- and

pathogen-associated molecular patterns (MAMPs and PAMPs), and have the ability to initiate and support robust immune responses through the formation of inflammasomes and the activation of NF-κB, IRF, and MAPK pathways. Functions such as the enhancement of MHC transcription and presentation implicate NLRs in adaptive immunity, and their roles in reproduction, indicate a broader responsibility of this gene family than previously suspected. The potency of NLRs in inducing immune defenses is vital for the host, but can also provide serious problems when dysregulation or malfunction occurs. GWAS have found many SNPs in NLR genes associated with a plethora of inflammatory and autoimmune pathologies. Research is vastly expanding contemporary knowledge on the functions and roles of NLRs, but several NLRs still remain poorly characterized and understood. Specifically, it remains unclear how NLRs can interact with various and structurally diverse ligands. It is hypothesized that upstream receptors or effectors dictate the activation of NLRs. Alternatively, NLRs might employ co-receptors or dimerize with additional sensors to achieve their functions. Further description of the roles of NLRs in initiating and perpetuating human disease, as well as the role of NLRs at the steady state, will prove vital to gaining a comprehensive understanding of many human pathologies and will provide novel targets and therapies for patients afflicted with these diseases.

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NOD-like receptors in lung diseases

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The lung is a particularly vulnerable organ at the interface of the body and the exterior environment. It is constantly exposed to microbes and particles by inhalation. The innate immune system needs to react promptly and adequately to potential dangers posed by these microbes and particles, while at the same time avoiding extensive tissue damage. Nucleotide-binding oligomerization domain-like receptors (NLRs) represent a group of key sensors for microbes and damage in the lung. As such they are important players in various infectious as well as acute and chronic sterile inflammatory diseases, such as pneumonia, chronic obstructive pulmonary disease (COPD), acute lung injury/acute respiratory distress syndrome, pneumoconiosis, and asthma. Activation of most known NLRs leads to the production and release of pro-inflammatory cytokines, and/or to the induction of cell death. We will review NLR functions in the lung during infection and sterile inflammation.

Keywords: NOD-like receptors, inflammasome, lung, pneumonia, lung injury

INTRODUCTION

The respiratory tract constitutes a large surface of the body with the outside environment that is exposed to high volume airflow and large numbers of inhaled microbes and particles. The microflora of the upper respiratory tract consists of non-pathogenic bacteria but also frequently comprises potential pathogens such as *Streptococcus pneumoniae* and *Staphylococcus aureus* (1). The distal bronchi and the alveoli have long been considered sterile, however more recently microbes that are either aspirated from the upper respiratory tract (2) or constantly reside in the lower airways (3, 4) have been found by culture-independent approaches.

Inflammatory disorders of the respiratory tract involving the innate immune system include both infectious and non-infectious diseases. Lower respiratory tract infections, or pneumonia, generally develop when facultative pathogenic microbes that colonize the upper respiratory tract are aspirated or airborne pathogens are inhaled. The World Health Organization (WHO) estimates 429 million cases of acute lower respiratory tract infections in 2004, making it the third leading cause of death world-wide (5). Moreover, non-infectious and chronic lung diseases substantially contribute to morbidity. Chronic obstructive pulmonary disease (COPD) is mainly caused by tobacco smoke and can exacerbate during acute infections, ranks as the number four leading cause of death in most industrialized countries (5). Acute lung injury (ALI) and its severest form, called acute respiratory distress syndrome (ARDS), can develop after infectious as well as non-infectious insults (6). Another potentially life-threatening disorder is allergic asthma, which is characterized by airway hyperresponsiveness due to allergen-triggered airway inflammation causing chronic recurrent airflow obstruction. Long-term exposure to silica, asbestos, or coal particles can cause chronic occupational lung disease called pneumoconiosis.

The innate immune system is a key player in various infectious and non-infectious disorders of the lung (7–9). It senses infections, sterile tissue damage, and probably any disturbance of host cell and tissue integrity by so-called pattern-recognition receptors (PRRs). PRRs comprise different protein families such as the transmembrane Toll-like receptors (TLRs) and the intracellularly located nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (10–12). PRRs recognize conserved microbial molecules, referred to as pathogen-associated molecular patterns (PAMPs) (13). However, recent evidence suggests that recognition of disturbed host cell integrity and danger signals might also play a role in the immune responses to invading pathogens (see below). In contrast to the original paradigm (13) it is now well-accepted that PRRs also sense non-microbial ligands generated during sterile tissue damage, often called damage-associated molecular patterns (DAMPs) (14, 15). Moreover, some PRRs can additionally respond to large particles and therefore appear to be key mediators in pneumoconiosis (16–18).

The NLR family comprises 22 members in humans and even more in mice. Most NLRs share common structural characteristics including a C-terminal leucine-rich repeat (LRR) domain, often involved in ligand recognition, a central NOD, and a variable N-terminal effector domain (10). Based on the type of effector domains that is either a caspase recruitment domain (CARD), a pyrin domain (PYD), or a baculoviral inhibitor of apoptosis protein repeat (BIR) domain, the NLR family can be further divides into five subfamilies. The NLRA subfamily consist of only one member, the transcription factor CIITA, of which at least one splice variant expresses a CARD (**Figure 1**). CIITA is involved in transcriptional activation of genes encoding major histocompatibility complex class II [for detailed discussion of this unique NLR protein we refer to Ref. (19)]. The NLRB group of NLRs expresses a BIR domain and consists of NAIP1–7 in mice and NAIP in humans. The NLRC subfamily includes the CARD-containing molecules NOD1, NOD2, and NLRC3–5, whereas the 14 known NLRP proteins (NLRP1–14) express a PYD. NLRX1 is the only member of the NLRX subgroup, and the only NLR protein that is localized in mitochondria (10, 20). Whereas some NLR proteins

Name	Domains	Activator	Adapter/ binder	Function	Expression (lung)
NOD1	CARD NOD LRR	mDAP-Tri-[h]/Tetrapeptide [m]	Rip2, ATGL16	NF-κB activation, autophagy	yes
NOD2	CARD CARD NOD LRR	MDP and other PG motifs	Rip2, ATGL16	NF-κB activation, autophagy	Myeloid cells, monocytes, bronchial epithelial cells
NLRC3	NOD LRR	?	TRAF6	Negative TLR regulator?	?
NLRC4	CARD NOD LRR	flagellin, T3SS components	ASC, caspase-1	Inflammasome formation	macrophages
NLRC5	NOD LRR	virus	?	Transcription MHC class I related genes	high
NLRX1	MT NOD LRR	RNA?	UQCRC2, MAVS, TUFM	ROS production, autophagy, negative regulator of TLR and MAVS-dep. signaling	yes
NLRP1	human: PYD NOD LRR CARD mouse: ? NOD LRR CARD	LF [m], MDP [h]	ASC, caspase-1	Inflammasome formation	leukocytes and epithelial cells
NLRP2	PYD NOD LRR	?	ASC?	Inflammasome formation?	yes
NLRP3	PYD NOD LRR	pore-forming toxins, nucleic acids, ATP, uric acid, hyaluronan…	ASC, caspase-1	Inflammasome formation	Myeloid cells monocytes, DC
NLRP4	PYD NOD LRR	?	Beclin-1	Autophagy and negative reg. of NF-κB	?
NLRP6	PYD NOD LRR	?	ASC	Inflammasome formation, negative TLR regulator	yes [h]
NLRP7	PYD NOD	microbial acylated lipopeptide	ASC	Inflammasome formation	?
NLRP10	PYD NOD LRR	?	?	Negative NF-κB regulator, DC migration	?
NRLP12	PYD NOD LRR	Lipopeptide (*Yersinia pestis*)	ASC	Negative TLR regulator	myeloid cells
NAIP [h]	BIR BIR BIR NOD LRR	TTSS needle protein (CprI) flagellin?	NLRC4	Inflammasome formation	yes
NAIP2 [m]	BIR BIR BIR NOD LRR	T3SS components (PrgJ)	NLRC4	Inflammasome formation	yes
NAIP5 [m]	BIR BIR BIR NOD LRR	flagellin	NLRC4	Inflammasome formation	yes
CIITA	CARD NOD LRR	?	?	MHCII regulation	lymphocytes, endothelial cells

FIGURE 1 | Summary of the main characteristics of the NLRs. [h] and [m] symbolized a characteristic specific to human or mouse. For more details, refer to the main text.

function as *bona fide* PRRs, other family members act as adaptor molecules or regulators of signal transduction.

In this review article we discuss the current knowledge about NLR expression and function in the lung in different pulmonary diseases. We have grouped the NLRs based on functional similarities and summarize major pathways and common principles of function.

NOD1 AND NOD2

NOD1 and NOD2 were the first NLR proteins to be discovered (21–25). In the lung, NOD1 is expressed in various cell types including lung epithelial cells, endothelial cells, human airway smooth muscle cells, and different types of leukocytes (26–29). NOD2 has been found in alveolar macrophages, neutrophils, and bronchial epithelial cells (30–32). NOD1 responds to bacterial cell wall peptidoglycan containing *meso*-diaminopimelic acid found predominantly in Gram-negative bacteria (33, 34). NOD2 recognizes the muramyl-dipeptide (MDP) MurNAc-L-Ala-D-isoGln, which is conserved in peptidoglycans of the majority of bacteria (35, 36). Other peptidoglycan motifs can be recognized by NOD1 and NOD2, for details refer to review (37).

Ligand recognition by both receptors leads to signal transduction through Rip2 kinase with downstream activation of MAP kinases and the transcription factor NF-κB, leading to activation of genes encoding different cytokines, chemokines (e.g., IL-8), and antimicrobial peptides. Both NOD signaling cascades are regulated by small GTPases such as Rac1, however conflicting evidence exists as to whether this regulation enhances or reduces NOD-dependent NF-κB activation (38–40). A recent study suggested that Rac1 is activated upstream of NOD1, and that NOD1 essentially senses GTPase activation rather than the peptidoglycan fragments directly (41). NOD1 and NOD2 can recruit the GTPase

ATG16L1 and subsequently stimulate autophagy, a highly conserved bulk degradation system with antimicrobial activity against intracellular pathogens (42).

Among the studied lung pathogens, NOD1 responds to *Chlamydophila pneumoniae*, *Legionella pneumophila*, *Klebsiella pneumoniae*, *Haemophilus influenzae*, and *Pseudomonas aeruginosa* (32, 40, 43–47), whereas NOD2 senses *S. pneumoniae*, *S. aureus*, *Escherichia coli*, *C. pneumoniae*, and *Mycobacterium tuberculosis* (30–32, 44, 48–50) (**Figure 2**). Accordingly, *Rip2*$^{-/-}$ mice – and to a lesser extend also *Nod1*$^{-/-}$ and *Nod2*$^{-/-}$ mice – display impaired chemokine production, neutrophil recruitment, and reduced antibacterial defense in response to pulmonary *C. pneumoniae* or *L. pneumophila* infection (32, 44). NOD2 is also required for efficient antibacterial innate and adaptive immunity in the chronic phase of pulmonary *M. tuberculosis* infection (51), and polymorphisms in the human *NOD2* gene have been associated with resistance or susceptibility to tuberculosis (52). Of note, mycobacteria express N-glycolylated MDP that has a stronger NOD2-activating potential compared to the MDP (53). NOD2 controls inflammatory responses to *S. aureus* pneumonia (49), and it is also required to clear pneumococcal colonization of the upper respiratory tract by CCR2-dependently recruited monocytes/macrophages. It was shown that professional phagocytes produce CCL2 after LysM-mediated bacterial digestion and subsequent NOD2-dependent detection of *S. pneumoniae*-derived peptidoglycan (48). Similarly, NOD1 controls neutrophil-dependent clearance of nasopharyngeal colonization with encapsulated *H. influenzae* in mice, whereas it is redundant for non-encapsulated strains (46). NOD1 might critically regulate microbial competition in the upper respiratory tract as *H. influenzae* derived peptidoglycan fragments activate NOD1, which instructs neutrophils to clear co-colonizing *S. pneumoniae* (54). Finally, one study implicated NOD2 in antiviral immunity to RSV and influenza virus infections (55).

Importantly, NOD proteins might also indirectly regulate immune responses in the respiratory tract. An elegant study by

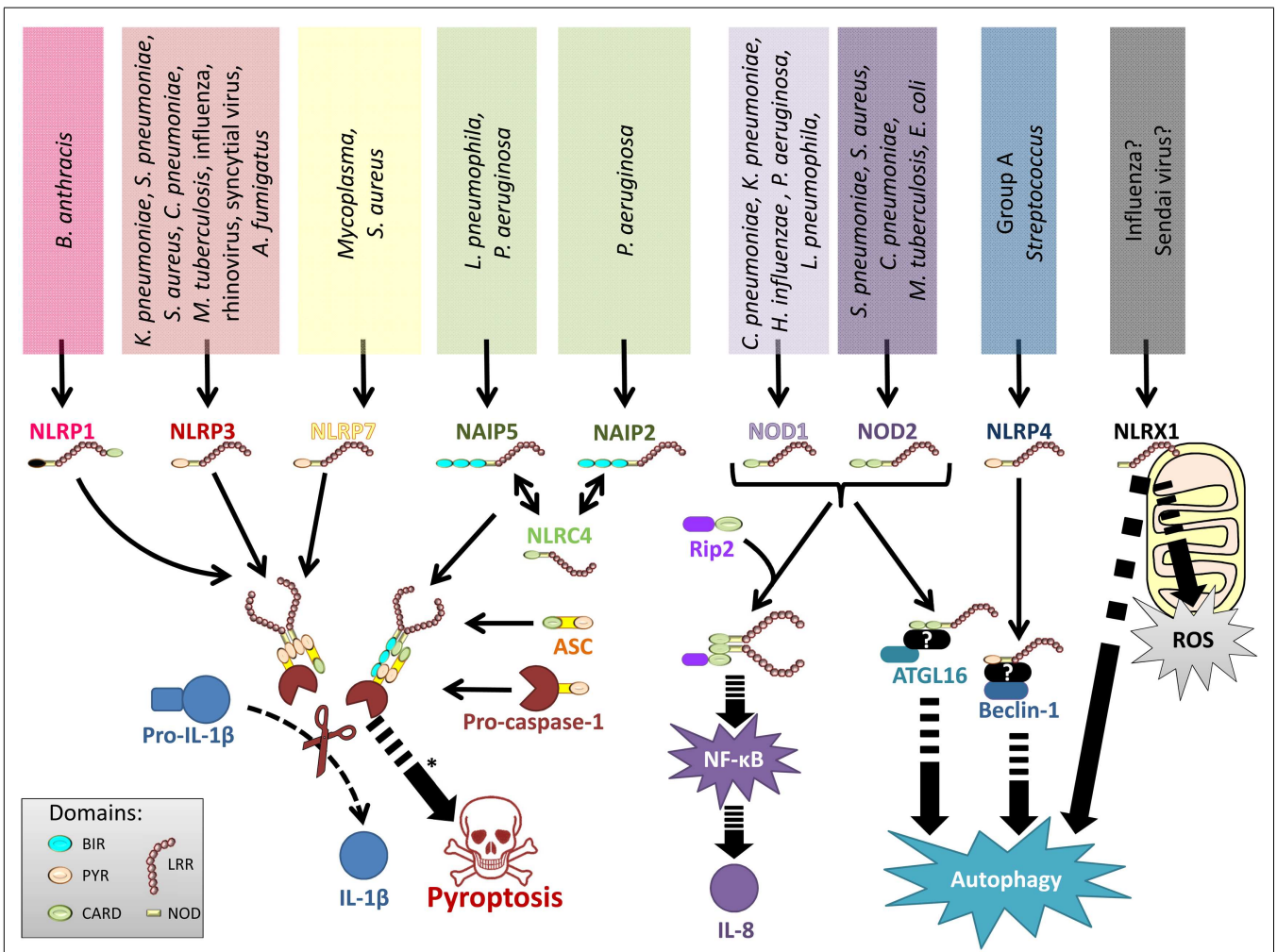

FIGURE 2 | Representation of NLRs involved in lung infections. Various lung pathogens can be sensed by different NLR. These NLRs either form inflammasomes to regulate IL-1 family cytokines and pyroptotic cell death, stimulate production of NF-κB-dependent inflammatory mediators, regulate autophagy, or control ROS production.

Weiser and colleagues showed than intestinal microbiota-derived NOD1 ligands translocate into the circulation and the bone marrow, where it enhances protective neutrophil functions in the periphery. This NOD1 induced neutrophil activation is required for efficient clearance of *S. pneumoniae* or *S. aureus* from the respiratory tract (56). NOD2 regulates the composition of the intestinal microbiota in mice (57, 58) and one might speculate about a similar function in shaping microbial communities in the upper respiratory tract.

Finally, NOD1 and NOD2 have been implicated in granulomatous and allergic lung diseases. For example, a genetic variation in *NOD1* was found to be associated with increased susceptibility to sarcoidosis in a Japanese cohort (59), and *NOD2* polymorphisms were associated with severe pulmonary sarcoidosis in Caucasian patients (60). *NOD1* as well as *NOD2* polymorphisms have been associated with increased risk of developing allergy and allergic asthma (61–64). Moreover, intranasal delivery of NOD2 ligands was shown to inhibit airway tolerance to antigens by modulating the Treg/Th2-cell balance (65). However, the function of NOD1/2 in these diseases remains ill-defined as compared to their well-established role in host defense.

NLRP PROTEINS

The NLRP subgroup of NLRs comprises 14 proteins of which NLRP1, NLRP3, NLRP6, NLRP7, and NLRP12 form multiprotein complexes termed inflammasomes, consisting of one or two NLR proteins, the adapter molecule ASC and pro-caspase-1 (20). Inflammasomes serve as platforms for autocatalytic caspase-1 activation, which in turn critically regulates IL-1β and IL-18 production by processing their zymogens proIL-1β and proIL-18, and induce an inflammatory form of cell death called pyroptosis. Inflammasome activation has also been implicated in the production of eicosanoids (66). A number of NLRPs, such as NLRP6 and NLRP12 exert inflammasome-independent functions, like negative regulation of innate immune signaling pathways (as discussed below).

NLRP1

NLRP1 was the first NLR protein to be described as forming an inflammasome (67). In humans, NLRP1 is abundantly expressed in myeloid cells, lymphocytes, and respiratory epithelial cells (68). A biochemical study showed that purified human NLRP1 can form an active inflammasome with ASC and caspase-1 in presence of MDP and ribonucleoside triphosphates (69). Nevertheless, it has so far not been clearly confirmed that MDP can trigger NLRP1 inflammasome formation in human cells.

Mice possess three genes encoding NLRP1, which are present in tandem on chromosome 11: *NLRP1a*, *1b*, and *1c* (70). Depending on the genetic background, one, two, or three of these NLRP1s can be expressed. Mouse NLRP1b senses lethal toxin (LT) of *Bacillus anthracis*, leading to inflammasome activation (70, 71). Using cells from wild-type and NLRPP1$^{-/-}$ mice, it was shown that LT but not MDP could trigger the NLRP1 inflammasome assembly (72). LT consists of two components: protective antigen (PA) and lethal factor (LF). PA mediates cytosolic uptake of LF, which has endopeptidase activity and cleaves several MAPK kinases [reviewed in Ref. (73, 74)]. This way *B. anthracis* blocks early immune responses by abrogating TLRs and NOD2 signaling (75). The expression of NLRP1b and potentially NLRP1a in macrophages, depending on the mouse background, leads to resistance to LT (70, 72, 75, 76).

It is unclear if NLRP1 mediated LT resistance exists in human cells, however it was mentioned in a recent review that the authors had never observed LT resistance in macrophages isolated from healthy human subjects (77). Studies in rats and mice, which also present cell death induced by LT depending on NLRP1, showed that LF-mediated cleavage of the N-terminal domain of NLRP1 leads to caspase-1 activation and IL-1β release (71, 78, 79). Interestingly, a recent study indicated that a direct cleavage of murine NLRP1b is sufficient to induce inflammasome activation in the absence of LF, and proposed that NLRP1 might function as a sensor of protease activity of multiple pathogens (79).

NLRP3

Expression of NLRP3 is strongly induced by inflammatory cytokines and TLR agonists in myeloid cells (68, 80). Moreover, low level expression has also been found in human bronchial epithelial cells (81). Similar to the other inflammasomes, the NLRP3 inflammasome mediates caspase-1-dependent processing of proIL-1β as well as proIL-18 into their mature forms and stimulates pyroptosis (20).

The NLRP3 inflammasome responds to a broad range of microbial and non-microbial agents. Among lung pathogenic microorganisms, *K. pneumoniae*, *S. pneumoniae*, *S. aureus*, *C. pneumoniae*, *M. tuberculosis*, *L. pneumophila*, influenza virus, human rhinovirus, RSV, and *Aspergillus fumigatus* have been shown to induce NLRP3 activation (82–99). It is generally accepted that those microbes or their molecules do not directly interact with NLRP3, but instead microbe-induced disruption of host cell physiology is sensed by NLRP3. The exact nature of the NLRP3 activating signal remains somewhat elusive although production of reactive oxygen species (ROS) (16, 100), mitochondrial dysfunction (101, 102), potassium efflux (103, 104), calcium mobilization (105), have been implicated in NLRP3 inflammasome activation during infection. Most pathogens stimulate ROS production in host phagocytes, which might be involved in NLRP3 activation (85, 93). Furthermore, *S. aureus*, *S. pneumoniae*, *M. tuberculosis*, and influenza virus disturb the cell membrane and/or the intracellular ionic concentrations by their pore-forming toxins, secretion apparatus or ion channel proteins (82, 86–88, 96, 106). Other pathogens might activate NLRP3 through an incompletely defined mechanism upstream of NLRP3 that senses microbial RNA (107), and Gram-negative bacteria stimulate a non-canonical caspase-11 inflammasome (108–111). Interestingly, NLRP3 inflammasome activation by non-pathogenic bacteria that do not actively disrupt host cell integrity is dependent on bacterial viability. Live but not dead bacteria contain significant amounts of mRNA, the recognition of which triggers NLRP3 inflammasome formation. This response requires the adaptor protein TRIF, but it remains unclear whether prokaryotic mRNA can directly activate NLRP3 or if it is the result of a proximal signaling event. Detection of bacterial mRNA is a key mechanism employed by the host immune system to sense the presence of viable and thus infectious microbes and thereby to scale the level of infectious threat (112, 113). These

findings underscore the role of NLRP3 as a sensor of microbial (and non-microbial) danger signals.

Several *in vivo* infection models have highlighted the central role of NLRP3 in host defense. NLRP3 was required for efficient antimicrobial responses against *S. pneumoniae*, *K. pneumoniae*, and influenza A virus *in vivo* (84, 85, 87, 88). Interestingly, the known susceptibility of aged mice toward influenza infection has been attributed to a reduced expression of NLRP3, ASC, and caspase-1 (114). It remains to be studied whether a similar mechanism contributes to the elevated susceptibility of elder humans to community-acquired pneumonia (115). Importantly, NLRP3 activation may also contribute to ALI, which was observed in a mouse model of *S. aureus* pneumonia (96). The net effect of NLRP3 during pneumonia might thus dependent on the pathogen load, the virulence of the pathogen and/or the expression of inflammasome components, as well as the susceptibility of the patient to pulmonary damage.

Of note, NLRP3 (and even more pronounced the NLRP6; see below) inflammasome activation in the gut shapes the intestinal microbiota (116). The commensal microflora in turn induces expression of NLRP3, proIL-1β, and proIL-18 in the lung (117). This microflora driven host gene regulation is beneficial since antibiotic depletion of the resident microbiota resulted in markedly elevated susceptibility to influenza A virus infection in mice (117).

Importantly, the NLRP3 inflammasome responds to a vast range of sterile stimuli, particularly so-called DAMPs released by dying cells including ATP, uric acid metabolites, biglycan as well as hyaluronan (106, 118–121). Experimental studies in mice suggest activation of NLRP3 by some of those DAMPs might have important functions in the pathogenesis of ALI/ARDS, COPD/emphysema, and lung fibrosis.

Efficient pulmonary gas exchange critically depends on the integrity of the fragile lung barrier composed of the alveolar epithelium and the endothelium of the pulmonary microvasculature. ALI and ARDS can develop in the course of pneumonia, sepsis, as a result of mechanical ventilation and hyperoxia, aspiration of gastric content, or major trauma (6). ALI and ARDS are characterized by a disrupted lung barrier, resulting in interstitial and alveolar edema, impaired gas exchange, and in severe cases organ failure and death. In addition, lung fibrosis may develop as a long term consequence of ALI/ARDS. Bleomycin treatment as a mouse model of acute inflammation and fibrosis results in uric acid- and ATP-release by dying cells that stimulated NLRP3 activation and IL-1β production and IL-1R-mediated inflammation, remodeling, and fibrosis (122–124). Bleomycin induced inflammation and fibrosis can be rescued by treatment with IL-1R antagonist (Anakinra) (123), allopurinol (impairs uric acid synthesis), uricase (degrades uric acid) (122), and apyrase (degrades ATP) (124). Moreover, it has been suggested that hyperoxia leads to NLRP3 inflammasome activation, secretion of pro-inflammatory cytokines, epithelial barrier dysfunction, and cell death (125, 126). Mechanical ventilation was shown to enhance IL-18 levels in the lung and serum, and inhibition of caspase-1 or IL-18 reduced ventilation-induced lung injury (127). Human ARDS patients express increased mRNA levels of inflammasome-related genes and IL-18 protein in their peripheral blood (127).

NLRP3 inflammasomes might also contribute to pathogenesis of chronic pulmonary disorders such as COPD and emphysema. Concentrations of uric acid is increased in broncho-alveolar fluid (BALF) of smokers and individuals with COPD as compared to healthy controls (128). COPD patients also have reduced levels of IL-1R antagonist (IL1RA) compared to controls (129). The mouse model of elastase-induced emphysema depends on uric acid, NLRP3, ASC, IL-1R, and MyD88 as critical mediators of inflammation, alveolar wall destruction, and fibrosis (130). Conflicting data exist regarding the contribution of the NLRP3 pathway in tobacco smoke-induced pulmonary inflammation. Whereas the study by Doz et al. indicated that smoke-induced inflammation is mediated by TLRs, the purinergic receptor P2X7, caspase-1, and IL-1R (131, 132), Pauwels et al. reported in another study that smoke-induced pulmonary inflammation occurs independently of the NLRP3 inflammasome (133). Transgenic overexpression of mature IL-1β in the lung epithelium of mice evokes a phenotype that closely resembles COPD, including inflammation, emphysema, airway fibrosis, and mucus cell metaplasia (134). Finally, *H. influenzae* infection induces NLRP3 expression and activation in human lung tissue, which might be a mechanism of infection-triggered COPD exacerbations (135). These studies together indicate an important role of caspase-1 and IL-1β in COPD and emphysema.

Conflicting evidence exists regarding the role of NLRP3 inflammasome-dependent IL-1β production in experimental asthma. Whereas ovalbumin-induced airway inflammation requires NLRP3 and IL-1β, house dust mite allergens induce pathology in an NLRP3-independent fashion (136, 137).

Pneumoconiosis is an occupational lung disease resulting from long-term exposure to silica, asbestos, or coal particles. It is characterized by pulmonary inflammation as well as fibrosis, which may be driven by NLRP3 inflammasome activation. It has been shown that engulfment of silica or asbestos crystals by resident macrophages leads to NLRP3 inflammasome activation and IL-1β production (16–18). It was suggested that crystal-induced inflammasome formation is a consequence of phagolysosomal disruption and leakage of enzymes such as cathepsin B into the cytoplasm (18). *Nlrp3*$^{-/-}$ and *Asc*$^{-/-}$ mice are protected from silica or asbestos-induced granuloma formation and fibrosis (16, 17). In contrast, mesothelioma development, a serious long term consequence of asbestosis, appears to be independent of NLRP3 (138). A recent case-control study in a Chinese population suggested that a NLRP3 polymorphisms may confer increased risk for coal workers' pneumoconiosis (139).

Taken together, NLRP3 is a key sensor of disturbed cell and tissue integrity during infectious and non-infectious pulmonary disorders.

NLRP4

NLRP4 has been proposed to be involved in reproduction in mammals (140–142). Nevertheless, NLRP4 expression in humans is found in various organs including the lung (142–144). *In vitro*, this NLR has the feature of a negative regulator of inflammatory responses by lowering NF-κB activation and IFNβ production (143, 144). Another particularity of NLRP4 is that its PYD is structurally different compared to the one in other NLRs, leading to the absence of interaction of NLRP4 with ASC (145). Besides, it has

been described that NLRP4 negatively controls autophagy during group A streptococcal infection by interacting with the autophagy regulator Beclin-1 (146). However, in the absence of conditional gene-targeted mice it is hard to predict a functional contribution of NLRP4 to pathologies in the lung.

NLRP6

NLRP6 has been indicated to fulfill anti-inflammatory functions by inhibiting NF-κB signaling downstream of, e.g., TLRs in macrophages and mouse (147). Moreover, elegant studies by the Flavell's laboratory showed that NLRP6 can form an inflammasome in intestinal epithelial cells that appears to sense components of the gut microflora and in turn regulates the composition of this flora through IL-18 (116). Related or unrelated to these mechanisms, NLRP6 has also been implicated in wound healing of the intestinal mucosa (148). NLRP6 have so far been mainly described in intestinal epithelial cells, neutrophils, and macrophages (116, 147) but our own unpublished data show expression of this protein also in activated murine alveolar epithelial cells (data not shown). The function of NLRP6 in the lung has, however, not been studied yet.

NLRP7

The NLRP7 gene is only present in humans and it is expressed in peripheral blood mononuclear cells (PBMCs) upon LPS and IL-1β stimulation (149). Gene silencing experiments in human monocytes and macrophages recently indicated that NLRP7 responds to bacterial lipopeptides and *Mycoplasma* as well as *S. aureus* infections by forming an inflammasome (150). Its precise function during bacterial infections remains unknown, and no data is available regarding its role in pulmonary physiology.

NLRP12

NLRP12 is expressed mainly in myeloid cells (151–153). Its expression is reduced by TLR stimulation and TNFα (151, 154) NLRP12 has been described as a negative regulator of classical and non-classical NF-κB activation downstream of TLR or cytokine receptors, by interacting with IKK and NIK (151, 155, 156). Furthermore, NLRP12 has been indicated to form an inflammasome, however this has so far only been observed upon *Yersinia pestis* infection (157). Finally, NLRP12 might play a role in adaptive immunity by controlling migration of DCs to the draining lymph nodes (153). *NLRP12*$^{-/-}$ mice did not respond differently to *M. tuberculosis* and *K. pneumoniae* lung infections and allergic airway inflammation wild-type mice in (158, 159), suggesting a functional redundancy with other NLRs, or a minor contribution of NLRP12 to inflammatory processes in the lung.

NLRC4 AND NAIP PROTEINS

NAIP5 and NLRC4 are expressed in the cytosol of bone-marrow and alveolar macrophages. A polymorphism in *NAIP5* (also called Bircle) has long been known to affect resistance of inbred mice toward *L. pneumophila* (160, 161). Whereas most mice strains are resistant against *L. pneumophila* infection due to a functional NAIP5, A/J mice expressing a NAIP5 that differs in 14 amino acids or *NAIP5*$^{-/-}$ mice allow *L. pneumophila* replication (162–164). This NAIP5-mediated resistance against *L. pneumophila* is dependent on detection of flagellin (162, 165), and on pyroptosis of the infected macrophage as well as effects on the trafficking of the *Legionella*-containing vacuole (166, 167). Similarly, NLRC4 is well known for mediating caspase-1-dependent responses to *L. pneumophila* and other flagellated bacteria (168–171). NLRC4, however, also respond to bacteria that express a type 3 secretion system (T3SS) including, for example, *P. aeruginosa* (172–176). Of note, the *Pseudomonas* T3SS effector protein ExoU can inhibit this inflammasome activation (176). One study suggested that NLCR4 is partially involved in the production of IL-1β and inflammasome-independent cytokines upon *K. pneumoniae* infection *in vivo* (177), even though *K. pneumoniae* does neither express flagella nor T3SS.

It is now clear that murine NLRC4 forms together with either NAIP5 (and possibly NAIP6) or NAIP2 two different inflammasomes that recognize flagellin or T3SS rod proteins, respectively (178, 179). These inflammasomes appear to regulate IL-1β/IL-18 through ASC and pyroptosis independently of ASC (175, 180, 181).

The exact function of human NAIP is currently incompletely understood. We and others suggested that hNAIP can detect and restrict flagellated *Legionella* (182–184), whereas others indicated recognition of bacterial T3SS needle proteins by hNAIP (178).

NLRC3 AND 5

NLRC3 is expressed in macrophages as well as lymphocytes and has been suggested to function as a negative regulator of the early TLR signaling (185). NLRC5 is highly expressed in mouse and human lung tissue. Its expression can be further induced by IFNγ and LPS in macrophages (186–189). NLRC5 protein has been described to inhibit IL-1β, TNFα, and IL-6 productions in macrophages upon viral infection. Its most prominent role is probably in adaptive immunity by enhancing the transcription of MHC class I related genes (186, 187, 189–197). In light of these data, NLRC5 involvement in pulmonary infection such as influenza infection will be an interesting area of study.

NLRX1

NLRX1 is ubiquitously expressed and located at the mitochondria due to an N-terminal mitochondrial targeting sequence, although the precise location (matrix or outer membrane) is still controversial (198, 199). The C-terminal LRR domain has been shown to bind to RNA but not to DNA by (200). Silencing of NLRX1 expression or knockout at the exons 4–5 in mice leads to exacerbated immune responses *in vivo* upon TLR stimulation and influenza or Sendai virus infections (201, 202). Knockout of the first four exons or exon 3, however, had no influence on the immune response to Sendai and influenza virus infections (203, 204). Instead, NLRX1 might function as an inducer of mitochondrial ROS production (205). This is consistent with the finding of two different groups that NLRX1 interacts with a protein of the mitochondrial respiratory chain (198, 203). Finally, NLRX1 has been indicated to regulate autophagy (206). Further work is clearly needed to clarify the function and exact mode of action of this unique NLR protein in general and in the lung in particular.

CONCLUDING REMARKS

Nucleotide-binding oligomerization domain-like receptors proteins are without doubt key players in the innate immune

responses to infectious and sterile inflammatory diseases of the lung, although many functions of several NLR family members, particularly in the lung, are still unknown. Many NLRs respond in functional cooperation with other innate sensors to invading microbes, particles, and endogenous danger signals after tissue damage. In similarity to possibly most immune receptors they can exert beneficial or detrimental functions, depending on the magnitude and the context of their activation. Increasing knowledge on specific activators and inhibitors of these pathways might help to manipulate them therapeutically in the not-so-distant future.

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Beneficial and detrimental roles of NLRs in carcinogenesis

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Inflammation plays a critical role in tumorigenesis and can contribute to oncogenic mutations, tumor promotion, and angiogenesis. Tumor-promoting inflammation is driven by many factors including the presence of the pro-inflammatory cytokines interleukin (IL)-1β and IL-18. One major source of IL-1β and IL-18 secretion is through the activation of inflammasomes. Inflammasomes are multi-protein complexes that upon activation lead to the processing and secretion of IL-1β and IL-18 mediated by the cysteine protease caspase-1. Several inflammasomes, including NLRP3, NLRC4, and NLRP6, have been implicated in tumorigenesis. However, inflammasomes play divergent roles in different types of cancer reflecting the complexity of inflammation during tumorigenesis. Understanding the role of inflammasome activation during specific stages of tumorigenesis and also during cancer immunotherapy will help identify novel therapeutic targets that could improve treatment strategies for cancer patients. Here we will discuss recent advances in understanding the mechanism by which NLRs regulate carcinogenesis.

Keywords: inflammasomes, NLR, cancer, interleukin-1, interleukin-18

INTRODUCTION

In order to protect us from infection, the immune system has employed an arsenal of pattern recognition receptors (PRRs) capable of recognizing a wide variety of microbial pathogens and viruses. PRRs are also activated under conditions of cell stress through the detection of damage associated molecular patterns (DAMPs) (1, 2). However, it is not well understood if and how the immune system has evolved to recognize overexpressed or mutated self-antigens in the context of cancer. Various PRRs have been implicated in cancer, either employing a protective or detrimental role. However, it remains unknown what causes the activation or suppression of PRRs within the tumor microenvironment. The activation of PRRs by tumor-related signals, in many cases, leads to the release of pro-inflammatory cytokines which can be beneficial by leading to proper activation of antigen presenting cells and subsequent T cell activation. However, it is well established that chronic inflammation can play roles during all stages of tumorigenesis and can be associated with poor clinical prognosis, depending on the type of pro-inflammatory cytokines and cancer (3–5). Here we describe the current literature on the role of PRRs, with a special focus on nucleotide-binding leucine-rich repeat (NLR) proteins, in cancer and how they might be utilized in tumor immunotherapy (summarized in **Table 1**).

The NLR family is comprised of over 22 members in humans (18, 19). NLR proteins are characterized by the presence of three homologous domains: a central nucleotide-binding and oligomerization (NACHT) domain, a C-terminal leucine-rich repeat domain (LRR), and an N-terminal effector domain (18, 19). As the name indicates, the NACHT domain is important for oligomerization, the LRR domain is important for ligand sensing, and the N-terminal effector domain recruits downstream signaling molecules (20, 21).

Within the NLR family, NLRP1, NLRP3, and NLRC4, as well as the PYHIN family member Absent in Melanoma 2 (AIM2), have been shown to form large multi-protein complexes termed inflammasomes. There is evidence that NLRP6 may also form a functional inflammasome (9); however, additional studies are required to confirm this. Inflammasomes are composed of an NLR protein (or AIM2), an adaptor protein apoptosis-associated speck-like protein containing a Card domain (ASC), and the cysteine protease caspase-1. Inflammasome activation is a two-step process requiring a priming step and an assembly step. The priming step results in the transcription of pro-IL-1β and pro-IL-18 along with certain inflammasome components (22, 23). The second step, which can be triggered by a variety of stimuli, results in the assembly and activation of inflammasomes. Inflammasome activation leads to the cleavage of pro-caspase-1 and its subsequent activation, which in turn cleaves pro-IL-1β and pro-IL-18 into their mature forms that can be secreted from the cell. Additionally, caspase-1 activation can lead to an inflammatory form of cell death known as pyroptosis (18, 23).

In addition, there are also reports of non-canonical inflammasome activation independent of caspase-1. For example, the receptor dectin-1 activates caspase-8 leading to processing and secretion of IL-1β upon sensing fungi or mycobacteria (24). Non-canonical inflammasome activation of caspase-11 has also been shown to be important for IL-1β secretion, caspase-1 activation, and macrophage cell death in response to *Escherichia coli*, *Citrobacter rodentium*, and *Vibrio cholera* (25). These data demonstrate

Table 1 | Role of NLRs in cancer.

NLR	Type of cancer	Possible mechanism	Reference
NLRC4	Plays a protective role in mouse models of colitis-associated colorectal cancer (CAC)	NLRC4 may regulate apoptosis in colonic epithelial cells	Hu et al. (6), and Sadasivam et al. (7), and Hu et al. (8)
NLRP6	Plays a protective role in CAC	Absence of NLRP6 leads to an increase in *Prevotellaceae* in the intestines and increased inflammation	Elinav et al. (9), Hu et al. (10), Normand et al. (11), and Chen et al. (12)
NLRP3	Plays a protective role in CAC	NLRP3 drives secretion of IL-18 which leads to activation of STAT1 and tumor suppression	Allen et al. (13) and Zaki et al. (14)
	Promotes tumor formation in a mouse model of fibrosarcoma Promotes pulmonary metastasis in intravenous B16F10 and RM-1 prostate mouse models	Presence of NLRP3 leads to an decrease in NK cells and $CD11b^{+}Gr\text{-}1^{int}$ myeloid cells leading to increased tumorigenesis	Chow et al. (15)
NLRP12	Plays a protective role in CAC	Absence of NLRP12 leads to dysregulation of canonical or non-canonical NF-κB signaling leading to increased inflammation	Zaki et al. (16) and Allen et al. (17)

important roles of various other pathways in processing of IL-1β. Importantly, non-canonical inflammasomes may also play a role in cancer-related inflammation.

Within the past 10 years, cancer-related inflammation was added to the list of cancer hallmarks (26, 27). Inflammation has been linked to tumor initiation, progression, angiogenesis, metastasis, tumor cell proliferation and survival, and alterations in the anti-tumor adaptive immune response. Sources of tumor-related inflammation include bacterial and viral infections, environmental irritants and obesity, and tumor-elicited or therapy-induced inflammation. Pro-inflammatory cytokines, such as IL-1β and IL-6, are critical mediators for inflammation-promoted tumorigenic effects and play critical roles in tumorigenesis (3, 4, 26, 27). For future development of therapeutic strategies, it will be crucial to understand how NLRs and inflammasomes are activated during tumor progression and how pro-inflammatory cytokines downstream of their activation impact tumorigenesis.

NLRC4

NLRC4

The PRR NLRC4 contains an N-terminal caspase activation and recruitment domain (CARD), a central NACHT domain, and a C-terminal LRR as depicted in **Figure 1A** (28, 29). The CARD domain present in NLRC4 allows for direct interaction with pro-caspase-1 (28). However, optimal caspse-1 activation requires the adaptor protein ASC (30–32). NLRC4 is classically understood to recognize a number of Gram-negative bacteria including *Salmonella enterica*, *Anaplasma phagocytophilum*, *Pseudomonas aeruginosa*, and *Legionella pneumophila*, which in turn leads to subsequent activation of the NLRC4 inflammasome (31, 33–35). More specifically, cytosolic flagellin and proteins with structural homology to flagellin such as PrgJ, a component of type III secretion systems, have also been shown to activate the NLRC4 inflammasome (36–38). The NLRC4 inflammasome works in concert with neuronal apoptosis inhibitor protein (NAIP) 2 to recognize PrgJ-like proteins and NAIP5 to recognize cytosolic flagellin (39–41). NAIP proteins, like NLRC4, have a C-terminal LRR domain, a central NACHT domain, and an N-terminal baculovirus IAP repeat (BIR) instead of the CARD domain (42).

NLRC4 AND CANCER

NLRC4 along with caspase-1 has been shown to regulate tumorigenesis in a mouse model of colitis-associated colorectal cancer (CAC) (6). In this model, cancer was induced by administration of azoxymethane and dextran sodium sulfate (AOM-DSS) to mice. Interestingly, $Nlrc4^{-/-}$ and $caspase\text{-}1^{-/-}$ mice exhibited increased tumor formation compared to wild-type mice. However, this phenotype was specific to colorectal cancer and not DSS colitis indicating that increased colonic inflammation was not the driving force for the enhanced tumorigenesis seen in $Nlrc4^{-/-}$ and $caspase\text{-}1^{-/-}$ mice and instead was likely due to a cell intrinsic mechanism. Although a definitive mechanism was not determined in the study, it is speculated that NLRC4 may be playing a role in alterations in colonic epithelial cell apoptosis as p53 activation has been linked to NLRC4 gene expression (7). Such a notion was further supported by the generation of wild-type and $Nlrc4^{-/-}$ bone marrow chimeras. $Nlrc4^{-/-}$ mice receiving a wild-type bone marrow transplant had similar tumor loads as $Nlrc4^{-/-}$ mice receiving $Nlrc4^{-/-}$ bone marrow in the CAC model, but exhibited significantly higher tumor burdens than wild-type mice receiving either wild-type or $Nlrc4^{-/-}$ bone marrow (8). This observation indicates that NLRC4 plays a CAC-suppressive role confined to cells other than in the hematopoietic compartment.

An alternative hypothesis that has been proposed involves the role of NLRC4 in the regulation of commensal microbiota. NLRC4 is important for detecting bacterial pathogens in the intestines without becoming activated by the presence of commensal organisms (44). Interestingly, $Nlrc4^{-/-}$ mice have alterations in their microflora compared to wild-type mice (9), indicating that NLRC4 is in some way important for the regulation of intestinal microbiota. The differences in microbiota seen in $Nlrc4^{-/-}$ mice did not lead to enhanced susceptibility to DSS-induced colitis (9). In

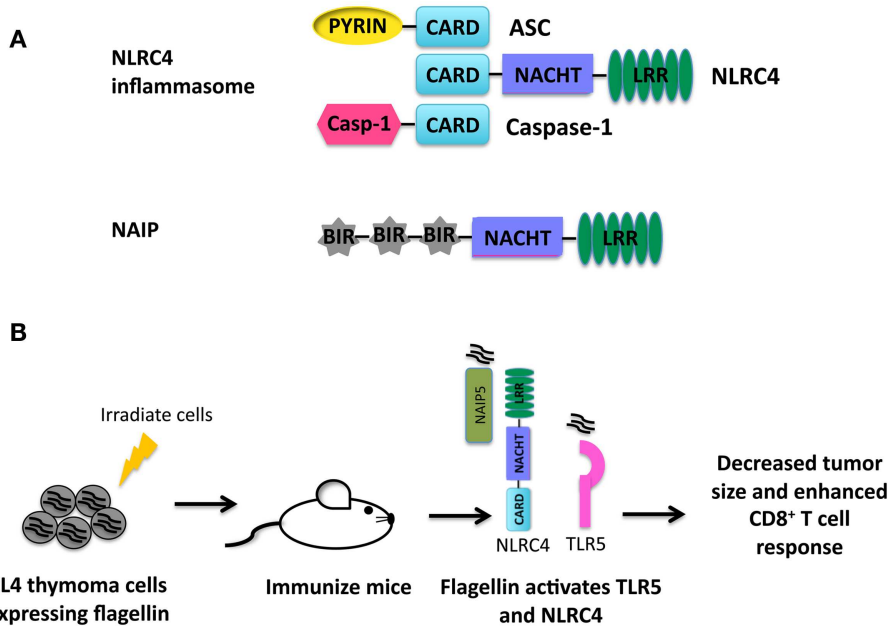

FIGURE 1 | (A) Structure of the NLRC4 inflammasome and NAIP proteins. **(B)** Summary of immunotherapy utilizing activation of NLRC4 (43). EL4 thymoma cells were transduced to express flagellin from *S. typhimurium*. Mice were then immunized with irradiated EL4 cells expressing flagellin. Flagellin is recognized by TLR5 and NLRC4 in the presence of tumor antigens leading to production of pro-inflammatory cytokines and an enhanced anti-tumor $CD8^+$ T cell response.

concert with these data *Nlrc4*$^{-/-}$ mice co-housed with wild-type mice, which leads to transmission of the intestinal microbiota, exhibited no differences in CAC tumorigenesis (10). Therefore indicating that alterations in the microbiota in the absence of NLRC4 are not correlated with progression of CAC.

NLRC4 AND TUMOR IMMUNOTHERAPY

Activation of NLRC4 results in the potent secretion of pro-inflammatory cytokines necessary for priming an effective adaptive immune response. Priming an immune response is especially problematic in a tumor environment where antigens are altered or are simply overexpressed self-antigens and the immune milieu is generally suppressive (45). A novel set of experiments were performed integrating the activation of TLR5, NAIP5, and NLRC4 by bacterial flagellin into tumor immunotherapy (43). In these studies, B16 melanoma cells and EL4 thymoma cells expressing flagellin from *Salmonella typhimurium* were generated. Both EL4 and B16 cells expressing flagellin were unable to establish tumors *in vivo*. In addition, EL4 and B16 cells expressing flagellin also induced a potent anti-tumor response from CD4 and CD8 T cells. Immunization of mice with irradiated EL4 cells expressing flagellin protected mice during subsequent challenge with live EL4 cells (**Figure 1B**). These data indicate that activation of TLR5, NAIP5, and NLRC4 during therapeutic strategies including tumor cell vaccination could be beneficial.

NLRP6

NLRP6

The structure of NLRP6 is comprised of an N-terminal Pyrin domain, a central NACHT domain and a C-terminal LRR as seen in **Figure 2A** (46). It is unclear if NLRP6 is able to form a functional inflammasome like NLRP1, NLRP3, NLRC4, or AIM2. From over-expression studies in human embryonic kidney (HEK) 293T cells, NLRP6 was recruited to ASC speck-like structures. In addition, COS-7L cells co-transfected with plasmids encoding pro-caspase-1, ASC, and NLRP6 were shown to secrete IL-1β (46). Additionally, *in vivo* studies suggest that NLRP6 may be forming an inflammasome (9). However, whether NLRP6 is able to be recruited to an inflammasome complex and cleave IL-1β when expressed at basal levels has not been determined.

NLRP6 has no identified ligand; however, it was recently shown to play a significant role as a negative regulator of inflammatory signaling during bacterial infection (47). *Nlrp6*$^{-/-}$ mice had decreased mortality and bacterial burdens when challenged with *Listeria monocytogenes* and *S. typhimurium*, accompanied by increased neutrophil influx. Additionally, *Nlrp6*$^{-/-}$ macrophages had increased NF-κB and ERK activation in response to bacterial infection. These data highlight NLRP6 as a negative regulator of inflammation with a role to potentially dampen pathology and damage to the host.

NLRP6 AND CANCER

NLRP6 is highly expressed in the duodenum, ileum, and colon, which prompted studies to determine the role of NLRP6 in the intestine (11). Like NLRC4, NLRP6 was shown to negatively regulate colitis and CAC in mice (9–12). In the AOM-DSS CAC model, *Nlrp6*$^{-/-}$ mice had increased pathology and tumor numbers compared to wild-type mice. *Nlrp6*$^{-/-}$ colons exhibited increased expression of pro-inflammatory cytokines indicating an inability of mice to control inflammation within the intestine.

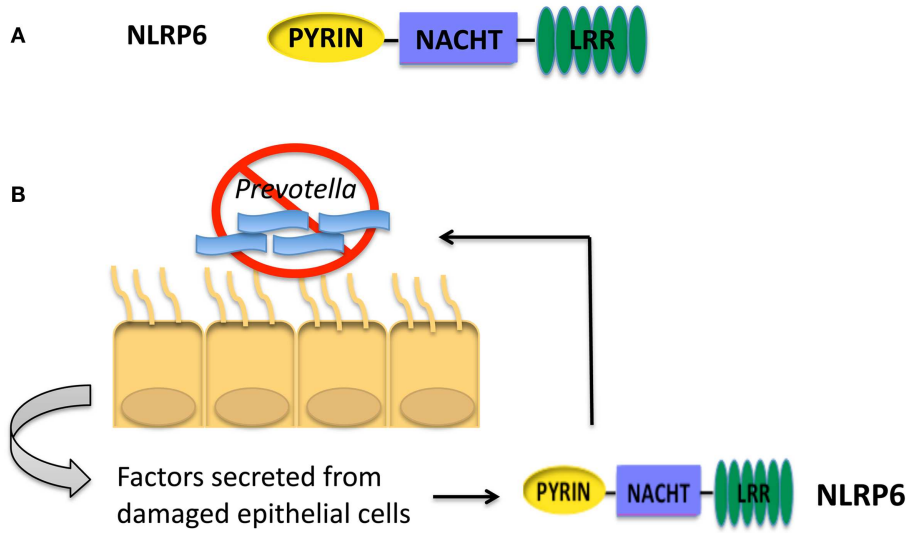

FIGURE 2 | (A) Structure of NLRP6. **(B)** NLRP6 is important for controlling the growth of *Prevotella* in the intestines of mice (9). Interactions between intestinal epithelial cells and pathogenic species of microbiota will lead to epithelial cell damage. Factors secreted from damaged epithelial cells activate NLRP6. Activation of NLRP6 leads to processing and secretion of IL-18 and a decrease in *Prevotella*. The presence of *Prevotella* is correlated with colitis and colorectal cancer in humans.

Moreover, *Nlrp6*$^{-/-}$ mice exhibited an increased level of epithelial proliferation and a decreased ability to heal wounds (11, 12). These data demonstrate a unique role for NLRP6 in controlling intestinal inflammation, preserving the integrity of the intestinal epithelial barrier, and wound healing in the intestine. Without NLRP6, the intestine is more susceptible to epithelial damage, which in turn increases inflammation and susceptibility to CAC.

In other studies focusing on the role of NLRP6 and colitis, *Nlrp6*$^{-/-}$ mice were shown to have significantly altered intestinal microbiota (9). Of note, *Nlrp6*$^{-/-}$ mice had an increase in the phylum Bacteroidetes family *Prevotellaceae* as described in **Figure 2B**. The increased inflammation and colitis seen in *Nlrp6*$^{-/-}$ mice was attributed to the presence of *Prevotellaceae*, and these colitis-conferring bacteria could be transmitted to co-housed wild-type mice leading to enhanced disease. The role of *Prevotellaceae* was not examined in the mouse model of CAC. However, wild-type mice co-housed with *Nlrp6*$^{-/-}$ mice exhibit similar tumor score indicating that the microbiota present in *Nlrp6*$^{-/-}$ mice promotes enhanced CAC (10). In addition, due to the increased inflammation present in the intestine colonized with *Prevotellaceae*, it could be hypothesized that the presence of *Prevotellaceae* might lead to an increased susceptibility to colorectal cancer. Interestingly, *Prevotellaceae* was also shown to be prominent in the microbiota of patients with inflammatory bowel disease and was significantly increased in colorectal cancer patients (48–50), thus providing a link between *Prevotellaceae* and colorectal cancer. However, it is unknown whether the increased inflammation due to presence of *Prevotellaceae* leads to inflammatory bowel disease and subsequent increased risk for colorectal cancer, or if *Prevotellaceae* can directly increase the risk for colorectal cancer independent of inflammatory bowel disease. Additional studies need to be performed to clarify the role of *Prevotellaceae* in the development of colorectal cancer.

NLRP6 AND TUMOR IMMUNOTHERAPY

Although there is no described tumor immunotherapy directly involving NLRP6, this area of research represents an interesting potential. As mentioned above, NLRP6 is a negative regulator of inflammation during bacterial infections. However, the role of NLRP6 as a negative regulator of sterile inflammation has not been explored. Chronic inflammation plays a crucial role in the initiation and progression of cancer (3, 4). If NLRP6 has a more global role in suppressing inflammation, it is possible that NLRP6 may be important in dampening tumor-promoting inflammation. Alternatively, inflammation is important for activating dendritic cells for proper antigen presentation and T cell activation (45). NLRP6 has the potential to alleviate inflammation that is crucial for an effective T cell response. Further studies of NLRP6 will need to be pursued to determine if it contributes to tumor progression or protection.

Alterations in microbiota have been shown to impact the severity of inflammatory bowel disease and colorectal cancer in humans and mice (48–50). The microbiota is very pliable making it an ideal therapeutic target. With the development of probiotics, fecal transplants, and antibiotic treatments, there are a number of ways to treat dysbiosis in the intestine. Preventative treatments to alter the microbiota of patients with inflammatory bowel disease or dysbiosis may decrease levels of inflammation present in the intestine, thereby decreasing the risk of developing colorectal cancer. Moreover, treatment of dysbiosis in colorectal cancer patients may alleviate symptoms. Future studies should include determining if mutations in NLRP6 are correlated with colorectal cancer and dysbiosis in humans, specifically with the increased

Prevotellaceae. If a correlation exists between NLRP6 and colorectal cancer in humans, NLRP6 may serve as a valuable biomarker and therapeutic target.

NLRP3

NLRP3

NLRP3 contains an N-terminal Pyrin domain, a NACHT domain, and a C-terminal LRR as seen in **Figure 3A** (18). NLRP3 is expressed by a number of cells including epithelial cells, neutrophils, macrophages, and dendritic cells (51, 52). Upon activation, NLRP3 forms a complex with ASC, and caspase-1 leading to pyroptosis and the release of inflammatory cytokines. A number of stimuli are able to activate the NLRP3 inflammasome. Pathogens including *Candida albicans*, *Staphylococcus aureus*, and *Influenza*, among others, have been shown to activate the NLRP3 inflammasome (53–55). Additionally, host-derived stress or danger signals are able to activate the NLRP3 inflammasome, including extracellular ATP and monosodium urate crystals (55–57). Exposure to environmental irritants including silica and asbestos will also lead to activation of the NLRP3 inflammasome (58–60). It is hypothesized that the numerous diverse NLRP3 agonists converge on a common pathway that results in NLRP3 inflammasome activation. Currently, potassium and calcium fluxes along with the generation of reactive oxygen species (ROS) and mitochondrial dysfunction have all been shown to be required for NLRP3 inflammasome activation (61–65).

Cancer cells also exhibit altered metabolic activity in order to support increased proliferation and survival, and can sometimes display increased ROS production (68, 69). Thus, it could be hypothesized that dysfunctional mitochondria in cancer cells activate the NLRP3 inflammasome, leading to an increase in tumor-promoting pro-inflammatory cytokines. NLRP3 activation by mitochondria-associated factors from cancer cells is not well studied and is an area that warrants further investigation.

NLRP3 AND CANCER

NLRP3, like NLRC4 and NLRP6, is important for prevention of CAC development in the AOM-DSS model (13, 14). *Nlrp3*$^{-/-}$ mice had increased polyp numbers and size and worsened pathology compared to wild-type mice. This phenotype was also seen in *Asc*$^{-/-}$ and *caspase-1*$^{-/-}$ mice, indicating that the NLRP3 inflammasome is important in suppressing CAC development. Importantly, it was shown that the presence of NLRP3 in hematopoietic cells was necessary for this the tumor-suppressing effect in response to AOM-DSS challenge (13). Furthermore, IL-18 levels were dramatically reduced in the colon of *Nlrp3*$^{-/-}$ and

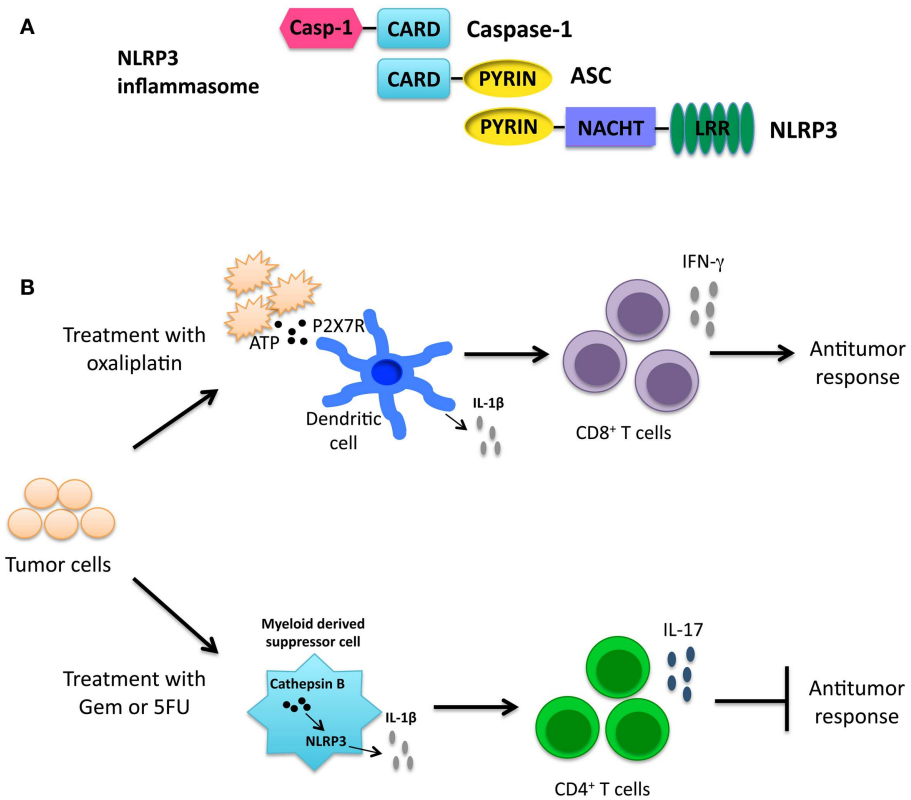

FIGURE 3 | (A) Structure of the NLRP3 inflammasome. **(B)** Cancer therapies activating the NLRP3 inflammasome have opposing roles. Treatment with the chemotherapeutic drug oxaliplatin leads to the release of ATP from dying cancer cells. ATP interacts with the P2X7 receptor on dendritic cells leading to activation of the NLRP3 inflammasome. IL-1β secreted by dendritic cells primes anti-tumor CD8$^+$ T cells and promotes the anti-tumor response (66). In contrast, gemcitabine (Gem) and 5-fluorouracil (5FU) activate the NLRP3 inflammasome via release of cathepsin B in myeloid derived suppressor cells leading to IL-1β secretion. IL-1β promotes CD4$^+$ T cells to secrete the cytokine IL-17, which blunts the anti-tumor response (67).

caspase-1$^{-/-}$ mice. Treatment of *caspase-1*$^{-/-}$ mice with recombinant IL-18 led to a reduction in disease demonstrating a crucial role of IL-18 in protection against CAC development (14). Partial amelioration of disease in *caspase-1*$^{-/-}$ by administration of exogenous IL-18 was also demonstrated in a separate study (70). In concert with these data, *Myd88*$^{-/-}$, *Il18*$^{-/-}$, and *Il18r*$^{-/-}$ mice were also shown to be more susceptible to DSS-induced colitis and colon cancer (71).

IL-18 was proposed to have a role in IFN-γ mediated activation of STAT1, which is known to have a role in tumor suppression (72). These data imply that the absence of NLRP3 results in decreased IL-18 production and STAT1 signaling that are necessary for protection against CAC potentially by playing a role in epithelial repair. Treatment with recombinant IL-18 did not fully alleviate symptoms indicating that some other mechanisms may be at play. It should be noted that in another study utilizing the AOM-DSS model, *Nlrp3*$^{-/-}$ mice exhibited a similar tumor load as WT mice (6). The difference in phenotypes may be attributed to alterations in mouse intestinal microbiota or differences in experimental procedures. However, whether NLRP3 is important for protection in colitis-associated cancer still remains unclear.

In contrast to the protective role of NLRP3 in CAC, NLRP3 was shown to promote tumor formation in a chemical-induced fibrosarcoma model (15). *Nlrp3*$^{-/-}$ mice treated with methylcholanthrene (MCA) exhibited prolonged tumor-free survival compared to wild-type mice. In concert with this data, *Nlrp3*$^{-/-}$ mice challenged intravenously with B16F10 melanoma and RM-1 prostate carcinoma cells had significantly fewer metastasis compared to wild-type mice. Reduced pulmonary metastasis was also seen in *Nlrp3*$^{-/-}$ mice using orthotopic transplant of E0771 mammary adenocarcinoma cells (15). The decrease in tumor formation was attributed to an increase in natural killer (NK) cells and CD11b^{+}Gr-1^{int} myeloid cells seen in *Nlrp3*$^{-/-}$ mice. The CD11b^{+}Gr-1^{int} myeloid cells secreted CCL5 and CXCL9 that were important for recruiting NK cells into the tumor microenvironment (15), demonstrating a role for NLRP3 in the suppression of NK cell activation and promotion of a suppressive tumor environment.

In an interesting *in vitro* study of late stage metastatic melanoma cell lines, HS294T and 1205Lu cells were shown to constitutively produce IL-1β in culture (73). In contrast, non-metastatic melanoma cell lines secreted less or no IL-1β. HS294T and 1205Lu cells also expressed NLRP3, ASC, and caspase-1 and secreted factors that resulted in increased macrophage chemotaxis and angiogenesis (73). These data merit further study on the role of inflammasome activation in metastatic tumor cells, and how inflammasome activation is altered in tumor cells as they progress from non-metastatic cells to ones with metastatic capacity.

NLRP3 AND IMMUNOTHERAPY

As inflammation has been shown to be both beneficial and detrimental for cancer, it is fitting that NLRP3 activation in immunotherapy can also be beneficial and detrimental. Chemotherapy-induced cell death was shown to result in both priming and activation of the NLRP3 inflammasome. Priming occurred through TLR4 by high mobility group box-1 (HMGB1) protein; ATP released from dying cells then lead to activation of the NLRP3 inflammasome (66). NLRP3 activation in dendritic cells by oxaliplatin-treated tumor cells resulted in the release of IL-1β that was critical for priming of anti-tumor CD8^{+} T cells as depicted in **Figure 3B**. This knowledge is useful in formulating a therapeutic setting where chemotherapeutic drugs known to activate the NLRP3 inflammasome may be used to improve prognosis.

In contrast, the chemotherapeutic agents gemcitabine (Gem) and 5-fluorouracil (5FU), which have been shown to deplete myeloid derived suppressor cells (MDSCs), activated NLRP3 in MDSCs resulting in a diminished anti-tumor response (67, 74, 75). Treatment with Gem or 5FU resulted in the release of cathepsin B that was shown to activate NLRP3 with the subsequent secretion of IL-1β. IL-1β then led to an enhanced IL-17 production by CD4^{+} T cells as depicted in **Figure 3B**. Interestingly, treatment of *Il17a*$^{-/-}$ mice with 5FU resulted in decreased tumor size (67). Taken together these findings demonstrate a role for NLRP3 activation in skewing a Th17 response and leading to a decreased anti-tumor response. It should be noted that 5FU treatment combined with IL-1R-blocking antibody lead to decreased tumor size. Although treatment with IL-17-blocking antibodies was not included in this study, combining IL-1R or IL-17 blocking antibodies with chemotherapeutic treatment may lead to a better outcome for some patients.

Another cancer immunotherapy is the use of dendritic cell vaccinations. Interestingly, the presence of NLRP3 led to a decrease in survival during B16F10 melanoma challenge and subsequent vaccination with B16-pulsed dendritic cells (76). The decrease in vaccine efficacy was due to a significant increase in tumor-infiltrating MDSCs and a decrease in CD8^{+} effector T cells. How NLRP3 recruits MDSCs into the tumor environment is unknown. MDSCs are very potent immunosuppressive cells that are associated with increased tumor growth (77). Determining the role that NLRP3, or other NLRs, may play in MDSC recruitment will be an important area of research to explore.

NLRP12

NLRP12

NLRP12, also known as Monarch-1, is composed of an N-terminal Pyrin domain, an NACHT domain, and a C-terminal LRR (78) as shown in **Figure 4A**. NLRP12 was shown to associate with ASC in an overexpression system; however, whether NLRP12 forms a functional inflammasome has not been well documented (79). NLRP12 is highly expressed in granulocytes in the bone marrow, and also macrophages in the spleen (80). Currently, NLRP12 is viewed as a regulator of inflammation. However, NLRP12 has been shown to have opposing roles in activation of NF-κB. Transient transfection of 293T cells with NLRP12 and ASC constructs led to transcription of an NF-κB luciferase reporter, demonstrating a role for NLRP12 in the activation of NF-κB (79). NLRP12 seems to inhibit the non-canonical NF-κB activation in the human monocytic cell line THP-1 by the binding of NLRP12 to NF-κB inducing kinase (NIK) and leading to the degradation of NIK (81). Interestingly, in humans, mutations in NLRP12 have been associated with a periodic fever syndrome (82, 83). When HEK293T cells were transfected with NLRP12 constructs harboring these mutations, an increase of NF-κB activation was seen (82). Missense mutations in NLRP12 in periodic fever syndrome were also associated

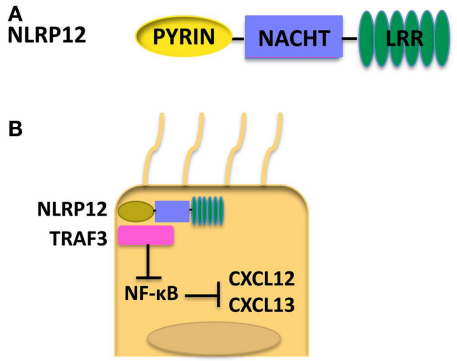

FIGURE 4 | (A) Structure of NLRP12. **(B)** When NLRP12 is present in intestinal epithelial cells it interacts with TRAF3 leading to stabilization of TRAF3 levels. Stabilization of TRAF3 leads to regulation of the non-canonical NF-κB pathway and diminished levels of the chemokines CXCL12 and CXCL13, both of which are upregulated in human cancers. Regulation of the non-canonical NF-κB pathway by NLRP12 leads to decreased inflammation and tumorigenesis in the colon (17).

with increased caspase-1 activation and had no effect on NF-κB (83, 84). These data further demonstrate NLRP12 as a potential negative regulator of inflammation and imply that NLRP12 may play a causal role in certain human disease.

NLRP12 has also been implicated in the control of bacterial pathogens. Both NLRP12 and IL-18 were shown to be crucial for control of *Yersinia pestis* infection (85), but NLRP12 was dispensable during infections with *Klebsiella pneumoniae* and *Mycobacterium tuberculosis* (80).

NLRP12 AND CANCER

The role of NLRs in colorectal cancer is well studied. NLRP12, like NLRP3, NLRP6, and NLRC4, plays a protective role during colorectal tumorigenesis. *Nlrp12*$^{-/-}$ mice exhibited an increase in tumor numbers in the AOM-DSS CAC model. Additionally, colons from *Nlrp12*$^{-/-}$ mice had increased tissue damage, pro-inflammatory cytokine production, and ERK, STAT3, and NF-κB activation (16). The increased tumorigenesis was attributed to a lack of NLRP12 in hematopoietic cells. Bone marrow-derived macrophages from *Nlrp12*$^{-/-}$ mice exhibited an increase in phosphorylation of p105/NF-κB1 in response to lipopolysaccharide stimulation demonstrating a role for NLRP12 in regulation of the canonical NF-κB pathway (16). It was hypothesized that dysregulation of the canonical NF-κB signaling in *Nlrp12*-deficient hematopoietic cells leads to increased inflammation and tumorigenesis.

In a more recent study, NLRP12 expression in non-hematopoietic cells was shown to play a protective role in CAC (17). *Nlrp12*$^{-/-}$ mice exhibited increased inflammation and tumor numbers. Interestingly, both *in vitro* and *in vivo* NLRP12 deficiency resulted in an increased activation of the non-canonical NF-κB pathway. *Nlrp12*$^{-/-}$ mice expressed elevated levels of CXCL12 and CXCL13, and also increased phosphorylation of ERK (17). It was hypothesized that NLRP12 negatively regulates non-canonical NF-κB signaling via interactions with NIK and TRAF3. In the absence of NLRP12, there is an increased level of inflammation and cancer-promoting chemokines as depicted in **Figure 4B**. Discrepancies seen in canonical versus non-canonical NF-κB signaling *in vitro* may be due to the nature of the stimulus used. NLRP12 was shown to downregulate canonical NF-κB signaling in response to stimulation with TLR agonists whereas, in response to stimulation with TNF-α or CD40L, NLRP12 regulates non-canonical NF-κB signaling. These data suggest NLRP12 may modulate both canonical and non-canonical NF-κB signaling depending on the upstream stimuli.

It is possible that a common pathway exists which NLRs converge on leading to protection against colorectal cancer. This likely includes maintenance of epithelial barrier integrity. Inflammation, as a result of altered pro-inflammatory signaling or altered microbiota, leads to the breakdown of the epithelial barrier in the intestine. When the barrier between immune cells and the microbiota dissolves, PRR ligands are in abundance and activate cells in the intestine, leading to the release of more pro-inflammatory cytokines and creating a cycle of inflammation that leads to development and progression of cancer.

NLRP12 AND IMMUNOTHERAPY

The possibility of targeting NLRP12 as a therapeutic treatment has not been explored. NLRP12 is crucial for the down-regulation of NF-κB signaling. Activation of NF-κB has been correlated with enhanced tumor cell survival and growth (86). As NLRP12 is a regulator of NF-κB, it would be beneficial to determine the role of NLRP12 in human cancer. Additionally, it may serve as a more specific target in immunotherapy compared to NF-κB.

INTERLEUKIN-1β

IL-1β AND CANCER

As mentioned above, inflammasome activation leads to the processing and secretion of IL-1β. IL-1β is a potent pro-inflammatory cytokine associated with tumor growth and angiogenesis (87). In a study utilizing a B16 melanoma model, IL-1β-deficient mice had remarkably reduced subcutaneous tumor size and lung metastasis compared to wild-type mice. Additionally, IL-1β-deficient mice and wild-type mice treated with an IL-1R blocking antibody had significantly reduced angiogenesis as measured by microvessel density compared to wild-type mice (88). In concert with these data, IL-1β- and IL-1α-deficient mice had decreased tumor numbers and tumor size in the MCA model of fibrosarcoma. Additionally, IL-1R antagonist (IL-1Ra)-deficient mice had increased tumor size (89). Expression of IL-1β by tumor cells also contributes to tumor growth and angiogenesis. Mice challenged with Lewis lung carcinoma cells (LLC) transduced with human IL-1β exhibited increased tumor size and vasculature compared to LLC cells alone (90). These studies are critical in demonstrating a role for IL-1β in promoting growth of solid tumors, angiogenesis, and metastasis.

IL-1β AND IMMUNOTHERAPY

IL-1 expression is enhanced in a number of cancers including lung, colon, melanoma, and breast (91, 92). Studies in mouse models have demonstrated therapeutic promise for treatment with IL-1 blocking antibodies and also combining IL-1 blocking antibodies with traditional anti-cancer immunotherapies (67, 87, 88).

Anakinra is an IL-1R antagonist that is used to treat a number of autoinflammatory disorders (91). Anakinra in combination with dexamethasone has shown promise in slowing myeloma proliferation in patients with smoldering myeloma (93). Due to the strong correlation between IL-1β and tumor progression, it is worthwhile to continue to explore Anakinra and other IL-1R antagonists as tumor immunotherapy options.

INTERLEUKIN-18

IL-18 AND CANCER

Along with IL-1β, the cytokine IL-18 is also cleaved by caspase-1 into its mature form and plays both beneficial and detrimental roles in the progression of cancer. In B16 melanoma models IL-18 acts as both an immunosuppressive and prometastatic factor. In an intravenous model of B16 melanoma IL-18 was shown to upregulate PD-1 expression on NK cells. Knock-down of IL-18 led to reduced pulmonary metastasis and increased NK cell function (94). Other studies showed that administration of IL-18 binding protein, which blocks IL-18, before injection of B16 melanoma into the spleen reduced metastasis to the liver. Mechanistically it was shown that treatment with IL-18 binding protein also reduced adhesion of B16 melanoma cells to hepatic sinusoidal endothelial cells leading to decreased metastasis (95). In concert with these data, administration of exogenous IL-18 led to an increase of adherent melanoma cells to sinusoidal endothelial cells (96). In humans an increase in IL-18 is correlated with various types of cancer including ovarian carcinoma, head and neck squamous carcinoma, breast cancer, and others (97–100). These data clearly demonstrate a pro-tumorigenic role of IL-18 both in humans and mice. Additionally, IL-18 may serve as a valuable biomarker for certain types of cancer.

In contrast, IL-18 plays a protective role during development of AOM-DSS CAC as mentioned above. *IL-18*$^{-/-}$ and *IL-18r*$^{-/-}$ mice were more susceptible to colon polyp formation and treatment of *caspase-1*$^{-/-}$ mice with recombinant IL-18 leads to amelioration of disease (14, 70, 71). It was also shown that administration of IL-18 induced anti-tumor immunity in mice bearing B16 melanoma tumors expressing B7-1 (CD80). However administration of IL-18 to mice bearing B16 melanoma tumors alone had no effect on tumorigenesis (101). Therefore the role of IL-18 may vary depending on the types of tumor and the therapies it is combined with.

CONCLUSION

It is clear that NLRs possess roles far from just pathogen recognition. NLRs play crucial roles in both promoting and dampening inflammation associated with tumors. Although the role of NLRs is best characterized in colorectal cancer, NLRs likely play a role in many other types of cancer. Further research needs to be performed to determine what specifically in the tumor environment leads to activation or down-regulation of NLRs. Danger signals released from dying cells have been shown to activate the NLRP3 inflammasome and it is possible that cancer cells release specific ligands capable of activating other NLRs. As some NLRs, like NLRP12, seem to be regulators of inflammation it could be hypothesized that mutations in these genes could correlate with tumor initiation and progression. A deeper understanding of the role of NLRs in the stages of cancer from initiation to metastasis will aid the development of new therapeutic strategies.

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Licensing adaptive immunity by NOD-like receptors

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The innate immune system is composed of a diverse set of host defense molecules, physical barriers, and specialized leukocytes and is the primary form of immune defense against environmental insults. Another crucial role of innate immunity is to shape the long-lived adaptive immune response mediated by T and B lymphocytes. The activation of pattern recognition receptors (PRRs) from the Toll-like receptor family is now a classic example of innate immune molecules influencing adaptive immunity, resulting in effective antigen presentation to naïve T cells. More recent work suggests that the activation of another family of PRRs, the NOD-like receptors (NLRs), induces a different set of innate immune responses and accordingly, drives different aspects of adaptive immunity. Yet how this unusually diverse family of molecules (some without canonical PRR function) regulates immunity remains incompletely understood. In this review, we discuss the evidence for and against NLR activity orchestrating adaptive immune responses during infectious as well as non-infectious challenges.

Keywords: NLR, dendritic cell, NLRP3 inflammasome, NLRP10, vaccine response, autoimmunity, Th2 response, asthma

INTRODUCTION

When pathogens or injuries threaten the body, two different branches of the immune system work together to restore homeostasis: the innate and adaptive immune systems. They monitor the tissues of vertebrates and use different tactics to recognize and overcome threats. These two branches are inherently different in the sensors and mechanisms employed in order to provide either immediate protection with broad specificity (innate immunity) or delayed and prolonged protection with exquisite specificity (adaptive immunity). Further, the two branches must effectively coordinate a response in order to prevent excessive or inappropriately targeted inflammation. As Charles Janeway wrote in 1992, "the immune system evolved to discriminate infectious non-self from non-infectious self" (1). We will use this paradigm to develop a broadened classification system of five adaptive immune response types and then review how nucleotide-binding oligomerization domain-containing receptors (NOD-like receptors or NLRs) potentially regulate the response to each. These five categories are based on the origin of the target antigen affected by the adaptive immune response: (1) foreign pathogenic targets (i.e., antimicrobial immunity); (2) foreign non-pathogenic targets (i.e., allergies); (3) self targets (i.e., autoimmunity); (4) altered self targets (i.e., tumor immunity); and (5) foreign self targets (i.e., commensal homeostasis). The role of NLRs in each category is likely different, as the initiation of immunity as well as the outcome for the host are different (e.g., beneficial protection to pathogens versus loss of tolerance resulting in self-destruction).

PATTERN RECOGNITION RECEPTORS IN INNATE AND ADAPTIVE IMMUNITY

The innate immune system consists of barriers, networks of soluble mediators, and myeloid-derived executioner (effector) cells including macrophages, dendritic cells (DCs), and granulocytes. It utilizes evolutionary conserved receptors to survey the extracellular and intracellular environment for pathogenic elements and injury. These molecules include pattern recognition receptors (PRRs) and as the name suggests, they are able to recognize a variety of common molecular motifs called "pathogen/microbe associated molecular patterns" (PAMPs/MAMPs) derived from microbes, and "damage associated molecular patterns" (DAMPs) derived from mislocalized or damaged host molecules during states of cell stress (2). PRRs exist in transmembrane, secreted, and cytosolic forms. Toll-like receptors (TLRs) are located on the cell surface and in endosomal compartments where they can recognize extracellular or phagocytosed pathogens (3). C-type lectin receptors (CLRs) can be found in both membrane-bound and secreted forms, and bind carbohydrate-based PAMPs and DAMPs (4). RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and other nucleic acid sensing PRRs along with NLRs are located exclusively in the cytosol and nucleus, where they detect pathogens or processes that breach the cell membrane (2, 5–7). NLRs respond to a wide variety of PAMPs and DAMPs as well as intracellular and extracellular signals generated by other arms of the innate and adaptive immune system. Following PRR activation, various signaling cascades are induced that initiate or shape the appropriate inflammatory response and, mostly through the action of DCs, activate T and B lymphocytes of the adaptive branch of the immune system. As the highly specific antigen receptors on lymphocytes can sense an almost infinite diversity of antigens, a crucial distinction between an "appropriate" and an "inappropriate" target for the adaptive immune response is made during this step.

NLR FAMILY ARCHITECTURE AND FUNCTIONS

The array of cellular responses regulated by NLRs is striking and includes transcription (e.g., of MHC molecules), enzymatic activity (e.g., of caspases), and positive and negative regulation of

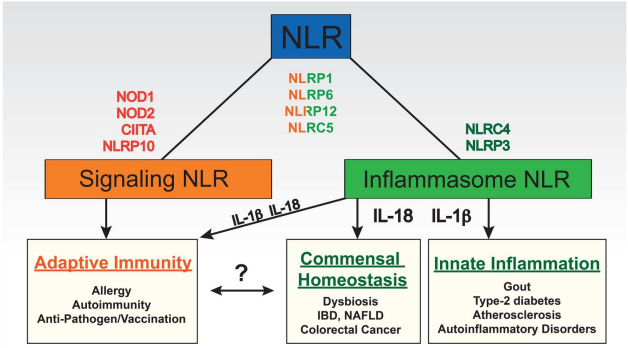

FIGURE 1 | Functional categories of NLRs based on their roles in shaping immune responses. Some NLRs have been found to play primary roles in regulating particular pathways (e.g., transcription) or signaling cascades ("signaling NLRs") such as NOD1, NOD2, NLRP10 (putative signaling NLR), and CIITA. Others regulate the formation of inflammasomes ("inflammasome NLRs") such as NLRP3 and NLRC4. Some do both such as NLRP1, NLRC5, NLRP6, and NLRP12. The immune consequences of these broad categories can be quite different. Although IL-1β and IL-18 derived from inflammasome activity can regulate particular aspects of adaptive immunity, direct roles of each of these cytokines in promoting non-lymphocyte based inflammatory reactions or commensal flora in the gut have also been identified. The latter has been implicated in shaping the adaptive immune system and therefore we linked these two categories; however, no direct role has yet been identified for an NLR in this link. On the other hand, NLRs involved in signaling cascades that in particular effect dendritic cell function as antigen presenting cells have a more obvious direct effect on adaptive immunity. IBD, inflammatory bowel disease; NAFLD, non-alcoholic fatty liver disease.

intracellular signaling cascades (e.g., NF-κB pathway) (**Figure 1**). This latter category of signaling NLRs primarily modulates pathways relevant to the innate immune response or its regulation of adaptive immunity. Signaling through NOD1 and NOD2, after sensing various bacterial peptidoglycan fragments, results in the activation of pro-inflammatory NF-κB and MAPK pathways, and the induction of autophagy (8–11). Extensive work on the biochemistry, ligands, and role of NOD1 and NOD2 in the innate and adaptive immune response has been done and is the topic of an accompanying review by Boyle et al. (12). NLRP10 and NLRP12 are required for the migration of antigen presenting cells (APCs) and thus have a role in defining the onset of adaptive immunity as will be discussed further in subsequent sections (13, 14). The NLRs CIITA and NLRC5 are induced by cytokines and act as transcriptional activators of MHC molecules, thereby potently regulating adaptive immunity. CIITA controls the transcription of MHC class II molecules and related proteins necessary for the presentation of antigen to $CD4^+$ T cells; NLRC5 does the same for MHC class I molecules and related proteins (15, 16) although it appears to have a broader function in pathogen sensing as well (17–19). In addition, several NLRs appear to have functions unrelated to the recognition of pathogens or damage, such as tissue homeostasis and embryonic development; however, further research is needed to unravel their exact roles (20).

Some NLRs perform more than one of these functions and for a majority of NLRs no known function has yet been identified. Therefore NLRs are classified by their structure, which consists of three distinct parts: a central nucleotide-binding domain (NBD), a carboxy-terminal leucine-rich repeats (LRR), and an amino-terminal effector domain. The effector domain instigates activity and divides NLRs into four subfamilies: the NLRA family, with an acidic domain, the NLRB family, with a baculoviral inhibitory repeat (BIR) domain, the NLRC family, containing a caspase activation and recruitment domain (CARD) and the NLRP family, which has a pyrin domain. Only NLRP1 and NLRP10 do not have this basic structure (21, 22). The only domain common to all NLRs is the central NBD, which has ATPase activity and is thought to induce oligomerization of the NLR proteins following activation (5).

NLR ACTIVATION

The classic paradigm for the function of a pattern recognition receptor was established through the study of TLRs. TLRs sense a foreign motif (e.g., a PAMP) on a pathogen through receptor ligation of the LRR domain, resulting in the induction of an inflammatory response and enhanced antigen presentation to lymphocytes [reviewed in Ref. (6)]; however, this paradigm does not fit the NLRs. Except for a few members like NOD1, NOD2, and NAIP5 (23–27), there is limited direct evidence that the LRR actually recognizes their respective agonists (28). It has been difficult to precisely define how NLRs bind ligands, activate, and oligomerize, although from electron microscopy data, a wheel-like structure analogous to many of the other oligomerized STAND (signal transduction ATPases with numerous domains) proteins appears to form (29–31). A recent crystal structure of part of NLRC4 in the inactive state suggests that the activity of this NLR is indeed regulated by the LRR domain along with the adjacent helical domain HD2 (32). Yet it had been previously demonstrated that instead of directly interacting with its ligands, NLRC4 senses PAMPs with the help of adaptor NLRs NAIP2 and NAIP5, which associate with PrgJ and flagellin of flagellated bacteria, respectively, and control the oligomerization of NLRC4 after ligand binding (25, 33).

Despite the many known stimuli, including insoluble crystals, bacterial toxins, and extracellular ATP, the mechanism by which NLRP3 is activated is only recently becoming clear. Because of the diversity of these ligands, it is likely that they activate NLRP3 through a shared mechanism involving desequestration of a host-derived trigger (34, 35). Sensing of increased cytosolic reactive oxygen species (ROS), intracellular calcium fluxes (36–38), potassium efflux (39–42), protein kinase (PKR) binding (43), or the release of contents from phagolysosomes have all been proposed as mechanisms. Numerous recent studies have honed in on mitochondrial-derived triggers regulating the cellular processes listed above or release of NLRP3 ligands from mitochondria including ATP, DNA, ROS, or mitochondria-associated adaptor molecule (MAVS) (38, 44–51). Given the importance of the mitochondria in regulating cell death, the potential triggering of NLPR3 activity by mitochondrial-derived signals suggests an interesting and central role for this organelle in integrating numerous cellular insults into a set of cell fate outcomes, some of which might be NLR-dependent (52). Biochemical identification of NLRP3 and other NLR-specific ligands, whether PAMPS, DAMPS, or more traditional signaling pathway molecules, will

greatly facilitate our understanding of how NLRs shape both innate and adaptive immunity.

INFLAMMASOME COMPLEXES

After recognition of a PAMP or DAMP, a number of NLRs (NLRP1, NLRP3, NLRP6, NLRP7, NLRP12, NLRC4, NLRC5, NAIP2, NAIP5) form multi-protein complexes called inflammasomes (19, 33, 53–56). The signature events of a functional inflammasome are the activation of caspase-1 and subsequent cleavage of the pro-inflammatory cytokines IL-1β and IL-18 into their bioactive forms. Inflammasomes are thought to consist of multiple copies of an NLR that, after ligand sensing, recruit the protease pro-caspase-1. In most inflammasomes, these proteins are oligomerized by an adaptor protein called ASC (apoptosis-associated speck-like protein containing a CARD). ASC consists of both a pyrin domain and a CARD domain, which enable it to interact with the pyrin domain of the NLR and the CARD domain of pro-caspase-1. NLRC4 and mouse NLRP1b do not need ASC to form an inflammasome, but when ASC is recruited, the production of cytokines following NLRC4 signaling is much more efficient (57–59). However, since no NLR inflammasome structure has yet been solved (30, 60), there is still debate on the exact composition of inflammasomes. A "non-canonical" pathway resulting in inflammasome function has recently been described, in which caspase-11 activation by cytosolic Gram-negative bacteria such as *Escherichia coli* and *Citrobacter rodentium* enhances caspase-1 inflammasome activity or instigates cell death (61–63).

HOW NLRs MIGHT SHAPE THE FIVE CATEGORIES OF ADAPTIVE IMMUNE RESPONSES

DENDRITIC CELLS IN INNATE AND ADAPTIVE IMMUNITY

The numerous pathogens we encounter everyday do not normally cause disease because most are quickly eliminated by our innate immune system. Under certain circumstances, some pathogens can evade our first line of defense and our adaptive immune system must use alternative tactics to combat the invading pathogen. APCs are critical for initiating this adaptive immune response by processing antigen and presenting it to naive T cells. Among APCs, DCs are thought to be the most potent as they temporally express requisite T cell co-stimulatory molecules, are readily motile and are widely distributed throughout the body forming a remarkable network of sentinel cells (64, 65). Under the steady state, DCs patrol the body seeking out evidence of invasion or malfunction. They express a variety of PRRs, including TLRs, NLRs, RLRs, and CLRs; PRRs are one major pathway DCs use to recognize either foreign invaders (containing PRR-stimulating PAMPs) or self molecules that have become altered (e.g., dying or transformed cells containing DAMPs). PRR activation dramatically impacts DC function by altering antigen presentation, phagocytic and macropinocytic capacity, and migratory properties. This allows DCs in peripheral tissues to transmit information about infection or tissue damage to the distal secondary lymphoid organs where naïve T-cells await stimulation.

The role of TLRs in shaping adaptive immunity via modulating DC function is well-studied, while the role of NLRs in adaptive immunity is less well understood. Recent studies suggest that the NLRs NOD1 and NOD2 might influence T cell differentiation via enhancing cytokine production from DCs in synergy with TLRs (66, 67). Work from our group and others demonstrated that particular NLRs can regulate DC migration during inflammation and infection (13, 14, 68). Antigen presentation depends critically on CIITA and NLRC5 while inflammasome activation results in IL-1β and IL-18 production, which can have both autocrine effects on DC maturation as well as shape the differentiating T cell cytokine profile (**Figure 2**). Despite these few examples, most of the work on NLRs has not clearly elucidated how DCs are affected by NLR activity. We will discuss those studies that have addressed the role of NLRs in shaping each of the five categories of adaptive immunity defined above and highlight findings regarding DCs when known.

CATEGORY 1: ADAPTIVE IMMUNITY TO FOREIGN PATHOGENIC TARGETS

Unlike the well-studied TLRs, which detect extracellular or endosomal PAMPs, the NLRs respond to pathogens that have reached the cytosol or the accompanying cell damage caused by breaching the cell membrane. Accordingly, NLRs can respond to particular pathogens that might evade detection by TLRs or other PRRs. For those NLRs that have been studied, much of the work regarding immune consequences of NLR activity has either used *in vitro* systems or evaluated the acute innate immune responses *in vivo*. Therefore knowledge regarding how NLRs shape adaptive immunity during infection is largely incomplete. In this section we will discuss only those studies that addressed regulation of adaptive immune responses to bacterial, viral, parasite, or fungal pathogens.

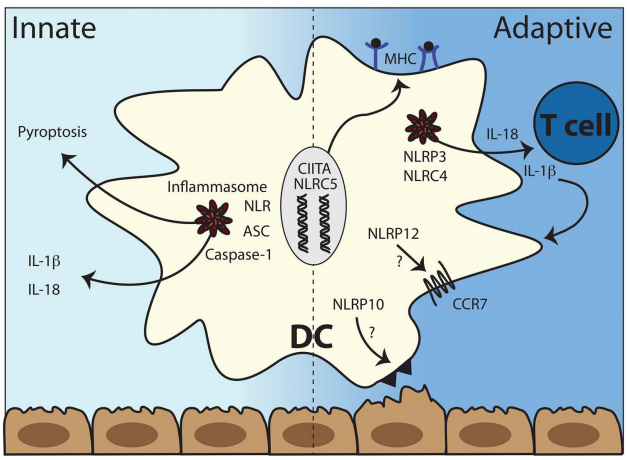

FIGURE 2 | The multiple roles of NLRs in dendritic cell function. Left panel: the recognition of intracytoplasmic triggers leads to the formation of inflammasomes and subsequent cleavage and release of the pro-inflammatory cytokines IL-1β and IL-18 and a specific kind of cell death called pyroptosis. Right panel: antigen presentation depends critically on CIITA and NLRC5 while inflammasome activation results in IL-1β and IL-18 production, which can have both autocrine effects on DC function as well as shape the differentiating T cell cytokine profile. NLRP10 and NLRP12 both are involved in DC migration from peripheral tissues to draining lymph nodes although potentially via different mechanisms. DC, dendritic cells; ASC, apoptosis-associated speck-like protein containing a CARD; NLR, NOD-like receptor; CCR7, C–C chemokine receptor type 7, MHC, major histocompatibility complex (class I left and class II right).

BACTERIA

Nucleotide-binding oligomerization domain-containing receptors are well suited to detect live cytosolic bacteria via PAMP recognition such as flagellar proteins or the cellular effects invading bacteria induce such as pore formation by toxins or secretion systems. The exotoxin pneumolysin (PLY) from *Streptococcus pneumoniae* is an important virulence factor responsible for forming pores in cell membranes (69). It was previously shown that PLY was crucial for caspase-1 activation and subsequent IL-1β and IL-18 production during pneumococcal infections (70, 71). In line with this, a recent study demonstrated that PLY activation of the NLRP3 inflammasome synergized with TLR agonists on *S. pneumoniae* to enhance secretion of IL-1β, IL-1α, and TNF-α by DCs and promote IL-17A and IFN-γ production by splenocytes (72, 73). Accordingly, NLRP3-deficient mice were more susceptible to pneumococcal pneumonia, suggesting that the inflammasome and its components contribute to the protective immune response to this bacterium.

A particularly crucial NLR poised to detect intracellular pathogens and orchestrate the subsequent adaptive immune response is NLRC5. Although NLRC5 has been reported to have a variety of functions in the innate immune response, its role in transcriptional regulation of MHC class I gene expression is essential for driving $CD8^+$ T cell priming to intracellular bacteria such as *Listeria monocytogenes* (18). NLRC5 expression is enhanced by PAMP as well as IFN-γ stimulation of hematopoietic cells and it can move from the cytosol into the nucleus where it binds and transactivates mouse and human class I genes as well as associated genes such as β2 microglobulin and TAP1 (74, 75). Therefore NLRC5 has a fundamental role in antigen presentation. The role of NLRs is less clear in another intracellular bacterial infection, *Mycobacterium tuberculosis (Mtb)*. *Mtb* readily infects macrophages *in vitro*, which subsequently secrete IL-1β and IL-18 in a NLRP3 inflammasome-dependent manner (76–79). As signaling through the IL-1 receptor and MyD88 are required for protection during *Mtb* infection, inflammasome regulation of protective immunity seemed likely. Using heat-killed *Mtb* as part of the potent adjuvant complete Freund's adjuvant (CFA), Shenderov and colleagues indeed demonstrated a profound impairment of Th17 differentiation in ASC- and caspase-1-deficient mice secondary to reduced IL-1β stimulation of T cells (80). Yet *in vivo* with a live infection, IL-1β levels in the lung are unaffected by loss of ASC or caspase-1 and bacterial burden and mortality in inflammasome-deficient mice are not significantly different from WT mice (76, 77, 79). Although IL-1β processing and secretion are, under most circumstances, inflammasome dependent, IL-1α is often secreted in an active form in an inflammasome-independent fashion (81). As both cytokines activate IL-1R1 and either cytokine alone is sufficient for protection during *Mtb* infection, it is perhaps not surprising that NLRP3 and the associated inflammasome components are dispensable for protective immunity (82).

Although numerous studies on NLRC4 have described its role in the innate immune response to flagellated bacteria, only a few investigations evaluated the role of NLRC4 in generating adaptive immunity to bacteria. Flagella from *Salmonella, Yersinia*, and *Pseudomonas* activate the NLRC4 inflammasome in splenic $CD8\alpha^+$ DCs to release IL-1β and IL-18; IL-18 can stimulate IFN-γ production from non-cognate memory $CD8^+$ T cells in the spleen thereby enhancing host resistance to bacterial infection (83). Caspase-1 regulated IL-1β and IL-18 production from DCs was also crucial for both Th1 and Th17-dependent protective immunity during *Helicobacter pylori* infection or even following vaccination, although the relevant NLR involved remains unknown (84). Caspase-1-, ASC-, and IL-18-deficient mice are all more susceptible to infection with the intracellular bacterium *Anaplasma phagocytophilum* as a result of inadequate Th1 activity. Partially via the inflammasome-forming activity of NLRC4, enhanced IL-18 secretion (presumably by APCs in the spleen) promotes Th1 differentiation and IFN-γ production – a crucial cytokine in regulating *A. phagocytophilum* bacterial loads (85). In fact one evasion mechanism used by the pathogen *Salmonella* is to downregulate NLRC4 expression to prevent the inflammasome response and thereby promote bacterial persistence and dissemination (86). Therefore NLRC4 and its adaptors are well poised to detect intracellular bacteria and mount an important inflammasome-dependent IL-18 driven adaptive immune response.

VIRUSES

Toll-like receptor- and RIG-I-like receptor-mediated recognition of viral infections results in the production of a number of viral resistance pathways and in particular the secretion of type I IFNs; in contrast, NLRs do not directly regulate IFNα or IFNβ and in fact inflammasome-driven pathways can be inhibited by these cytokines. Most NLRs do not directly recognize viral motifs but rather respond to cell disruption induced during viral infection with inflammasome-dependent cytokine secretion (87–89). The most extensively studied virus that activates inflammasome pathways is the respiratory pathogen influenza. Much like the pore forming bacteria described above, influenza can activate the NLRP3 inflammasome via insertion of a proton-selective ion channel, which it uses to change ionic gradients in intracellular compartments during viral entry and propagation, but also alerts NLRP3 to presence of the virus (90).

It is well known that IL-1 cytokines are crucial to the Th1 and $CD8^+$ T cell adaptive immune response to influenza; accordingly, ASC and caspase-1 are also needed for protective adaptive immunity (91, 92). A recent paper showed that DCs from elderly mice exhibited decreased expression of ASC, NLRP3, and caspase-1 compared with DCs from infected young mice and the concomitant blunted IL-1β response resulted in enhanced morbidity and mortality in influenza-infected elderly mice (93). A similar study demonstrated that TLR3 or NLRP3 stimulating adjuvants enhanced the efficacy of influenza virus-like particle vaccines in aged mice (94). These studies suggest that suppressed NLRP3 inflammasome activity during aging impairs protective adaptive immunity to influenza. Yet the course of influenza infection in different studies using NLRP3-deficient mice widely varied with some studies showing enhanced influenza susceptibility and others showing no difference (92, 95, 96). It was suggested that these opposing results might be due to different doses of influenza virus inoculation or the different viral strains used in these studies (92), but an alternative explanation might come from recent data on the role of IL-1 cytokines in regulating DC migration

during influenza infection. Pang et al. found that IL-1 signaling in pulmonary DCs was required for proper DC migration to lung draining lymph nodes and subsequent activation of influenza specific T cells; however, DC intrinsic activation of NLRP3 was not needed for antigen presentation or T cell priming (97). Therefore, much like the pathways described for *Mtb*, if inflammasome-independent IL-1α can be generated during infection, adaptive immunity should be minimally impacted in the absence of NLRP3, ASC, or caspase-1.

West Nile virus (WNV) is an emerging flavivirus capable of infecting the central nervous system (CNS) and mediating neuronal cell death. A recent study demonstrated that acute WNV infection induced IL-1β production *in vivo*, and that IL-1-R-, caspase-1-, and NLRP3-deficient mice exhibited increased susceptibility to WNV (98). This outcome was associated with increased accumulation of virus within the CNS and reduced anti-viral activity of effector $CD8^+$ T cells. This study indicates that IL-1β signaling and the NLRP3 inflammasome are important for host control of virus replication in neurons and WNV-induced pathology in the CNS.

PARASITES

Although the NLRP3 inflammasome is activated during many parasitic infections (e.g., *Leishmania major*) or by byproducts of parasitic infections (e.g., malarial hemozoin) and its effect on the acute innate immune response is well documented, the role of NLRP3 or other NLRs in the adaptive immune response to parasites has not been extensively studied (99, 100). The role of the NLRP3 inflammasome in shaping the T cell response to *Schistosoma mansoni* has been studied (101); the authors found that both ASC- and NLRP3-deficient mice failed to upregulate IL-1β in the liver and all parasite-specific helper T cell responses (Th1, Th2, and Th17) were reduced after infection compared to wild type controls. Surprisingly, these impaired T cell responses were correlated with smaller liver granulomas and attenuated immunopathology in the inflammasome-deficient mice, suggesting enhanced adaptive immunity to *Schistosoma* by NLR activation is not necessarily beneficial to the host.

FUNGI

It has been reported that *Candida albicans* hyphae activate NLRP3 (102, 103) and NLRP3-deficient mice succumb to both disseminated Candidiasis (102, 104) and mucosal Candidiasis (103), suggesting that the inflammasome is crucial for anti-fungal host defense. Caspase-1- and ASC-deficient mice indeed have impaired Th1 and Th17 responses during *Candida albicans*, leading to increased fungal outgrowth and reduced survival (105). Further, exogenous IL-18 was able to restore Th1 immunity to *Candida* in caspase-1-deficient mice (106), indicating that inflammasome-derived cytokines direct protective adaptive immune responses during invasive fungal infection.

NLRP10 has a critical role in DC localization *in vivo*; in its absence, impaired DC migration results in a profound helper T cell priming defect in a number of immunization models (14). In line with this, NLRP10-deficient mice have increased susceptibility to disseminated Candidiasis, as indicated by decreased survival and increased fungal burdens, secondary to impaired induction of *Candida*-specific Th1 and Th17 responses (68). In fact, adoptive transfer of *Candida*-specific primed T cells from WT mice rescues NLRP10-deficient mice infected with *Candida*. Therefore NLRP10, by regulating the movement of DCs presumably in the spleen in this case, can dramatically regulate pathogen-specific adaptive immunity. It still remains to be determined how NLRP10 activity is triggered during *Candida* infection and the molecular function of NLRP10 that regulates DC migration.

CLINICALLY APPROVED VACCINE ADJUVANTS

The development of vaccines against infectious pathogens has been and continues to be one of the most important medical interventions in global health. Many of our current vaccines do not induce adequate immunity unless co-administered with an adjuvant, which helps initiate adaptive immunity by stimulating the innate immune system. Although many pathogen-derived adjuvants used in animal models (e.g., CFA containing heat-killed mycobacteria or immunostimulatory oligodeoxynucleotides containing CpG motifs) have the capacity to stimulate various TLRs and NLRs, almost all clinically approved adjuvants for human use in fact do not contain any pathogen-derived motifs. Instead the most commonly employed adjuvants in human vaccines are aluminum hydroxide, which is a crystalline mixture and MF59, which is an oil-in-water emulsion. We and a number of other groups hypothesized that these adjuvants might trigger a DAMP-dependent rather than PAMP-dependent innate immune response thereby accounting for their immunostimulatory properties. Indeed alum is a potent activator, like many other insoluble crystals, of the NLRP3 inflammasome (107–111); however, whether triggering this innate immune pathway subsequently instructs the adaptive immune response during vaccination remains unclear (112). Different groups using the same mice with similar models have observed opposing phenotypes, possibly suggesting that multiple factors influence whether NLRP3 is relevant to the adjuvant function of alum. Some studies, including our own, have suggested that induction of adaptive immunity following alum-based vaccination requires a functional NRLP3 inflammasome via maturation of DCs (107, 109, 110); however, other groups found that NLRP3-deficient mice had intact T and B cell responses following vaccination with alum and antigen (108, 111, 113). Further, numerous other pathways downstream of alum immunization have now been proposed to be required for its adjuvant function, including DNA released during cell death, lysosomal permeabilization resulting in cathepsin B and S release, extracellular ATP, and uric acid release (114–117). Surprisingly, all of the above stimuli have also been directly implicated in NLRP3 inflammasome activation, yet in all cases NLRP3 was not required for successful immunization, leaving us without consensus on the mechanism by which alum regulates adaptive immunity. As alum has proven to be a great adjuvant for almost 100 years in that it works in most people, it is likely that it has multiple routes to induce immunity. In isolated systems used by different groups using murine models we have uncovered some, but perhaps not all, of those mechanisms.

A similar story has emerged for the oil-in-water adjuvant MF59 (118, 119). Ellebedy et al. showed that although NLRP3 is dispensable for the adjuvant effect of MF59, ASC is crucial for

the induction of antigen-specific IgG following vaccination with H5N1 in combination with MF59 (118). Indeed, activation of both germinal center B cells and $CD4^+$ T cells are significantly reduced in ASC-deficient mice, but not in NLRP3- or caspase-1-deficient mice. As it has been shown that DCs take up both antigen and MF59 and transport them to draining lymph nodes (120, 121), the markedly reduced secretion of inflammatory cytokines by ASC-deficient DCs upon stimulation with MF59 may be responsible for impaired T and B cell responses (118). Consistent with these findings, Seubert et al. also demonstrated that NLRP3 is not required for induction of adaptive immune responses to *Neisseria meningitides* with three particulate adjuvants: alum, MF59, and CFA (119).

Apart from traditional adjuvants, nanoparticles have been extensively studied as vehicles to co-deliver antigens and adjuvants for effective vaccination (122). Using a biocompatible polyester, poly(lactic-co-glycolic acid) (PLGA), loaded with antigen Demento et al. demonstrated that LPS-modified particles are preferentially internalized by DCs compared to uncoated nanoparticles and elicit NLRP3 inflammasome activity (123). Further, inhibition of endocytosis and lysosomal destabilization diminished inflammasome activity, suggesting that the rupture of lysosomal compartments by nanoparticles is a crucial trigger for inflammasome activation (123, 124). A more recent study found that porous silicon nanoparticles enhance phagocytosis and subsequent activation of DCs as well as IL-1β production (125). Administration of these nanoparticles also enhances DC migration to draining lymph nodes and T cell priming. However, adaptive immunity was either not evaluated in these studies or only partially affected in the absence of NLRP3, again leaving us with a vaccine formulation that can activate the inflammasome but might not absolutely require its activity for the initiation of adaptive immunity.

Nucleotide-binding oligomerization domain-containing receptors not only perform a major role in innate immune responses, but also have defined roles in generating effective adaptive immune responses against various infections. However, a number of important questions remain unanswered, including the mechanism by which NLRs detect molecules of microbial origin as well as how NLRs cooperate with other PRRs to mount an effective immune response against pathogens. It is possible that some NLRs serve as direct receptors of PAMPs, while others instead detect the perturbation of the host microenvironment by pathogens (42). Undoubtedly, understanding how NLRs recognize microbial products and initiate subsequent adaptive immune responses will provide new insights into vaccine design and vaccination strategies against infectious diseases.

CATEGORY 2: ADAPTIVE IMMUNITY TO FOREIGN NON-PATHOGENIC TARGETS

The diversity of molecules that can act as allergens is immense with the only unifying theme being the allergic type 2 immune response induced; in most cases this immune response is driven by Th2 lymphocytes [for review, see (126)]. Allergens are foreign, non-self molecules, and as such could trigger innate immune responses. Yet as most [but not all (126)] of these molecules are considered inherently non-pathogenic, it is unclear if they trigger classic PRR pathways. Many allergens indeed have enzymatic activity or are pathogen-derived (e.g., helminths) or can mimic PAMPs (e.g., Derp 2) and this might be how the innate immune system is tricked into initiating adaptive immunity (127, 128). Are NLRs involved in sensing allergens as pathogenic and determining the subsequent maladaptive immune response? Given that some allergens can directly induce tissue damage along with previous implications of IL-1 and IL-33 in Th2-mediated inflammatory responses, the prediction that NLRs play a role in the development of allergic diseases seems obvious. However, the evidence in favor of this argument is limited (129).

IL-33 is a prime candidate in the development of allergy as it clearly promotes Th2 (as well as ILC2) responses (130–133). Early work in the inflammasome field suggested that IL-33 was cleaved by caspase-1 and so this cytokine was grouped with the other inflammasome-regulated cytokines IL-1β and IL-18 and further postulated to be one mechanism by which inflammasome activity could regulate Th2 development (107). However, more recent work indicates that active IL-33 is in fact released from necrotic cells and functions more like a DAMP; if IL-33 is instead cleaved by caspase-1 or by caspase-3 during apoptosis it becomes inactive and fails to bind its receptor T1/ST2 (134–136). Therefore, the data supporting a role for IL-33 in the development of Th2-driven allergic inflammation indicates that this cytokine can function like a DAMP and shape the immune response but does not implicate a role for inflammasome activity in such a process.

LUNG

Similar to the diversity of antigens that can serve as allergens, there is a spectrum in the pathophysiology of the major allergic lung disease, asthma. The classic form is triggered by inhaled allergens that trigger degranulation of mast cells and basophils in the lung in an IgE-dependent manner and results in airway constriction and mucus production along with tissue inflammation dominated by activated eosinophils. Although the IL-1 gene cluster on chromosome 2 and promoter polymorphisms in IL-18 have been weakly associated with susceptibility to asthma (137, 138), the mechanistic link between IL-1, inflammasome activity and Th2 induction is uncertain partly due to the range of allergic airway disease models employed. As many murine allergy models involve intraperitoneal administration of antigen with the potent adjuvant aluminum hydroxide, the role of NLRs during the T cell sensitization phase to allergens in lung draining lymph nodes is difficult to determine (139); indeed intraperitoneal injection models have demonstrated a range of effects in IL-1- or IL-1 receptor-deficient or inflammasome-deficient mice (113, 140, 141). In contrast, when inhalational allergens are administered with nitrogen dioxide, urban particulate matter, or with presumed low-level PAMP contamination, a more significant role for IL-1 pathways and inflammasome molecules has been observed in some (142–145) but not all allergic airway disease models (113, 141). One caveat to these latter systems however, is that they can have a significant Th17-driven inflammatory response and as some studies have demonstrated a significant role for inflammasome-derived IL-1β on Th17 but not Th2 induction, the observed differences in inflammation might be due to loss of the Th17 component (143, 146). Nevertheless, inflammasome activity likely via IL-1 secretion appears to modulate particular aspects of adaptive immunity to inhaled triggers.

GASTROINTESTINAL

The disease corollary of this type of allergic adaptive immune response in the gastrointestinal system is food allergies. Outside of one study on subjects with a specific clinical subset of food allergies demonstrating an association with *Nlrp3* polymorphisms (147), there has been no link yet established between NLRs and the development of adaptive immunity to food antigens.

SKIN

The other primary organ affected by allergic adaptive immunity is the skin. Disorders such as atopic dermatitis (in some cases referred to as eczema) and allergic contact dermatitis (included under contact hypersensitivity) are manifestations of an often Th1/Th2 mixed adaptive immune response to unclear targets, but can include prototypical allergens such as house dust mite antigens or small molecule contact allergens. Accordingly, the inflammasome-dependent cytokines IL-1β and IL-18 have been implicated in promoting aspects of both a Th2 (elevated IgE, mast cell, and basophil degranulation with overproduction of type 2 cytokines) and a Th2- and IgE-independent innate inflammatory response (basophil and mast cell degranulation) (148, 149). IL-18, traditionally thought of as a Th1-promoting cytokine, has been implicated in the development of atopic dermatitis; transgenic mice expressing either human caspase-1 or IL-18 in keratinocytes develop spontaneous atopic dermatitis-like lesions with elevated mast cell activity (148). House dust mite cysteine proteases can directly activate the NLRP3 inflammasome in keratinocytes, which has been one proposed mechanism for induction of caspase-1 activity (150); however, mast cell chymase can also directly cleave pro-IL-18 independently of caspase-1 (151). Further work is needed to clarify if particular NLRs are indeed needed to direct the adaptive immune response to skin antigens in atopic dermatitis, but clearly pathways downstream of particular NLRs appear relevant.

Contact hypersensitivity is triggered by contact allergens (haptens, most often small lipophilic molecules that bind to self proteins) that induce a Th1/CD8+ T cell driven neutrophilic inflammation. An important role for IL-1β in the development of contact hypersensitivity has been known for many years (152) and a role for inflammasome-forming NLRs seems likely, especially given the expression of many NLRs and associated inflammasome components in the skin (153). Multiple studies have suggested that IL-1β directly enhances Langerhans cell activity in priming T cells to contact allergens (154, 155). However, subsequent work on this unique cell has indicated that it is primarily derived from embryonic fetal liver monocytes and locally maintained in contrast with classical DCs; further, Langerhans cells migrate significantly slower (e.g., on the order of 3–4 days) to draining LNs than typical DCs (156, 157). Recent studies instead suggested a regulatory role of Langerhans cells to skin-derived antigens (157). Therefore, the exact role of Langerhans cells in initiating adaptive immunity to contact sensitizers is not clear. Yet separate studies using caspase-1, ASC, or NLRP3-deficient mice all support a requirement for inflammasome activity in the generation of immunity to skin sensitizers (153, 155, 158, 159). This implies that inflammasome-regulated IL-1β might target cells other than Langerhans cells during sensitization in the skin such as dermal DCs; alternatively, inflammasome-dependent signals beyond IL-1β might directly modulate the lymphocyte response to contact sensitizers.

Beyond regulating inflammasome-induced cytokine secretion, NLRs can play a more direct role in regulating sensitization of T cells via affecting DC maturity and migration. Recently two NLRs were described to regulate the movement of DCs in an inflammasome-independent manner and gene knockouts of each had dramatic but specialized effects on allergic models *in vivo*. Arthur et al. demonstrated that hapten-induced contact hypersensitivity was defective in the absence of inflammasome-forming NLRP12 potentially via regulation of DC and neutrophil motility but independent of inflammasome activity (13). However, subsequent work from the same group indicated that NLRP12-deficient mice had no defect in T cell sensitization to antigens inhaled or administered intraperitoneally in two different allergic airway disease models (141), suggesting that NLRP12-dependent DC migration might be specific to skin DCs. NLRP10 was described by a number of groups including our own to regulate DC-dependent T cell priming as well as NOD1 signaling and inflammasome activity [please see (160) for review]. Our work suggests that sensitization to inhaled or subcutaneous antigens is defective in the absence of NLRP10 secondary to a DC migration defect; however T cell priming following topical sensitization was not evaluated (14). Therefore whether the NLRP10 and NLRP12 phenotype are complementary versus overlapping has yet to be determined. But interestingly both NLRP10 and NLRP12 were identified as susceptibility loci from GWAS studies of patients with atopic dermatitis (161, 162). NLRP10 is most highly expressed in the skin and knockdown of human NLRP10 in primary dermal fibroblasts attenuates innate immunity mediated by NOD1 (163). Therefore, further work is necessary to clarify if NLRP10 regulates adaptive immunity to skin antigens via hematopoietic cellular changes or rather acts in the barrier function of the skin.

CATEGORY 3: ADAPTIVE IMMUNITY TO SELF TARGETS

Autoimmunity is a pathologic process wherein T and B lymphocytes are activated by self molecules and induce damage to host tissues. As tolerance is built into the education of T and B cells, an active process must overcome tolerance to induce lymphocyte sensitization to self molecules; the innate immune system plays one key role in that process. Much of the early work on NLRs and inflammasomes followed the discovery that constitutively active mutants of NLRP3 were responsible for a subset of periodic fever syndromes now grouped under the term autoinflammatory diseases [for review, see (164)]. This NLRP3-dependent collection of rare syndromes, called the Cryopyrin-associated periodic syndromes (CAPS), is characterized by self-limited bouts of inflammation of the joints and skin with more systemic inflammation depending on the subtype; all are accompanied by fever and elevated levels of IL-1β (165). In fact, these disorders are now successfully treated by blocking IL-1β activity (166). It is important to recognize that these disorders do not include self-reactive T or B cells and therefore are distinct from autoimmune diseases (167). Although both autoinflammatory and autoimmune diseases involve recurrent episodes of inflammation (sometime with similar cytokine profiles) triggered by self molecules, the cells that drive inflammation and the nature by which they do so in the

two disease categories are different. Therefore for the remainder of this section, we will only consider the role of NLRs in the adaptive immune response to self targets and therefore autoimmune diseases. In contrast to the clear mechanistic link between autoinflammatory diseases and NLR activity, the link between autoimmunity and NLR activity is more tenuous (168).

How would NLR activity drive autoimmunity? One obvious link between inflammasome-forming NLRs and adaptive immunity is the potent cytokine IL-1β. IL-1β enhances expansion and survival of T cells (169), promotes differentiation of potentially pathogenic Th17 cells (170, 171) and can promote APC migration, thereby potentially enhancing antigen presentation (97) (**Figure 2**). Yet IL-1β cannot be sufficient for driving autoimmunity as autoreactive T and B cells are not a part of the pathophysiology of autoinflammatory diseases (165, 167). Indeed there is only limited evidence that NLR triggering induces a complete DC maturation program the way TLR activation transforms naïve DCs into potent APCs. Yet as discussed previously, particular NLRs such as CIITA, NLRC5, NLRP10, and NLRP12 regulate key steps in the antigen presenting function of DCs (34) and might regulate adaptive immunity to self in this way.

MULTIPLE SCLEROSIS

Perhaps the strongest link between an inflammasome-forming NLR and an autoimmune process is the link between NLRP3 and multiple sclerosis (MS). MS is a Th1 and Th17-driven response directed against myelin-producing oligodendrocytes in the CNS (172). Overexpression of IL-1β, IL-18, and caspase-1 has long been recognized in samples from MS patients (173–175). Studies in animal models further strengthened a connection between inflammasome activity and MS. A murine model with a clinical phenotype similar to MS, experimental autoimmune encephalomyelitis (EAE) exists. However this model relies on a highly artificial induction step to induce CNS-reactive T cell priming using CFA administered with peptides from the CNS (14). As *Mycobacterium* can activate multiple NLRs including NOD1, NOD2, and NLRP3 (66, 80, 176), one might expect to observe differences in adaptive immune-mediated disease when the adjuvant contains *Mycobacterium* as does CFA. Despite bypassing the natural immunization process, this animal model has been useful to dissect the immune pathways governing the autoimmune destruction of the CNS. Indeed caspase-1-deficient mice have reduced disease severity and delayed onset of paralysis (177). Further, caspase-1 dependent IL-1β and IL-18 from activated DCs promotes Th17 and γδ T-cell activation directed against the CNS (178, 179).

Once the link between caspase-1 and NLRP3 was identified, an obvious question was whether this particular NLR regulates the adaptive immune response to the CNS. Yet the answer remains ambiguous. One group using two different models of EAE found that NLRP3-deficient mice had normal $CD4^+$ T cell activation to CNS antigens but poor Th17 and Th1 differentiation. This was accompanied by reduced adaptive immune cell infiltration of the CNS and improved clinical outcomes (180, 181). Consistent with this finding, another group subsequently reported that NLRP3-sufficient DCs were required in order to program the primed autoreactive $CD4^+$ T cells to traffic into the CNS (182). In contrast to the above work, a third group demonstrated, using a similar EAE model that NLRP3 deficiency did not affect the development of paralysis and presumably retained autoreactive T cell activity (183). Instead this latter study proposed an inflammasome-independent role for the adaptor ASC in the autoimmune disease process; however, subsequent work from this group demonstrated a second mutation in their strain of ASC-deficient mice in the guanine nucleotide-exchange factor Dock2, which regulates actin polymerization during lymphocyte migration and DC antigen uptake (184). Therefore, the findings from the 2010 paper might be affected by a non-inflammasome related mutation in the ASC-deficient mice used. Nevertheless, the findings in NLRP3-deficient mice should have been unaffected and therefore still suggest NLRP3-independent induction of adaptive immunity in EAE. To date, no clear mechanism explains the discrepancy in results between groups using NLRP3-deficient mice; however, a recent paper by Inoue et al. suggests that the dose of *Mycobacterium* used in the CFA adjuvant determines whether a NLRP3-dependent or -independent response is observed in EAE models (185). Since, in all of the papers discussed above the dose of *Mycobacterium* used was not specified, it remains unclear if a difference in mycobacterial doses explains the above discrepancy. Therefore the question remains whether an inflammasome-dependent step is required to initiate adaptive immunity to CNS self antigens.

We recently described another NLR crucial in the early stages of T cell priming against self-molecules in the CNS in a non-inflammasome-dependent manner. NLRP10 regulates the movement of antigen-laden DCs and in a standard EAE model, NLRP10 deficiency almost completely abrogated the priming of IL-17 and IFN-γ producing $CD4^+$ T cells to CNS peptides and the associated demyelination (14). In this case, IL-1β and IL-18 production were not altered but rather autoreactive T cells were not activated because the crucial step of antigen presentation in secondary lymphoid organs failed. Again, given the use of CFA in this model, one can only conclude that DC movement and T cell priming are NLRP10-dependent in MS if the immune system is triggered in an analogous way during the natural course of disease.

DIABETES

In contrast to MS/EAE, no other autoimmune disease has been as clearly linked to the activity of an NLR. Type 1 diabetes (T1D) is driven by T cells specific for beta cells in the pancreas ultimately resulting in destruction of the islets and is accompanied by systemic production of islet-specific antibodies. Although there is mounting evidence that NLRP3 inflammasome activity promotes insulin resistance in models of type 2 diabetes (T2D) (186–188), there is scarce evidence indicating that the same is true for autoimmune T1D. In fact, the unaltered development of spontaneous diabetes in NOD mice lacking caspase-1 argues against a role for inflammasome activity in the pathogenesis of autoimmune diabetes (189). Yet various human studies have suggested that particular polymorphisms in NLRP1 and NLRP3 confer risk for T1D (190, 191). Further, IL-1β is known to promote beta cell secretory dysfunction and apoptosis and this has prompted clinical trials of IL-1 blockade in both T1D and T2D. Unfortunately though, this approach was shown to be ineffective in new onset type 1 diabetics in a recent multicenter randomized

double-blind placebo-controlled trial (192). Older work using the mouse NOD model suggested that IL-1R blockade in fact does not alter insulitis or the autoimmune process, although glycemic control was improved (193). Therefore, inflammasome activity and IL-1β in particular might play a more significant role in insulin sensitivity and beta cell dysfunction (T2D) rather than regulating autoimmunity to beta cells (T1D).

RHEUMATOID ARTHRITIS

The pathology in rheumatoid arthritis (RA) is driven by a T-cell-mediated immune response directed at the synovial lining of the joints and is accompanied by autoreactive antibodies systemically. Unlike some of the other autoimmune diseases discussed above, IL-1β plays a less significant role in the pathology of RA (194) and therefore the role of NLRs and inflammasomes in driving the autoimmune process has been less extensively studied. A handful of studies specifically looking for polymorphisms in NLRs or associated inflammasome components has indeed found some associations with NLRP1 and NLRP3 polymorphisms and RA susceptibility (195, 196). Yet two groups using two different mouse models of antigen-induced arthritis reported that the adaptive immune response directed at the joints was NLRP3, NLRC4, and caspase-1 independent (197, 198). Interestingly, both reported dependence instead on the inflammasome adaptor ASC. But as described above, one of these groups subsequently discovered an unintended mutation in a non-inflammasome pathway in the ASC-deficient mice relevant for antigen presentation by DCs and T cell activation (184). Given that both groups observed T cell and DC intrinsic defects in their strain of ASC-deficient mice, it is again possible that the above finding stems from an ASC-independent defect. Yet other data from these papers suggests that NLRP3, NLRC4, and caspase-1 do not appear to play a significant role in the adaptive immune response in RA models.

LUPUS

Systemic lupus erythematous (SLE or lupus) affects a wide range of organs primarily driven by immune complexes of nuclear antigens bound by autoantibodies, which initiate inflammation and tissue destruction. The primary nuclear antigen, double stranded DNA (dsDNA) can potentially act as a DAMP to trigger many PRRs including TLR7 and TLR9 (199), NLRP3 (200), and AIM2 (201) and each of these receptors has been implicated in the pathogenesis of lupus. In the case of the latter two PRRs, inflammasome function has been suggested from older studies demonstrating a crucial role for IL-1β (but not IL-1α) in the development of anti-nuclear antibodies and lupus disease manifestations (202). Indeed a recent elegant study by Shin et al. identified how NLRP3 is triggered by dsDNA; human monocytes produce IL-1β only when stimulated with dsDNA in combination with anti-dsDNA antibodies, the complexes known to trigger inflammation in lupus (200). Further, supernatants from these stimulated monocytes promoted IL-17 production from memory $CD4^+$ T cells. This suggests that the complexes formed in lupus patients can activate myeloid cells via NLRP3 and thereby reinforce T cell activation. It remains unclear though if this process and the NLRP3 inflammasome are required for initiation of the self-directed adaptive immune process. In fact a recent limited genetic analysis of *Nlrp3* (and *Aim2*) failed to find an association of particular single nucleotide polymorphisms (SNPs) with SLE predisposition; instead, polymorphisms in *Nlrp1*, including one in the promoter region, were associated with SLE in a Brazilian population (203).

Interestingly, non-synonymous coding-region and promoter polymorphisms in *Nlrp1* have been recurrently identified in genetic screens for a number of autoimmune diseases including vitiligo (melanocytes in the skin and hair targeted), Addison's disease (cortex of the adrenal gland targeted), type 1 diabetes (beta cells in the pancreas targeted), systemic sclerosis (nuclear antigens targeted), RA (synovium in joints targeted), and SLE (nuclear antigens targeted) (190, 191, 195, 203–206). Frustratingly, there has been little work done to identify how these various polymorphisms influence autoimmunity susceptibility outside of a possible effect on transcription level of *Nlrp1* (195). Indeed little is known about the function of NLRP1 *in vivo*, in part due to the divergence of *Nlrp1* genes between humans and mice. But of all NLRs discussed, NLRP1 is the most widely implicated in susceptibility to autoimmunity in human studies. Until a mechanistic understanding of these SNPs is discovered, the association with autoimmunity does not provide a paradigm in which to develop a role for NLRP1 in the initiation of adaptive immune processes directed against self molecules.

CATEGORY 4: ADAPTIVE IMMUNITY TO ALTERED SELF TARGETS

As tumors are derived from host cells that have lost the ability to regulate their appropriate growth, there is little to mark them as foreign or dangerous to the immune system. Outside of the aberrant loss of particular inhibitory signals to immune cells or altered forms of a self molecule, one signal that might alert the innate immune system to the loss of homeostasis is excessive cell turnover and damage to surrounding tissue, leading to the release of DAMPs. Therefore NLRs might be a relevant pathway to detect unchecked proliferation in tumors and initiate an immune response to these altered host-derived cells. However, several studies have suggested that IL-1β may have a role instead in promoting tumorigenesis (207, 208), since reducing or eliminating IL-1β can prevent tumor metastases and progression (209, 210), thus suggesting that inflammasome signaling may play a more complex role in the tumor-immune system interaction.

COLON

Most studies of NLR function in tumorigenesis have focused on colonic inflammation and tumor models. NLRs are involved in the maintenance of gut homeostasis and dysregulation of this fine-tuned balance can lead to chronic inflammation (please see Category 5: Adaptive Immunity to Foreign Self Targets), which is believed to create a tumor-promoting condition for intestinal cancer. Accordingly, mice deficient in caspase-1, ASC, NLRP3, NLRP6, NLRP12, and NLRC4 all have increased colitis and subsequent development of colorectal cancers in gut irritant models. In most of these studies caspase-1 processed IL-18 is a key regulator of these processes (211–214). Although most of these chronic models did not evaluate potential alterations in anti-tumor adaptive

immunity, one study showed that in the absence of NLRP3 and caspase-1, reduced IL-18 in a colorectal tumor model led to diminished IFN-γ levels in the colon (214). However, the cellular origin of this IFN-γ was not identified and therefore it remains unclear if anti-tumor adaptive immunity was in fact altered. In contrast, IL-18 has been implicated in promoting tumor metastasis from the lung, but in a T-cell and B-cell independent fashion; tumor-derived IL-18 promoted increased expression of the inhibitory receptor PD-1 on NK cells and prevented effective immunosurveillance (215). Therefore there might be a very different effect of inflammasome activity outside of the gut, wherein secreted IL-1β and IL-18 actually suppress tumor surveillance by innate cells but perhaps ultimately promote adaptive immunity to tumor antigens.

TUMOR VACCINES

Chemotherapy is one of the most commonly used treatments for cancer patients. It has been proposed that chemotherapy works via eliminating immunosuppressive cells and by directly inducing tumor-cell death (216, 217). Ghiringhelli et al. demonstrated that chemotherapy-treated dying tumor cells can activate the NLRP3 inflammasome in DCs and that a functional NLRP3 inflammasome in DCs is required for tumor-specific CD8+ T cell priming (217). In this study, the authors found that NLRP3- and caspase-1-deficient mice are unable to prime $CD8^+$ T cells, unless exogenous IL-1β is provided. Furthermore, they found that HMGB1 from dying tumor cells is critical for IL-1β release by DCs. In contrast, van Deventer et al. found that NLRP3 activity actually impairs anti-tumor DC-based vaccination by enhancing the accumulation of tumor-associated myeloid-derived suppressor cells (MDSC) thereby inhibiting the cytotoxic T cell response (218). Tu et al. also found enhanced MDSCs in gastric tumors induced by overexpression of IL-1β in the stomach; however, the enhanced tumor development was independent of T and B lymphocyte responses (219). In line with these studies, Bruchard et al. demonstrated that although chemotherapy depleted MDSCs and increased the survival of tumor-bearing mice by generating tumor-specific $CD8^+$ T cells, it triggered the release of cathepsin B from MDSCs leading to IL-1β secretion (220). IL-1β promoted IL-17 production from $CD4^+$ T cells, which in turn attenuated the anti-tumor effect of chemotherapy via an IL-17-dependent proangiogenic effect (220). These conflicting results may be due to the ability of particular drugs to induce immunogenic tumor-cell death (221) or differences in the form of tumor antigens used in these studies, [i.e., particulate (217) versus soluble (218)], which might be taken up by different DC subsets or recognized differently by the innate immune system.

Although TLR ligands have been tried as adjuvants to enhance vaccination efficiency for many years, very limited work has been done with NLR ligands. In a recent study, Garaude et al. demonstrated that introducing the bacterial protein flagellin, which activates TLR5, NAIP5, and NLRC4, into tumor-cell lines induced a potent $CD8^+$ T cell anti-tumor adaptive immune response and thereby helped eliminate injected tumors. They additionally found that priming of tumor-specific $CD4^+$ and $CD8^+$ T cells by DCs was promoted by the dual triggering of TLR5 and NLRC4 (222). Therefore, under specific situations, NLR triggering, possibly in combination with other PRRs, crucially regulates adaptive immunity to altered host cells in tumors.

CATEGORY 5: ADAPTIVE IMMUNITY TO FOREIGN SELF TARGETS

Up to 100 trillion microbes inhabit the human intestinal tract, which is 10-fold the number of human cells in the body. This ecosystem consists of fungi, bacteria, archaea, and viruses; most of these organisms are not pathogenic, but rather commensal, meaning that both the host and the organism benefit from co-habitation. Since these organisms function as a part of the human body and the immune system tolerates their presence, they can be thought of as an immune target that is both foreign but also self. A majority of these microbes can be found in the colon, where they contribute to the energy we extract from food, defend the mucosa against invading pathogens, induce the production of protective mucus and antimicrobial peptides as well as influence host immunity (223, 224). The microbiota is needed to adequately develop gut-associated lymphoid tissues by recruiting IgA-producing plasma cells and $CD4^+$ T cells to the lamina propria and directing the development both of local lymphocyte subsets (224–226) including Th17 and Treg populations, as well as distal B cell and T cell zones in lymph nodes and the spleen (227). Accordingly, changes in the microbiota are believed to contribute to the development of intestinal diseases as well as systemic metabolic disorders.

To date NLRs, which are clearly present and highly active in the gut, have not been implicated in educating these intestinal lymphocyte populations. However, their activity can have a profound impact on the composition of the microbiota and loss of NLRs with the ensuing dysbiosis can impact both local and systemic immunity (12, 227). The gut immune system must be able to tolerate commensal microbes, while still being able to keep microbes from coming in close proximity to the epithelial cell layer and inducing damage; the ability of NLRs to identify pathogens that have breached cell membranes makes them well suited to act at this level of barrier function (**Figure 1**). Several examples have been described: decreased NLRP3 expression and defective NOD2 have been associated with Crohn's disease, in which the microbiota is believed to contribute to the intestinal inflammation (10, 228, 229); NLRP3 and NLRP6 deficiency lead to a high susceptibility to dextran sodium sulfate (DSS)-induced colitis (54, 230) and NLRP6 was shown to impact the composition of the microbiota leading to an intestinal dysbiosis that resulted in spontaneous intestinal inflammation (54). Huber et al. found an important role for NLRP3 and NLRP6 in regulating tissue repair and tumorigenesis in the colon through IL-18-dependent downregulation of dendritic cell-derived IL-22 binding protein (231). Conversely, inflammasome regulation of adaptive immunity at distal sites can be influenced by the gut microbiota. Ichinohe et al. demonstrated that commensal bacteria were critical for the induction of adaptive immune responses (including $CD4^+$ T cell, $CD8^+$ T cell, and antibody responses) to respiratory influenza infection by providing "signal 1" or the priming signal for inflammasome substrates pro-IL-1β and pro-IL-18 (232).

However, the impact of NLRs on the induction of adaptive immune responses in the intestine needs further elucidation. We have previously proposed a "two-hit model" of PRR triggering to set thresholds for adaptive immunity in which DCs are primed by TLR activation but licensed by NLR activation (34). This model could also work to maintain homeostasis in the gut, in which the presence of microorganisms could lead to activation of TLRs, but activation of NLRs by DAMPS would be the decisive step in initiating an adaptive immune response.

CONCLUSION

Although a tremendous amount of work has been done resulting in significant advances regarding our understanding of NLR function over the past decade, there remains limited evidence that NLRs directly regulate adaptive immune responses. In ways distinct from TLR-driven pathways, NLRs indeed regulate aspects of DC migration, antigen presentation, and production of particular pro-inflammatory cytokines that can shape developing T cell responses (**Figure 2**). We have previously argued that this potentially represents a division of labor between two of the major subsets of PRRs, the TLRs and NLRs (34), although some NLRs can instigate inflammatory processes that overlap or modulate TLR-driven pathways to impact adaptive immunity. Yet for a majority of NLRs, including those identified through unbiased genetic screens such as NLRP1 (see Category 3: Adaptive Immunity to Self Targets), we do not know how these innate immune molecules function to instruct immunity. Given the location of these molecules and the distinct set of insults that stimulate their activity, it is not surprising that they have thus far been found to fulfill specialized but delimited functions in the intricate interplay between the innate and adaptive branches of the immune system.

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NLRC5, at the heart of antigen presentation

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Nucleotide-binding domain and leucine-rich repeat containing receptors (NLRs) are intracellular proteins mainly involved in pathogen recognition, inflammatory responses, and cell death. Until recently, the function of the family member NLR caspase recruitment domain (CARD) containing 5 (NLRC5) has been a matter of debate. It is now clear that NLRC5 acts as a transcriptional regulator of the major-histocompatibility complex class I. In this review we detail the development of our understanding of NLRC5 function, discussing both the accepted and the controversial aspects of NLRC5 activity. We give insight into the molecular mechanisms, and the potential implications, of NLRC5 function in health and disease.

Keywords: NLRC5, antigen presentation, transcription, innate signaling, NLR, MHC class I

INTRODUCTION

Nucleotide-binding domain and leucine-rich repeat (LRR) containing proteins (NLRs) play pivotal roles as intracellular pattern recognition receptors (PRRs) mediating detection of invading pathogens and triggering innate immune responses. Most NLRs are involved in either NF-κB signaling or interleukin (IL)-1β and IL-18 processing (1, 2). Nonetheless, functions beyond pathogen recognition have been reported for several NLRs [reviewed in Ref. (3)]. In mammals, NLRs are a large protein family, including 22 members in humans (4). They have a common tripartite structure, which consists of an N-terminal effector domain, a central nucleotide-binding domain and a C-terminal LRR-containing region [reviewed in Ref. (3, 5)]. The N-terminal effector domain of NLR proteins is in most cases either a caspase recruitment domain (CARD) or a pyrin domain that link to different signaling pathways [reviewed in Ref. (6, 7)] (**Figure 1**).

In this review, we focus on the NLR CARD containing 5 (NLRC5), a recently characterized family member involved in the regulation of major-histocompatibility complex (MHC) class I transcription. In particular, we will discuss the current understanding of NLRC5 expression patterns; the function of NLRC5 *in vitro* and *in vivo*; and how NLRC5 is implicated in immunological function and disease.

THE MHC CLASS II TRANSCRIPTIONAL ACTIVATOR: A PARADIGM FOR NLRC5 FUNCTION

The best example of an NLR protein that does not function as a PRR is the MHC class II transcriptional activator (CIITA). In contrast to other NLR proteins, CIITA harbors a unique N-terminal transcription activation domain (AD) followed by a proline/serine/threonine-rich region (P/S/T) [reviewed in Ref. (8)]. CIITA was originally discovered through an expression cloning approach using an MHC class II deficient clone of the Burkitt Lymphoma B cell line Raji (9–12). Steimle and colleagues identified a cDNA that complemented MHC class II expression, and termed the encoded protein CIITA (12).

Major-histocompatibility complex class II molecules are expressed constitutively on professional antigen presenting cells (APCs) and display antigens of exogenous origin. Phagocytosed antigens undergo endosomal degradation and are loaded onto MHC class II molecules in the so-called "MHC class II compartment." Peptide-MHC class II complexes are then transported to the cell surface, where foreign antigens activate $CD4^+$ T cells [reviewed in Ref. (13)] (**Figure 2**). Not surprisingly, CIITA loss of function mutants are associated with a severe immunodeficiency called bare lymphocyte syndrome (BLS). This condition is characterized by a lack of MHC class II expression, deficiency in helper T cells, and impaired humoral and cytotoxic responses (12).

In humans, CIITA transcription is tightly regulated and can be induced by the differential use of four alternative promoters, pI–pIV. However, the relevance of pII, which is used at low rate, remains unclear and only pI, pIII, and pIV are conserved in mice (14). Each promoter initiates transcription at unique sites, resulting in four isoforms of CIITA that differ in their N-terminus. pIII is mainly used in B cells, activated human T cells, and plasmacytoid dendritic cells (pDCs) (8, 14). By contrast, pI is active in DCs and results in a transcript that encodes an N-terminal CARD-domain. This isoform is the most efficient at inducing MHC class II expression *in vitro* (15), though its physiological role remains elusive (16). Interferon (IFN)-γ leads to high induction of CIITA expression, mainly through activation of pIV (14, 17).

In addition to CIITA, MHC class II transcription requires the DNA-binding factors regulatory factor X (RFX) 5, RFX-AP, and RFX-ANK (or RFX-B) (18–21). These RFX proteins specifically

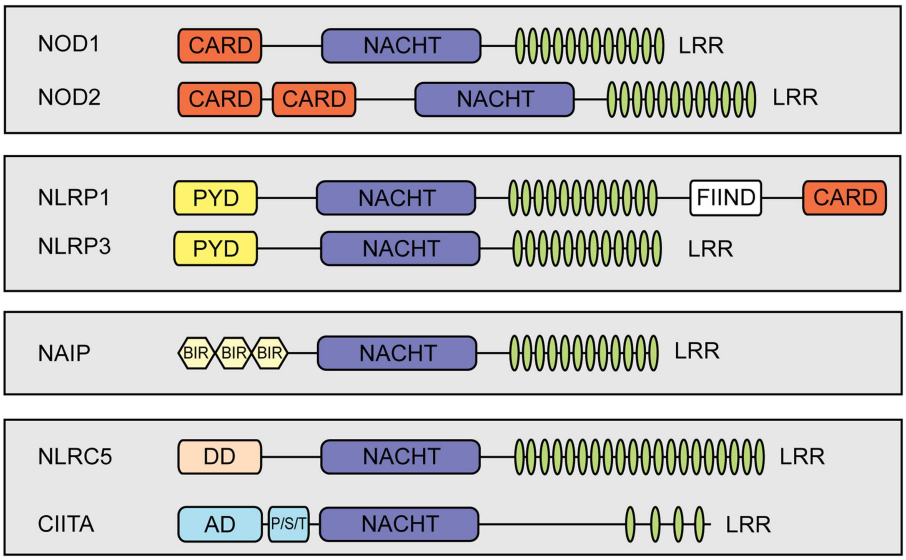

FIGURE 1 | Schematic representation of members of the mammalian NLR-family. NLRs share a tripartite domain architecture and can be subdivided based on the identity of their N-terminal effector domain which links to different cellular signaling pathways. All NLRs contain a central nucleotide-binding domain (NACHT) that mediates oligomerization. In addition, most NLRs contain putative ligand-sensing leucine-rich repeats (LRRs) and a variable N-terminal effector domain. The effector domain can be a pyrin domain (PYD), a caspase recruiting domain (CARD), or a baculovirus inhibitor of apoptosis repeat (BIR). Additional abbreviations: FIIND, function to find; DD, death-domain different from a typical CARD and PYD; AD, activation domain; P/S/T, proline/serine/threonine-rich protein domain.

bind to the X1-box inside the conserved SXY motif of the MHC class II promoters (22) (**Figure 3**). They assemble, together with X2BP (23, 24) and NF-Y factors (25, 26), into a DNA-binding platform known as the enhanceosome. CIITA itself does not directly bind DNA but rather uses the enhanceosome to dock to the MHC class II promoter to activate gene transcription by recruiting the general transcription machinery and histone-modifying enzymes [reviewed in Ref. (27, 28)] (**Figure 3**). CIITA not only governs the transcriptional regulation of the MHC class II α-chain and β-chain, but also regulates expression of other genes in the MHC class II locus, including the invariant chain (Ii), human leukocyte antigen (HLA)-DM, and HLA-DO. Interestingly, CIITA has also been reported to control expression of IL-4, Fas-ligand (CD95L), collagen A2, and some viral proteins [summarized in Ref. (29)].

By contrast to the presentation of exogenous antigens by MHC class II molecules to $CD4^+$ T cells, endogenous peptides are presented by MHC class I to $CD8^+$ T cells. Differently from MHC class II, MHC class I gene expression can be regulated by NF-κB and IFN-sensitive response element (ISRE) binding sites located within the promoter regions, which are absent in MHC class II promoters. In particular the classical MHC class I molecules HLA-A and HLA-B can be induced by NF-κB and are highly transactivated by IFN-γ (30, 31) (**Figure 3**). However, transcription of MHC class I genes, which are expressed in almost all nucleated cells, also occurs in the absence of NF-κB or interferon regulatory factor (IRF) signaling. MHC class I levels differ between tissues and cell types, indicative of a complex regulatory network (32). Interestingly, an SXY motif is also present in MHC class I promoter regions (33) (**Figure 3**) and several groups reported an involvement of CIITA in MHC class I expression *in vitro* via the enhanceosome (34–37). However, this could not be validated *in vivo*, as *Ciita*-deficient mice show normal MHC class I expression, suggesting the presence of additional factors driving basal MHC class I expression through the SXY regulatory region (38).

Intriguingly, alignment studies of the human NLR-family performed for the nucleotide-binding domain and the LRR region revealed a close homology between human CIITA and human NLRC5, suggesting that the latter might exhibit a function analogous to CIITA (39–42). Although, NLRC5 shows a typical tripartite NLR domain organization, it should be mentioned that it contains a rather unusual long LRRs and harbors a N-terminal effector domain with a predicted death-domain (DD) fold different from regular DD, DED, PYD, or CARD domains (39, 43, 44).

NLRC5 EXPRESSION AND REGULATION

Recent insights into the expression of NLRC5 have shown constitutive levels in several tissues. mRNA is abundant in lymphoid organs such as lymph nodes and spleen, and – particularly in humans – in the lung and the intestinal tract. This implies a role for NLRC5 in the immune response and may suggest a specific role at mucosal surfaces (41, 43, 45). Taking a closer look at hematopoietic cells, high levels of *NLRC5* transcripts were observed in T and B cells, and lower levels in $CD14^+$ leukocytes. A detailed analysis of NLRC5 expression at the protein level in murine tissues and primary hematopoietic cells confirmed these observations (46).

NLRC5 is induced by several stimuli including type I and II IFNs (41, 42, 46, 47). To better understand the transcriptional regulation of *NLRC5*, Kuenzel and co-workers analyzed the promoter region of the human *NLRC5* gene using a bioinformatic approach. They identified putative *cis*-elements that might

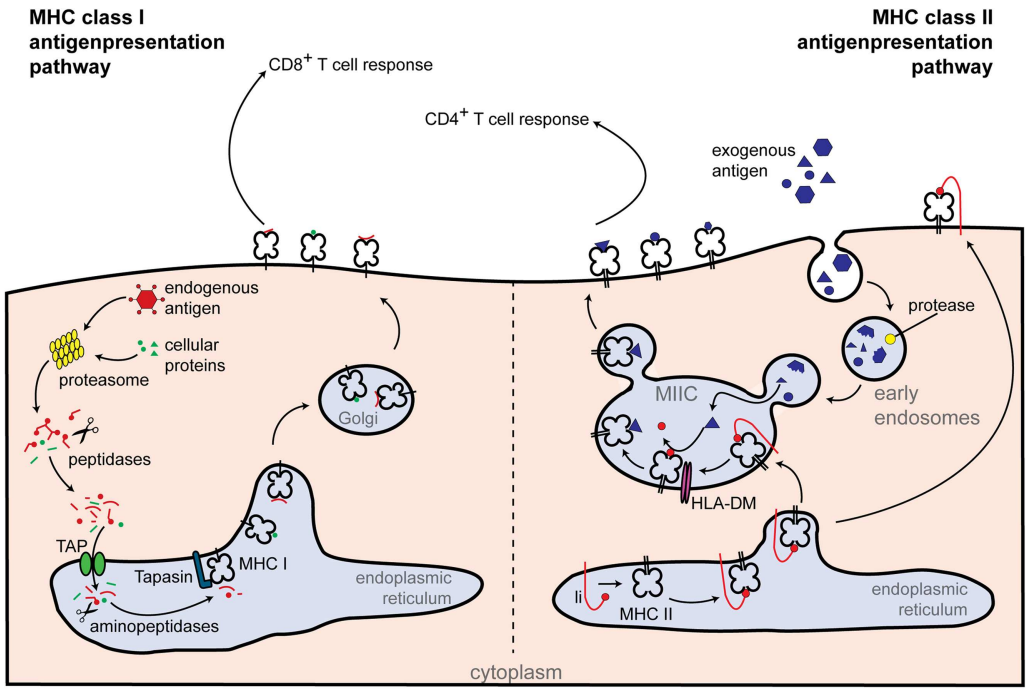

FIGURE 2 | Classical routes of antigen presentation by MHC class I and II molecules. Class I antigen presentation (left-hand side): proteasomes generate peptides from all proteins present within the cell. Peptide fragments are transported to the endoplasmic reticulum, where they are further trimmed by aminopeptidases and loaded onto the MHC class I molecule. MHC class I-loaded complexes are transported to the cell surface, where they are presented to $CD8^+$ T cells. Class II antigen presentation (right-hand side): extracellular antigens are taken up within phagosomes by APCs. Phagosomes fuse with lysosomes, which contain proteolytic enzymes that cleave the phagocytosed proteins into small peptides. Newly synthesized MHC class II molecules from the endoplasmic reticulum are delivered to the phagolysosomes and loaded with peptide. Peptide-loaded MHC class II complexes are transported to the cell surface, allowing antigen presentation to $CD4^+$ T cells. Additional abbreviations: Ii, MHC class II-associated invariant chain; MIIC, MHC class II compartment; TAP, transporter associated with antigen processing.

regulate *NLRC5* expression (47). These regulatory elements consist of two signal transducer and activator of transcription (STAT) consensus-binding sites at position −1336 and −452 that, according to Genomatix, would be specific for the transcription factors STAT1 or STAT3 (48). A potential NF-κB consensus-binding site, partially overlapping with the STAT binding site at position −1336, was identified in the human promoter (47). Kuenzel and co-workers showed that IFN-γ-mediated *NLRC5* promoter transactivation was partially abrogated in the absence of either STAT binding site. Accordingly, in different hematopoietic mouse cells and human HeLa cells, NLRC5 expression was increased early upon treatment with both type I and II IFNs in a STAT1-dependent manner (42, 46). However, it is important to point out that an ISRE binding site, which can be bound by STAT and IRF complexes, has been predicted in the promoter of NLRC5 (44, 47). Therefore, IFN-mediated *NLRC5* induction could be dependent on this additional regulatory sequence. Thus, the importance of these predicted binding sites in NLRC5 maintenance or induction still awaits thorough characterization.

Innate immune stimuli such as polyinosinic-polycytidylic acid (Poly I:C) and lipopolysaccharide (LPS) efficiently induce NLRC5 expression in bone marrow-derived macrophages (BMDMs) through the autocrine effect of type I IFNs. This was demonstrated with the use of *Ifnar*- and *Stat1*-deficient cells (46). In addition the use of *Trif* (TIR-domain-containing adapter-inducing IFN-β)-deficient and *Myd88* (myeloid differentiation primary response gene 88)-deficient BMDMs showed that upregulation of NLRC5 by LPS was dependant on the toll-like receptor (TLR) 4 adaptor protein Trif (46). Moreover, inflammatory stimuli such as IFN-γ, poly I:C, or Sendai Virus (SeV), a virus inducing strong IFN responses, also enhanced NLRC5 expression in human primary dermal fibroblasts, the epithelial carcinoma cell line HeLa, and the colon carcinoma cell lines CaCo2 and HT29 (43, 47).

Since several isoforms have been described for *CIITA*, it is conceivable that *NLRC5* could also possess different mRNA variants. Indeed, six isoforms varying in their C-terminal LRR sequences have been reported in databases (www.uniprot.org; Q86WI3). Isoform 3, which is missing the entire LRR region, was found to be mainly expressed in $CD8^+$ and $CD4^+$ lymphocytes. Isoforms 4 and 5, which both lack exon 25, were detected at low levels in THP-1 cells (43). Whether these different NLRC5 isoforms have any biological relevance remains to be established. While for some NLRs, such as NOD1 and NOD2, the LRR region has been involved in sensing pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) (49, 50), it can also have autoinhibitory functions (1). Thus the different isoforms of NLRC5 deserve further investigation, as they could either diversify its potential ligand binding ability or alter its autoregulatory activity.

NLRC5 CONTROLS BASAL MHC CLASS I GENE EXPRESSION

Given the phylogenetic proximity between CIITA and NLRC5, Meissner and co-workers used overexpression and RNA interference (RNAi)-mediated knockdown to study the capacity of NLRC5 to activate MHC gene transcription (42). They found that NLRC5 overexpression induced classical MHC class I molecules in HEK293T and in Jurkat cells. This finding has been subsequently confirmed and extended to both primary cells and other cell lines (42, 46, 51–56). Interestingly, human NLRC5 is able to restore MHC class I expression in the murine melanoma cell line B16F10, which is defective for MHC class I expression (53). NLRC5 knockdown in THP-1 cells, poly I:C-treated Jurkat T cells, HeLa cells, and human dermal fibroblasts confirmed that NLRC5 contributes to MHC class I expression in these cells (42, 53). Moreover, HLA-B and NLRC5 expression correlate in several human tissues supporting the function of NLRC5 as a transactivator of MHC class I genes (53), although discordant results from lung tissue suggests the existence of additional control mechanisms.

Classical MHC class I molecules are highly polymorphic and consist of a heavy chain (encoded by the genes HLA-A, -B, and -C) and the β_2-microglobulin chain. Peptide fragments presented in MHC class I mainly derive from cytosolic proteins degraded by the 26S proteasome which can be supported by additional subunits forming the so-called "immunoproteasome" (57). Peptides are then transported into the endoplasmic reticulum (ER) by the peptide transporter associated with antigen processing (TAP), in which the MHC class I molecules are present as membrane-bound proteins stabilized by a subset of chaperones. Following further aminopeptidase-mediated cleavage, peptides are loaded onto the MHC class I molecule by the peptide-loading complex, which includes TAP1, TAP2, and tapasin. Antigen-loaded MHC class I molecules are transported via the Golgi-network to the cell surface, where they are detected by specific receptors on $CD8^+$ T cells, as depicted in **Figure 2** [reviewed in Ref. (13)]. Interestingly, NLRC5 also controls the expression of MHC class I-related genes, such as β_2-microglobulin, TAP1, and the immunoproteasome subunit low molecular mass poylpeptides 2 (LMP2) (42).

The forced expression of NLRC5 also leads to induction of the non-classical MHC class I genes HLA-E, F, G, less polymorphic molecules that are mainly involved in Natural Killer (NK) cell inhibition (42). Altogether, these data indicate NLRC5

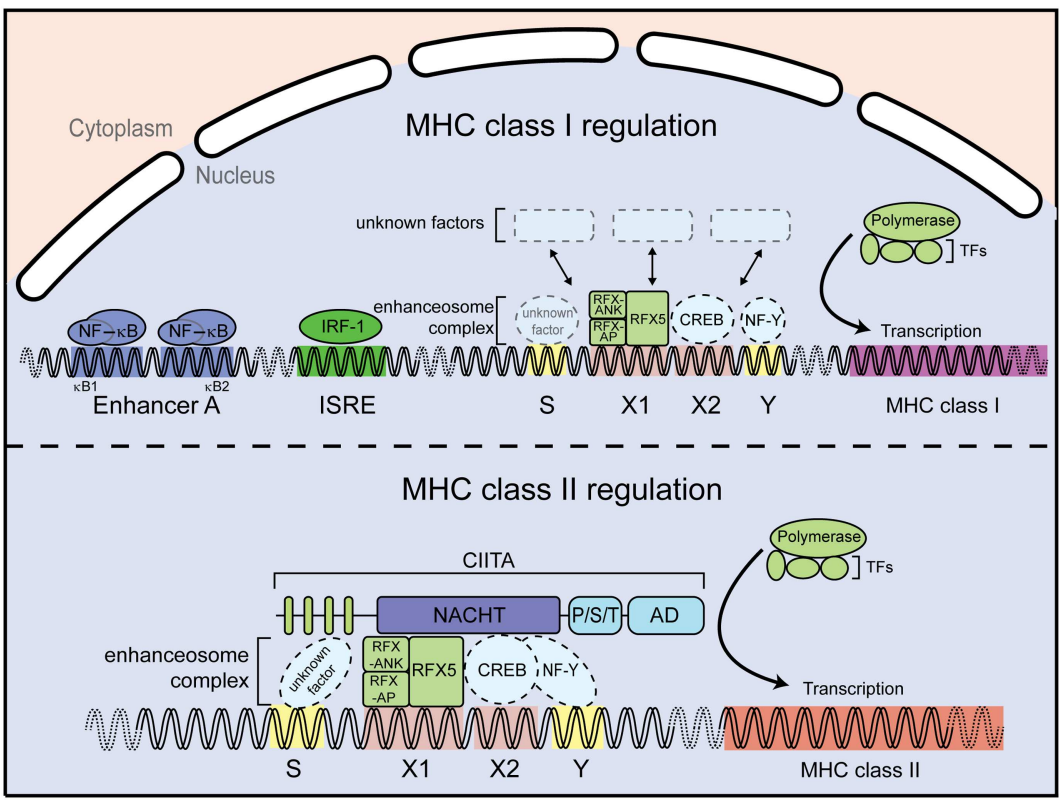

FIGURE 3 | Major-histocompatibility complex class I and II promoters. MHC class I gene regulation (upper panel). Expression of MHC class I genes is regulated by several elements in the promoter region. For instance, the enhancer A sequence allows binding of the transcription factor NF-κB; the interferon stimulated response element (ISRE) serves as an active site for interferon regulator factor 1 (IRF1). In addition, a conserved SXY module is found in the proximal MHC class I gene promoters. MHC class II gene regulation (lower panel). The MHC class II gene contains an SXY module that facilitates assembly of the so-called enhanceosome. The enhanceosome is a protein complex containing regulatory factor X (RFX) proteins, cAMP response element-binding protein (CREB), and the nuclear factors Y (NF-Y). The assembly of this protein complex constitutes a platform for the recruitment the class II transactivator CIITA. Additional abbreviation: RFX-AP, RFX-associated protein; RFX-ANK, RFX-associated ankyrin-containing protein; TFs, transcription factors.

to be a key player in the process of MHC class I-mediated antigen presentation.

MOLECULAR ASPECTS OF NLRC5 TRANSCRIPTIONAL REGULATORY FUNCTION

NLRC5 TRANSCRIPTIONAL REGULATORY ACTIVITY DEPENDS ON THE ENHANCEOSOME

As discussed above, CIITA-dependent MHC class II activation requires the enhanceosome complex, which binds to the SXY motif of the MHC class II promoter region [reviewed in Ref. (8, 27)]. A similar region in the MHC class I promoter was shown to be important for enhanceosome-dependent MHC class I activation (36) (**Figure 3**). Importantly, NLRC5 has been shown to occupy the promoter of MHC class I genes in the region encompassing the SXY module by chromatin immunoprecipitation assays (42, 46). Mutation of the X1- and X2-motifs, as well as the absence of selected enhanceosome components, abolished NLRC5-dependent MHC class I activation, demonstrating that NLRC5-mediated MHC class I activation is dependent on components of the enhanceosome (53, 58). Consistent with this, ankyrin repeat dependent interaction of RFX-ANK and NLRC5 has been reported (58). In addition, Meissner and colleagues identified the S-motif as important for NLRC5-dependent, but not CIITA-dependent, transactivation (**Figure 3**) (58). The S-motif has been implicated in enhanceosome-dependent regulation of MHC transcription (59). However, its precise role in NLRC5-dependent gene regulation still needs to be established.

Altogether, these results reveal that NLRC5 exerts transcriptional regulation in an enhanceosome-dependent manner. A future challenge is to understand the epigenetic mechanisms underlying NLRC5-dependent transactivation of MHC class I gene promoters; the first insights into this important aspect are just starting to emerge, as recently reviewed (44).

MOLECULAR MECHANISMS OF NLRC5 NUCLEAR LOCALIZATION

The transcriptional function of NLRC5 requires an ability to shuttle into the nucleus. NLRC5 strongly accumulates in the nucleus upon inhibition of nuclear export, while at steady-state conditions only a minor portion is detectable within the nucleus (41, 42, 46, 47, 53). In contrast, CIITA is evenly distributed between cytoplasm and nucleus (60, 61). An N-terminally located nuclear localization sequence (NLS) is responsible for the nuclear shuttling of NLRC5 (42, 52, 53). However, this process is also reported to depend on the Walker A motif that constitutes the nucleotide-binding site of the central nucleoside triphosphatase (NTPase) domain (see **Figure 1**) (52, 53). Of note, disruption of the Walker A box in CIITA was also shown to diminish MHC transactivation and nuclear shuttling (62, 63). However, these results should be interpreted cautiously, as mutation of the Walker A site might affect overall protein structure.

NLRC5 and CIITA show close sequence homology in their LRR regions (40). However, the LRR regions differentially affect their subcellular localization. For CIITA it has been shown that several structural features of the LRRs are important for nuclear import (60, 61, 64). In contrast the LRR region of NLRC5 is dispensable for nuclear import, but appears to be involved in nuclear export. NLRC5 constructs lacking the LRR region showed nuclear localization even in the absence of blockage of nuclear export (52, 53).

Paradoxically, forced nuclear localization of NLRC5 (by fusion of a strong viral NLS) results in reduced MHC class I transactivation potential (52, 53). This could mean that the transcriptional activity of NLRC5 might depend on cytoplasmic modifications that cannot be carried out correctly if the protein only resides briefly in the cytoplasm. Of note, NLRC5 runs as a double band in SDS-gel electrophoresis, which might be indicative of such a post-translational modification, although its nature remains to be established (46, 47). Alternatively, enhanced nuclear import of NLRC5 might interfere with the formation of NLRC5 protein–protein complexes in the cytosol that confer transcriptional activity upon translocation to the nucleus.

HINTS FROM NLRC5-ANALOGOUS PROTEINS IN MAMMALIAN AND PLANT CELLS

As discussed above, our understanding is that NLRC5, similarly to CIITA, is active without any DAMP or PAMP stimulus; its activity relying mainly on the expression level. This is in sharp contrast with the current idea of NLR activation, in which a DAMP or PAMP is needed to induce a conformational change and activate these proteins. It remains to be established if the LRRs of NLRC5 have PAMP or DAMP binding capacity that might further regulate activity. However, in the case of both NOD1 and NOD2, for which a clear activator has been defined, high expression levels are sufficient to confer autoactivation (65).

Although it has been presumed that plants and animals use different immune mechanisms to detect pathogens, there are interesting studies showing remarkable similarities in the structure and function of the receptors that recognize microbial antigens [reviewed by Beutler (66)]. In fact, plants harbor Nucleotide-Binding-Leucine-Rich Repeat (NB-LRR) proteins, which have a similar structure to NLRs and also function as intracellular PRRs to ensure immunity against invading pathogens. Many of these NB-LRR proteins are present both in the cytoplasm and nucleus and function as transcriptional regulators [reviewed by Padmanabhan and Dinesh-Kumar (67)]. Recently, it was shown that two plant NB-LRR proteins [Barley mildew A (MLA) and *Nicotiana tabacum* TIR-NB-LRR immune receptor N] enhance pathogen-mediated defense gene transcription by forming a complex with specific plant transcription factors (68, 69). The similar function of CIITA and NLRC5 to these plant proteins makes it tempting to speculate that convergent evolution might also have selected similar regulatory mechanism (70).

IN VIVO NLRC5 FUNCTION AND RELEVANCE IN HEALTH AND DISEASE

LESSONS FROM KNOCKOUT MOUSE MODELS

The involvement of NLRC5 in MHC class I regulation was confirmed in *Nlrc5*-deficient mice by several independent studies (46, 51, 54–56). These works also enabled to determine the contribution of NLRC5 to MHC class I expression in different cell types. The greatest decrease in MHC class I expression was observed in $CD4^+$ and $CD8^+$ T cells, NK, and NKT cells; a significant defect was seen in B cells; and a more

moderate defect in DCs and macrophages (46, 51, 54). The most prominent lack of MHC class I expression was therefore noticed among immune cells, where NLRC5 is mainly expressed at the steady-state (43, 46). However, analysis of MHC class I expression in thymic epithelial cells (TECs), revealed that *Nlrc5*-deficiency decreased their MHC class I display (46), indicating that NLRC5 also participates in MHC class I expression in non-hematopoietic tissues; a function that deserves further investigation.

Major-histocompatibility complex class I is not only the key molecule for $CD8^+$ T cell activation and function but is also essential for thymic selection and peripheral maintenance of naïve $CD8^+$ T cells. In line with that, two reports detected slightly reduced total $CD8^+$ T cell levels in the spleen of *Nlrc5*-deficient mice under steady-state conditions (46, 56). This strongly suggests that the diminished MHC class I levels encountered in knockout animals affects $CD8^+$ T cell homeostasis. In addition, the decreased surface MHC class I levels described above in *Nlrc5*-deficient TECs suggests that *Nlrc5*-deficiency might alter thymic selection and – potentially – the TCR repertoire.

The use of knockout animals also highlighted the ability of *Nlrc5*-deficient cells to increase their surface MHC class I levels following treatment with inflammatory stimuli such as IFNs or LPS, both in T cells and macrophages (46, 51, 54). In fact, although the defect in MHC class I expression observed in *Nlrc5*-deficient cells was maintained after stimulation, both control and knockout cells were able to substantially augment their MHC class I levels. This reiterates the existence of *Nlrc5*-independent mechanisms that regulate MHC class I (**Figure 3**).

NLRC5 IN INFECTIONS

Given the recent discovery of NLRC5, we have just started to gain insight into the significance of this NLR in pathological conditions. Based on current knowledge one would have predicted it to be important in viral infections. However, the only information to date on the effects of *Nlrc5*-deficiency in this context come from an acute model of VSV viral infection in which no significant differences were observed between control and knockout mice (55). It would be extremely valuable to assess the ability of knockout animals to control less acute viral infections in which the adaptive immune response is activated.

Nonetheless, two studies clearly show that NLRC5 is important in the control of infection by *Listeria monocytogenes*, a Gram-positive motile bacterium that primarily infects monocytes and macrophages. One week post-inoculation, *Nlrc5*-deficient mice showed severely reduced numbers of IFN-γ-producing $CD8^+$ T cells (51, 56). In agreement with this observation, bacterial clearance was affected and an increased bacterial burden observed in the liver and spleen of knockout animals. Surprisingly, the difference in restriction of *L. monocytogenes* infection was observed as early as 1 day after infection, indicating that besides the impaired $CD8^+$ T cell-mediated response, an early defect in the innate response occurs in the absence of NLRC5 (56). The authors explained this by the observation of a partial defect in IL-1β production in *Nlrc5*-deficient mice upon infection. Future work aimed at dissecting the contribution of NLRC5 to the innate and the adaptive responses will be extremely important.

NLRC5 AND TUMOR SURVEILLANCE

Because MHC class I surface expression was strongly diminished among *Nlrc5*-deficient lymphoid cells, the efficiency of CTLs in killing these cells was evaluated. In agreement with their reduced ability to present antigen, *Nlrc5*-deficient target T cells were eliminated less than control T cells by cognate CTLs *in vitro* (46). Although this observation still awaits confirmation *in vivo*, it has long been known that several malignancies, and in particular, hematological tumors, often lose MHC class I expression. This may enable more efficient evasion of immunosurveillance mechanisms. Mutations or epigenetic silencing that mostly target β2M or single HLA genes have been identified in these tumors (71). As such, NLRC5 mutation or silencing that results in down-regulation of classical MHC class I in lymphoid malignancies could favor tumor development. Interestingly, the screening of a handful of lymphoid tumor cell lines suggested that NLRC5 is indeed expressed at low levels in several of these. In any case, NLRC5 expression level strongly correlates with the expression of a number of MHC class I genes in human tumor cell lines (Broad-Novartis Cancer Cell Line Encyclopedia). Future studies aimed at evaluating NLRC5 expression and mutations in a larger number of lymphoid tumor cells, and in primary tumors will contribute to delineate the potential importance of NLRC5 in tumor progression.

Displaying reduced MHC class I expression to escape CTL-mediated immunosurveillance can result in increased NK cell-mediated lysis. In fact, NK cells kill cells lacking the expression of MHC class I molecules, according to the "missing-self" hypothesis (72). For now studies addressing the role of NLRC5 in NK cell biology have yet to be performed.

EMERGING FUNCTIONS OF NLRC5 IN ANTIVIRAL AND INFLAMMATORY RESPONSES

The first characterization of NLRC5 showed that it can activate ISRE- and IFN-γ activating sequence (GAS)-containing promoters in human HeLaS3 cells (47). However, NLRC5 overexpression in other human cell lines failed to strongly activate important pro-inflammatory pathways such as NF-κB, mitogen-activated protein kinase (MAPK) or type I IFN (41, 43). Several studies reported that that overexpression of human NLRC5 actually resulted in an inhibition of NF-κB, activator protein 1 (AP1), and ISRE signaling; as well as TANK-binding kinase 1 (TBK1)- and SeV-mediated IFN-β promoter activation in HEK293T cells (41, 43, 45). The ability of NLRC5 to bind components of the NFκB and type I IFN pathway, namely IκB kinase (IKK)-α, IKK-β, retinoic acid-inducible gene 1 (RIG-I), and melanoma differentiation-associated protein 5 (MDA5) (45), led to the suggestion that NLRC5 overexpression interfered with these factors to inhibit inflammatory signaling. In spite of this, two independent studies using RNAi, revealed that human NLRC5 positively contributes to type I IFN expression upon viral infection in human cells (47, 53). This observation contrasts with the results of NLRC5 overexpression in HEK293T cells and highlights the difficulty in interpreting functional studies based on NLR overexpression (73).

In cells from *Nlrc5* knockout mice, PRR-mediated IFN and NF-κB signaling were also shown to be affected in embryonic fibroblasts, peritoneal macrophages, and – to a lesser extent – in BMDMs

(55). However, no differences were observed in BMDMs and dendritic cells in complementary studies using three independently generated *Nlrc5* knockout mouse strains (46, 54, 74). In particular, work by Kumar and colleagues clearly showed that myeloid cells derived from *Nlrc5*-deficient mice showed no significant changes in the production of IFN-β or IL-6 after treatment with RNA viruses, DNA viruses, and bacteria (74). Moreover, serum IFN-β, IL-6, and IL-1β levels after polyI:C injection were also comparable between control and knockout mice. This discrepancy remains to be addressed by future works.

Many NLRs form multimeric complexes called inflammasomes that act as scaffold for the activation of the inflammatory caspase-1, which in turn cleaves and hence activates IL-1β and IL-18 (1). Several reports show that NLRC5 is able to enhance inflammasome-mediated IL-1β processing in human and murine cells. In human HEK293T cells ectopically expressing pro-IL-1β and pro-caspase-1, NLRC5 expression led to dose dependent IL-1β release (74). In isolated murine macrophages and dendritic cells derived from *Nlrc5*-deficient animals, activation of the NLRP3 and Aim-2 inflammasome was normal, although *Francisella tularensis*-mediated IL-1β release, that depends on Aim-2, might be affected (74). Also in human myeloid THP-1 cells, bacterial- and PAMP-induced IL-1β secretion, but not pore forming toxin-dependent activation of the inflammasome, was reduced upon silencing of NLRC5 expression (75). This was suggested to be mediated by interactions of NLRC5 with NLRP3 and the inflammasome adaptor protein ASC (56, 75). Despite the reported discrepancies, the observed effects of NLRC5 expression on the inflammasome and, more precisely, on NLRP3-specific stimuli deserve further research.

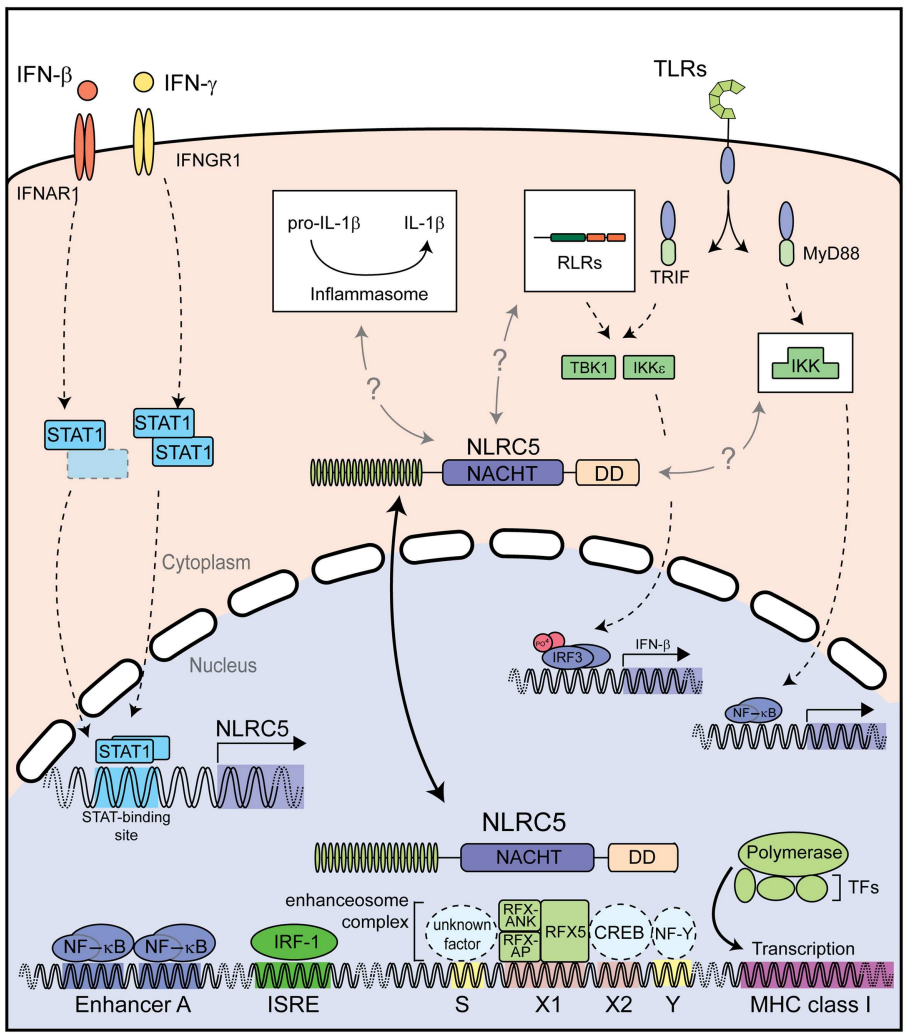

FIGURE 4 | Described functions of NLRC5. Both type I and II IFNs strongly induce NLRC5 expression through STAT1 activation. NLRC5 can then shuttle into the nucleus and bind to the enhanceosome on MHC class I gene promoters, resulting in their expression. NLRC5 has also been shown to act as a modifier of NF-κB activity and type I interferon responses induced by PRRs recognizing microbial nucleic acids. Additional abbreviation: IRF, interferon regulator factor; ISRE, interferon stimulated response element; CREB, cAMP response element-binding protein; IKK, IκB kinase complex; IKKε, IκB kinase subunit epsilon; NF-Y, nuclear factor Y; RFX5, regulatory factor X 5; RFX-AP, RFX-associated protein; RFX-ANK, RFX-associated ankyrin-containing protein; RLRs, RIG-I-like receptors; TBK1, TANK-binding kinase 1; TFs, transcription factors; TLRs, toll-like receptors.

Taken together, NLRC5 appears to have distinct functions depending on its cytoplasmic or nuclear location (**Figure 4**). In the cytoplasm NLRC5 might influence canonical innate immune pathways, including type I IFN signaling and NF-κB signaling; at least in some cell types and species (41, 43, 45, 47). On the other hand, nuclear NLRC5 is a master regulator of MHC class I gene expression both in human and murine cells (42, 46, 53, 55, 56, 58). Additional studies are needed to elucidate the detailed cytoplasmic function of NLRC5, particularly with regard to the potential functional differences between humans and mice.

CONCLUDING REMARKS

A substantial amount of evidence supports NLRC5 as the long sought after transcriptional regulator of MHC class I in human and murine cells, particularly in the hematopoietic lineage. However, emerging evidence suggests additional roles for this NLR in innate immune responses; roles we are only starting to understand and which require further investigation. We are beginning to gain insight into the molecular mechanisms underlying the transcriptional regulatory function and physiological relevance of NLRC5. In spite of the little understanding we have to date of NLRC5-mediated activities, its role as transcriptional regulator of MHC class I anticipates that important functions in health and disease await discovery.

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Pyrin- and CARD-only proteins as regulators of NLR functions

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Upon activation Nod-like receptors (NLRs) assemble into multi-protein complexes such as the NODosome and inflammasome. This process relies upon homo domain interactions between the structurally related Pyrin and caspase-recruitment (CARD) domains and adaptor proteins, such as ASC, or effector proteins, such as caspase-1. Although a variety of NLRP and NLRC complexes have been described along with their activating stimuli and associated proteins, less familiar are processes limiting assembly and/or promoting dissociation of NLR complexes. Given the importance of limiting harmful, chronic inflammation, such regulatory mechanisms are significant and likely numerous. Proteins comprised of a solitary Pyrin domain (Pyrin-only) or CARD domain (CARD-only) posses an obvious potential ability to act as competitive inhibitors of NLR complexes. Indeed, both Pyrin-only proteins (POPs) and CARD-only proteins (COPs) have been described as regulators of caspase-1 and/or NLR-inflammasome activation and not surprisingly as factors mediating pathogenesis. Although clear examples of pathogen encoded POPs are currently limited to members of the poxviridae, the human genome likely encodes three POPs (POP1, POP2, and a potential POP3), of which only POP2 is known to prevent NLR:ASC interaction, and three COPs (COP/Pseudo-ICE, INCA, and ICEBERG), initially described for their ability to inhibit caspase-1 activity. Surprisingly, among eukaryotic species POPs and COPs appear to be evolutionarily recent and restricted to higher primates, suggesting strong selective pressures driving their emergence. Despite the importance of understanding the regulation of NLR functions, relatively little attention has been devoted to revealing the biological impact of these intriguing proteins. This review highlights the current state of our understanding of POPs and COPs with attention to protein interaction, functions, evolution, implications for health and disease, and outstanding questions.

Keywords: pyrin, CARD, NLR, inflammasome, NF-kappaB, inhibitors

INTRODUCTION

Inflammation is a non-specific, physiological response of the immune system to infection and injury. Acute inflammation occurs within a few minutes following the injury of tissues. This process is initiated by tissue-associated cells, such as resident macrophages, that release inflammatory mediators, increase permeability of the blood vessels, and subsequently recruit phagocytes to the affected sites, eliminating not only invading organisms but damaged tissues as well. Although acute inflammation is normally self-limiting and beneficial for host defense and healing, excessive inflammation or prolonged (chronic) inflammation is deleterious and a cardinal, if not causative, feature of many diseases.

Among the mediators of inflammation, IL-1β is a prominent pro-inflammatory cytokine produced primarily by myeloid cells that efficiently stimulates the expression of other gene products, such as IL-6 and acute phase proteins associated with inflammation, thus initiating a self-amplifying cytokine network (1). IL-1β is also key to the fever response and vascular changes accompanying inflammation. It is well-known that IL-1β, together with the closely related cytokine IL-18, is matured through catalytic processing by caspase-1, an event associated with the assembly of multi-protein, caspase-1 activating complexes known as inflammasomes. A closely related protein complex, the NODosome, also promotes inflammation through activation of transcription factors promoting expression of the proform of IL-1β as well as other inflammatory cytokines including TNFα and IL-6 [reviewed in (2)]. Moreover, an increasing number of publications highlight the importance of various specific inflammasome/NODosome complexes in not only normal inflammatory responses, but in human pathologies ranging from metabolic disorders to autoimmune diseases and cancer [reviewed in (3–5)].

Most inflammasomes (and all NODosomes) result from the activation of intracellular sensor proteins belonging to the nucleotide-binding, leucine-rich repeat (LRR), or NOD-like receptor family (NLR). NLR agonists include pathogen-derived molecules [e.g., lipopolysaccharide, muramyl dipeptides (MDPs), and flagellin] as well as "sterile" substances (e.g., asbestos, silica, cholesterol, or uric acid crystals) [reviewed in (6, 7)]. The non-NLR dsDNA sensor AIM2 also seeds an inflammasome complex (8). In all cases, formation of these active complexes requires

homotypic domain interactions involving Pyrin domains (PYD), caspase-recruitment (CARD) domains, or both.

Given the broad significance of inflammasomes, understanding their regulation is a topic of critical importance. Beyond regulation of expression and preventing inflammasome assembly/activation, mechanisms that limit that assembly or favor disassembly of these complexes have obvious implications for controlling inflammasome-mediated inflammation. Interestingly, two groups of proteins, Pyrin-only proteins (POPs) and CARD-only proteins (COPs) have been recently described as negative modulators that impact and likely regulate inflammasome assembly and/or caspase-1 dependent production of IL-1β. NODosomes also rely upon CARD domains and their activation may also be regulated by COPs. Although not well-studied and underappreciated, these molecules are known, or strongly implied, to interfere with either inflammasome adapter molecule interaction or downstream recruitment of caspase-1. In addition, POPs and COPs, like other PYD and CARD-containing proteins, can influence the activation of NF-κB.

NOD-LIKE RECEPTORS

Cells are able to recognize and respond to a large array of common molecules by virtue of a set of diverse, but limited, pattern recognition receptors (PRRs). PRRs have been characterized into four groups, Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), AIM2-like receptors (ALRs), and NLRs. While TLRs, a family of classical transmembrane proteins well-known as important for recognizing either extracellular or membrane-encased foreign organisms (9), form the front line of innate immune sensors, NLRs constitute a second, intracellular line. The NLR family consists of intracellular soluble proteins that sense cytosolic pathogen-associated molecular patterns (PAMPs) as well as a range of environmental- and host-derived stress signals, also known as danger-associated molecular patterns (DAMPs). Conserved tripartite-domain proteins, NLRs contain a central nucleotide-binding and oligomerization domain (NOD, NBD, NACHT), C-terminal LRR similar to those of TLRs that may serve as a "sensor domain," and a N-terminal effector domain. NLRs are thought to be synthesized in an auto-repressed, inactive form where an intramolecular interaction between the LRR and NACHT domains is proposed to block NACHT-mediated oligomerization, thus inhibiting NLR auto-activation (10). Upon binding (or responding to) respective ligands, the LRRs are thought to undergo a conformational change allowing NACHT-dependent oligomerization and recruitment of appropriate adaptor proteins, leading to NODosome or inflammasome assembly (**Figure 1**).

NODOSOMES

The NLRC (NOD) and NLRP (NALP) subfamilies which contain a CARD or PYD N-terminal effector domain, respectively, are the most studied NLRs (11). NOD1/NLRC1 (CARD 4) and NOD2/NLRC2 (CARD 15) were among the first NLRs to be described (12, 13). NOD1 and NOD2 both detect muropeptides released from bacterial peptidoglycan (14). NOD1 binds to diaminopimelic acid (DAP) (strictly in Gram-negative bacteria), whereas NOD2 directly interacts to MDP (found in both Gram-positive and Gram-negative bacteria) (15–18). Upon specific ligand recognition, NACHT domain oligomerization initiates recruitment of the CARD-containing kinase RIP-2 through a homotypic CARD–CARD interaction, leading to the formation of NODosomes (**Figure 1**). RIP-2, in turn, activates the IκB kinase (IKK) complex followed by the subsequent release and nuclear translocation of NF-κB (12, 19). Beyond NF-κB activation, NOD1 recruitment of RIP-2 is believed to activate c-jun kinase (JNK) (20, 21).

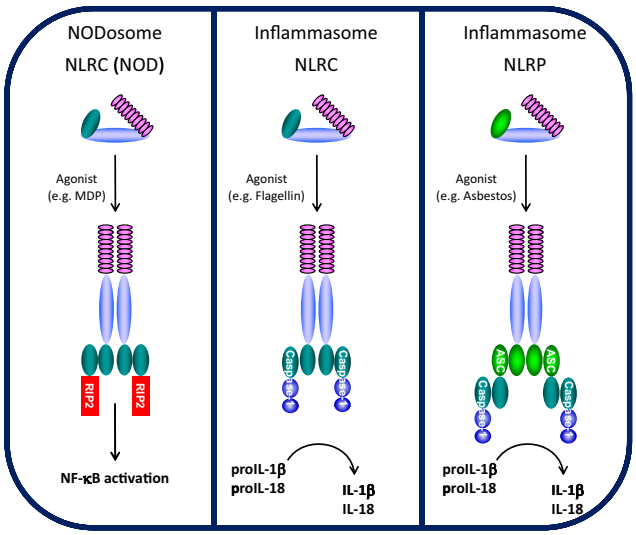


FIGURE 1 | NODosome and inflammasome complexes assembled through CARD–CARD and PYD–PYD homotypic interactions. CARD domain-dependent direct recruitment of RIP-2 by NLRCs (e.g., NOD2/NLRC2) leads to NF-κB activation (left panel) and CARD domain-dependent direct recruitment of Caspase-1 by NLRC4 leads to IL-1β/IL-18 processing (middle panel), while PYD domain-dependent recruitment of the adaptor ASC (PYCARD) leads to CARD domain-dependent recruitment of Caspase-1 and IL-1β/IL-18 processing (right panel). (Domains: purple, LRRs; light blue, NBD/NACHT; teal, CARD; light green, PYD).

INFLAMMASOMES

NLRPs represent the largest NLR subfamily and are characterized by the presence of an N-terminal PYD effector domain (11, 22). Activation of various NLRPs by specific agonists leads to assembly of a multi-protein inflammasome complex. During inflammasome activation, the activated NLRP recruits the bipartite PYD-CARD domain protein ASC (also known as PyCARD) though a PYD–PYD interaction and the CARD domain of ASC subsequently recruits the CARD domain of caspase-1 (4) (**Figure 1**). In the assembled inflammasome, proximity-induced auto-activation of the catalytic domain of caspase-1 results in the mature, fully active caspase-1 followed by proteolytic cleavage and release of IL-1β and IL-18 (23, 24). Unlike NODosomes, the critical interaction to assemble NLRP inflammasomes is the PYD–PYD interaction with ASC. Two typical types of NLRP inflammasomes have been identified (25, 26). The NLRP1 inflammasome is composed of NLRP1, the adapter protein ASC, ASC-recruited caspase-1, and caspase-5 which is recruited through a NLRP1-specific C-terminal

CARD domain (27). The NLRP3 inflammasome is believed to represent the arrangement of most NLRP inflammasomes and contains NLRP3, ASC, and caspase-1, but does not recruit caspase-5 (28). One other class of NLR-inflammasome is represented by NLRC4 (Ipaf/Naip5). By virtue of its N-terminal CARD domain, NLRC4 which is activated by flagellin, is believed to directly recruit caspase-1 through a CARD–CARD interaction, independent of ASC (29, 30) (**Figure 1**). Despite differences in the mode of caspase-1 recruitment, all of these inflammasomes control the processing and activation of the pro-inflammatory cytokines IL-1β and IL-18.

HOMO-DOMAIN INTERACTION

Pyrin domains and CARD domains are members of the larger death domain (DD) fold superfamily characterized by the highly similar secondary structure of an antiparallel, six α-helical bundle (31). Like their close cousins, members of the DD and death effector domain (DED) family which interact homotypically (32), it is widely held that PYD- and CARD-containing proteins act exclusively in similar fashion.

PYRIN DOMAINS

The PYD domain is a conserved sequence motif found in more than 20 human proteins with putative functions in apoptotic and inflammatory signaling pathways (33). Studies reporting PYD structures are rare, due to the limited solubility of these domains (31). However, several PYDs including those from ASC, POP1, and NLRP3 have been structurally characterized. All have distinct positively and negatively charged surface patches in their structure, leading to the proposal that electrostatic interactions are critical for PYD interaction (33–36). Notably, the three-dimensional structure of the human ASC PYD has helped clarify how this adaptor protein binds to the PYD of NLRP3 as well as that of POP1 (33, 37). The PYD domain of ASC is a highly bipolar molecule with most of the positively charged side-chains located in helices 2 and 3 and the connecting loop, while most of the negatively charged side-chains reside in helices 1 and 4 and the immediately adjacent regions, suggesting that, in analogy to CARD domains, charge-charge interactions may play an important role in PYD domain interactions (38). In fact, the negative residues on the ASC PYD Asp6, Asp10, Asp51, and Asp54 play important role in the interaction between ASC and POP1. Consistently, at least two positively charged amino acids in POP1, including Lys21 and Arg41, were required for this association (39).

CARD DOMAINS

CARD domains are thought to homotypically interact in a fashion similar to PYDs. Structural studies of adaptor proteins such as RAIDD and the NLR-like apoptosome protein Apaf-1 revealed that CARD domains also contain distinct basic and acidic patches (38, 40–42), suggesting an electrostatic nature for CARD–CARD interaction. Indeed, the acidic surface of the Apaf-1 CARD located in helices 2 and 3 is necessary for interaction between Apaf-1 and caspase-9 (38, 42). In addition, hydrophobic interactions are also an important driving force underlying this particular CARD/CARD interaction (42).

REGULATION OF NLR ASSEMBLY BY POPs AND COPs

Based on our current knowledge of NLR assembly, it is likely that one powerful way to modulate the assembly of NODosomes and inflammasomes is the disruption of PYD and CARD homodomain interactions. The growing POPs and COPs families (as discussed in detail below) are potential modulators of inflammasome and NODosome assembly and are likely important to understanding NLR-associated diseases.

Pyrin-only proteins and COPs are relatively short proteins of approximately 90 amino acids composed essentially of only a PYD or CARD domain. Accordingly, they are structurally and functionally related, and as expected, homotypic interactions appear key to their inhibitory effects in regulating NLR assembly. The currently known POPs and COPs and their known or likely roles in regulation of NLR complexes are depicted in **Figure 2**.

PYRIN-ONLY PROTEINS

To date, two POPs have been characterized based following initial identification in the human genome using bioinformatic mining. These include POP1 (*PYDC1*) and POP2 (*PYDC2*). An evolutionary analysis of POP2 suggests the possibility of a third POP within the NLRP2P pseudogene locus (43). Both appear to have resulted from either gene duplication (POP1) or retrogene insertion (POP2) from PYD-encoding genes already present in an ancestral primate genome (43, 44) and thus do not represent lateral gene transfer of viral PYD sequences. Both POPs exhibit NF-κB modulating activity, but contrary to numerous reviews which incorrectly ascribe inflammasome inhibitory properties to POP1, only POP2 is known to inhibit inflammasome assembly. Curiously,

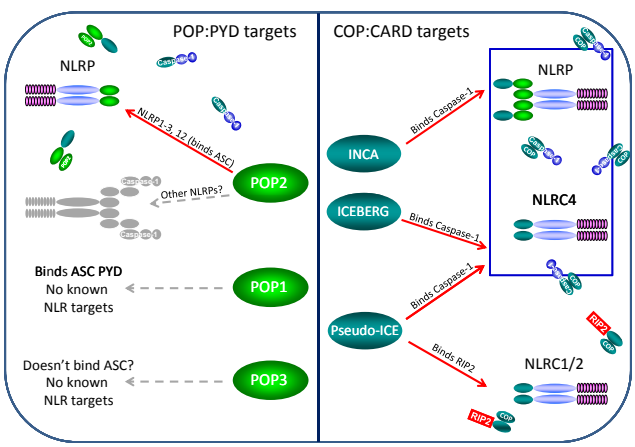


FIGURE 2 | Known and likely targets for human POPs and COPs. Left panel, only POP2 is known to limit assembly of certain NLRP inflammasomes but whether POP2 restricts all NLRP inflammasomes is unknown. POP1 may be able to inhibit some NLRP:ASC interactions, but these remain unknown. While POP3 may likely be unable to bind ASC, whether it might interact with an NLRP PYDs remains to be determined. Right panel, INCA, ICEBERG, and COP/Pseudo-ICE all bind pro-Caspase-1 and should inhibit all NLRP and NLRC inflammasome, however, no specific inflammasome targets have been examined. However, only COP/Pseudo-ICE interacts specifically with the CARD of RIP-2, thus it may be a singular regulator of NODosomes.

certain myxoma viruses belonging to the poxviridae employ POPs (vPOPs) as pathogenic determinants.

POP1

In an early bioinformatics screen for Pyrin-containing sequences, Stehlik et al. discovered the first POP1, initially designated ASC2 due to the high degree of similarity with the ASC PYD domain (44, 45). The POP1 gene is located on chromosome 16p12.1 and consists of two exons interrupted by a 580 bp intron, where the entire PYD is encoded in a single exon yielding an 89 amino acid sequence which is 64% identical (88% similar) to the PYD domain of ASC (44). Expressed predominantly in monocytes, macrophages, and granulocytes, POP1 is implicated in regulating inflammation. POP1 inhibits NF-κB activation by a variety of stimuli, including TNFα, IL-1β, Nod1, and Bcl-10, in HEK-293, COS-7, and Hela cells (44). POP1 interacts with the PYD of ASC and also suppresses NF-κB activity stimulated by co-expression of ASC and pyrin or NLRP3. The capacity of co-transfected POP1 to inhibit NF-κB activation induced by various proteins including TRAF2, TRAF6, the TRAF-binding kinase TBK1, the IKK complex constituents IKKα and IKKβ, and by the IKK-related kinase IKKi, revealed that POP1 modulates NF-κB activation at the level of the IKK complex, as reported for ASC and for NALP4/NLRP4 (46, 47). Consistently, POP1 associates with the IκBα kinases IKKα and IKKβ, inhibiting their kinase activity. These associations are very unlikely to be the result of homotypic domain interactions as IKKs lack PYD. Instead, a more likely possibility is that POP1 contains small IKK-inhibiting sequence similar to the 23 amino acid NEMO binding domain (48). It is anticipated, although not yet demonstrated, that POP1 should inhibit IKK activity downstream of the NODosome activation of RIP-2. Although POP1 enhanced ASC-mediated IL-1β activation (44), in a later study, POP1 did not inhibit either ASC-dependent or NLRP3 inflammasome processing of IL-1β (49). Thus, no evidence to date supports a role for POP1 in inhibiting an inflammasome, consistent with a structure-predicted inability to regulate NLRP3 inflammasome activation (39). Taken together, it is likely that POP1 targets NF-κB activity via inhibition of IKK, but does not act as an inhibitor of ASC-dependent NLRP inflammasomes. Despite the strong molecular evidence for the function of POP1, there have been no studies of its function using primary human or POP1-expressing mouse myeloid cells.

POP2

Following the discovery of POP1, a second human POP (POP2) was identified simultaneously by two independent groups (50, 51). POP2 is encoded by a 294 nucleotide, single exon gene located on human chromosome 3q28 and produces a protein of 97 amino acids with high similarly to the PYDs of NLRP2 and NLRP7 (78% similarity, 67% identity to the PYD of NLRP2) (50, 51). POP2 is expressed in human testis, primary peripheral blood leukocytes, monocytic cell lines, and is induced in human primary monocytes or monocytic cell lines by a variety of stimuli (PMA, LPS, or TNF-α) (49, 50), implicating a function in inflammation and host immunity. Despite the absence of a canonical nuclear localization signal sequence, POP2 displays both cytoplasmic and nuclear expression patterns in transfected cells, suggesting that POP2 may function in either, or both, compartments (50). As postulated from observations with POP1 and other PYD-containing proteins, POP2 diminishes NF-κB activation. As anticipated, POP2 blocks TNF-α-mediated NF-κB activation (50). However, POP2 inhibits the activity of transfected NF-κB p65/RelA, indicating that POP2 acts distally in the NF-κB cascade at the level of p65, a function that POP1 and other PYDs apparently lack. Recent work reveals that POP2 acts to limit the transactivation potential of the C-terminal transcriptional activation domain 1 (TAD1) of p65/RelA (49). How this is accomplished is unclear, but one possibility is a blockade of one or more of the kinases responsible for necessary phosphorylation events within TAD1 of p65 such that transactivation potential is reduced. In addition, although POP2 interacts with ASC, POP2 inhibition of NF-κB p65 is ASC-independent (49).

The interaction of POP2 with ASC leads to formation of a peri-nuclear "speck" structure similar to that observed with co-expression of ASC with Pyrin, POP1, various NLRPs, and upon NLRP3 inflammasome activation (50, 51). Moreover, POP2 also interacts with several PYD-containing NLRs (NLRP1, 2, 3, and 12) (50, 51). Indeed, POP2 nearly abolishes the interaction between ASC and the NLRP3-PYD as well as inhibiting ASC interaction with NLRP1 and NLRP12 although to a lesser extent (50). Thus, POP2 may be a broad acting competitive inhibitor of inflammasomes. This notion is supported by recent experiments revealing that POP2 effectively inhibits the non-NLR AIM2 inflammasome (unpublished observation). Co-expression of POP2 with ASC and the disease-associated NLRP3 R260W mutant impairs activation of caspase-1 in a dose-dependent manner (51). NLRP3 R260W (like other disease producing NLRP3 variants) interacts more readily/efficiently with ASC leading to caspase-1 activation, even without an agonist (51), suggesting that POP2 limits on NLRP3 inflammasome activity may not be overcome by activating mutations. POP2 also impairs activation of the NLRP2 inflammasome (51). These *in vitro* observations demonstrate the broad potential of POP2 to disrupt NLRP-ASC interactions and have significant implications for the *in vivo* role of POP2.

By generating POP2 truncation mutants with inter-helical stop codons to maintain the integrity of the remaining helices, Atianand et al. probed the specific portions of POP2 required for its function. In this study, the first α-helix of POP2 (residues 1–19) was shown to be both necessary and sufficient for inhibiting transactivation by NF-κB and for restricting inflammasome assembly (49). The first α-helix of POP2 has no basic residues but contains three acidic residues Glu^6, Asp^8, Glu^{16} (49). Consistent with the structural data showing the importance of electrostatic surface patches (EPSPs) in PYD–PYD interactions (39) mutation of these acidic residues markedly impairs the ability of POP2 to disrupt inflammasome function. NF-κB inhibition is seemingly unaltered by these mutations, implicating other elements of the first helix which might reside on the opposite helical face, although this remains to be established. The ASC adaptor protein has a bipartite charge distribution, with both positive and negative EPSPs (39). Although the negative EPSP of ASC was demonstrated to be a POP1 binding site, the presence of necessary acidic residues in the first α-helix of POP2 (and another contributing acidic residue in helix 4), leads to the proposal that helices 1 and 4 of POP2 interact with the positive EPSP on helix 2 and 3 of the ASC PYD. Interestingly,

the positive ASC helix 2/3 EPSP was also proposed as the binding site of NLRP3 (39), suggesting that POP2 inhibits NLRP3 inflammasome assembly through competition with NLRP3 for this site on ASC.

OTHER HUMAN POPs

Our evolutionary analysis of POP2 suggests the possibility of a third POP (POP3) within the human genome, encoded by an open reading frame within the NLRP2P pseudogene (43). Based on sequence similarity, a protein produced by this ORF would be anticipated to possess the NF-κB inhibitory properties of POP2, but lack the ability to inhibit the NLRP3 inflammasome as the acidic residues known to be important for inhibition are non-charged, non-polar. Unfortunately, the details of this additional potential POP protein encoding gene await an initial description.

vPOPs

Some viral proteins, such as Myxoma virus protein M013 and Shope fibroma virus protein (SFV-gp013L) have also been described to inhibit PYD-dependent inflammasomes and impair NF-κB activity (52–54). Johnston et al. identified the M13L gene which encodes the PYD-containing protein M013 in Myxoma virus, a rabbit-specific poxvirus that is the causative agent of the lethal disease myxomatosis (52). Notably, in the absence of the M013 vPOP, rabbits readily clear the viral infection and survive, indicating the key role of M013 in pathogenesis. Interestingly, genes encoding additional viral POPs closely related to M13L were found in other poxviruses, including Yaba-like disease virus, Tanapox virus, Shope fibroma virus (gene S013L, protein gp013), Mule deer poxvirus, and Swinepox virus, suggesting the conservation of PYD proteins which likely benefit viral replication and virulence among diverse poxvirus genera (52, 53, 55). As with human POPs, the vPOPs M013 and gp013L interact directly with ASC through PYD–PYD interaction, thus preventing activation of NLRP3, consequently reducing protective bioactive IL-1β, IL-18 production (52, 53). Interestingly, the first 22 amino acids of M013 vPOP were required for ASC binding, formation of peri-nuclear specks, and subsequent inhibition of caspase-1 activation as seen with the first α-helix (19 residues) of human POP2 (49, 52). Both M013 and SFV-gp013L vPOPs impact NF-κB activity. While the SFV-gp013L vPOP enhances activation of NF-κB independently of ASC and NLRs (53), M013 vPOP inhibits NF-κB, binds directly to host NF-κB1, inhibits the translocation of p65 to the nucleus (54), reminiscent of human POP2 (50). Interaction between M013 vPOP and NF-κB1 may interfere with NF-κB1/p105 degradation thus preventing p50 release, formation and subsequent nuclear translocation of the active p50/p65 heterodimer (54). Accordingly, infection of THP-1 cells with M013-deficient myxoma virus led to rapid secretion of cytokines such as TNFα, IL-6, and MCP-1 (54). While the competitive binding of M013 vPOP to ASC is required for the disruption of NLRP3 inflammasome, as with POP2 this interaction was dispensable for NF-κB inhibition (56). The sequence and functional similarity between the first helices of POP2 and M013 suggests the use of similar dual mechanisms to provide the presumed beneficial control of inflammation by POP2 but prevent protective anti-viral inflammatory responses through M013.

SUMMARY

Among the identified human POPs, POP2 is the only one with a demonstrated capacity to restrain assembly and function of NLR complexes, although both POP1 and POP2 impact NF-κB signaling. Two distinct interaction surfaces on the first α-helix of POP2 likely account for its unique ability to both limit NF-κB activation and restrict inflammasome formation. Thus, POP2 is likely a multifunctional and broadly acting regulator of inflammatory signal pathways in higher primates, while in contrast, POP1 inhibits upstream aspects of NF-κB signaling. The biological role of these POPs, although intriguing and of highly probable significance, remains less clear. Similar to human POP2, the vPOP M013 also has a dual role in impairing NF-κB activation and inflammasome formation, representing a viral strategy to circumvent protective innate inflammatory responses. Future investigation is needed to clarify the mechanism(s) by which human as well as viral POPs modulate NF-κB activity.

CARD-ONLY PROTEINS

The human genome also contains three COPs, COP/Pseudo-ICE (*CARD16*), INCA (*CARD17*), and ICEBERG (*CARD18*) which most likely arose through gene duplication and as with the POPs are restricted to higher primates (57). These proteins were initially identified and described due to sequence similarity with caspase-1 and serve as non-enzymatic decoys regulating caspase-1 activity. To date, there are no clear demonstrations of viral COPs.

COP/PSEUDO-ICE

The 16th human CARD (CARD 16) was identified as a 97 amino acid protein consisting of a CARD region (residues 1–91) with 97% identity to the CARD of caspase-1 (58). This protein is encoded on chromosome 11p22, in the same region where caspase-1, caspase-4, caspase-5, and ICEBERG (see below) reside, and is composed of three exons separated by introns of 631 and 844 bp respectively, such that the majority of the coding sequence and the entire CARD domain derive from exon 2 (57–59). Since CARD16 contains essentially only a CARD domain, this protein was named COP or Pseudo-ICE due to its high sequence identity with the CARD domain of caspase-1 [also known as Interleukin-1β converting enzyme (ICE)] (59). To avoid confusion between COP and the generic group of COP proteins, we will refer to this protein as COP/Pseudo-ICE. COP/Pseudo-ICE is expressed mainly in placenta, spleen, lymph node, and bone marrow and in the THP-1 cell line (58, 59). Binding caspase-1, RIP-2, and self-associating through its CARD domain, COP/Pseudo-ICE inhibits RIP-2-mediated oligomerization of caspase-1, thus blocking the activation of caspase-1 and subsequent release of mature IL-1β (58, 59). Overexpression of COP/Pseudo-ICE can trigger NF-κB induction and enhanced TNF-α-induced NF-κB activation via a mechanism dependent on the IKK complex (59).

INCA

The newest indentified COP named INCA (Inhibitory CARD/CARD17) is located on human chromosome 11q22, between COP/Pseudo-ICE and ICEBERG (57, 60). The predicted INCA cDNA sequence is composed of four exons and three introns 628, 1092, and 6778 bp in length (60). The open reading frame

spans the first to the third exon, but as with COP/Pseduo-ICE, exon 2 encodes most of the open reading frame including the CARD domain. Exon 4 is non-coding and thus functions as a 3′-untranslated region (60). Like COP/Pseudo-ICE and ICEBERG, although INCA contains 110 amino acids, the essential CARD domain consists of the first 91 residues and shares 81% sequence identity with the CARD of caspase-1. INCA is expressed in a wide variety of human tissues with highest expression in brain, heart, spleen, lung, and salivary gland. In general, in tissues expressing INCA, caspase-1 is also expressed, with the exception of the salivary gland. Interestingly, INCA and caspase-1 are coordinately upregulated upon stimulation with INF-γ in the monocytic cell lines THP-1 and U937, however, LPS and TNF-α which also induce caspase-1, fail to upregulate INCA (60). INCA does not interact with the NF-κB-activating kinase RIP-2, but like COP/Pseudo-ICE, INCA can self-associate, bind to the pro-domain of caspase-1, and cross-associate with the other COPs (60). Like ICEBERG which also fails to bind RIP-2, INCA was completely incapable of activating NF-κB itself and does not inhibit NF-κB activation induced by well-known factors such as TNF, caspase-1, COP/Pseudo-ICE, or RIP-2. However, INCA significantly reduces the release of mature IL-1β from THP-1 cells (comparable to COP/Pseudo-ICE), probably due to its interaction with caspase-1 (60).

ICEBERG

EST clone AA046000 contains a 273 bp open reading frame that codes for a 90-residue protein named ICEBERG (59) which is 53% identical to the CARD domain of human caspase-1 (61). ICEBERG is detected mainly in placenta and in many human cell lines and its expression is upregulated by LPS and TNF in THP-1 monocytes (61). ICEBERG can self-associate, bind to the pro-domain of caspase-1, and cross-associate with another COP/Pseudo-ICE through its CARD domain. Although unable to interact with RIP-2, ICEBERG clearly binds to caspase-1 through charge–charge interaction between their CARD domains; not surprisingly ICEBERG is unable to activate NF-κB (59, 61). However, RIP-2 independently binds and activates caspase-1 directly via CARD–CARD interaction (61, 62) and ICEBERG blocks this interaction by binding caspase-1, a competitive inhibition that disrupt oligomerization of RIP-2 and caspase-1 and consequently inhibits IL-1β production.

SUMMARY

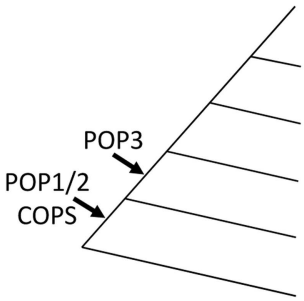
All three human COPs interact with caspase-1 and thus, when expressed are anticipated to influence all instances of inflammasome-elicited IL-1β and IL-18 production. Only COP/Pseudo-ICE targets events mediated through the CARD domain of RIP-2. Why three independent regulators are required and how they differ is largely unknown and unstudied, but their common expression in the placenta and monocytic cells may suggest roles in development as well as immunity.

EVOLUTIONARY HISTORY OF POPs/COPs

In the battle between pathogens and host immunity, pathogens, typically viruses, manifest sophisticated mechanisms to escape the detection and control of host immune systems. In contrast, the host defense incessantly develops strategies to eliminate pathogens without becoming hyper-responsive and causing harm to itself. Evolution plays a pivotal role in these both processes. Poxviruses are excellent examples for studying genome evolution. They accumulate point mutations at relatively low rates, whereas gene duplications, losses, gain by horizontal gene transfer, and recombination between different viral species occur frequently (55). These events led to the appearance of several groups of genetic elements known as host range genes thought to be important for host adaptation and subverting the host anti-viral response [listed in (55)]. Of these host range genes, M013 vPOP protein was proposed to be an evolutionary factor based on its high sequence similarity with pyrin, particularly within the first 50 residues and its role as a competitive inhibitor of ASC interactions (52, 53). It is possible that M013 vPOP is the product of ancestral capture, recombination, and re-assortment events that occurred during co-evolution of the virus and its host. Interestingly, phylogenetic analyses showed that, together with Myxoma virus, other poxviruses belonging to the largest subgrouping of Chord poxviruses possess M013 orthologs. These include Yaba-like disease virus, Tanapox virus, Shope fibroma virus, Mule deer poxvirus, and Swinepox virus (55), suggesting a shared evolutionary ancestor that may have acquired a host PYD domain prior to the divergence of this group of viruses.

Among mammals, the human genome encodes at least two and possibly three POPs (POP1, POP2, and the putative POP3) and syntenic orthologs are present in the genomes of the non-human primates *Pan troglodytes* (chimpanzee), *Macaca mulatta* (rhesus macaque), *Pongo abelii* (orangutan) but the NLRP2P locus (POP3) and a functional POP2 appear to be absent in that of marmosets (43). Interestingly, POPs were not found in the genomes of mice, rats, and domestic animals, even though most human NLRPs are similar to those in other species (11, 43). Taken together, these observations support the current hypothesis that POPs are evolutionary recent developments in the mammalian genome and may be limited to hominids and Old World primates. The evolutionary history of POPs and COPs are summarized in **Figure 3**.

Although the selective pressures driving the appearance of human POPs are unknown, their origins and evolutionary pathways have been investigated. The POP1 gene is located on chromosome 16p12.1, the same chromosomal band as the ASC gene, approximately 14 kb away and the PYD of POP1 is most closely related to that of ASC (88% similarity and 64% identity) (44). This close proximity as well as the strong sequence similarity suggests that POP1 arose by gene duplication (44). More interestingly, phylogenetic analysis revealed that both POP2 and NLRP2P arose from the same ancestor, an NLRP2/7-like gene (designated Nlrp2 in mouse), most likely through retrogene insertion events during the course of primate evolution resulting in two distinct, and seemingly non-functional, pseudogenes (43). Curiously, during higher primate evolution, POP2 gained a functional promoter, lost the remnants of the non-PYD portions of the ancestral NLRP2/7-like gene, and acquired a new polyadenylation sites (43). Despite the sequence similarity, NLRP2 is adjacent to NLRP7 on chromosome 19 in human and chimpanzee genomes, whereas POP2 and NLRP2P are located on chromosome 3 and chromosome X, respectively. The distal location and the absence of



		POPs			COPs	
	POP1	POP2	POP3	COP/Pseudo-ICE	INCA	ICEBERG
Human	+	+	+	+	+	+
Chimpanzee	+	+	+	+	+	- ?
Orangutan	+	+	+	ND	ND	ND
Rhesus macaque	+	+	+	+	+	+
Marmoset	+	+*	-	ND	ND	ND
Mice	-	-	-	-	-	-

FIGURE 3 | The evolution of POPs and COPs. POPs and COPs emerged very recently during mammalian evolution and are restricted to primates. All currently known POPs are present as intact coding regions in the complete genomes of Old World primates and hominids but are absent from the complete genomes of mice, non-primates, and, for at least POP2 and POP3, New World primates. All currently known COPs are primate restricted [an ICEBERG locus has been reported for the treeshrew a primate ancestor, see (63)]. Arrows indicate the relative timing of the retro-transposon insertion of POP2/3 and the duplication events leading to POP1/COPs in an ancestral primate genome. (ND, not determined; *, non-functional).

introns as well as the presence of stop-codon disrupted NBD and LRR sequences in the founding primate genomes strongly implicates the retro-transposition of NLRP2/7-like mRNAs yielding processed pseudogenes.

Chimpanzee POP2 is identical to human POP2 at both the DNA and protein level (43). Macaque POP2 is highly conserved, but not identical to the human sequence. Although macaque POP2 retains both functions of the human protein, it is a less robust inhibitor which may result from an additional C-terminal 41 amino acid stretch absent from human and chimpanzee POP2 (43). Among the New World primates, the marmoset genome contains an intron-less pseudogene sequence without a start codon with remnants of not only the PYD, but the nucleotide-binding domain and portions of the LRR sequences, which share identity with NLRP2/7. Under strong selective pressure during divergence of New World and Old World primates, the NBD and LRR sequences were lost to produce a functional POP2, retained in the common ancestor of macaques and higher primates. Further pressure during the emergence of hominids purified the developing POP2 PYD sequence to yield a version of POP2 with stronger inhibitory activities on NF-κB and inflammasomes which is invariant between humans and chimpanzees (43).

The other NLRP2/7-derived pseudogene named CLRX.1/NOD24 (NLRP2P) also contains an open reading frame that may encode POP3, but unlike POP2 it contains a conserved stop codon that would preclude PYD helices 4 through 6.

Similar to POPs, the three mammalian COPs (COP, INCA, ICEBERG) are also found only in humans and primates (63). No ortholog has been identified in the same locus in mouse and rat genome (57). All three COPs are highly homologous with the CARD domain of caspase-1 and are located on the same chromosome adjacent to caspase-1 (58, 60, 61), suggesting that COPs emerged by gene duplication. While putative orthologs of COP and INCA were identified in human, chimpanzee, and Rhesus macaque, ICEBERG was only found in human and Rhesus monkeys despite the earlier divergence of these monkeys from the human-chimpanzee lineage (57), perhaps owing to the incompleteness of the chimpanzee genome at this locus on chromosome 9. Nevertheless, like POPs, COPs appear restricted to hominids and primates, suggesting that strong selective pressures, perhaps acting to control inflammation at both level of cytokine gene transcription and processing of IL-1β by inflammasome activated caspase-1, drove the emergence of at least six independent genes with largely unexplored and unexplained biological roles.

BIOLOGICAL IMPACT

Given the key role of IL-1β in inflammation, it is not surprising that defective control of the inflammasome appears to be a feature of many inflammatory diseases. In fact, the initial description of the NLRP3 inflammasome and clinical interest in defective control of inflammasome activation resulted in large part from genetic analysis of families with autoinflammatory diseases. These include familial Mediterranean fever (FMF), Muckle-Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS), neonatal-onset multisystem inflammatory disease/chronic infantile neurological cutaneous, and articular syndrome (NOMID/CINCA) (64–67). Of these, FMF is the first known disease with associated mutations in the Pyrin protein, a small non-NLR protein composed of an N-terminal PYD domain and a C-terminal PRY-SPRY domain (68). Like NLRPs, Pyrin associates with ASC and may form an inflammasome (69, 70), however, most disease-associated Pyrin mutations occur in the PRY-SPRY domain which is functionally uncharacterized. Unexpectedly, the other hereditary fever syndromes, including FCAS, MWS, CINCA, which range from the very mild FCAS to the severe and chronic NOMID/CINCA, are all attributable to NLRP3 (CIAS1/Cryopyrin). Even though being distinct clinical entities, the majority of disease-associated

mutations are located in exon 3 which encodes the NACHT (NBD/NOD) domain (66, 67). It is likely that these mutations cause spontaneous oligomerization of the NACHT domain due to decreased interaction with the LRR which are thought to inhibit auto-activation of NLR-inflammasomes (10) which in turn results in overproduction of IL-1β and IL-18, leading to chronic, excessive inflammation. Other inflammation-related diseases have been reported. Blau syndrome and Crohn's disease are associated with the CARD-containing NLR, NOD2/NLRC2 with NACHT domain mutations occurring in Blau syndrome (71), while LRR domain mutations of NOD2 are reported for Crohn's disease (13, 72). Moreover, through rapidly growing clinical and NLR-deficient mouse studies, specific NLRs have been linked to numerous diseases, including NLRP1 [various autoimmune diseases (e.g., psoriasis, systemic lupus erythematosus, rheumatoid arthritis, Celiac disease, and type 1 diabetes), NLRP3 (e.g., gout, atherosclerosis, type II diabetes, rheumatoid arthritis, Alzheimer's, and cancer of the colon and skin), NLRP6 (e.g., fatty liver disease, inflammatory bowel disease, and gastric cancer), NLRP12 (atopic dermatitis), and NLRC4 (e.g., gastric cancer and inflammatory bowel disease); reviewed in (5, 73)]. The non-NLR PYD-containing protein AIM2 also forms an inflammasome and is implicated in allergy [reviewed in (5)]. The importance of inflammasomes in complex diseases, the majority of which have underlying inflammatory etiologies, underscores the need for tight control of inflammasome activity as well as other inflammatory signaling pathways to maintain homeostasis.

In humans, POPs and COPs very likely represent a mechanism for restraining pro-inflammatory activation events, for terminating inflammasome signals, or both depending on the specific protein. Given that POP2 disrupts downstream activation of the NLRP3 and likely other NLR-inflammasomes and concomitant release of IL-1β, POP2 may be critically important in establishing the normal limits of inflammasome activation. This has significant implications for inflammasome-associated diseases. For example, mutations in POP2 involving the acidic amino acids of the first α helix that are important for NLRP3 inflammasome inhibition may result in diseases similar to those seen with NLRP3 mutations. Likewise, mutations that diminish expression of POP2 may have broad effects allowing more ready and sustained activation of various NLRPs with attendant increased pathologic inflammation. Gain of function mutations might provide a protective benefit for NLRP-associated diseases, although diminished inflammatory responses might similarly predispose individuals to infection or interfere with wound healing. Similarly, COPs directly binds caspase-1 and therefore interfere with the interaction of caspase-1 with ASC (in inflammasomes) and/or with RIP-2 (in NODsomes). Consequently, COPs potentially target NLRCs such as NOD1, NOD2, and NLRC4 as well as NLRPs, and in principle any pathway requiring a CARD:CARD domain interaction. The combined regulatory potential of POPs and COPs within NLR biology is thus staggering. Further, the evolutionary evidence suggests that it is critical for human survival. Despite the implications, little attention has been devoted to understanding these small, seemingly insignificant proteins.

OUTSTANDING QUESTIONS

In light of the profound biological consequences of NLR auto-activation (chronic inflammation), tight control of NLR protein functions is critical to provide the delicate balance between the initiation and perpetuation of immune responses and anti-inflammation mechanisms. POPs and COPs are preeminent candidates as they are able to control of both NF-κB and inflammasome activation. However, studies of the mechanism(s) by which they interfere with these processes are few. Whether POPs and COPs represent an array of modulators with overlapping functions or are discrete, sufficient, regulators of different aspects of NF-κB signaling, and/or inflammasome activity is unknown. Several outstanding questions are of immediate importance to the field. Beyond more immediate questions such as which tissues and cells express POPs and COPs and under what conditions, the question of what roles POPs and COPs play in normal human physiology and pathology is the most urgent. Mouse models of POP/COP *in vivo* function need to be developed to study these primate-restricted proteins. Gene association/single nucleotide polymorphism studies of human inflammation-related disease without specific NLR mutations are also likely informative. Whether POPs and COPs are broadly acting regulators or specific to particular pathways remains unclear, but the answer certainly resides at the intersection of mechanistic studies aimed at understanding the distinctions between family members and the insights provided from *in vivo* systems. More broadly, the presence of POPs and COPs in humans implies fundamental differences in some, if not many, aspects of NLR biology between mice and humans that at present remain mysteries. Finally, lessons learned from the study of POPs and COPs may lead to additional biologics that can specifically target dysregulated inflammatory processes mediated through PYD and CARD domain-containing protein complexes.

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An update on PYRIN domain-containing pattern recognition receptors: from immunity to pathology

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Cytosolic pattern recognition receptors (PRRs) sense a wide range of endogenous danger-associated molecular patterns as well as exogenous pathogen-associated molecular patterns. In particular, Nod-like receptors containing a pyrin domain (PYD), called NLRPs, and AIM2-like receptors (ALRs) have been shown to play a critical role in host defense by facilitating clearance of pathogens and maintaining a healthy gut microflora. NLRPs and ALRs both encode a PYD, which is crucial for relaying signals that result in an efficient innate immune response through activation of several key innate immune signaling pathways. However, mutations in these PRRs have been linked to the development of auto-inflammatory and autoimmune diseases. In addition, they have been implicated in metabolic diseases. In this review, we summarize the function of PYD-containing NLRPs and ALRs and address their contribution to innate immunity, host defense, and immune-linked diseases.

Keywords: PYRIN domain, innate immunity, pattern recognition receptor, Nod-like receptor, NLR, AIM2-like receptor, ALR, inflammasome

INTRODUCTION

The innate immune system relies on germline-encoded pattern recognition receptors (PRRs) to detect threats against tissue homeostasis. In response to pathogen infection, tissue damage or environmental stress, inflammatory mediators including cytokines, type I interferons (IFNs), and anti-microbial factors are produced. While Toll-like receptors (TLRs) utilize their TIR domain and RIG-I-like receptors (RLRs) and NLRCs their CARD for downstream signaling upon activation, NLRPs and AIM2-like receptors (ALRs) recruit signaling adaptors through their PYRIN domain (PYD). Active NLRPs and ALRs trigger multiple innate immune effector pathways, but by far the best established function of these PYD-containing proteins is the assembly of inflammasomes, which are large multiprotein platforms that form in response to infection and tissue damage and are responsible for the activation of inflammatory caspases, in particular caspase-1 (1, 2). Thus, a necessity of these PRRs is to be able to promote the clustering of inflammasome adaptors, which is essential for induced proximity-mediated activation of caspase-1 (3). Active caspases then induce inflammatory cell death (pyroptosis), maturation, and/or secretion of the leaderless pro-inflammatory cytokines IL-1β and IL-18, and contribute to the release of the related IL-1α (4, 5) as well as the stress-associated danger signal HMGB1 (6, 7). Furthermore, there is increasing evidence for a broader contribution of inflammasomes to unconventional protein secretion (8), to lipid biogenesis and to the release of inflammatory lipids (9–11). Although not as well-established and in many cases derived from overexpression studies, these proteins have also been linked to transcriptional responses, through activation of NF-κB, IRFs, and MAPKs to regulate pro-inflammatory and anti-microbial gene expression, autophagy, and to affect adaptive immune responses.

PYRIN DOMAIN

The PYD, also referred to as PAAD or DAPIN, is a protein binding domain belonging to the death domain superfamily (12). The structure of several PYDs has been determined, which revealed a bundle of 5- to 6-α-helices. PYDs display distinct negatively and positively charged surface patches, which are indicative of electrostatic interactions to occur during PYD-PYD interactions, reminiscent to other death domain folds (13–18). NLRPs and ALRs both encode an N-terminal PYD, but while NLRPs are further composed of a central nucleotide binding NACHT domain and varying copies of C-terminal leucine-rich repeats, ALRs rather contain one or two copies of the oligonucleotide binding HIN-200 domain at the C-terminus. The PYD is the effector domain required for downstream signaling, while evidence supports a role of the LRR and HIN-200 domain in ligand recognition (19–21). The current model for both PRR families is that ligand recognition promotes a conformational change (15, 21–23), which allows nucleotide binding by the NACHT domain and consequently, enables NLRP oligomerization (24–27), while ALRs cluster alongside the DNA staircase (21). Ultimately, this exposes the PYD in NLRPs and ALRs, thus enabling the recruitment of ASC by homotypic PYD–PYD interactions and clustering of ASC. In the context of inflammasomes, the recruitment and clustering of ASC then triggers its interaction with pro-caspases-1 (3, 28) and -8 (29, 30) and their activation by induced proximity. The precise order of events is still elusive and a recent model proposed spontaneous self-oligomerization of the ASC-PYD, which subsequently facilitates its interaction with NLRP3 and potentially also other PYD-containing PRRs (31). Hence, this model suggests that PYDs contain a dual binding interface (31). The influence of NLRPs on other signaling pathways is even less well understood, but might

also occur through these adaptors (32, 33). In contrast to ASC-mediated inflammasome activation in response to KSHV (34), the ALR IFI16 promotes induction of IFN-β through connecting to the common pathway leading to IRF-3 phosphorylation through the adaptor STING (stimulator of IFN genes) (35).

Only 14 NLRPs and 4 ALRs are encoded in humans, while both families are amplified to 34 and 13 members, respectively, in mice (**Figures 1A,B**). However, the precise function of most family members is still unknown (36–39). Besides NLRPs and ALRs, the PYD is also present in the inflammasome adaptor protein ASC, the regulatory PYD-only proteins (POPs) and Pyrin (**Figure 1C**) (12). Below, we will specifically discuss the mechanism of activation and function of NLRPs and ALRs, and how defects within these proteins are involved in immune-related disorders.

NOD-LIKE RECEPTORS

NLRP1

NLRP1 (**Figure 1A**) is also known as NALP1, NAC, DEFCAP, CARD7, and CLR17.1 and has initially been linked to caspase-9 activation within the apoptosome (40).

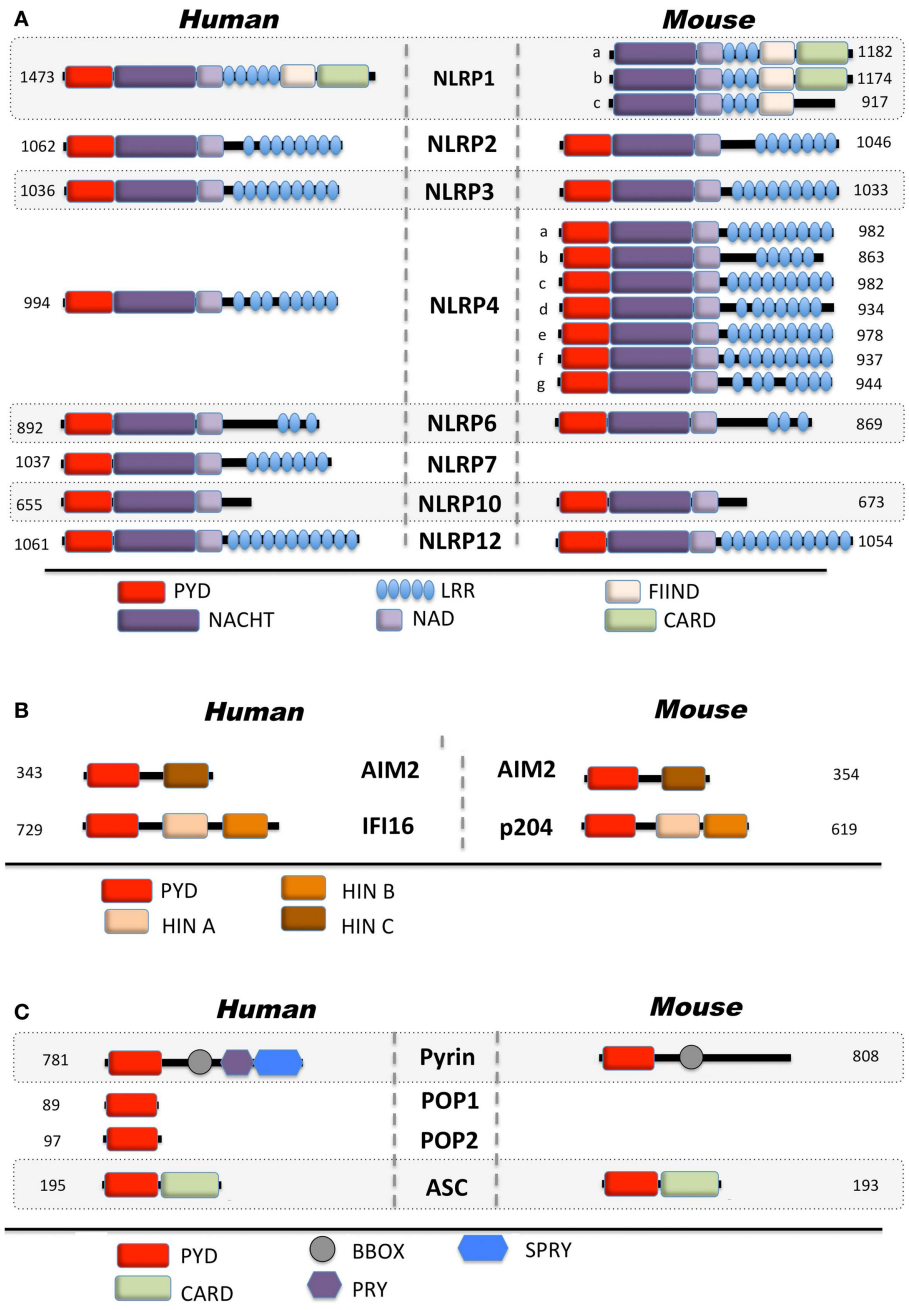

FIGURE 1 | Domain architecture of PYD-containing proteins involved in innate immunity. Depicted are human and mouse **(A)** Nod-like receptors, **(B)** AIM2-like receptors, and **(C)** regulatory proteins.

Inflammasomes were first discovered in 2002 with the initial observation that NLRP1 is able to assemble in an ASC, caspase-1, and caspase-5-containing large inducible protein complex responsible for the autocatalytic activation of caspase-1 in THP-1 cells (1). However, caspase-5 is not recruited to other inflammasomes (25, 41), which is likely due to the unique domain structure of NLRP1. In addition to the common tripartite domain organization of NLRPs, NLRP1 also encodes a C-terminal function to find (FIIND) domain and a CARD, which enables direct caspase-5 recruitment (**Figure 1A**). Despite its early identification, the *in vivo* function of NLRP1 however remains largely elusive, at least partially due to several key differences between mice and human, which limits the relevance of *in vivo* mouse models. In contrast to human NLRP1, mouse NLRP1 lacks the PYD and exists in three tandem paralog genes (Nlrp1a, Nlrp1b, and Nlrp1c) (**Figure 1A**). While the PYD is crucial for the recruitment of ASC and subsequently of caspase-1, the C-terminal CARD directly recruits caspase-5, which is necessary for full caspase-1 activation in human cells (1). However, analysis of the first *in vitro*-reconstituted inflammasome with purified recombinant human proteins demonstrated that the core inflammasome components NLRP1 and caspase-1 are sufficient for promoting caspase-1 activation in the presence of NTPs and MDP as a specific agonist (25). In this context, ASC was not necessary, but addition of ASC increased the efficiency of caspase-1 activation. Similar results have also been observed *in vivo* for murine NLRP1b (22). In contrast, a recent analysis suggested that caspase-1 is directly recruited to the C-terminal CARD of NLRP1 and that the PYD is dispensable for inflammasome activation (42). This model could therefore explain NLRP1 inflammasome activation of human and mouse NLRP1, in spite of mouse NLRP1 lacking the PYD. Although the role of the PYD in human NLRP1 is still elusive, the presence of ASC, facilitated by PYD–PYD interaction, could enable an increase in NLRP1-mediated caspase-1 activation in addition to CARD mediated caspase-1/5 recruitment. Additional insights into the molecular mechanism of NLRP1 inflammasome activation came from studies showing that the FIIND domain resembles the autoproteolytic ZU5-like domain found in PIDD, which contains a LRR and a death domain and is part of the caspase-2-activating PIDDosome (43). Accordingly, the FIIND domain in NLRP1 also undergoes autoproteolytic cleavage, which is required for inflammasome activation and congruently, NLRP1b^{V988D}, which disrupts the protein conformation required for autoproteolysis, or NLRP1b^{S984A}, which disrupts the catalytic serine residue, results in deficient caspase-1 activation without impairing NLRP1 oligomerization (42–44). This step is further regulated by splicing, since an alternative transcript lacking exon 14, which contains the FIIND cleavage site, is deficient in autoproteolytic processing (42). Moreover, rat NLRP1 activation by the *Bacillus anthracis* virulence factor lethal toxin (LTx), a metalloproteinase composed of the pore-forming antigen (PA) and a lethal factor (LF), also cleaves NLRP1, but within the N-terminal domain, suggesting that NLRP1 potentially has several protease cleavage sites (45, 46). Both steps appear necessary for caspase-1 activation, and a possibility could be that the FIIND has partial autoproteolytic activity, and cleavage of NLRP1 by LT might increase this activity (46). Accordingly, a C-terminal fragment of NLRP1b containing the CARD and 56 adjacent amino acids is sufficient for caspase-1 activation with the 56 adjacent amino acids being required for oligomerization (47). However, LTx-mediated cleavage of NLRP1b is still controversial, since another study failed to observe LTx-mediated cleavage of NLRP1b, although LTx was required for autoproteolysis (44).

A first glimpse into the functional importance of NLRP1 was discovered, when genetic mapping identified NLRP1b as the gene responsible for LTx sensitivity in mice. In mice, only NLRP1b, and none of the two other paralogs (NLRP1a and NLRP1c), confer susceptibility to LTx (**Figure 2**) (48). The exact role of LTx in this context during *B. anthracis* infection is, however, a matter of controversy, since *in vitro* cell death and *in vivo* end-stage LTx-induced disease and death appear to not be linked (49). Furthermore, different mechanisms have been reported for LTx and spores, with the latter promoting an inflammasome response in LTx susceptible and resistant macrophages (50). A similar protective response has also been reported in response to *Toxoplasma gondii* infection, where NLRP1b activation ensured selective elimination of the niche for pathogen proliferation, cytokine release, and effective spreading of danger signals to neighboring cells (51).

Several studies observed NLRP1/NLRP1b sensing of MDP (22, 25, 52). However, while the recently generated NLRP1b deficient mice demonstrated impaired inflammasome response to LTx, the response to MDP was intact and rather NLRP3-dependent (53). Furthermore, NLRP1b has been suggested to sense energy stress in fibroblasts, as a consequence of starvation (54). In particular, NLRP1b senses the reduction of intracellular ATP levels and the subsequent activation of the AMP-activated protein kinase (AMPK). Congruently, a mutation of the ATP binding pocket within the NACHT of NLRP1b yielded a constitutively active inflammasome, suggesting that ATP binding might inhibit, rather than activate NLRP1b, in contrast to what has been reported for human NLRP1 (25, 55).

Underlining its functional importance, further control mechanisms besides RNA splicing may regulate the activity of the NLRP1 inflammasome. The anti-apoptotic proteins Bcl-2 and Bcl-X_L were reported to specifically inhibit NLRP1 activation by blocking ATP binding (52, 55). Both proteins appear to bind to the LRR of NLRP1 with their loop region, suggesting that different domains are responsible for their NLRP1 inflammasome-suppressing activity compared to their apoptosis-suppressing activity. Furthermore, recent evidence suggests that NLRP1 may provide a more effective immune response by associating with NOD2 (22). Finally, there is evidence that the anti-inflammatory omega-3 (ω-3) polyunsaturated fatty acids attenuate NLRP1b through interaction of NLRP1b with β-arrestin-2, the downstream scaffold for GPR120 and GPR40 (56).

NLRP2

Although NLRP2 (**Figure 1A**), also known as PYPAF2, NALP2, PAN1, and CLR 19.9 failed to affect activation of NF-κB or caspase-1 in initial *in vitro* studies (57), it was later shown to inhibit cytokine-induced NF-κB activation. Subsequently, it was shown that PYD-mediated interaction of NLRP2 with ASC resulted in the abrogation of the expression of NF-κB target genes in the monocytic THP-1 cell line (58). Highly expressed in T-cells, NLRP2 was also found to inhibit NFAT and AP-1, in addition to NF-κB,

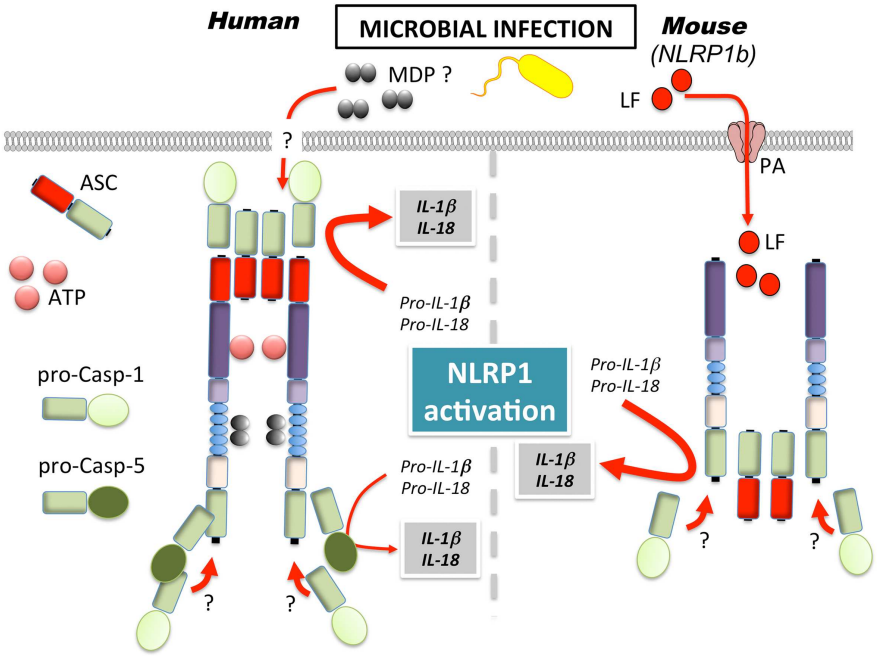

FIGURE 2 | Mechanism of NLRP1 activation in human and mice. MDP, muramyl dipeptide; LF, lethal factor; PA, protective antigen.

following TCR activation by anti-CD3 and anti-CD28 antibodies or PMA/ionomycin (59). Besides its transcriptional regulation, biochemical studies in THP-1 cells, suggesting that NLRP2 could assemble into an ASC and caspase-1-containing inflammasome (41). NLRP2 does not contain a FIIND domain, but CARD8 (also known as Cardinal and TUCAN), which is the only other FIIND domain-containing protein besides NLRP1, is recruited to NLRP2 via its NACHT (41). In a manner similar to NLRP1, the FIIND domain of CARD8 is also autoproteolytically cleaved, potentially to promote downstream signaling (43). The *in vivo* function of CARD8 and its role in inflammasome activation, however, is still poorly defined, since CARD8 is does not exist in mice (60). NLRP2 is highly expressed in human astrocytes within the central nervous system and, similar to NLRP3, appears to assemble in an ASC- and caspase-1-containing inflammasome in response to exogenous ATP, as shown by gene silencing (61). In this context, NLRP2 may directly interact with the $P2X_7R$ and pannexin-1, suggesting a direct effect on the NLRP2 inflammasome, rather than the indirect effect that is observed for NLRP3. However, these findings will need further corroboration, in particular *in vivo*.

NLRP3

NLRP3 (**Figure 1A**), also known as Cryopyrin, NALP3, PYPAF1, CIAS1, CLR1.1, is the best-studied member of the NLRP family. It was initially discovered by positional cloning in the search for the genetic cause of a group of auto-inflammatory diseases, now referred to as Cryopyrinopathies or Cryopyrin-associated periodic syndromes (CAPS) (62). While initial overexpression studies suggested that NLRP3 affects NF-κB activation, NLRP3-deficient mice displayed defects restricted to inflammasome activation (63–66). In contrast to other Nod-like receptors (NLRs), NLRP3 is activated by, and responds to a diverse set of stimuli originating from microbes pathogen-associated molecular patterns (PAMPs) (**Figure 3**) or from environmental and endogenous danger signals danger-associated molecular patterns (DAMPs), which can be of either soluble or particulate matter (**Figure 4**). Microbial activators include various Gram-positive and -negative bacteria (*Listeria monocytogenes*, *Staphylococcus aureus*, *Vibrio cholera*, *Neisseria gonorrhoeae*, and others) (64, 67–71), fungi (*Candida albicans*, *Saccharomyces cerevisiae*) (72), RNA and DNA viruses (adenovirus, influenza virus, Sendai virus, MCMV) (73–75), as well as protozoa (*Plasmodium malariae*) (76–78). The fact that NLRP3 also senses sterile environmental and endogenous stress signals, and promotes inflammatory responses further expands the repertoire of NLRP3 reactivity. Environmental triggers include the particulates alum (79–83), asbestos (84, 85), silica (81, 84, 85), skin irritants (trinitrochlorobenzene, trinitrophenylchloride, and dinitrofluorobenzene) (66, 86), and even UVB radiation (87). An increasing complexity of endogenous danger signals is now also known to activate NLRP3, since the discovery that monosodium urate crystals (MSU) and pyrophosphate dihydrate (CPPD) crystals are able to activate NLRP3 (65). Other known NLRP3-inducing crystals are cholesterol, amyloid deposits (88, 89), hydroxyapatite crystals (90), and hyaluronan (91). In addition to these crystalline danger signals, NLRP3 also senses non-crystalline stress signals, including ATP (64), high glucose (92), and saturated fatty acids (93). The mechanism that causes NLRP3 activation in response to so many different stimuli is still controversial and more discussed below.

Basic concepts of NLRP3 inflammasome activation

Based on the diverse structural nature of NLRP3 agonists, the current model assumes that intermediate factors may be involved in

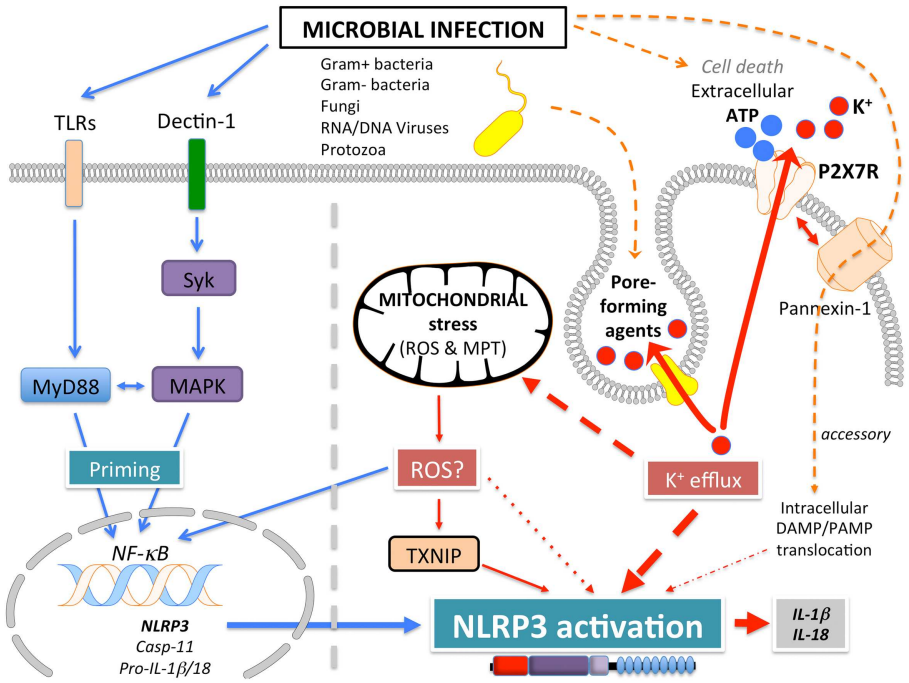

FIGURE 3 | Mechanism of NLRP3 activation in response to pathogen infection.

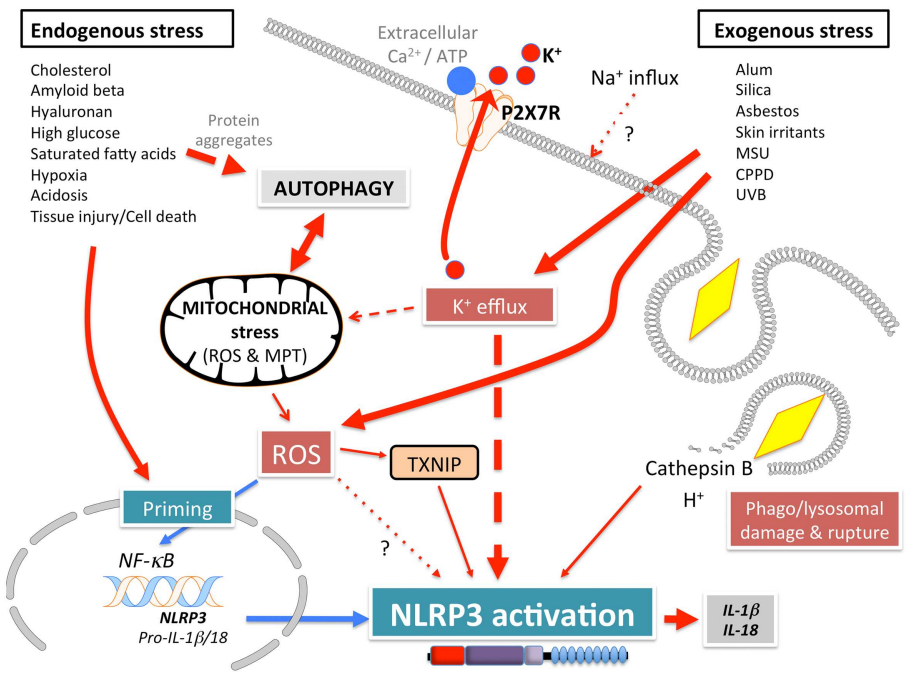

FIGURE 4 | Mechanism of NLRP3 activation in response to endogenous and exogenous danger signals.

sensing of these activators, rather than a direct receptor-ligand interaction. Among all NLRPs, an essential *in vivo* function of the LRR in NLRP activation has only been shown for NLRP3. In contrast to many *in vitro* studies showing that deletion of the LRR renders the NLRP constitutively active, likely because of a lack of autoinhibition, the absence of the LRR *in vivo* renders NLRP3

unresponsive to MSU and ameliorates MSU-induced inflammation in mice (19). Activation of NLRP3 does not fit into a unifying model (94), but it is well-established that NLRP3 activation employs a two-step mechanism.

Signal 1: activation of NLRP3, especially in mouse myeloid cells, requires a "priming" step. While it was initially believed that this step is necessary for providing the cytokine substrates, in particular proIL-1β, which is highly inducible by NF-κB, it was subsequently proposed that induction of NF-κB is necessary for transcription of NLRP3 itself (95, 96). This proposal was based on the observation that ectopic expression of NLRP3 uncouples NLRP3 activation from priming (95). In addition to NLRP3 expression, priming has been shown to potentiate NLRP3-specific activation of caspase-1 at short time points that do not affect NLRP3 expression levels and furthermore, also potentiates NLRP3 inflammasome activity following ectopic NLRP3 expression (97). The mechanism behind this observation is likely TLR4-MyD88-dependent deubiquitination of NLRP3 by BRCC3, which is essential for its activation (98–100).

Signal 2: subsequently, a specific activating step (signal 2) triggers NLRP3 activation and assembly of the NLRP3 inflammasome. Three main activating mechanisms have been proposed: (1) K^+ efflux, (2) mitochondrial dysfunction and generation of mitochondria-derived reactive-oxygen species (ROS), and (3) phagolysosomal destabilization in response to particulates (**Figures 3** and **4**).

(1) ATP is released into the extracellular space after tissue injury and cell death. The extracellular ATP then triggers the purogenic $P2X_7R$, which is an ATP-gated K^+ ion channel, that facilitates K^+ efflux, which activates the NLRP3 inflammasome (64, 101, 102). Although the interaction of $P2X_7R$ with the hemichannel protein pannexin-1 was initially proposed to allow influx of PAMPs/DAMPs into the cytosol through a 900 kDa pore, based on pannexin-1 blocking peptides (103). However, this scenario is not any longer considered to play any role in NLRP3 activation, since pannexin-1-deficient macrophages exhibit no defect in NLRP3 activation (104). Similarly, microbial pore-forming toxins (such as hemolysins) on the cell surface or on phagolysosomal membranes trigger K^+ efflux and NLRP3 activation (105). The precise mechanism by which low K^+ levels affect NLRP3 activation is not understood. While K^+ efflux in NLRP3 activation is well-established, Ca^{2+} mobilization and Ca^{2+}-mediated signaling has also been linked to NLRP3 activation, but this is controversial (75, 106–108). ATP induced Ca^{2+} signaling is regulated by the calcium-sensing receptor (CASR), phospholipase C-mediated generation of inositol-1,4,5-trisphosphate, IP3R mediated release of Ca^{2+} from the ER, and store-operated Ca^{2+} entry (SOCE) mediated influx of extracellular Ca^{2+}, which is important for NLRP3 inflammasome activation by extracellular ATP. Hence, caspase-1 and IL-1β processing and release are also controlled by PLC, IP3R, and SOCE (75, 106–108). In addition to ER stores, Ca^{2+} influx has also been proposed to occur through the plasma membrane channel TRPM2 (108). However, the involvement of Ca^{2+} in NLRP3 activation has been recently disputed and linked to the precipitation of insoluble particulates, which then activates NLRP3 in a K^+ efflux-dependent manner (102).

(2) A second mechanism proposed to contribute to NLRP3 activation, involves mitochondria and generation of ROS (92, 109, 110). However, involvement of mitochondria and mitochondria-derived molecules, including mROS in NLRP3 inflammasome activation is controversial with arguments found for and against throughout the literature. ATP-mediated ROS production is necessary for caspase-1 activation (111) and initial studies linked NADPH oxidase-produced ROS to NLRP3 activation (76, 85). Interaction of NLRP3 with the thioredoxin (TRX)-interacting protein TXNIP through its LRR, has been proposed as a mechanism, since NLRP3 agonists caused ROS-dependent dissociation of TXNIP from TRX (92). However, subsequent studies in chronic granulomatous disease (CGD) patients disproved these earlier observations. CGD patients lack $p22^{phox}$, which is essential for the proper function of the NADPH oxidase Nox1-4, but CGD macrophages showed either no defect in IL-1β release (112), or even an increased caspase-1 activity and IL-1β release (113, 114). This is in agreement with the finding that ROS actually inhibit caspase-1 through reversible oxidation and glutathionylation of two redox-sensitive cysteine residues (C^{397} and C^{362}), which is in contrast to an earlier study. Furthermore, the crystal structure of the NLRP3 PYD revealed that it is unique in containing a disulfide bond between C^8 and C^{108}, which could be important for redox potential-dependent regulation (13). Mitochondria are the other main source for ROS, and mitochondria have been linked to NLRP3 activation through mROS generation and as a platform for inflammasome assembly. While mROS are necessary for homeostasis, cellular stress including hypoxia, acidosis, changes in intracellular ionic milieu and membrane damage are known to promote release of mROS (115, 116). It has also been proposed that all NLRP3-activating stimuli induce apoptosis in target cells, thereby causing opening of the voltage dependent anion channel (VDAC), decreases the mitochondrial membrane potential ($\Delta\Psi$), generation of mROS, which in turn promotes mitochondrial permeability transition (MPT) and cytosolic release of mitochondrial DNA leading to NRLP3 activation (92, 110, 117). Accordingly, inhibiting VDAC1 and 2, but not VDAC3 decreased NLRP3 activation (110). Furthermore, defect mitophagy or autophagy, and consequently, accumulation of damaged mitochondria, causes NLRP3 activation and elevated IL-1β levels (109, 110, 118, 119). However, autophagy is also involved in degrading ubiquitinated inflammasomes through recruiting the autophagic adaptor p62 (119). Moreover, it has also been proposed that mitochondrial damage does not contribute to NLRP3 activation, but can occur in response to NLRP3-activating stimuli at later time points (102). Additional support for a significance of mitochondria as a platform facilitating NLRP3 activation is supported by studies showing that ER-localized NLRP3 is redistributed to mitochondria upon activation (110). This transport has been shown to occur by a dynein-mediated movement of mitochondria in response to reduced NAD^+ levels caused by defect mitochondria. This

facilitates inactivation of sirtuin 2, an NAD^+-dependent α-tubulin deacetylase, and consequently, accumulation of acetylated α-tubulin necessary for mitochondrial movement (120). However, mitochondrial ASC and NLRP3 localization is also controversial. Yet another study proposed that the CARD-containing RLR adaptor MAVS is necessary for full NLRP3 inflammasome activation through targeting NLRP3 to mitochondria, which requires a short peptide within the PYD (121). However, MAVS appears to be only necessary for non-crystalline activators, suggesting that other adaptors might be involved in crystalline responses. However, this finding is controversial and has only been partially reproduced in the context of Sendai virus infection (122).

Altogether, there is widely conflicting information of the involvement of mitochondria and mROS to NLRP3 activation. Analyses of various mitochondria-targeted drugs suggested an involvement of mitochondria and mROS dependent and independent mechanisms (123). But a recent study suggested that, rather than acting on the signal 2 of NLRP3 inflammasome activation, ROS might only be necessary for inflammasome priming through NF-κ activation or deubiquitination (95, 98). Yet, these studies have also been disputed and attributed to the use of high concentrations of ROS inhibitors and proposed that ROS do not play any role in signal 1 and 2 (102).

(3) Reactive-oxygen species are also generated upon lysosomal rupture and leakage of lysosomal contents in the cytosol, as a consequence from the digestion of particulate matter or infection. Phagolysosomal destabilization itself, rather than the absorbed particulate matter, seems to be perceived as the danger signal leading to NLRP3 activation (81, 89). Abnormal release of H^+ into the cytosol, either from lysosomal rupture or from the activation of a proton-selective ion channel, such as the M2 channel upon infection with Influenza virus (124), activates NLRP3. The lysosomal-derived protease cathepsin B is one of the lysosomal factors that activate NLRP3 (81, 89). However, this finding was dependent on a chemical cathepsin B inhibitor, while cathepsin $B^{-/-}$ macrophages do not show defects in caspase-1 activation (76), suggesting off target effects of this inhibitor (125).

A recent study aimed to provide an explanation for these diverse NLRP3-activating mechanisms, by essentially demonstrating that all tested NLRP3-activating stimuli act through promoting K^+ efflux and subsequent Na^+ influx, and that K^+-free medium alone is sufficient to activate NLRP3 in the absence of any other agonist (102). This study further suggested that neither mitochondrial perturbation nor the generation of ROS directly contributes to NLRP3 activation (102).

Special considerations for NLRP3 inflammasome activation and alternative upstream pathways

Several co-factors have been proposed to affect NLRP3 activation in response to all or select stimuli, which, however, in some cases are not as well-established. According to the universal NLR model, NLRP3 likely exists in an inactive, auto-inhibited conformation, which is aided by the interaction with the ubiquitin ligase SGT1 and the heat shock chaperon HSP90 (126). This is in agreement with the above described finding that deubiquitination of NLRP3 is essential for its activation (98–100). Yet another mechanism to maintain an inactive conformation or to prevent oligomerization, has been proposed to be interaction with cAMP via its NACHT. Ca^{2+} signaling through CASR during NLRP3 activation then causes depletion of intracellular cAMP levels and promotes NLRP3 activation (106). Yet another player regulating NLRP3 inflammasome activation, is the double-stranded RNA-dependent protein kinase (PKR), which phosphorylates NLRP3, but also interacts with other NLRs and ALRs (127). Once activated, oligomerization via its NACHT domain also requires ATPase activity and ATP hydrolysis (24). NLRP3 oligomerization is necessary for ASC clustering, which, however, in response to non-crystalline stimuli, may require PYD-mediated interaction with tetrameric guanylate binding protein 5 (GBP5) to facilitate oligomerization (128). Activation of NLRP3 is also inhibited by anti-inflammatory ω-3 polyunsaturated fatty acids through binding of the downstream scaffold β-arrestin-2, as also shown for NLRP1 (56). Furthermore, LRRFIP2 inhibits NLRP3 inflammasome activation by recruiting the pseudo caspase-1 substrate Flightless-I through NACHT-LRR interaction (129).

Although, $NLRP3^{-/-}$ and $ASC^{-/-}$ mice are less sensitive to LPS-induced shock, this only occurs at lower LPS doses and only provides partial protection (64, 130, 131). Contrary, $caspase\text{-}11^{-/-}$ mice are fully protected from LPS-induced shock (132). In response to selective Gram-negative *Escherichia coli*, *Citrobacter rodentium*, *Salmonella typhimurium*, or *V. cholera*, or upon cytosolic delivery of LPS, caspase-11 is required for full activation of caspase-1 within the NLRP3 inflammasome, which is referred to as the non-canonical inflammasome pathway (132–136). In the presence of NLRP3, ASC and caspase-1, caspase-11 favors secretion of the pro-inflammatory cytokines IL-1β and IL-18. However, in their absence, caspase-11 drives pyroptosis, IL-1α, and HMGB1 secretion. In particular, caspase-11 activation upon infection by *Salmonella* renders cells more susceptible to pyroptosis, which is even detrimental to the host in the absence of caspase-1 (136). Similar to NLRP3, a priming step is necessary to up-regulate caspase-11 transcripts. A TRIF-type I IFN-dependent transcriptional response has been initially proposed (135, 136). However, subsequent studies disputed a TRIF-specific mechanism, but nevertheless highlighted the necessity for TLR-mediated priming to up-regulate caspase-11 (137, 138). However, the LPS sensor upstream of caspase-11, however, is still elusive.

NLRP4

The function of NLRP4 (also known as NALP4, PAN2, PYPAF4, RNH2, and CLR19.5) (**Figure 1A**) in innate immunity is still poorly understood. It has not been linked to inflammasome activation, but overexpression studies indicated that NLRP4 modulates NF-κB activation in response to pro-inflammatory cytokines, including TNFα and IL-1β (139). Recently, NLRP4 has been proposed to modulate type I IFN signaling and autophagy, based on gene silencing and overexpression (140, 141). In response to Group A *Streptococcus* (GAS) infection, NLRP4 inhibits the initiation of autophagy through interaction with beclin-1. Interestingly, all other tested NLRs, including NLRC4, NLRP3, and NLRP10 also

interacted with beclin-1, potentially indicating this is a more common mechanism of NLRs (140). NLRP4 further interacted with the class C vacuolar protein-sorting complex to inhibit phagolysosomal maturation (140), suggesting that NLRP4 and possibly other NLR family members play a role in autophagosome maturation following bacterial infection. Yet, during viral infection, NLRP4 has been proposed to play a regulatory role within the type 1 IFN pathway in response to dsDNA and dsRNA (141). NLRP4 targets the central type IFN signaling component TBK1 for K48-linked polyubiquitination and degradation, through recruiting the E3 ubiquitin ligase DTX4 to TBK1, resulting in loss of IRF-3 activity. Only the NACHT of NLRP4 is required for this activity. While humans encode only NLRP4, mice encode seven paralog genes, NLRP4a-g, with at least NLRP4b and NLRP4g also inhibiting type I IFN production (141).

NLRP6

Initial overexpression studies suggested that NLRP6 (also known as NALP6, PAN3, PYPAF5, CLR11.4) (**Figure 1A**) mediates activation of NF-κB and caspase-1 in the presence of ASC (57). A subsequent study hinted at a function of NLRP6 within the intestinal epithelium, based on transcriptional profiling (142), and it is now evident that NLRP6 might function differently in myeloid cells and in intestinal epithelial cells. Three recent studies in NLRP6-deficient mice confirmed a role for NLRP6 in the regulation of intestinal host-microbiota (**Figure 5**) (143–145). NLRP6-deficient mice develop an increased sensitivity to DSS-induced colitis and colitis-induced tumorigenesis, suggesting a protective role of NLRP6 against intestinal inflammation and inflammation-induced cancer (143, 145). Although, it was previously suggested that NLRP6 is mostly expressed in the non-hematopoietic compartment, bone marrow chimera demonstrated the requirement of hematopoietic cells for this function (143). These studies further elute to a function of NLRP6 in intestinal epithelium self-renewal during steady state and during repair after inflammation through suppressing inflammation and associated colorectal carcinogenesis (143, 145, 146). NLRP6 is essential in regulating the interplay between host and gut microflora. Mice deficient in the NLRP6, or a potential NLRP6 inflammasome, although the latter is only based on overexpression data, develop a transferable colitogenic intestinal microbiota due to failure to produce IL-18, a necessary cytokine for the restriction of *Prevotellaceae* and *TM7* species in the steady state and upon DSS treatment through induction of CCL5 and IL-6 (144, 146). These results support the idea that NLRP6-driven IL-18 production from the epithelium is the major contributor to prevent the development of the colitogenic phenotype, as opposed to IL-18 secreted from the hematopoietic compartment. IL-18 is at least partially responsible for the down-regulation of IL-22BP during inflammation, allowing IL-22 to improve epithelial cell repair, while IL-22BP increases again at the end of regeneration with the decrease of IL-18 (147). In addition to restricting colitogenic microbiota species, NLRP6 also functions downstream of TLR signaling to dampen anti-microbial host defense. Rather than contributing to elimination of infections, NLRP6 has a deleterious role within the hematopoietic and the non-hematopoietic compartments and, accordingly, NLRP6$^{-/-}$ mice show increased resistance to infection by extracellular *E. coli*, intracellular *L. monocytogenes* and *S. typhimurium*, and display increased circulatory monocytes and neutrophils upon infection (148). Mechanistically, NLRP6 acts as an inhibitor of MAPK and the canonical NF-κB

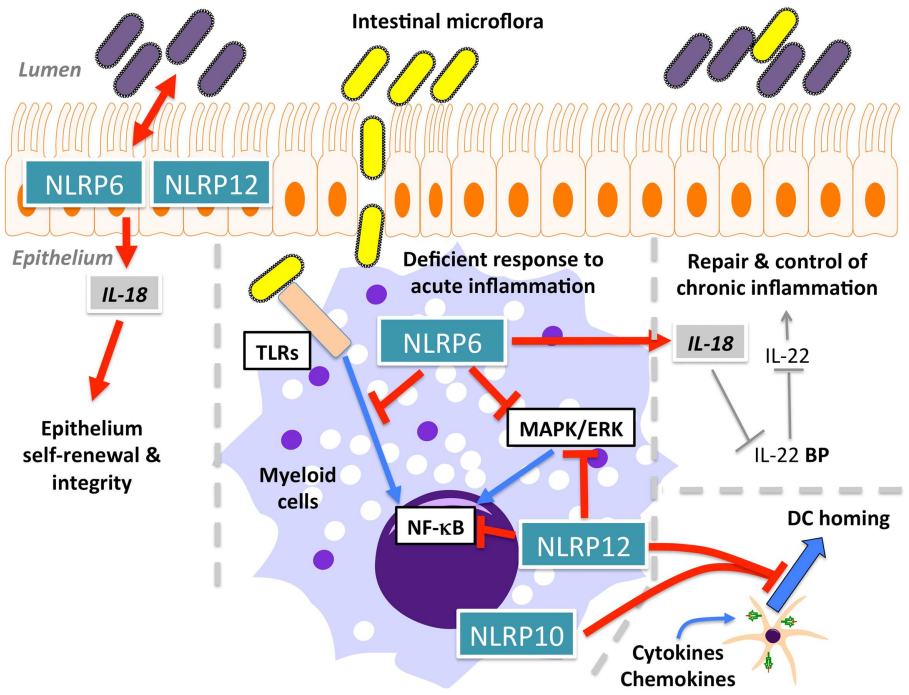

FIGURE 5 | Function of NLRP6, NLRP10, and NLRP12 in intestinal homeostasis and dendritic cell (DC) homing.

pathway activated by TLR, but not NLR ligation (148). A potential explanation could be that the full extent of the immune response is required to defend against systemic infection, whereas a more controlled immune response might be required in the case of localized inflammation in the gut. Thus, NLRP6 may play a regulatory role in both scenarios by providing protection against chronic inflammation, but consequently being deleterious during acute infections.

NLRP7

NLRP7 (also known as NALP7, PAN7, PYPAF3, NOD12, CLR19.4, HYDM) (**Figure 1A**) is one of four NLRPs, which exist in humans, but not in mice. Although, earlier overexpression studies NLRP7 failed to observe effects on NF-κB and caspase-1 activation (57), several studies since then reported modulation of these pathways by NLRP7. However, conflicting reports describe NLRP7 as either an activator or inhibitor of caspase-1 (**Figure 6**). NLRP7 has been proposed to directly interact with pro-caspase-1 and pro-IL-1β, without affecting NF-κB (149). It was also proposed that NLRP7 affects secretion of IL-1β and TNFα in PBMCs isolated from patients harboring NLRP7 mutations, which affected its localization to the microtubule-organizing center and the Golgi apparatus, and was associated with a down-regulation of intracellular pro- and mature IL-1β (150). NLRP7 also interacts with FAF-1, which also interacts with several other NLRPs and promotes apoptosis and inhibits NF-κB activation (151). However, modulation of NF-κB was not observed following NLRP7 over expression nor on endogenous level following NLRP7 silencing (57, 71). Overall, there are several potential mechanisms by which NLRP7 could negatively regulate release of inflammatory cytokines (152). In contrast, there is also evidence for a pro-inflammatory role of NLRP7 through the formation of an ASC-containing inflammasome that is triggered in response to bacterial acylated lipoproteins (71). NLRP7 collaborates with NLRP3 and TLR2 in restricting intracellular growth of *S. aureus* and *L. monocytogenes* in human macrophages (71). Similar to NLRP3, NLRP7 also functions downstream of lysosomal damage, with the difference that NLRP7 appears to be only partially sensitive to K^+ efflux (71). Thus, NLRP7 might contribute to pro- as well as anti-inflammatory processes (152).

NLRP10

NLRP10 (also known as NALP10, PAN5, NOD8, PYNOD, CLR11.1) (**Figure 1A**) is the other structurally atypical NLRP besides NLRP1, since it lacks the typical C-terminal LRR. The LRR is essential for NLRP3 activation in response to specific agonists, such as MSU (19), while deletion of the LRR reliefs autoinhibition and renders the NLR active in several *in vitro* studies. Thus, one may predict that NLRP10 might not respond in a stimuli-dependent manner. Over expression studies proposed that NLRP10 oligomerizes with ASC and inhibits ASC-mediated NF-κB activation and apoptosis, as well as caspase-1-dependent IL-1β release (153). Direct caspase-1 inhibition only requires the NACHT domain of NLRP10, but inhibiting ASC-mediated apoptosis, NF-κB and caspase-1 activation required the PYD (154). In contrast to human NLRP10, mouse NLRP10 failed to reduce self-aggregation of ASC, which is required for inflammasome activation. However, transgenic mice ubiquitously expressing high levels of mouse NLRP10 recapitulated the inhibitory effects observed *in vitro*, and mice were more resistant to endotoxic shock *in vivo*

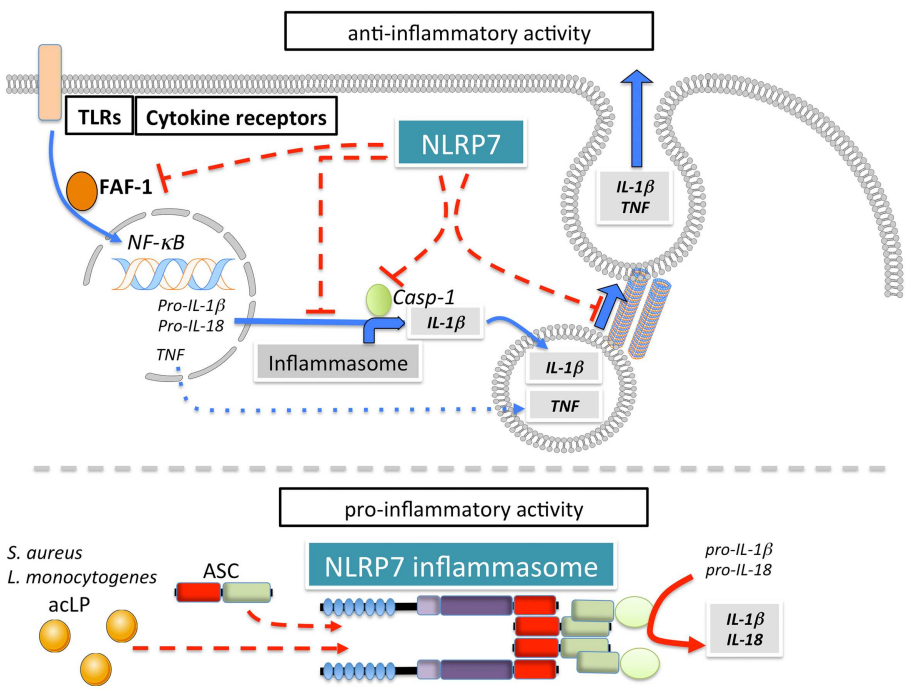

FIGURE 6 | Pro- and anti-inflammatory mechanisms of NLRP7.

(154). In contrast, NLRP10 contributes to host defense to *Shigella flexneri* in epithelial cells and fibroblasts by promoting secretion of IL-6 and IL-8 through induction of NF-κB and p38 signaling pathways, without affecting IL-18 release, arguing against an inflammasome role by gene silencing. This response required the ATPase activity and the PYD of NLRP10 (155). Mechanistically, NLRP10 interacts with signaling components of the Nodosome, including NOD1, RIP2, TAK, and IKKγ in response to *S. flexneri* infection (155). However, NLRP10$^{-/-}$ mice revealed a pronounced defect in mounting adaptive immune responses in the Th1/Th17-dependent experimental autoimmune encephalomyelitis (EAE) mouse model and Th2-dependent OVA- and Alum-driven asthma models (156). These defective Th cell responses were caused by a defective emigration of activated DCs from sites of inflammation to draining lymph nodes, loss of antigen transport, and subsequent priming of CD4^{+} T-cell, though their activation profile remained unaffected (**Figure 5**). Similar results were obtained in a *C. albicans* dissemination model, in which NLRP10$^{-/-}$ mice displayed increased susceptibility marked by defective Th1 and Th17 responses (157). In both studies, NLRP10$^{-/-}$ macrophages and DCs did not reveal any impact on inflammasome-dependent pathways, and thus above described observations might be caused from overexpression (156, 157). Although hereditary mutations in NLRP3, found in CAPS patients, have been shown to affect Th17 polarization in mice (158, 159), and since CAPS itself is a purely innate immune-driven disease, this is thus the first demonstration of a profound effect of an NLRP on adaptive immunity.

NLRP12

NLRP12 (also known as NALP12, PYPAF7, RNO2, PAN6, Monarch-1, CLR19.3) (**Figure 1A**) associates with ASC to form an inflammasome and to promote NF-κB activation, when overexpressed (160). It also enhances expression of the non-classical and classical MHC Class I genes (161). However, NLRP12 also antagonizes signals originating from TLRs and TNF receptor superfamily members upstream of IκBα within the canonical NF-κB signaling pathway by binding to IRAK-1 via its NACHT domain (162) and the non-canonical NF-κB signaling pathway by binding to NIK and inducing its proteasomal degradation (163). Like several other NLRPs, also NLRP12 is an ATPase, and ATP binding/hydrolysis is critical for its function (27). Similar to NLRP3, the interaction of NLRP12 with HSP90 is also important for its stability (164). NLRP12$^{-/-}$ mice recently revealed immune defects. NLRP12 is predominately expressed in DCs and neutrophils, and mice lacking NLRP12 display less severe inflammation in models of contact hypersensitivity (165). However, in contrast to *in vitro* studies, this effect was independent of inflammasome activation and antigen presentation and did not alter inflammatory cytokine levels (165). Similar to NLRP10$^{-/-}$ mice, NLRP12$^{-/-}$ mice also display defects in the migration of peripheral DCs and neutrophils to draining lymph nodes due to an impaired chemokine response (**Figure 5**) (165). In agreement with *in vitro* data showing that NLRP12 antagonizes NF-κB signaling pathways, NLRP12$^{-/-}$ mice were more susceptible to intestinal inflammation, colitis and the associated colorectal tumorigenesis, due to a failure to resolve pro-inflammatory non-canonical NF-κB, ERK, and AKT signaling, which resulted in elevated levels of pro-inflammatory cytokines and chemokines (**Figure 5**). Overall, this suggests an important role for NLRP12 in maintaining intestinal homeostasis (166, 167). Although these functions are uncoupled from inflammasome activation, NLRP12 and NLRP3 inflammasomes do contribute to the host defense against *Yersinia pestis* through IL-18 and subsequent IFN-γ production. Surprisingly, NF-κB activation was not affected in this study (168). Thus, dependent on the context and cell type, NLRP12 either promotes or antagonizes immune and inflammatory responses, which has also been observed for several other NLRPs.

AIM2-LIKE RECEPTORS

The ALRs AIM2 and IFI16 belong to the PYHIN protein family, which is named after their domain architecture, typically consisting of an N-terminal PYD and one or two C-terminal hematopoietic IFN-inducible nuclear protein with 200-amino acids (HIN-200) domains (**Figure 1B**). The HIN-200 domain contains partially conserved repeats, which assemble into an oligonucleotide/oligosaccharide-binding fold (OB-fold), which facilitates DNA binding. The OB-fold is a common DNA binding motif, which allows numerous proteins to directly recognize and bind single- and double-stranded DNA (20, 169). While AIM2 preferentially binds dsDNA (170, 171), IFI16 can bind to ssDNA and dsDNA, but only duplex DNA and not the single-stranded form of a Vaccinia virus-derived oligonucleotide was able to induce an IFI16-dependent IFN-β response (35, 172). While only four human *PYHIN* genes exist, this gene cluster is amplified in mice and contains at least 13 predicted and diverse members with only AIM2 being conserved between man and mice (36–38). However, co-localization of several mouse PYHIN proteins with ASC and/or STING, suggests their involvement in inflammasome and/or type I IFN responses (36).

AIM2

AIM2 or PYHIN4 was initially identified in a human malignant melanoma cell line, where the absence of AIM2 caused increased cell growth and has subsequently been mostly studied in the context of cancer (173). However, a connection between AIM2 and innate immune responses was made when AIM2 was found to recruit ASC to form an inflammasome (**Figure 7**) (170, 171, 174, 175). The DNA binding capability of the OB-fold within the HIN-200 domain of AIM2 (20) was confirmed to recognize synthetic cytoplasmic dsDNA as well as dsDNA from various pathogens including *Francisella tularensis* (174, 176–178), *L. monocytogenes* (178), Vaccinia virus (170, 174, 178), and MCMV (178), but not DNA from herpes simplex virus type I (HSV-1) and *S. typhimurium* (178). Reminiscent to NLRs, AIM2 activation relies on promoting clustering of ASC and consequently, also caspase-1, but in the case of AIM2, requires the presence of dsDNA (174). Structural analyses proposed that simultaneous binding of multiple AIM2 molecules through electrostatic interaction to the sugar-phosphate backbone of the DNA helix may facilitate the assembly of AIM2 inflammasomes along the DNA staircase (21). *In vivo* experiments also confirmed the importance of AIM2 in host defense, since AIM2$^{-/-}$ mice are unable to limit *F. tularensis* replication, similar to caspase-1$^{-/-}$ mice, and thereby failed to control *F. tularensis* infections (176, 177). AIM2 was also crucial

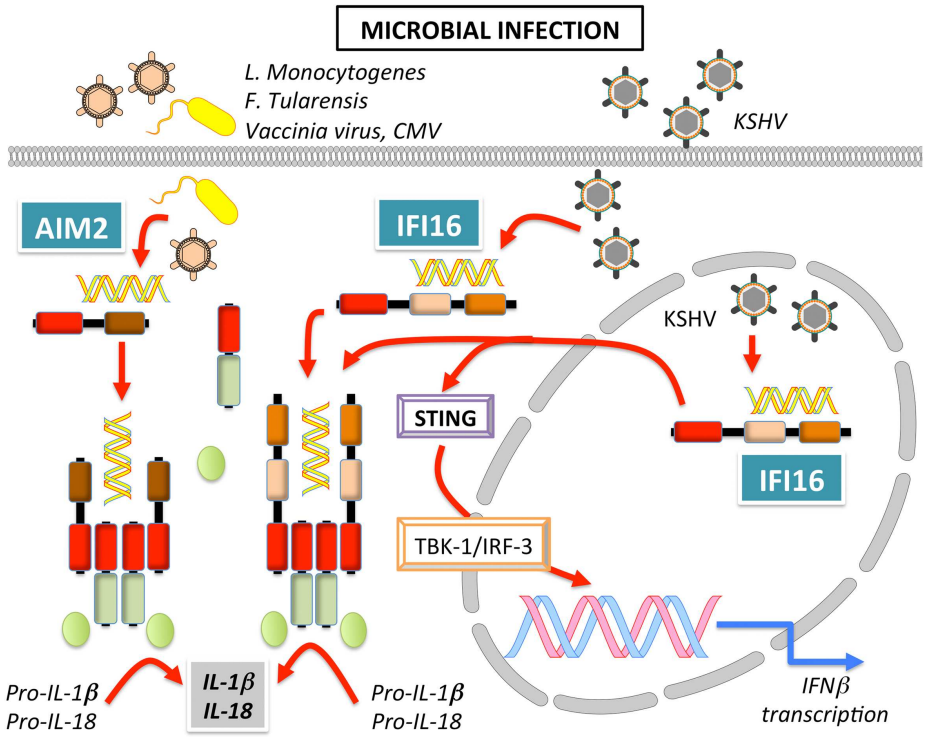

FIGURE 7 | Signaling of AIM2 and IFI16 leading to inflammasome activation and induction of IFNβ in response to bacterial and viral infection.

for innate immune responses to MCMV *in vivo*, since the serum levels of IL-18 and the linked production of IFN-γ by NK cells was significantly reduced in the absence of AIM2, which, however, caused an increased splenic virus titer (178). Interestingly, even though cytosolic DNA and some cytosolic bacteria such as *F. tularensis* and *L. monocytogenes* induce an IFN-β response and AIM2 expression is induced by type I IFN, IFN-β signaling is still intact in AIM2$^{-/-}$ macrophages, where it is even enhanced (176, 177, 179, 180). Moreover, type I IFN priming is essential for AIM2-dependent activation of caspase-1, inflammasome-mediated cell death and the release of IL-1β and IL-18 (176, 179). The HIN-200 protein, p202, negatively regulates AIM2 through competition for DNA binding in mice, but due to lacking a PYD, it cannot form an inflammasome (152), but since this protein does not exist in human, alternative regulatory mechanisms may exist. The antimicrobial cathelicidin peptide LL-37 can compete with AIM2 for DNA binding in psoriatic lesions (181).

IFI16

IFI16 or PYHIN2 was the first human IFN-inducible PYHIN protein identified in myeloid cells (182). Of the three IFI16 isoforms (A, B, and C), the B form is most abundantly expressed (183). IFI16 is also able to bind and recognize DNA to promote transcriptional regulation of genes involved in innate immunity, including type I IFN. Cytosolic DNA recognition promotes recruitment of STING to IFI16 and subsequent NF-κB and TBK-1-dependent IRF-3 activation (**Figure 7**) (21, 35). Besides this transcriptional response, IFI16 also recruits ASC to form an inflammasome upon recognition of latent viral DNA in the nucleus (34, 172), as well as in the cytoplasm (35, 184) (**Figure 7**). Curiously, in the steady state, IFI16 localizes mostly to the nucleus, but IFI16 is able to efficiently launch an immune response in the presence of both, nuclear and cytoplasmic DNA. The subcellular localization of IFI16 might determine its function as an IFN-β inducer in the cytoplasm, or an inflammasome-activating PRR in the nucleus. Thus, the immune response following DNA exposure may depend on the cellular or tissue micro-environment, since the function of IFI16 can shift from a transcriptional activator leading to IFN expression to a PRR that causes caspase-1 dependent IL-1β and IL-18 processing in inflammasomes (185). Moreover, one could predict the existence of a regulatory mechanism that restrains IFI16 and AIM2 inflammasome activation in the cytosol upon contact with self-DNA during cell division, since during this process nucleic acids are temporarily exposed to the cytoplasm.

NLRPs IN INFLAMMATORY, IMMUNE, AND METABOLIC DISEASES

As discussed above, PYD-containing PRRs play central roles in key innate immune pathways and are necessary for host defense against a wide range of pathogens and to initiate wound healing of damaged tissue following sterile insults. However, there is now compelling evidence that dysregulated activation of these PRRs, leading to either excessive or impaired activation, causes or contributes to immune-linked diseases. Below we briefly summarize the contribution of NLRPs to auto-inflammatory-, autoimmune-, and metabolic diseases.

AUTO-INFLAMMATORY DISEASES

Auto-inflammatory diseases are generally characterized by recurrent episodes of inflammation and fever in spite of lack of an apparent stimulus and involvement of autoantibodies and autoreactive T cells, causing widespread systemic inflammation which affects multiple tissues and organs (186).

NLRP3

Initially a genetic linkage between hereditary point mutations in NLRP3 and auto-inflammatory conditions, now referred to as Cryopyrinopathies or CAPS, was discovered (51). These mutations are gain of function mutations, mostly localizing to the NACHT domain, which create a constitutive active NLRP3 (164). Mutations prevent the inactive conformation of NLRP3 and promote activation in the absence of any specific agonist. Knock-in of CAPS mutations into mice revealed that the disease symptoms are caused primarily by excessive production of IL-1β, but also by pyroptosis in myeloid cells. However, due to IL-1β signaling, mice also show hyperactive Th17 responses (158, 159, 187, 188). Since IL-1β also drives Th17 differentiation in humans (189, 190) it was not surprising that CAPS patients also display significantly increased IL-17 serum levels as well as a higher frequency of Th17 compared to control subjects (191, 192).

Although not driven by hereditary mutations, endogenous crystalline danger signals similarly promote chronic and excessive inflammasome activation and cause crystalline arthropathies and related disorders. Calcium pyrophosphate, monosodium urate, and hydroxyapatite crystal depositions promote NLRP3 activation, excessive inflammation and eventually cause pseudogout, gout, and osteoarthritis (65, 90, 193). Hence, novel treatment regiments with IL-1β blockers have been proven effective (194). NLRP3 is similarly activated following phagocytosis of several other particulate matters. Silica and asbestos fibers activate NLRP3 and result in a non-resolving IL-1β-mediated inflammation, leading to lung fibrosis and ultimately to organ dysfunction in silicosis and asbestosis (84, 85). Cholesterol crystals are also sensed by NLRP3, which contributes to chronic vascular inflammation and ultimately the development of atherosclerosis (195). Similarly, amyloid-β fibrils and islet amyloid polypeptide (IAPP) activate NLRP3, which contributes to Alzheimer's disease and the progression of type 2 diabetes, respectively (88, 89). Even hemozoin crystals, which are generated during *Plasmodium* infection of red blood cells, trigger NLRP3 activation (76–78), although experimental cerebral malaria progresses independently of NLRP3 (196).

NLRP12

In addition to NLRP3, hereditary mutations in NLRP12 have also been linked to auto-inflammatory disease. Guadeloupe fever is clinically similar to CAPS, but is caused by NLRP12 mutations, which truncate the NACHT or delete the LRR (168). However, in contrast to CAPS, anti-IL-1β therapy provided only temporary clinical improvements in two patients, followed by relapse and re-activation of IL-1β secretion, possibly due to enhanced TNFα levels, which were observed in response to the treatment and may have lead to hypersecretion of IL-1β, which circumvented anti-IL-1β therapy (169).

NLRP1

Excessive NLRP1-induced IL-1β signaling and pyroptosis can also lead to deleterious organ-specific inflammatory events, such as acute lung injury (53). Moreover, as discussed later, polymorphisms of NLRP1 have been linked to an increased risk developing a number of autoimmune diseases. Although their pathogenesis has not yet been linked to excessive NLRP1 inflammasome activation in humans, it is of interest that analysis of one of these polymorphisms, $NLRP1^{M1184V}$, showed increased NLRP1 autoproteolysis and, consequently, activation of caspase-1 and release of IL-1β (42). Furthermore, *N*-ethyl-*N*-nitrosourea (ENU) mutagenesis screening in mice revealed that $NLRP1a^{Q593P}$, an activating mutation located within the linker connecting the NACHT and LRR, causes lethal systemic neutrophilia, thus linking NLRP1 mutations to hyper-inflammation (197). $NLRP1a^{Q593P}$-driven disease was dependent on IL-1β and caspase-1, but did not require ASC and caspase-11. Moreover, similar to hyperactive NLRP3 mutations, LPS priming of macrophages was sufficient for maturation of IL-1β in $NLRP1a^{Q593P}$ mutant macrophages (197). Interestingly, while the elevated IL-18 release due to $NLRP1a^{Q593P}$ mutation ameliorated the disease, $NLRP1a^{Q593P}IL18^{-/-}$ mice displayed increased neutrophilia, independently of IFN-γ, and an accelerated disease onset. IL-18 has emerged as a major intermediate in the crosstalk between the host and commensal microbiota. In this case, the onset and severity of $NLRP1a^{Q593P}$-driven disease was independent, although aggravated, by the presence of commensal microbiota. $NLRP1a^{Q593P}$ specifically caused cell intrinsic hematopoietic stem and progenitor cell defects and particularly manifested in reduced macrophage- and granulocyte-macrophage progenitor cell numbers, caused by pyroptosis, which is only evident in $Il1r^{-/-}$ mice in the absence of IL-1β-driven inflammation, and is exaggerated by hematopoietic stress (197). Thus, there is evidence that hereditary mutations in NLRP1 may also lead to excessive inflammasome activation, which is much better understood for NLRP3, as discussed below.

AUTOIMMUNE DISEASES

Although inflammasome activation is closely linked to innate immune responses, there is now increasing evidence for a role of inflammasomes in adaptive immunity. Although, IL-1β and IL-18 are prototypical cytokines produced by innate immune cells, both are also important for maintaining the Th1-Th17 vs. Th2 balance. Thus, inflammasomes play a role in initiating inflammatory events, but also in the perpetuation of autoimmune diseases characterized by a defect in the T-cell balance.

NLRP1

Strong evidence supports an etiologic role of NLRP1 in various autoimmune diseases, since NLRP1 variants have been associated with an increased susceptibility for Addison's disease, type 1 diabetes, Alzheimer's disease, celiac disease, Kawasaki disease, autoimmune thyroid disease, generalized vitiligo, systemic sclerosis, and rheumatoid arthritis (198–204). Little is known regarding the mechanism by which NLRP1 mutations affect autoimmunity. However, in generalized vitiligo high-risk NLRP1 haplotypes display elevated IL-1β processing (203), and in rheumatoid arthritis patients, NLRP1 transcripts are elevated (198). Similarly, fibrotic

patients display elevated IL-1β levels (205, 206), and systemic sclerosis patients produce considerably higher amounts of extracellular matrix upon exposure to IL-1β (207, 208). This is significant, since caspase-1 is necessary for unconventional protein secretion of numerous leaderless proteins in keratinocytes, which includes several proteins linked to fibrosis in response to UVB irradiation (8).

NLRP3

The most direct link of NLRP3 activation to adaptive immunity came from studies with mice harboring CAPS mutations, clearly providing evidence for an abnormal Th1/Th17-skewed immune response (158, 159, 209). Mice displayed spontaneous skin inflammation, consistent with a Th17-skewed response, and produced elevated levels of the Th17-related cytokines IL-17A, IL-21, and IL-22 and the Th17-specific transcription factor RORγt. This is in agreement with an activated phenotype driven by excessive IL-1β levels. In multiple sclerosis (MS), the prototypical Th1- and Th17-derived cytokines, IFNγ, and IL-17, respectively, play an important role. But this concept has been challenged recently by the discovery that only T helper-derived GM-CSF, and neither IFNγ nor IL-17, was essential during the effector phase of EAE, the animal model for MS (210, 211). NLRP3 inflammasome-derived IL-1β is essential for the production of GM-CSF (212), and accordingly, NLRP3 is involved in the pathogenesis of EAE and NLRP3-deficient mice show a strongly ameliorated pathogenesis (213, 214). Nevertheless, this finding is still controversial (215). Also the contribution of NLRP3 to allergic airway disease is still controversial. While some studies observed significantly attenuated airway inflammation, IgE production, and cytokine release in response to OVA in Nlrp3$^{-/-}$ mice (79, 216), others failed to observe any major contribution of NLRP3 (217, 218). Yet another link to adaptive immunity comes from the observation that NLRP3 mediates responses to aluminum hydroxide-containing particular adjuvant formulations (79, 82, 83). However, the precise contribution of NLRP3 to this adaptive immune response is still controversial (80).

NLRP10/NLRP12

NLRP10$^{-/-}$ and NLRP12$^{-/-}$ mice both show impaired DC migration to draining lymph nodes, which is independent of inflammasome activation. NLRP12 has been linked to atopic dermatitis and hereditary periodic fever in humans. Hence, NLRP12-deficient mice exhibited attenuated inflammatory responses in mouse models of contact hypersensitivity, which was attributed to a reduced capacity of DC and neutrophil migration and their inability to respond to chemokines *in vitro* (165). Similarly, NLRP10$^{-/-}$ mice displayed a profound impairment in T-cell-mediated immune responses due to the loss of antigen transport to the draining lymph nodes. The defective emigration of DCs from inflamed tissues lead to an almost complete loss of naive CD4^{+} T-cell priming. Hence, there is a critical link between innate immune stimulation, NLRP10 activity, and the immune function of mature DCs (156).

AIM2-like receptor

Evidence supports a role of AIM2, IFI16, and the regulatory p202 proteins (p202a and p202b) in the pathogenesis of Sjogren's syndrome and systemic lupus erythematosus (SLE) (219). In particular, p202 proteins have been linked to increased susceptibility for murine SLE and are regulated by AIM2 (220, 221). However, p202 genes are lacking from human. On the other hand, SLE and Sjogren's syndrome patients develop autoantibodies to IFI16 in 29 and 70% of all cases, respectively (222, 223), implying a causative link, which is significant due to the reported AIM2 inhibition by IFI16 (224). The most direct evidence shows a contribution of AIM2 to the pathogenesis of Lupus nephritis in an apoptotic lymphocyte DNA-induced SLE model (225). Nevertheless, mechanistic studies implicating ALRs in the pathogenesis of autoimmune disorders are still lacking.

METABOLIC DISEASES

NLRP3

Chronic low-grade metabolic inflammation (metaflammation) is an underlying cause for metabolic diseases. In obesity an excess of nutrients triggers inflammation, since the metabolic surplus induces the expression of inflammatory cytokines, including IL-1β. Hence, there are numerous obesity-related diseases, which include cardiovascular disease, atherosclerosis, insulin resistance, and type 2 diabetes mellitus (T2DM), which are linked to the NLRP3 inflammasome. The NLRP3 inflammasome can be triggered by oligomers of IAPP, which commonly form amyloid deposits in the pancreas during T2DM. In response to IAPP, inflammasome priming, which causes the transcriptional upregulation of IL-1β, requires a sufficient glucose metabolism and can be facilitated by minimally oxidized low-density lipoprotein (88). Subsequently, IL-1β causes apoptosis of insulin producing β-cells, which results in reduced insulin secretion over time and eventually leads to insulin resistance and T2DM (226, 227). Weight loss in obese individuals with T2DM correlates with reduced NLRP3 expression in adipose tissue. In addition, there is decreased inflammation and improved insulin sensitivity and glucose tolerance in adipose tissue macrophages (ATM) (228). Evidently, the lipotoxicity-associated increase of the intracellular saturated fatty acid palmitate and the metabolite ceramide, are also sensed by NLRP3, in particular following a high fat diet (93, 228). Accordingly, NLRP3$^{-/-}$ mice also show reduced hepatic steatosis and are protected against the accumulation of lipid deposits in the liver (228). Thus, NLRP3 is centrally involved in metabolic health. However, NLRP3, in concert with NLRP6, is also necessary for maintaining a healthy intestinal microbiota to prevent abnormal accumulation of bacterial PAMPs in the hepatic portal circulation.

NLRP6

Increasing evidence supports a profound impact of the intestinal microbiota to metabolic health and the intestinal microbiota of obese individuals differs from that of lean people and shows increased prevalence of *Prevotellaceae* (229). NLRP3 and NLRP6 are required for inflammasome-mediated surveillance of the gastrointestinal tract to prevent the spreading of colitogenic microbiota species, including *Prevotellaceae* and *TM7* (144, 230). Restricting these bacteria requires IL-18 and failure promotes CCL5-dependent colonic inflammation and increased TLR4 and TLR9 agonist influx into the portal vein, which eventually causes non-alcoholic fatty liver disease (NAFLD), a comorbidity associated with obesity, metabolic syndrome, and NASH progression (144, 230). Thus, NLRP3 and NLRP6 appear to have a specific

protective role within the gastrointestinal tract through production of IL-18, and accordingly, NLRP3$^{-/-}$ and NLRP6$^{-/-}$ mice are more susceptible to colon inflammation and colon cancer (142, 143, 145, 146).

NLRP12

Similar to NLRP6, NLRP12 dampens gastrointestinal inflammation and associated tumorigenesis, albeit through a distinct mechanism. Rather than through inflammasome-mediated IL-18 production, NLRP12 prevents intestinal inflammation through dampening NF-κB, ERK, and AKT activation and release of pro-inflammatory cytokines, chemokines, and tumorigenic factors from macrophages and intestinal epithelial cells (166, 167).

CONCLUSION

By now, the crucial role of PYD-containing PRRs in host defense is well-established. Although, these PRRs trigger many key innate immune pathways, their contribution to inflammasome activation is currently best understood. Nevertheless, it becomes increasingly recognized that not all PYD-containing PRRs assembly inflammasomes or even promote a pro-inflammatory response. However, the precise signaling mechanisms and in particular, the stimuli that trigger their activation, are largely elusive for most members. The tight affiliation of these PRRs with immune-based diseases further underscores their critical function in maintaining homeostasis, while at the same time opening up exciting avenues for developing novel therapies targeting these PRRs.

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Malarial pigment hemozoin and the innate inflammatory response

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Malaria is a deadly infectious disease caused by the intraerythrocytic protozoan parasite *Plasmodium*. The four species of *Plasmodium* known to affect humans all produce an inorganic crystal called hemozoin (HZ) during the heme detoxification process. HZ is released from the food vacuole into circulation during erythrocyte lysis, while the released parasites further infect additional naive red blood cells. Once in circulation, HZ is rapidly taken up by circulating monocytes and tissue macrophages, inducing the production of pro-inflammatory mediators, such as interleukin-1β (IL-1β). Over the last few years, it has been reported that HZ, similar to uric acid crystals, asbestos, and silica, is able to trigger IL-1β production via the activation of the NOD-like receptor containing pyrin domain 3 (NLRP3) inflammasome complex. Additionally, recent findings have shown that host factors, such as fibrinogen, have the ability to adhere to free HZ and modify its capacity to activate host immune cells. Although much has been discovered regarding NLRP3 inflammasome induction, the mechanism through which this intracellular multimolecular complex is activated remains unclear. In the present review, the most recent discoveries regarding the capacity of HZ to trigger this innate immune complex as well as the impact of HZ on several other inflammatory signaling pathways will be discussed.

Keywords: malaria, hemozoin, macrophages, signaling, inflammasome

INTRODUCTION

Malaria is an infection that affects 216 million individuals worldwide. Every year, approximately 700,000 people succumb to this devastating disease (1). The protozoan parasite *Plasmodium* is the etiological agent of malaria and it is transmitted during the blood meal of a female *Anopheles* mosquito (2). Of all the *Plasmodium* species infecting humans, *P. falciparum* is the most virulent and its pathology is characterized by severe anemia or the development of cerebral malaria, generally leading to death if left untreated (3). The *Plasmodium* life cycle within its mammalian host includes a non-pathological liver stage followed by red blood cell (RBC) invasion by merozoites, the infectious form of the parasite, which initiates the symptomatic intraerythrocytic cycle (4). Classical malaria paroxysms are characterized by periodic fevers and chills, which are synchronized with the release of merozoites from the infected RBC (iRBC) (5). Furthermore, in the case of *P. falciparum*, the sequestration and destruction of iRBC in the vasculature of lymphoid organs and the brain exacerbates this pathology (6). Disease severity was previously solely attributed to parasite virulence factors (5). However, recent studies have suggested that modulation of the immune system is involved in the development of pathology through the induction of a strong inflammatory response at the beginning of the acute phase, followed by a suppression of the host immune system at later time points (7).

MALARIA AND INFLAMMATION

Systemic hyperactivation in *P. falciparum*-infected patients is characterized by elevated levels of circulating nitrogen oxide reactive intermediates (8, 9) and by various cytokines, such as IFNα/γ (10–12), TNFα (5, 13–18), IL-1 (5), IL-6 (19), and the chemokine IL-8 (20). Furthermore, the levels of these cytokines and chemokines have been found to correlate with different manifestations of severe malaria (14, 16–19). Although the generation of these pro-inflammatory molecules favors reduction of the parasitic load, their exacerbated production seems to play a key role in the development of pathology. Both TNFα and IL-1β are considered to be important mediators of fever (3), and participate in the attachment of *P. falciparum*-iRBC to the vascular endothelium (21–23). And *in vivo* studies have demonstrated that the cytokines TNFα and IFNγ are essential for the development of cerebral malaria by inducing the expression of the adhesion molecule ICAM-1 and nitric oxide (NO) (24, 25). Finally, an *in vitro* study has shown that the induction of the pyrogenic molecules MIP-1α and MIP-1β by *Plasmodium* may play an important role in the initiation of fever (26).

During human or murine malaria, phagocytes [e.g., monocytes/macrophages (MØ), and to a lesser extent neutrophils (NØ)] have been demonstrated to play a crucial role in host defense by engulfing free parasites and *Plasmodium*-iRBC, and

by eliminating parasites through a respiratory burst-mediated mechanism (27, 28). During the early phase of the infection, the number of phagocytes increases and their activity intensifies (29–33). Moreover, since tissue and circulating MØ are the main source of cytokines during severe malaria (5), it seems that their contribution to the exacerbation of the inflammatory response is also important. For instance, *in vitro* studies have reported the production of several phagocyte-secreted molecules (e.g., IL-1, IL-6, IL-12, and TNFα) (3, 5, 14, 15, 34–40) by human and murine MØ upon contact with *Plasmodium*-iRBC or malarial antigens. Furthermore, the enhanced IFNγ production (19, 41), complement activation (42), and hypergammaglobulinemia observed during the course of acute malarial infection could stimulate cytokine release by MØ (43, 44). Additionally, the production of leukotrienes and reactive oxygen species (ROS) by phagocytes during infection seems to contribute to malaria pathogenesis (5).

HZ AND MALARIA

Although enhanced activation of the immune system has been reported during the early stage of the malarial infection, a markedly reduced or absent immune response is observed later during the acute phase of human and murine malaria (45, 46). The most well-studied mechanism explaining this phenomenon is the reduction of T cell proliferation and activity that occurs during malarial infection (47, 48). However, the reduction in T cell numbers is transient (49), and the restoration of their basal numbers does not restore their ability to specifically respond to malaria antigens (29), suggesting that other components of the immune system are also affected. Accordingly, various models of murine malaria have demonstrated that MØ functions (e.g., antigen presentation and microbicidal functions) (7, 10, 29, 50–54) are greatly altered during the course of infection, but the mechanisms involved in the functional modulation of MØ by *Plasmodium* are still incompletely understood. Several lines of evidence suggest that the parasite and its metabolites, principally hemozoin (HZ) and glycosylphosphatidylinositol (GPI), which are released into circulation during the intraerythrocytic cycle, could contribute to the activation and/or the suppression of the immune response (7, 52, 55, 56). The impact of HZ on host physiology and the host response has been a subject of increasingly intensive studies over the last 10 years, and already published data suggest that this metabolite could have an important role in malaria pathophysiology.

Hemozoin is a crystalline, brown pigment that is formed and sequestered in the digestive vacuole of *Plasmodium* as a product of hemoglobin (Hb) catabolism (57). The parasite digests up to 80% of the Hb in the host RBC, which it utilizes as an essential source of nutrients and energy (2). However, this process generates heme, which is highly toxic to the parasites. Since the parasite is unable to excrete the free heme and does not possess a heme oxygenase to recover the iron and detoxify the heme, it aggregates the heme into an insoluble crystal, HZ (58, 59). It was initially thought that this reaction was conducted by a heme polymerase (60). Some proteins have been proposed as candidates (61), but HZ formation does not require the use of a protein or a lipid (62–65), thus this aspect of HZ metabolism remains controversial (61).

In vivo, HZ crystals are remarkably uniform in size and shape; however, only certain synthetic protocols allow for the isolation of synthetic crystals with this morphology (58, 66, 67), with many synthetic protocols yielding material that is poorly crystalline (58, 68). HZ is composed of hematin molecules bonded by reciprocal iron–carboxylate linkages to form dimers, which are further connected via hydrogen bonds to form a triclinic crystal (69–71). Although HZ is remarkably thermally stable and insoluble, it has one of the highest concentrations of pro-oxidant sites of any condensed biomaterial, and therefore it may be the source of slowly released oxidation catalysts or the site of surface generated oxidation catalysts. Electron diffraction has been used to index the faces of HZ to determine the specific structures on each surface. The smallest, fastest growing faces are dominated by free propionic acid groups, while the larger faces of the crystal correspond to the hydrophobic flat porphyrin plane of the hematin dimers. Thus, these two pairs of faces on the HZ prisms contrast markedly in nature, with the former being very polar and hydrophilic, and the latter being hydrophobic and lipophilic.

In the past, HZ was only considered to be a metabolic waste, i.e., merely the byproduct of heme detoxification by the parasite (56). However, this molecule has been shown to sequester in various organs (e.g., liver, spleen, and brain), to be actively engulfed by phagocytes, and to modulate MØ functions, indicating that HZ can potentially contribute to the development of malaria immunopathogenesis (2, 26, 72–80). Following the rupture of *Plasmodium*-iRBC, HZ is released from the parasite digestive vacuole and is rapidly engulfed by phagocytes (29, 33, 56). In human and murine malaria, a large number of circulating phagocytes are loaded with HZ, as are phagocytes in the brain and lymphoid organs, such as the spleen (26, 29, 30, 56, 58, 81), where its presence seems to correlate with the severity of the disease. Although HZ is generated during malarial infection caused by all *Plasmodium* species, including the species-infecting mice (e.g., *P. chabaudi* and *P. berghei*), the amount released during severe or cerebral malarial infection due to *P. falciparum*, can be markedly more abundant and localized than compared to mild cases of malaria observed in individuals infected with *P. malariae*, *P. ovale*, or *P. vivax* (82).

HZ, IMMUNE CELLS, AND INFLAMMATION

Hemozoin accumulation occurs during erythrocyte rupture: merozoites, along with HZ, free heme, and other contents of the cytoplasm and digestive vacuole of the parasite are released. Many immune cells such as monocytes, macrophages, neutrophils, endothelial cells, and dendritic cells are able to interact with and internalize HZ and iRBC. Among these, the most well-characterized HZ-internalizing cells are the monocytes and macrophages. It has been well documented that human monocytes rapidly engulf HZ, which can fill up to 30% of their total cell volume. Furthermore, the consumed HZ can persist unmodified within the monocytes for long periods of time (83).

Accumulation of HZ in the phagocytic cells of the immune system is used in the diagnosis and prognosis of malaria. In pioneering studies, Laveran described the presence of the pigment granules not only in the iRBC, but also in phagocytes; in some cases, HZ could be detected in RBC that did not contain parasites (84). High levels of HZ within monocytes and neutrophils have been shown to correlate with disease severity. It was observed that adult patients who succumbed to *P. falciparum*

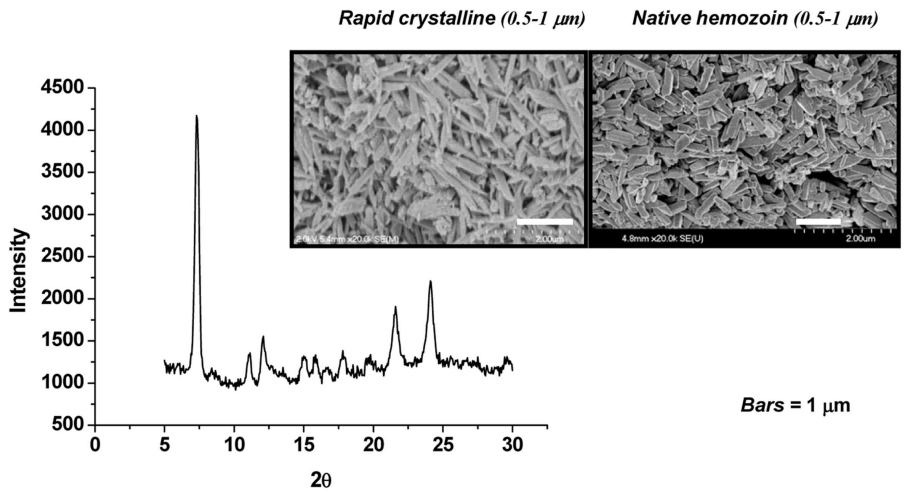

FIGURE 1 | Synthetic hemozoin analysis. Scanning electron micrographs of rapid crystalline HZ (rcHZ) and *Plasmodium falciparum*-native HZ. X-ray powder diffraction (XRD) analysis allows the determination of the quality of the crystal. Taken from Ref. (86).

infections presented with a higher proportion of HZ-containing neutrophils or monocytes than surviving patients (75). Additionally, it has been shown that children with cerebral malaria have more HZ-containing neutrophils than mildly infected or asymptomatic children (82). Furthermore, patients with severe malaria have iRBC and HZ-laden monocytes in their brain capillaries (85). The same profile of HZ accumulation within the organs has also been observed in the murine model of cerebral malaria (80). The presence of HZ in these immune cells corresponds with its immune modulatory activity.

The role of HZ in the modulation of host innate and inflammatory responses has been investigated by many researchers, using different HZ preparation protocols. HZ can be synthesized from hematin (sHZ) or native hemozoin (nHZ) can be purified from iRBC in culture (**Figure 1**). nHZ needs to be further treated to remove any proteins, lipids, and other materials from disrupted parasite that could have adhered to the highly amphiphilic molecule, in order to obtain a pure product. These HZ preparations have been used to gain a greater and more thorough understanding of the role of HZ in malarial pathology. Although sHZ and nHZ crystals of similar sizes are capable of inducing the same level of inflammatory mediators, sHZ with a smaller or larger crystal size will differently affect the production of pro-inflammatory cytokines. This is believed to occur because the smaller crystal sizes have a greater surface of interaction for a given amount of material (86).

Over the last 10–15 years, several groups have reported that HZ accumulation within MØ modulates several of their functions, and is associated with some malarial symptoms, such as fever, anemia, and splenomegaly (26, 88). It has been determined that human monocytes and murine MØ stimulated with HZ (purified from various species of *Plasmodium* or synthetically generated) produce large amounts of cytokines (IL-10, IL-12, IL-1β, and TNFα), chemokines (MIP-1α and MIP-1β), MIF erythropoietic inhibitor, and adhesion molecules (CD11/CD18) (4, 26, 45, 89). Consistent with these observations, we previously published the first report that *in vivo* inoculation of sHZ rapidly induces the generation of various pro-inflammatory mediators, including myeloid-related proteins (MRPs; S100A8 and S100A9), chemokines (MIP-1α/β, MIP-2, and MCP-1), and cytokines (90); strongly suggesting that HZ itself might play an important role in the development of malaria-related pathologies. Additionally, *in vitro* studies from our laboratory revealed that HZ significantly enhances IFNγ-induced NO production by MØ (91), an important inflammatory event that could favor cerebral malaria development. We also found that native *Pf*HZ- and sHZ-induced MØ chemokine expression is regulated by oxidative stress-dependent (92) and -independent mechanisms. Contrastingly, some *in vitro* studies have shown that MØ which have internalized HZ for a long period of time (over 24 h), exhibited inhibition of PKC (68) and NADPH oxidase (72), IFNγ-inducible class II (MHC-II) expression (45), LPS-induced functions (e.g., CD14, and TNFα) (34, 79, 93, 94), phagocytosis (72), microbicidal activity (74), and the respiratory burst (95). Despite these functional alterations and the possible toxic effects of oxygen radicals and lipoperoxidation triggered following HZ phagocytosis (72, 87, 88), the MØ and monocytes were able to remain viable for several days.

Many studies have reported that HZ induces TNFα gene transcription and expression. TNFα production has been shown to correlate with severe malaria, as it is found in higher concentrations in the serum of patients with severe malaria compared to those with mild malaria (96, 97). Supporting its importance in inflammatory-related processes, HZ-induced TNFα production by human monocytes was found to be inhibited by IgM purified from malaria patients, but not from healthy donors (98). Another important cytokine involved in malarial fever is IL-1β. HZ was found to induce IL-1β expression in an air pouch model (90), and in the liver when intravenously injected (86). Recently, several studies have reported that HZ induces IL-1β production by activating the inflammasome protein complex (99–101). The cellular and molecular mechanisms underlying the ability of HZ to activate the NLRP3/inflammasome complex will be further discussed later in

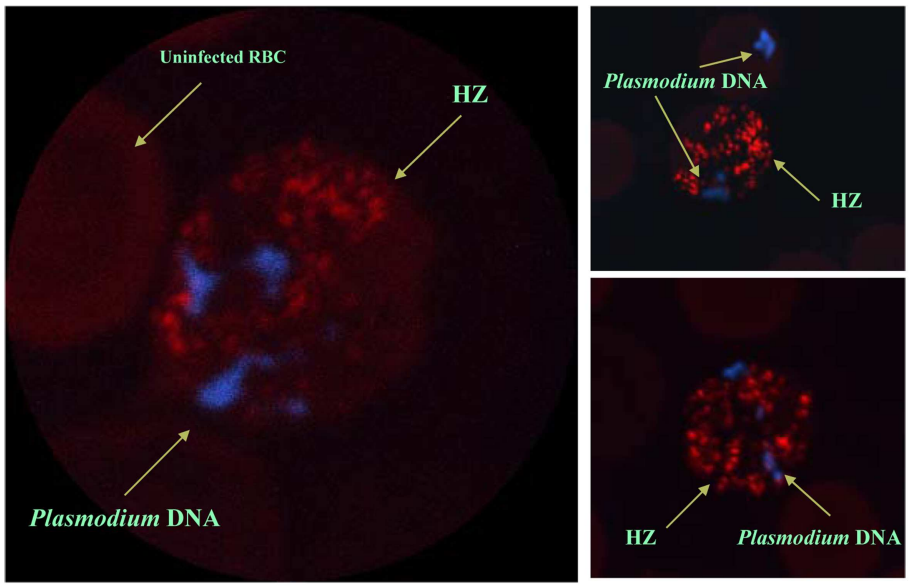

FIGURE 2 | *In situ* localization of *Plasmodium* hemozoin and parasitic DNA. Confocal pictures of RBC from *Plasmodium chabaudi* DK-infected mice. Selected images of schizonts and late trophozoites stages of iRBC. DAPI staining was used to visualize malarial DNA (blue). No staining was required to localize HZ since it autofluorescences (red). Even after merozoite release from the iRBC (see top right panel), malarial DNA was never in contact with hemozoin. Images were taken from Ref. (86).

this review. Furthermore, it was also found that HZ can induce the production of IL-6 by endothelial cells *in vitro* and that intraperitoneal administration of HZ can induce IL-6 production *in vivo* (100). Similar findings were also observed in a more controlled *in vivo* environment using an air pouch model (90).

Apart from cytokines, HZ also causes the release of various chemokines and the expression of chemokine receptors, as was briefly mentioned earlier. The engagement of a chemokine with its specific receptor triggers an intracellular signaling cascade that results in chemotactic recruitment of inflammatory cells, leukocyte activation, and antimicrobial effects (102). Using an air pouch model and intravenous injection (90), as well as murine macrophages (86, 103), it was shown that HZ induced the expression of various chemokines (MIP-1α/CCL3, MIP-1β/CCL4, MIP-2/CXCL2, and MCP-1/CCL2) and chemokine receptors (CCR1, CCR2, CCR5, CXCR2, and CXCR4). HZ was also found to augment the production of several β-chemokines in peripheral blood mononuclear cells (PBMC) (104), endothelial cells (100), and *in vivo* (26, 105). These results strongly support the role of HZ as a potent pro-inflammatory agent that could contribute to the immunopathology of malaria observed in humans and murine malaria.

HZ/PHAGOCYTE INTERACTION: FROM BASIC SIGNALING TO NLRP3 INFLAMMASOME

As mentioned above, HZ is capable of activating different cell types resulting in the release of several pro-inflammatory and anti-inflammatory mediators. However, the intracellular mechanisms underlying HZ-induced cellular events are still under investigation. An initial study revealed a synergism between HZ and IFNγ resulting in the induction of NO production. The generation of the microbicidal agent required the activation of extracellular signal-regulated kinase (ERK) 1/2 pathway, but was independent of an IFNγ-induced activation of the JAK2/STAT-1 pathway (91). However, both ERK and JAK2/STAT-1 signaling was found to be necessary to attain maximal NF-κB activation and iNOS promoter-binding capability (91). NF-κB was also shown to be greatly involved in HZ-induced chemokine expression (103). In addition to MAP kinases, HZ has recently been described to be capable of activating spleen tyrosine kinase (Syk), augmenting inflammasome activation and IL-1β production by THP-1 human monocytic cells and murine macrophages (99). In the same study, it was revealed that kinases downstream of Lyn/Syk, for instance, MAP kinase family members, might be involved in inflammasome activation, since inhibition of ERK, but not p38, decreased IL-1β production.

Despite the fact that HZ has been shown to be immunologically active *in vitro* and *in vivo*, the cellular receptors recognizing HZ remain elusive. However, the efficiency of HZ-induced signaling and phagocyte function seem to depend on its internalization and lipid raft integrity. It is well known that the cells of the innate immune system recognize pathogen-associated molecular patterns (PAMPs) or damage-associated molecular patterns (DAMPs) by expressing gene-encoded pattern recognition receptors (PRR), such as Toll-like receptors (TLRs), Nod-like receptors (NLRs), c-type lectin, and RIG-like helicases. TLRs can recognize *P. falciparum* GPI anchors (106), but the HZ-induced response is not dependent on TLRs (86, 100, 107, 108). Nevertheless, the ability of TLRs to sense HZ is still a controversial issue. It is important to be fastidious in the interpretation of these results, as the amphiphilic nature of HZ allows it to bind certain proteins, lipids, and nucleic acids; therefore, any *Plasmodium* molecules adhering to HZ during

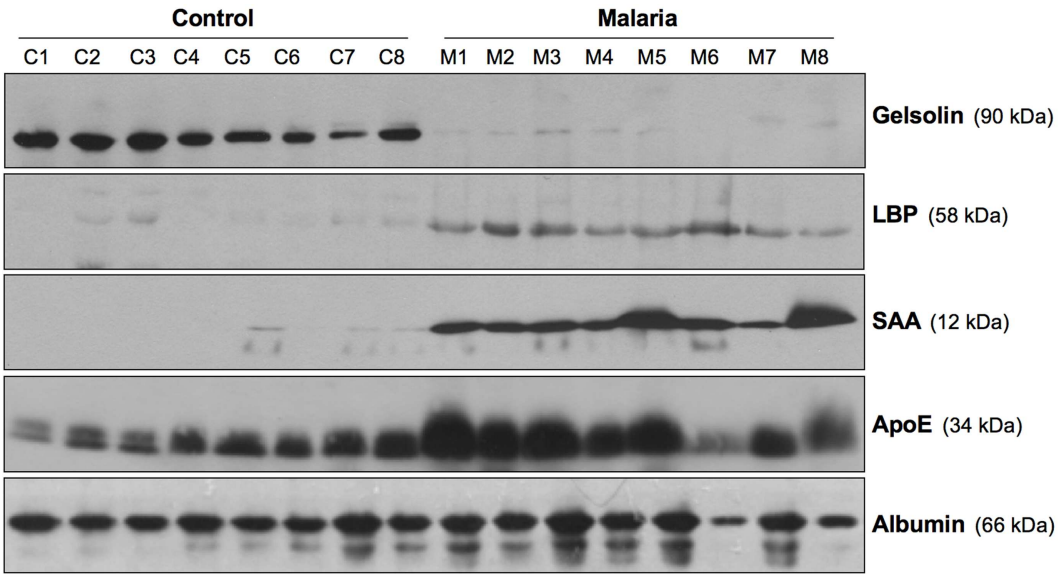

FIGURE 3 | Inflammatory biomarkers adhering to hemozoin. Sera biomarkers from malaria patients and healthy individuals were detected using Western blotting. The membranes were blotted using antibodies specific for gelsolin, LPS-binding protein (LBP), serum amyloid A (SAA), apolipoprotein E (ApoE), and serum albumin. C1–C8, control; M1–M8, malaria. Figure was taken from the supplemental data of Ref. (116).

the preparation of nHZ could be sensed by the TLRs. In this context, Parroche et al. (107) proposed that HZ was a carrier of a TLR ligand and that the immune response induced by HZ was from a possible contamination of HZ with *Plasmodium* DNA. However, different research groups have shown that synthetic and native HZ that are not contaminated by DNA (86, 109) are still very powerful immunogenic molecules (83). Furthermore, by using DNA staining and the natural red/green fluorescence of HZ, it was shown that *Plasmodium* DNA within iRBC never co-localizes with HZ, which is confined to the food vacuole (**Figure 2**) (86). In this regard, the ability of TLRs to recognize HZ is still unclear, as is the ability of other PRRs to recognize HZ, especially NLRs.

The NLR family of receptors is characterized by three domains: a leucine-rich repeat (LRR) domain in the N-terminus, a central nucleotide-binding domain, and a variable C-terminus. Based on the composition of the C-terminus and central domain, NLRs are divided into different subfamilies: the *NLRB subfamily* (which consists of one member, NAIP), the *NLRC subfamily* (NLRC4/IPAF and NOD1, which are NLR containing a CARD domain, and NOD2, which contains two CARD domains); and the *NLRP subfamily* (NLRP1 and NLRP3, NLR containing a pyrin domain). The members of each subfamily recognize different pathogen-associated molecules; for example: flagellin is recognized by NLRC4, anthrax lethal toxin by NLRP1, muramyl dipeptide and lysin-peptidoglycan by NOD2, meso-diaminopimelic acid-peptidoglycan by NOD1, and a vast spectrum of ligands (bacterial RNA, inorganic materials, gout-associated crystal-MSU, and microbes) by NLRP3. NOD1/NOD2 receptor stimulation has been shown to induce RIP2 kinase-dependent NF-κB activation, resulting in the transcription of pro-inflammatory cytokines (110–112). Recent findings suggest that certain pro-inflammatory events occurring during *P. berghei* ANKA infection may depend on NOD2 (113), however the role of HZ in this circumstance is still incompletely understood. Furthermore, several laboratories have made observations indicating that the NLRP3 inflammasome complex could be involved in a HZ-induced response.

The potential role of the NLRP3 inflammasome in HZ-triggered inflammatory events is of particular interest, because IL-1β is known to be integral to the fever episodes and pro-inflammatory processes observed during *Plasmodium* infection. However, the results regarding the participation of the inflammasome complex have been slightly inconsistent. Studies by our laboratory (99) and by Dostert et al. (101) showed that HZ-induced IL-1β production and neutrophil recruitment were dependent on the NLRP3 inflammasome. In partial agreement, Griffith et al. (100) showed that HZ-stimulated neutrophil recruitment into the mouse peritoneal cavity was dependent on NLRP3 inflammasome. Additionally, using a murine model, three different studies have demonstrated that NLRP3-deficient mice showed some level of protection against two different murine parasites, *P. berghei* ANKA and *P. chabaudi adami* DS (99, 101, 114). Nevertheless, this protection cannot be solely attributed to HZ, as during the course of *Plasmodium* infection, a number of factors from the pathogen and the host immune system will contribute to the outcome of the infection. Moreover, our study revealed that *Plasmodium*-infected NLRP3- and IL-1β-deficient mice have a lower body temperature compared to wild-type. This finding is consistent with the potential role of HZ during malarial infection, as it is released during iRBC lysis, which is usually followed by an episode of fever. Furthermore, the laboratory of Scharzwer recently reported that the attachment of fibrinogen to HZ imbued HZ with a greater capacity to activate host inflammatory functions (115). In this context, recent data from our laboratory (116) revealed that HZ interacts with a large number of inflammatory-related biomarkers

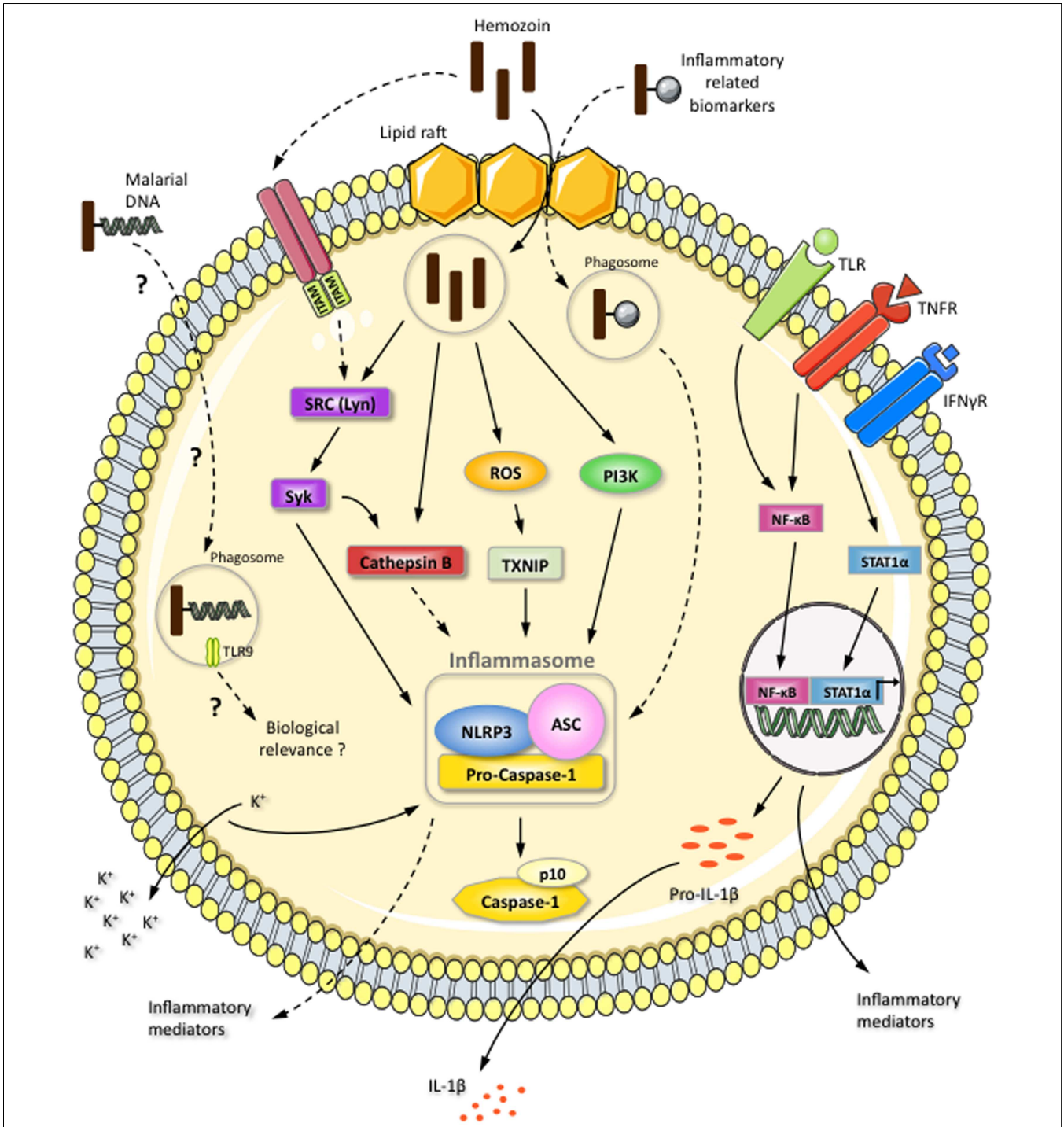

FIGURE 4 | Induction of NLRP3 inflammasome complex by the inorganic crystal HZ. HZ induces IL-1β production via the NLRP3/ASC inflammasome: activation of caspase-1 results in the cleavage of pro-IL-1β. Pro-IL-1β expression is resultant of the TLR4- and TNFα-activated NF-κB pathway or the IFNγ-stimulated STAT-1α pathway. The HZ-activated NLRP3 inflammasome is dependent on potassium efflux, ROS generation, HZ internalization, and cathepsin B activation. HZ internalization and the induction of SRC kinase signaling are mediated by lipid rafts. An ITAM-containing receptor could also be the starting point of SRC kinase cascade. HZ is internalized within a phagosome-like vesicle surrounded by LAMP-1. HZ activation of the SRC kinase Lyn leads to Syk phosphorylation. Subsequently, Syk positively modulates cathepsin B activation, which could result in the induction of the NLRP3 inflammasome. HZ is also capable of activating the NLRP3 inflammasome through PI3 kinase. The involvement of malarial DNA, which can adhere to HZ, in the activation of an intracellular receptor and its biological relevance are controversial and only reported for dendritic cells, which are present in limited numbers in the blood. HZ can interact with a large number of inflammatory-related biomarkers found in the circulation of *P. falciparum*-infected patients. However, the effect of these biomarkers on NLRP3 inflammasome activation is still unknown. Continuous arrows indicate a positive modulation. Dotted arrows indicate a hypothetical effect. Dotted arrows with a question mark indicate an unknown or controversial effect.

(e.g., fibrinogen, serum amyloid A (SAA), LPS-binding protein (LBP), and apolipoproteins) found in the circulation of *P. falciparum*-infected patients (**Figure 3**). The potential of these molecules to modify the interaction of HZ with immune cells is of great interest, as it could exacerbate the inflammatory events occurring during malaria.

The mechanisms underlying the activation of the NLRP3/inflammasome complex by HZ are currently under investigation. Three models have been proposed: potassium channel efflux, lysosome rupture, and/or ROS generation (117) (**Figure 4**). Two independent groups have illustrated the involvement of potassium efflux, phagocytosis, and ROS generation in inflammasome activation (99, 101). However, there is a discrepancy in whether cathepsin B is involved, depending on the approach used. Cathepsin B-deficient mice showed no effect (101), whereas cathepsin B-specific inhibitors were found to block inflammasome activation (99). Nevertheless, HZ-triggered inflammasome activation seems to involve at least two of the proposed models (potassium channel efflux and ROS generation) and cathepsin B activation. Disruption of the phagolysosome by HZ does not appear to occur, since HZ has been shown to be contained in vacuoles surrounded by LAMP-1, and cathepsin B has not been found in culture supernatant, which is generally the case for asbestos and silica (99), which not only disrupt the phagolysosome, but also kill the phagocytic cells. Of utmost importance, our study revealed that Lyn/Syk kinases are the upstream signaling partners in the activation of the NLRP3/inflammasome complex (**Figure 4**). The participation of these kinases in HZ-induced inflammasome activation is highly suggestive that an ITAM-containing receptor on the surface of the host cell could be the starting point for this biochemical cascade. However, it is also possible that HZ is capable of modulating the lipid raft environment, which could initiate the signaling cascade (**Figure 4**).

Finally, it is important to stress that inorganic crystals like asbestos, silica, and MSU not only disrupt phagolysosome integrity, but are also highly apoptotic and disruptive to cell integrity. Conversely, HZ is able to stay within host cells for long periods of time, from several days to weeks, without discernably affecting phagocyte viability (118). Moreover, HZ is markedly smaller than the other inorganic crystals mentioned above and is fully engulfed by the host cells, unlike the other crystals mentioned.

In conclusion, it is has been established that HZ is a powerful modulator of the innate immune response, which suggests that it has the potential to be detrimental or beneficial to the host depending on the stage of the infection. Furthermore, it has recently been demonstrated that HZ is sensed as a danger signal, resulting in the activation of the inflammasome (99, 101). However, there are contradicting results regarding the modulation of the immune response by HZ. These differences can be explained at least in part by the different cell types and incubation times used in various studies, and most prominently, by the quality of the HZ crystal utilized. Therefore, a unified method to generate sHZ crystals, which more closely resembles the ones naturally produced by *Plasmodium*, needs to be established. Additionally, ensuring that the resulting sHZ crystals possess the correct quality, size, and crystallinity by using X-ray powder diffraction (XRD) analysis would aid in attaining more reproducible data.

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NLRP7 and the genetics of hydatidiform moles: recent advances and new challenges

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NOD-like receptor proteins (*NLRPs*) are emerging key players in several inflammatory pathways in Mammals. The first identified gene coding for a protein from this family is *Nlrp5* and was originally called *Mater* for "Maternal Antigen That Mouse Embryos Require" for normal development beyond the two-cell stage. This important discovery was followed by the identification of other *NLRP*s playing roles in inflammatory disorders and of the first maternal-effect gene in humans, *NLRP7*, which is responsible for an aberrant form of human pregnancy called hydatidiform mole (HM). In this review, we recapitulate the various aspects of the pathology of HM, highlight recent advances regarding *NLRP7* and its role in HM and related forms of reproductive losses, and expand our discussion to other *NLRP*s with a special emphasis on those with known roles in mammalian reproduction. Our aim is to facilitate the genetic complexity of recurrent fetal loss in humans and encourage interdisciplinary collaborations in the fields of *NLRP*s and reproductive loss.

Keywords: *NLRP7*, hydatidiform mole, spontaneous abortions, reproductive loss, maternal-effect genes

HISTORICAL VIEW ABOUT HM

Hydatidiform mole (HM) is an aberrant human pregnancy with no embryo that has fascinated and puzzled scientists in all civilizations. The recognition and description of this condition is very ancient and appears in Hippocrates' manuals under the name "dropsy of the uterus" (1). In addition, a famous physician in the Byzantine period, Aetius of Amida, who was the private physician of Emperor Julian, wrote about moles and interestingly used the term inflammation to describe them, "an inflammation or strenuous walking" (2, 3). The etiology of HM continues to fascinate scientists in several aspects. HM is the only disease or tumor that may be formed by androgenetic, non-self-cells from a woman's sexual partner as opposed to all other tumors and cells in our body. Despite their common histopathological features, different HM tissues may have different parental contributions. Depending on its mode of formation, a HM's genotype might be diploid biparental, diploid androgenetic monospermic, diploid androgenetic dispermic, triploid dispermic, triploid digynic, tetraploid, aneuploid, or mosaic. These diverse possibilities generate an important diagnostic complexity and therefore continue to challenge scientists and clinicians in various disciplines. In this chapter, we review the pathology of HMs and describe recent advances in our understanding of its pathogenesis.

EPIDEMIOLOGY OF HM

The common form of HM is sporadic and not recurrent. The geographical distribution of its incidences varies widely, with a frequency of 1 in every 600 pregnancies in western countries (4) and 2–10 times higher frequencies in developing and undeveloped countries (5–7). Depending on populations and studies, 1–6% of women with a prior HM will develop a second HM (8–14). Cases in which a single family member has recurrent HM (RHM) are called singleton cases and those in which at least two women have one or several HM are called familial cases. Familial RHM is rarer and its exact frequency is not known.

CLINICAL AND ULTRASOUND MANIFESTATIONS

The clinical manifestation of moles has changed with the advances of medicine, largely because of the introduction of ultrasonography in the second half of the twentieth century as a routine exam to monitor all pregnancies starting from the eighth week of gestation. Consequently, many moles are now detected by ultrasound examination at the first gynecological visit or even earlier in cases of vaginal bleeding, which is the most common presenting symptom that would precipitate early medical consultation and diagnosis. Ultrasound indications of moles include the presence of echogenic structures in the placenta, the absence of a gestational sac, and/or the absence of fetal heart activity. These initial ultrasound observations are followed by a blood test of the human chorionic gonadotropin (hCG), the pregnancy hormone that is secreted mainly by syncytiotrophoblast cells of the chorionic villi (CV) into the intervillous space, whereupon it is carried to the maternal systemic blood circulation. HCG is much higher in women with molar pregnancies than in women with normal pregnancies of matching gestational stages, which is believed to be the consequence of the increased proliferation of syncytiotrophoblast cells. Depending on ultrasound findings, the gestational stage of the pregnancy, and the level of blood hCG, further ultrasound examinations and hCG follow-up tests may be required before a clinical decision is reached regarding the arrest of the pregnancy and the requirement of a surgical evacuation of the product of conception (POC). A non-viable pregnancy accompanied with

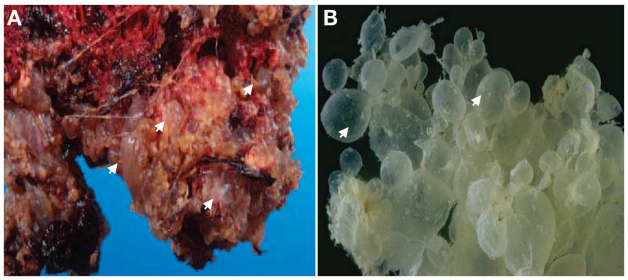

FIGURE 1 | Gross-morphology of HMs. **(A)** A photograph of gross-morphology of a HM directly after surgical evacuation. Note the presence of vesicles (only four are indicated by arrows) which represent edematic chorionic villi (CV) that have accumulated fluid and are covered with blood. **(B)** Another gross-morphology photograph of a HM after removing the blood. Note the hydropic degeneration of the CV and their appearance as a grape-like structure (only two are indicated by arrows). In **(B)** photo courtesy of Professor Edward C. Klatt, School of Medicine, Mercer University.

a high hCG test will necessitate dilatation and curettage suction of the POC. The evacuated tissues (**Figure 1**) are submitted for histopathological examinations and the diagnosis is made based on histopathological findings and criteria.

HISTOPATHOLOGY AND DIAGNOSIS

The original definition of HM was a pregnancy devoid of a fetus in which the chorion is replaced by grape-like vesicles. A long time ago, the severe form of this condition was believed to originate from pathologic ovaries (15) and was originally called "true moles" or "classical moles," which correspond to what we now call complete HMs. This classification evolved and other terms emerged later to describe milder forms of the same condition such as "transitional," "partial," and "incomplete" moles (16–18). The current histopathological classification of spontaneously arrested pregnancies includes three entities designated complete HM (CHM), partial HM (PHM), and non-molar spontaneous abortion (SA) (19). CHMs display circumferential trophoblastic proliferation affecting most CV (**Figure 2**) and do not contain extra-embryonic membranes (chorion and amnion), a fetal cord, fetal nucleated red blood cells, or any other embryonic tissue of inner cell mass origin. Both SAs and PHMs may contain extra-embryonic membranes (chorion and/or amnion), a fetal cord, fetal nucleated red blood cells, other embryonic tissues (cartilage, bones, etc.), or even a normal or an abnormal complete fetus (**Figure 2**). PHMs display mild and focal trophoblastic proliferation that can be observed on some CV and in several microscopic fields, whereas SAs do not display abnormal circumferential trophoblastic to warrant close hCG follow-up (**Figure 2**). The histopathological subdivision of spontaneously arrested pregnancies into CHMs, PHMs, and SAs has always been challenging and several scientists have noted the continuous variation in the molar degeneration (18). This challenge is more problematic nowadays because of the early evacuation of arrested pregnancies based on ultrasonography and before the manifestations of all their histopathological features. Consequently, there is a wide inter-observer and intra-observer variability in distinguishing non-molar SAs from PHMs and in distinguishing PHMs from CHMs (20–22). Practically, the difficulty for the pathologists is to know where to draw the lines of separation between the three entities due to the continuous spectrum of abnormalities and due to the fact that histopathology is a qualitative descriptive science (mild, excessive, focal, occasional, etc.) that lacks quantitative measurements to assess the degree and extent of trophoblastic proliferation.

KARYOTYPE AND GENOTYPE DATA

Karyotype and genotype analyses have shown that sporadic moles may have different genotypic types with the majority of CHMs being diploid androgenetic and the majority of PHMs being triploid diandric dispermic. Among androgenetic moles, the majority are monospermic and 10–20% are dispermic (23–27). In a minority of cases, some CHMs have been reported to be diploid biparental (25), triploid diandric dispermic (23), tetraploid triandric (3 paternal and 1 maternal sets of chromosomes) (28) or digynic (29), aneuploid, or mosaic with two cellular populations. Sporadic PHMs are mostly triploid diandric dispermic, but they have also been reported with diploid biparental, triploid digynic (29), triploid monospermic (30), tetraploid triandric (31, 32), or aneuploid genomes. Based on the major categories of complete and partial moles, different hypothetical models at the origin of moles' formation were proposed and have been accepted by the scientific community over the last 30 years. One of these models postulates that an androgenetic monospermic mole results from the fertilization of an empty oocyte by a haploid sperm that undergoes an endoreduplication of its genome to form the diploid androgenetic monospermic mole. Similarly, androgenetic dispermic moles would result from the fertilization of an empty oocyte by two spermatozoa, while triploid diandric (or dispermic) moles would result from the fertilization of a haploid oocyte by two different haploid spermatozoa. These accepted models were recently challenged by Golubovsky (33) who refutes the existence of empty oocytes at the origin of androgenetic moles. Instead, he proposes that dispermic fertilization followed by complex postzygotic abnormalities and diploidization is at the origin of the various genotypic types of moles as well as mixoploidies, trisomies, and various aneuploidies. These different models and their implications in the genesis of HMs are discussed below.

POST-EVACUATION hCG SURVEILLANCE AND MALIGNANCIES

Molar pregnancies are the most common gestational tumors and are benign in about 80% of cases. In these cases, hCG falls to non-pregnant levels after the surgical evacuation of the molar conception. However, in about 20% of cases in western countries, elevated hCG levels persist for several weeks post-evacuation or rise after falling down, which indicates the retention of some trophoblastic tissues. Such conditions are termed persistent gestational trophoblastic diseases (PGTDs) or gestational trophoblastic neoplasias (GTNs) and may necessitate a second surgical evacuation and/or chemotherapy treatments. GTNs occur most commonly after CHMs (15–29%), less frequently after PHMs (0.5–4%), and rarely after SAs, ectopic pregnancies, or normal pregnancies (34–36). Several classification systems of GTNs have been elaborated

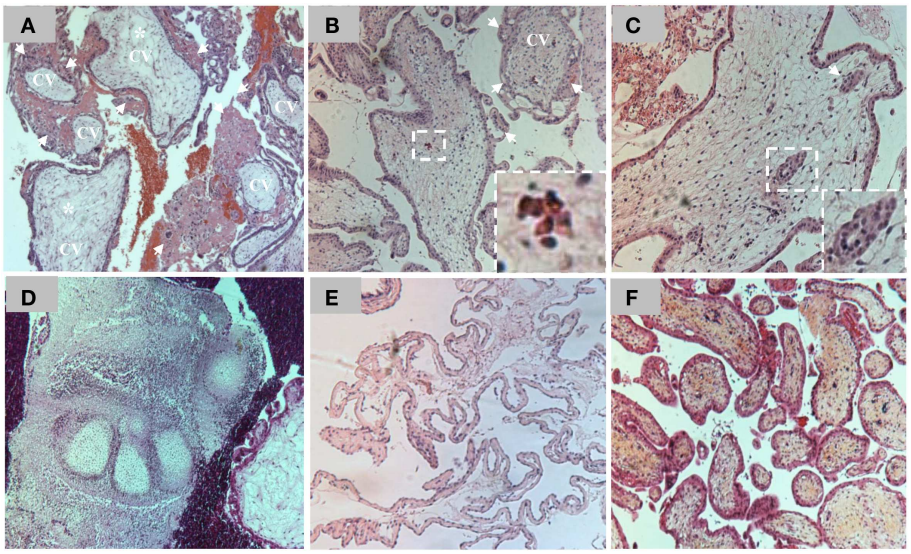

FIGURE 2 | (A) Hematoxilin and eosin (H&E) staining of a section of chorionic villi (CV) from a CHM. Note the presence of excessive circumferential trophoblastic proliferation around all CV (arrows) and the beginning of hydropic degeneration in two CV (asterisks). **(B)** H&E staining of a section of CV from a PHM. Note the presence of circumferential trophoblastic proliferation (arrows) around one chorionic villus (indicated by CV) while the others have no or few sprouts of trophoblastic proliferation (arrows). Note the presence of nucleated red blood cells inside the chorionic villus (on the right corner) in the conception that led to PHM. **(C)** One CV from a PHM displaying trophoblastic inclusions (arrows and magnified view on the right corner). **(D)** A view from a PHM showing phalanges of fetal foot. **(E)** Another view from a PHM showing fetal membranes. **(F)** H&E staining of a section of CV from a spontaneous abortion. Note the absence of trophoblastic proliferation around the CV.

over time and are used to help standardize and optimize treatments of these conditions. For good reviews on these topics see (37–39). In recent times, the most commonly used guidelines are those of the World Health Organization (WHO) and the Fédération Internationale de Gynécologie et d'Obstétrique (FIGO).

The most common malignant degeneration of HMs or GTNs are invasive moles and gestational choriocarcinomas (CCs). The diagnosis of invasive moles is based on persistent or rising levels of hCG and histopathological identification of CV within the myometrium (the deep layer of uterine tissues beneath the endometrium), maternal blood vessels, or within extrauterine tissues. Invasive moles affect approximately 20 and 2–4% of patients with CHMs and PHMs, respectively (34).

CCs may occur after any type of pregnancy in the following proportions: 35–60% after CHMs, 0.5–2% after PHMs, 15–20% after SAs, 1–2% after ectopic pregnancies, and 25–42% after normal pregnancies (40, 41). The diagnosis of CC is based on high hCG levels and both clinical and laboratory evidence demonstrating the presence of tumor cells in distant maternal tissues such as the lung, lower genital tract, brain, liver, kidney, gastrointestinal tract, and spleen. A definitive diagnosis of CC is based on histopathological findings demonstrating the presence of cytotrophoblastic and syncythiotrophoblast cells, without organized villous structures in distant maternal tissues (42). CCs are the most aggressive GTNs because of their ability to spread hematogenously. They may be fatal in the absence of appropriate follow-up and management. Again, both invasive HMs and CCs have higher frequencies in both developing and underdeveloped countries than in developed countries (40).

THE IMPORTANCE OF CROSSING OUR DISCIPLINE

Despite the ancient clinical recognition of HMs and the presence of several reports describing cases of recurrent moles (15, 43–46) no attempts were made to identify causative genes for the recurrent form until the report by Seoud et al. (47) that led to the mapping of the first maternal locus to 19q13.4 (48). At that time, only six other familial cases of RHMs had been reported in the English PubMed literature since 1980 (49–52). Consequently, we and others believed that the familial form of moles was extremely rare. However, this was not true and approximately 30 new familial cases have been reported since 1999 (47, 53–69) indicating that familial RHMs are not extremely rare as originally believed, but were probably under-reported. In addition, about 88 singleton cases of RHMs have been described since 1999. The importance of the case reported by Seoud and his collaborators (47) is in the fact that the authors crossed the boundaries of their disciplines, a common practice in many medical specialties, but a rare one in the field of Obstetrics and Gynecology. These authors sought the help of scientists from other disciplines at a time where small nuclear consanguineous families were an opportunity for gene mapping by homozygosity analysis. This original family as well as another (51) led to the mapping of the first maternal-effect locus responsible for recurrent moles to 19q13.4 (48) and opened a new avenue of research aimed at identifying maternal genes causing RHMs and recurrent fetal loss.

LESSONS FROM STUDYING EXTREME PHENOTYPES

One difficulty associated with homozygosity mapping and studying rare families is in narrowing down the size of the candidate

intervals. This was the case of 19q13.4 candidate region, which was originally four megabases and is a gene-dense region. Consequently, the identification of the causative gene, *NLRP7*, was tedious and required the screening of 80 different genes until the first causative mutations were identified (70). The mutations segregated in the studied families and each patient had two defective alleles, each inherited from one parent as expected for an autosomal recessive disease. Later, others and we confirmed the causality of *NLRP7* mutations in patients from different populations (54, 60, 63, 66, 67, 69, 71, 72), demonstrating that *NLRP7* is a major gene for RHMs. To date, approximately 42 different mutations have been reported in patients with two defective alleles (**Figure 3**) (73) Of these mutations, 65% are protein-truncating (stop codon, splice mutations, small insertions and deletions, and large rearrangements) and 35% are missense mutations, which are, respectively, higher and lower than the frequencies of these two categories of mutations observed in all human diseases, 56 and 44% (http://www.hgmd.cf.ac.uk/ac/index.php). Although, this difference is not statistically significant, it indicates that patients with RHMs and two mutations may represent the most severe phenotype of the disease.

The identification of *NLRP7* is therefore one of many examples where rare families segregating severe monogenic Mendelian forms of common conditions have led to the identification of causative genes [for an interesting review on the subject see (74)]. This raises an important question: do familial RHM cases with *NLRP7* mutations have more severe mutations than singleton cases? The originally reported families tended to have more protein-truncating mutations than singleton cases. However, this is no longer the case since reports of singleton cases with protein-truncating mutations have increased lately. This could be due to the fact that many singleton cases do not manifest as familial cases because of the small size of families in current societies and/or the lack of other female siblings who have tried to conceive. These factors may have prevented the familial manifestation of many singleton cases with inherited mutations from the two parents.

NLRP7 EXPRESSION

Before the identification of the causal link between *NLRP7* and RHMs, *NLRP7* transcripts were shown to be expressed in a large number of human tissues including liver, lung, placenta, spleen, thymus, peripheral blood leukocytes, testis, and ovaries (75, 76). After our group linked *NLRP7* to RHMs, we investigated its expression in oocytes and detected its transcripts in all stages of immature oocytes, fertilized eggs, and early embryo cleavage stages (70). These data were later confirmed in an interesting study that showed that *NLRP7* transcripts decrease progressively during oocyte maturation and reach their lowest level on day 3 post-fertilization, which corresponds to the morula stage, then increase sharply from day 3 to day 5, which corresponds to the blastocyst stage and the activation of the fetal genome transcription (77).

At the protein level, *NLRP7* expression was shown in all stages of growing follicles and in all these stages, its expression was restricted to oocytes (72). In another study reported by our group, we detected variable levels of *NLRP7* protein in seven analyzed hematopoietic cells: Epstein Barr virus transformed

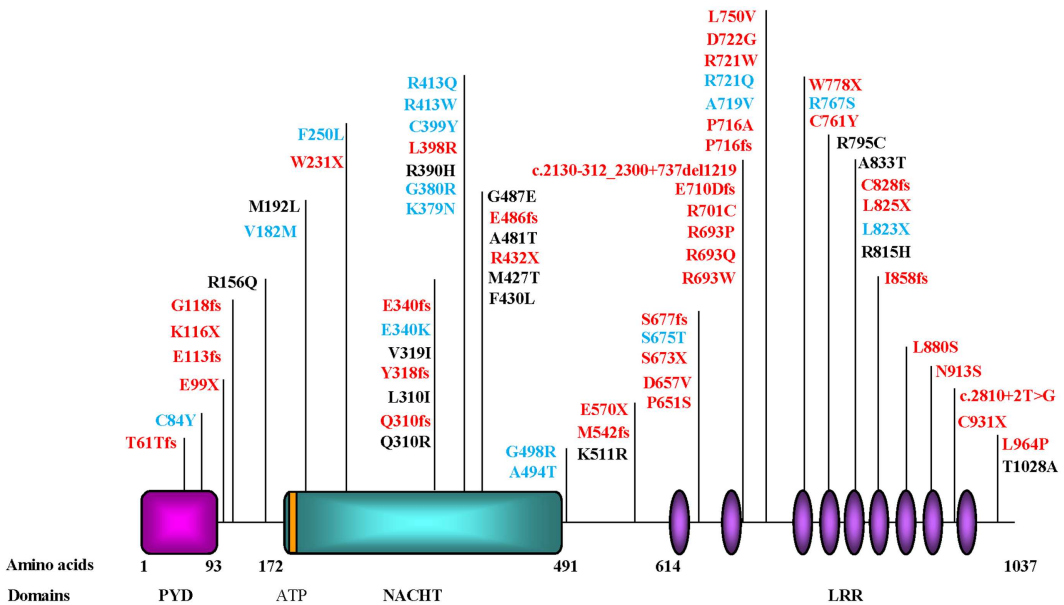

FIGURE 3 | Schematic representation of NLRP7 protein structure and identified mutations and variants in patients with hydatidiform moles and reproductive loss. PYD, stands for the pyrin domain, NACHT, stands for found in the NAIP, CIITA, HET-E, and TP1 family proteins; ATP for adenosine 5′-triphosphate binding motif; and LRR, for leucine rich repeats. The ATP binding domain is a small motif of 8 amino acids and starts at position 178. Mutations found in patients with two defective alleles are in red and include nonsense, frameshift, invariant splice site, and missense mutations. Variants found in patients in a heterozygous state and not in controls are mostly missenses and are in blue. Variants found in patients and in subjects from the general population are in black. Mutation nomenclature is according to the Human Genome Variation Society (HGVS) guidelines (http://www.hgvs.org/mutnomen/recs.html).

B-lymphocytes, BJAB, Raji and Ramos (all of B-cell origin), Jurkat (of T-cell origin), and THP1 and U937 (both of monocytic origin) (78).

NLRP7 DOMAINS

The *NLRP7* protein consists of three domains: (i) an N-terminal pyrin, (ii) a NACHT termed after four proteins containing an NTPase domain with significant similarities, neuronal apoptosis inhibitor protein (NAIP), MHC class II transcription activators (CIITA), incompatibility locus protein from Podospora anserine (HET-E), and mammalian telomerase-associated proteins (TP1); and (iii) a C-terminal stretch of 9 or 10 leucine rich repeats (LRRs) depending on splice isoforms (**Figure 3**).

The pyrin domain is a small domain (92 amino acids) found in all *NLRPs* and apoptotic proteins. The pyrin domain functions as an adaptor that helps to connect proteins of the programed death machinery. Pyrin domains can self-associate to form homodimers or associate with other proteins containing structurally related domains to form heterodimers. Domains known to interact with the pyrin domain include the death domain (DD), the death-effector domain (DED), and the caspase activation and recruitment domain (CARD). These pyrin-mediated associations result in the formation of protein complexes and networks that transmit signals from receptors to downstream effectors that function in various cell-death pathways (79). The NACHT domain has an ATP/GTPase-specific P-loop domain, which is a very ancient domain found in bacteria, plants, and all eukaryotes. NTPase domains are found in both apoptotic and anti-apoptotic proteins; they control programed cell-death during development by regulating cytochrome c efflux from the mitochondria, which stimulates apoptosis (80). The LRR domain is found in other proteins with divergent functions such as Toll-like receptors (TLRs), Ran GTPase, and RNAse inhibitor proteins. TLRs are components of the innate immune system, from which the LRR extends into the extracellular milieu where it senses extracellular danger signals and transmits the signals to cytoplasmic proteins. Ran GTPases are essential for transporting RNAs and proteins through the nuclear pore complex by interacting with shuttling transport proteins and changing their ability to bind or release cargo molecules. Finally, RNase inhibitor proteins bind RNAse A and angiogenin and regulate RNA degradation and angiogenesis (81).

KNOWN FUNCTIONS AND ROLES OF *NLRP7*

The most studied functions linked to the different NLRP domains are those involved in the activation of the innate immune system in response to various microbial and chemical products. With respect to *NLRP7*, four studies have addressed its functional roles to date and their results are recapitulated in **Table 1**. Using transient transfections, two studies showed that *NLRP7* down-regulates the intracellular level of mature IL1B (76, 78). While the first study showed that this is due to the down-regulation of pro-IL1B processing (76), the second, by our group, showed that this is due to the lower production of intracellular pro-IL1B (78). In addition, we found that in transient transfections, *NLRP7* inhibition of pro-IL1B production is mediated concomitantly by its three domains, with the strongest effect being mediated by the LRR, followed by the NACHT and the pyrin domains (78). In the study by Kinoshita et al., the authors showed that *NLRP7* binds pro-IL1B and pro-caspase 1 and inhibits IL1B secretion induced by caspase 1, ASC, or NLRP1-delLRR. They also showed that both recombinant mouse IL1B and LPS stimulation enhance *NLRP7* transcription, which in turn down-regulates IL1B secretion. They concluded that *NLRP7* is a negative feedback regulator of IL1B and consequently plays an anti-inflammatory role (76).

Part of the study conducted by our group was performed on *ex vivo* LPS-stimulated peripheral mononuclear blood cells from patients with one or two mutations in *NLRP7*. This experiment demonstrated the requirement of wild type *NLRP7* for normal IL1B secretion (78). Within monocytes, which are the main cells that secrete IL1B, *NLRP7* co-localized with the Golgi apparatus and microtubule organizing center (MTOC) (**Figure 4**) (78). Moreover, treatment of EBV lymphoblastoid cell lines with nocodazole, a drug that depolymerizes microtubules resulted in the fragmentation of *NLRP7* signal. This suggested that normal *NLRP7* associates with microtubules and that its mutations may impair cytokine secretion by disrupting microtubules structures and consequently affecting intracellular trafficking of IL1B vesicles. The role of *NLRP7* in IL1B secretion was confirmed in another independent study involving silencing *NLRP7* in macrophages using small interfering RNA (82). In this study, the effect of silencing eight other *NLRP*s was also tested, but only *NLRP7* knockdown significantly decreased IL1B secretion. This study by Khare et al. also confirmed the physical interaction between *NLRP7*, ASC, and caspase 1 via the pyrin domain, and that the LRR of *NLRP7* is required for sensing bacterial acylated lipopeptides.

Khare et al. (82) also revealed another function of *NLRP7* by demonstrating that *NLRP7* silencing promotes intracellular growth of *Staphylococcus aureus* and *Listeria monocytogenes*. A prior study implying a role for *NLRP7* in cellular proliferation, but in the opposite direction, was reported by Okada et al. (75), who showed that silencing *NLRP7* reduces the proliferation of human embryonal carcinoma cell lines, suggesting that the normal protein promotes cellular growth and has an oncogenic role. The mechanisms leading to both functions are currently unclear and need to be explored in future studies. However, from the HM perspective, we tend to believe in the role suggested by Khare et al. (82), because an important feature of molar tissues from patients with two *NLRP7* defective alleles, which are diploid biparental and obligate carriers of one mutated copy of *NLRP7*, is the excessive proliferation of their trophoblastic cells. This is in line with the data by Khare et al., and is a further indication that *NLRP7* mutations promote cellular growth.

UNDERSTANDING THE VARIABILITY OF A PHENOTYPE: BACK TO THE GENETIC COMPLEXITY OF REPRODUCTIVE LOSS

An important aspect of our understanding of any disease or system is to understand its variability and determine its extreme phenotypes with its most and less severe manifestations. Despite the fact that we named the 19q13.4 locus as responsible for RHMs, affected patients from the original family, MoLb1, experienced, in addition to their moles, other forms of reproductive loss, namely SAs, stillbirths, an early neonatal death, one malformed live birth, and two live births that led to healthy adults. This large variability

Table 1 | Recapitulation of the functional roles of *NLRP7* in different studies and cellular models.

(75)	(76)	(78)		(82)
	LPS or rm-IL1B induce *NLRP7* transcription in PBMC and THP1			
	Transient transfection in HEK293		***Ex vivo* PBMC**	**Macrophages**
	NLRP7 inhibits IL1B secretion induced by NLRP1-delLRR, IL1B, caspase 1, and ASC in transient transfection		**Cells with *NLRP7* mutations have low secreted IL1B**	***NLRP7* silencing reduces IL1B secretion in macrophages**
	***NLRP7* down-regulates pro-IL1B** and pro-caspase 1 processing **leading to lower intracellular mature IL1B**	***NLRP7* down-regulates pro-IL1B** production **leading to lower intracellular mature IL1B**	*NLRP7* mutations increase slightly pro-IL1B production	
	***NLRP7* interacts with** transfected **pro-caspase 1** and pro-IL1B		*NLRP7* and IL1B subcellular localization overlaps Cells with *NLRP7* mutations have low secreted TNF	***NLRP7* interacts with caspase 1** and ASC in HEK293 cells through the pyrin domain *NLRP7* Silencing does not affect IL6 or TNF secretion by macrophages
NLRP7 silencing with siRNA reduces cellular proliferation				*NLRP7* Silencing with siRNA increases intracellular bacterial growth *NLRP7* LRR is necessary to sense bacterial acylated lipopeptides

LPS stands for lipopolysaccharides; PBMC for peripheral blood mononuclear cells; NLRP1-delLRR stand for NLRP1 in which the leucine rich repeat is deleted; siRNA for small interfering RNA; rm-IL1B, indicates recombinant mouse IL1B. Conclusions obtained by at least two independent studies are in bold character.

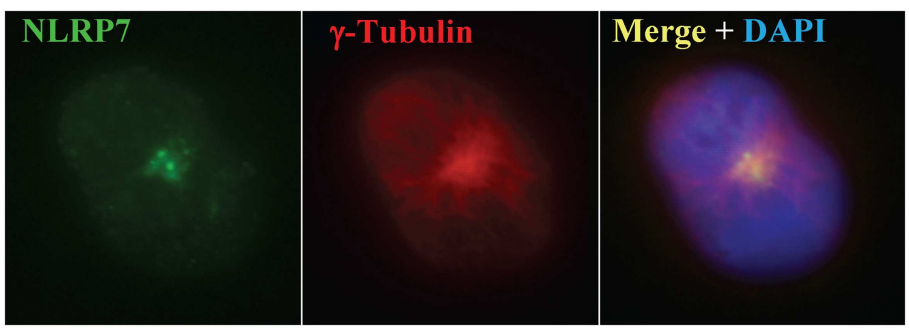

FIGURE 4 | *NLRP7* expression in monocytes using immunofluorescence. *NLRP7* stains two small dots specific for the microtubule organizing center, which is also revealed with γ-tubulin as previously reported (78).

in the reproductive outcomes of three patients from MoLb1 was intriguing because such variability is unusual in recessive diseases. However, this variability was not restricted to one family, but was observed, to a lesser extent, in other families studied by our group. Furthermore, this variability was in agreement with data from a large epidemiological study showing increased frequencies of moles, preterm births, stillbirths, and ectopic pregnancies in women with at least two SAs (83). These observations led us to extend our inclusion criteria for *NLRP7* sequencing to women with at least three SAs and no moles as well as to women with the sporadic, common, non-recurrent moles. This analysis showed that two of the 26 analyzed women with recurrent SAs (8%) and eight of the 64 analyzed women with a single HM (associated with and without other forms of reproductive losses) (13%) have novel *NLRP7* non-synonymous variants (NSVs), all missenses in heterozygous state, which were not found in a large number of control subjects from the same ethnicity of the patients (**Figure 5**) (84). One of the two patients with >3SAs and a missense mutation had a persistent gestational trophoblastic disease requiring chemotherapy after one of her miscarriages. Moreover, six of the patients

with one HM and a NSV in *NLRP7* had at least two other reproductive losses, in addition to their HMs, indicating their genetic susceptibility to recurrent reproductive loss. In addition, patients with one defective allele statistically had less severe reproductive outcomes and more live births than patients with two defective alleles (p-value = 2.809e−06) (**Figure 6**).

In conclusion, this analysis did provide a positive answer to our search for mutations in milder phenotype of RHMs. However, it raised challenging questions that all scientists working on complex traits are currently facing: how do we define a pathological NSV? And what tells us that these rare NSVs, found in heterozygous states in a so far believed autosomal recessive disease, have functional consequences on the protein and confer genetic susceptibility for reproductive loss?

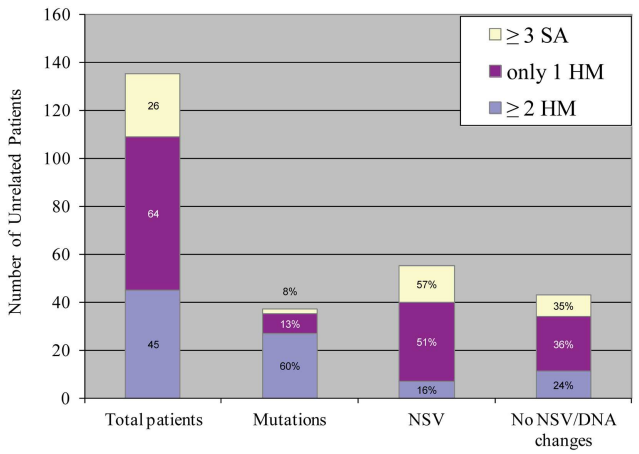

FIGURE 5 | Summary of *NLRP7* mutation and non-synonymous variants found in 135 unrelated patients with varying histories of reproductive wastage. HM stands for hydatidiform mole; SA, stands for spontaneous abortion; NSV, for non-synonymous variant. Mutations in *NLRP7* were most frequently observed in patients with at least two HMs, followed by patients with one HM, and then by patients with at least three SAs (84).

SIGNIFICANCE OF RARE *NLRP7* NSVs FOUND IN HETEROZYGOUS STATE IN PATIENTS

To date, a total of 17 rare NSVs, 16 missenses, and one nonsense, have been observed in heterozygous state in a total of 24 patients but not in controls (67, 85–89) (**Figure 3**). Some of these NSVs were later found in the 1000 Genomes database but at very low frequencies. Among patients analyzed in our laboratory, 19% of singleton cases with RHMs have one rare NSV in a heterozygous state. At this point in time, it is not clear whether these NSVs are pathologic or not. Consequently, such novel NSVs are not for clinical use and should not be reported to patients to predict the outcomes of future pregnancies. However, they cannot be ignored by scientists aiming at understanding the pathology of RHMs and its relationship to the sporadic common form of HMs, recurrent SAs, and other forms of reproductive loss.

To better understand the significance of these NSVs and elaborate strategies to investigate their pathogenicity, it is important to look at similar situations in other diseases with both rare severe recessive forms and common milder forms. A selection of such diseases is shown in **Table 2**. The best example is Parkinson disease (PD), for which several causative genes have been identified. Some of these genes are responsible for recessive forms of PD, while others are responsible for dominant forms. Among the causative genes for recessive forms, *PINK1* is responsible for an early onset form of PD and has two mutated alleles in several patients from familial and non-familial sporadic cases of PD. However, other patients were found to have single rare NSVs in heterozygous state. When compared to controls from the same ethnic group, patients with PD were found to have an excess of rare *PINK1* NSVs in heterozygous state. Consequently, these rare NSVs are believed to underlie the genetic susceptibility of these patients for PD (90–92). The same principle applies to other genes: *ATP13A2* responsible for a juvenile onset of PD (93), *GBA* responsible for Gaucher's disease (94, 95), *ABCA1* responsible for Tangier disease (96), and *MEFV* responsible for familial Mediterranean fever (FMF) (97). In most of these cases, patients with single heterozygous variants have a milder form of the same disease in terms of clinical severity

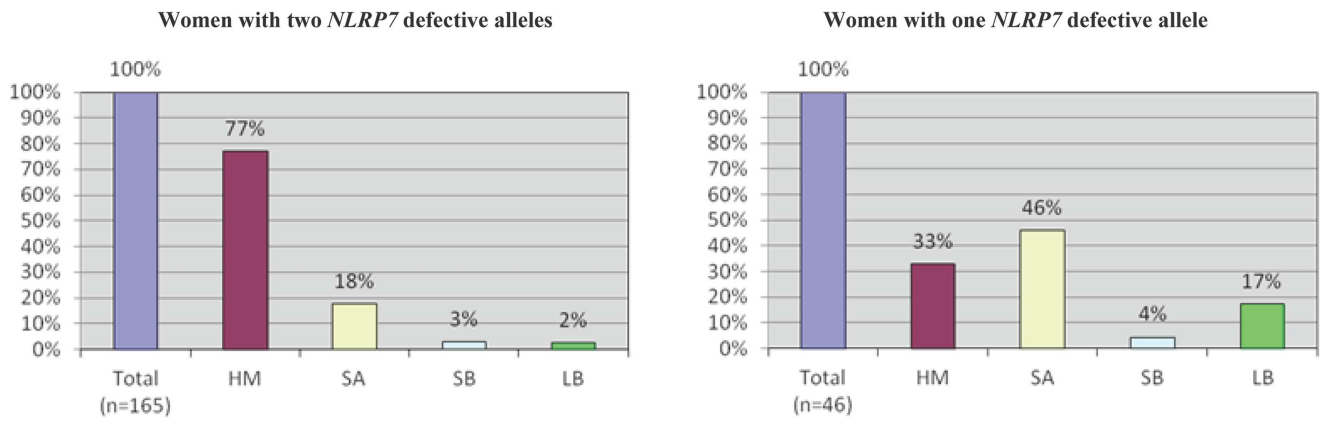

FIGURE 6 | A comparison of reproductive outcomes between women with two or one defective *NLRP7* allele. In both histograms, *n* indicates the total number of pregnancies from patients in either category. HM, hydatidiform mole; SA, spontaneous abortion; SB, stillbirth; and LB, live birth. A higher incidence of HMs and a lower incidence of live births are observed in patients with two defective alleles.

Table 2 | Examples of genes causing rare severe recessive diseases and confering susceptibility to common or related forms of the same disease.

Gene	Two defective alleles	Single mutated allele	Reference
PINK1	Autosomal recessive Parkinson diease (PD) with early onset	More rare variants in patients vs. controls (10 vs. 2) Milder phenotype and later onset in heterozygous relatives of severely affected patients in large pedigrees	(90, 92) (91)
ATP13A2	Juvenile onset Parkinson disease <21 years	Young onset Parkinson disease	(93)
GBA	Gaucher's disease	More rare variants in patients with PD vs. controls. This seems specific to some ethnic groups, e.g., Ashkenas, French	(94, 95)
MEFV	Familial mediterranean fever	In 15% of patients	(97)
ABCA1	Familial hypoalphalipoproteinemia	More rare variants in individuals with low HDL-C than in those with high HDL-C (16% vs. 2%)	(96)

or/and age of onset or have a related condition that include some of the features of the severe disease (93–97).

With respect to RHMs, the age of onset is not an appropriate indicator of severity; however, a severe genetic defect would translate into recurrence and would be expected to lead to the same genetic defect every time a patient tries to conceive. On the contrary, a milder genetic defect, which can be modulated by other environmental factors, would be expected to lead to more variability in the reproductive outcomes of the patients. This is exactly the conclusion we reached in the last analysis performed on three categories of patients (RHM, sporadic HM, and recurrent SA), which showed that patients with RHM have the highest frequency of *NLRP7* mutations (60%), and these patients had mostly two defective alleles, each. However, 13% of patients with one mole and other reproductive wastage had a single variant in a heterozygous state, while 8% of patients with at least three SAs had rare *NLRP7* variants in heterozygous state (**Figure 5**). Similar results were obtained from patients with sporadic HM and reproductive wastage in a different population (Tunisian) and again showed the presence of *NLRP7* variants in heterozygous state in 13% of the patients (59). Additional case-control studies designed to screen all *NLRP7* exons in patients with sporadic HM and recurrent SAs are needed to assess whether the burden of *NLRP7* mutations and rare NSVs is higher in patients than in ethnically matched controls. In the meantime, a number of other tests can be used to investigate the pathogenicity of encountered variants. These include (i) the absence of the variants in controls of matching ethnicity to the patients; (ii) the conservation of the changed amino acids throughout evolution; (iii) the predicted functional consequences of the identified variants using various algorithm; (iv) the segregation of the variants on different haplotypes when present with other known deleterious mutations; (v) the functional impact of the variants on the protein subcellular localization; and ideally (vi) the impact of the variants on the protein function in any type of cellular assays.

GENOTYPE OF HM TISSUES IN PATIENTS WITH *NLRP7* MUTATIONS

To date, the parental contribution to approximately 70 HM tissues from patients with two defective alleles in *NLRP7* have been characterized and all of them were found to be diploid biparental (55, 62, 63, 87, 98–100) with the exception of one tissue that was digynic (101). However, this is not the case for HM tissues from patients with single heterozygous rare *NLRP7* variants. In this category of patients, few HM tissues were genotyped; some were found to be diploid androgenetic monospermic (67, 85, 87, 89) and others were found to be triploid diandric dispermic (102). The reason for this difference is not yet clear and needs to be addressed in future studies. Such studies may also clarify whether specific single heterozygous rare *NLRP7* variants confer a genetic susceptibility to a specific genotypic type of moles. This would help elucidating the mechanisms of the formation of different genotypic types of moles. This is particularly important because the currently accepted mechanisms of mole formation are hypothetical and the emerging ideas propose a single model stemming from dispermic fertilization followed by postzygotic abnormalities (33).

NLRPs AND REPRODUCTION

NLrp5

Nlrp5 (originally called *Op1* then *Mater*, and lately *Nlrp5*) is the first *NLRP* gene shown to play a causative role in mammalian reproduction (103). *Nlrp5* was isolated from a mouse model of autoimmune oophoritis (also termed premature ovarian failure) generated by neonatal thymectomy. Female mice thymectomized in the third day after birth spontaneously develop autoimmune disorders characterized by organ-specific inflammation and lymphocyte infiltration (104). In some mouse strains, the predominant autoantibody is directed against the ovary where it reacts with NLRP5. To gain insights about the role of *NLRP5* in autoimmune oophoritis, the authors generated knockout null females, $NLRP5^{-/-}$, and found that these females ovulate normally and their oocytes fertilize *in vivo* with no apparent abnormalities. However, their embryos stop developing at the two-cell stage, a time at which major embryonic genome activation takes place. The role of *NLRP5* in preimplantation embryonic development was also confirmed in monkeys where its knockdown in MII oocytes resulted in a significant reduction in the number of embryos that reached the blastocyst stage (105). In mouse oocytes, *NLRP5* is part of specialized oocyte cytoskeletal structures (called cytoplasmic lattices) that are responsible for the distribution of organelles, maternal mRNA, and maternal proteins in the oocytes (106–108). Also, previous studies on *NLRP5* showed that within

oocytes, *NLRP5* localizes to mitochondria and nuclear pores and is implicated in oxidative stress during oocyte aging (109).

NLRP14

To date, a single study has implicated *NLRP14* in spermatogenic failure in humans based on the presence of one stop codon and four missense mutations, all of which were found in heterozygous state and each in a single patient and were not found in controls (110). However, no additional studies replicating the causal role of *NLRP14* or explaining its potential role in spermatogenic failure have been reported.

NLRP2

NLRP2 is the closest human gene to *NLRP7* in terms of protein homology and both genes are believed to have originated from the same mouse paralog during evolution (109, 111–113). *NLRP2* was shown to be responsible for a single familial case of Beckwith–Wiedemann syndrome (BWS) based on the presence of a frameshift mutation in a homozygous state in an unaffected mother and in her two children affected with BWS (114). The presence of a homozygous *NLRP2* mutation in the mother of two children with BWS is interesting because of the relationship between BWS and HM, and their association with reproductive loss and abnormal imprinting. However, since that report, no other cases of BWS were shown to have mutations in *NLRP2*, which makes this finding either a rare causal event occurring in a small minority of cases or a coincidental association. In addition, *Nlrp2* knockdown in murine oocytes at the germinal vesicle stage was shown to lead to embryonic arrest at the two-cell stage (115).

Nlrp4e

Recently a new study investigating the role of mouse *Nlrp4e* in female reproduction has been reported. In this study, *Nlrp4e* was found expressed in all follicular stages, unfertilized eggs, and early embryo cleavage stages. Again, *Nlrp4e* knockdown in fertilized eggs resulted in a reduced number of embryos that reach the blastocyst stage, which is an indication that maternal *Nlrp4e* is required for early embryo development (116).

CONCLUSION

Since the identification of *Nlrp5* and *NLRP7*, the list of *NLRP* genes with maternal-effects continues to grow. We expect this list to expand even further because of the presence of four additional *NLRPs* besides *NLRP4* and *NLRP2* that show oocyte-specific expression and have not yet been linked to reproduction in any organism: *NLRP8*, 9, 11, and 13 (112). All of these *NLRP*s are highly expressed in germinal vesicle oocytes and decrease during preimplantation development to reach their lowest levels at the blastocyst stage, which is in favor of their maternal-effect role.

With respect to *NLRP7*, we do not yet know the exact role of its protein in human oocytes. However, based on several observations, we believe that oocytes from patients with mutations are defective at several levels and are not able to sustain early embryonic development. Consequently, the embryos stop developing very early in these conceptions. Because these patients also have decreased cytokine secretion, we believe that they fail to mount an appropriate inflammatory response to reject these arrested pregnancies as normal women would. As a result, the retention of these dead pregnancies with no embryos to later gestational stages leads to the hydropic degeneration of CV. This, combined with the potential role of *NLRP7* mutations in enhancing proliferation, may lead to the three fundamental aspects of moles: aberrant human pregnancies with no embryo, abnormal excessive trophoblastic proliferation, and hydropic degeneration of CV. We believe that fully understanding the three aspects of the pathology of HM would greatly benefit from collaborations between scientists in various medical fields.

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Evolution and conservation of plant NLR functions

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In plants and animals, nucleotide-binding domain and leucine-rich repeats (NLR)-containing proteins play pivotal roles in innate immunity. Despite their similar biological functions and protein architecture, comparative genome-wide analyses of *NLRs* and genes encoding NLR-like proteins suggest that plant and animal NLRs have independently arisen in evolution. Furthermore, the demonstration of interfamily transfer of plant NLR functions from their original species to phylogenetically distant species implies evolutionary conservation of the underlying immune principle across plant taxonomy. In this review we discuss plant NLR evolution and summarize recent insights into plant NLR-signaling mechanisms, which might constitute evolutionarily conserved NLR-mediated immune mechanisms.

Keywords: NLR, NB-LRR, resistance protein, innate immunity, effector-triggered immunity

INTRODUCTION

Plants rely entirely on innate immunity to fight pathogens (1), as they do not have an adaptive immune system, including specialized immune cells, like higher animals. To achieve a specialized and targeted immune response, plants possess several lines of defense against pathogens. Plasma membrane localized pattern-recognition receptors recognize conserved pathogen molecules, such as flagellin and chitin and provide broad-spectrum pathogen resistance (2). However, host-adapted pathogens suppress this immune response by delivering effector molecules inside host cells (3, 4). As a counter mechanism, plants deploy the nucleotide-binding domain and leucine-rich repeats (NLR) family of intracellular receptors to detect the presence of effectors, triggering potent innate immune responses (5, 6). The former class of immunity is called "pattern-triggered immunity" (PTI), whereas the latter is called "effector-triggered immunity" (ETI), which is often associated with genetically programed host cell death (1).

The mechanism of effector recognition by plant NLRs has been well established. Plant NLRs utilize two major modes of effector recognition: a direct and an indirect recognition mode (5–8). In both cases, plant NLRs are kept in an inactive form by either intra- or inter-molecular interactions in the absence of cognate effectors (9). The difference lies within the mode of effector recognition: in case of the direct recognition, an effector is detected by direct physical interaction with its cognate NLR, whereas during the indirect recognition, a NLR senses modifications of host proteins caused by the cognate effector action. Experimental evidence supports that the indirect recognition enables a single NLR to recognize multiple effectors irrespective of effector structures when effectors target the same host protein (5, 6). However, detection of multiple effectors by a single NLR is not exclusive to the indirect recognition mode. Recently it was demonstrated that a single NLR can detect at least two sequence-unrelated effectors by direct binding (10).

Knowledge on signal initiation and transduction mediated by plant NLRs is rather sparse compared to the effector detection mechanism. However, through recent progress in plant NLR biology, the mechanisms of signal initiation and signaling relay are gradually being revealed. Furthermore, the demonstration of interfamily transfer of NLR functions across plant lineages implies evolutionary conservation of the underlying immune mechanisms. On the following pages, we will discuss plant NLR evolution and summarize recent insights into plant NLR-signaling mechanisms, which might hint at yet unidentified, evolutionarily conserved NLR-mediated immune signaling mechanisms. Furthermore, comparative genome-wide analyses of genes encoding NLRs and NLR-like proteins among various plant lineages give insights into the presumed history of plant NLR evolution and consequently important clues to elucidate NLR functions in innate immunity and possibly functions beyond innate immunity.

SURVEY OF *NLR* GENES IN LAND PLANTS: TOWARD A MODEL OF PLANT NLR EVOLUTIONARY HISTORY

EXPANDED *NLR* REPERTOIRES ACROSS PLANT LINEAGES

Similar to animal NLRs, plant NLRs are modular proteins that generally consist of three building blocks: a N-terminal domain, the central NB-ARC domain (named after Nucleotide-Binding adaptor shared with APAF-1, plant resistance proteins, and CED-4), and a C-terminal LRR (leucine-rich repeats) domain (11). The central domain of animal NLRs is also known as the NACHT domain (named after NAIP, CIITA, HET-E, and TP1) (12) which is structurally similar to the plant NB-ARC domain but distinctive of animal NLRs (13, 14). The utilization of either a TOLL/interleukin 1 receptor (TIR) domain or a coiled-coil (CC) domain at the N-terminus is a plant-NLR-specific feature and defines two major types of plant NLRs termed the TIR-type NLRs (TNLs) and the CC-type NLRs (CNLs), respectively. However, it

is often challenging to specify structures of N-terminal domains for a significant proportion of plant NLRs due to their structural diversity and lack of significant homology to validated protein structures. Thus, NLRs containing an N-terminus other than the TIR domain are sometimes designated as non-TIR-type NLRs (nTNLs) as a distinction to TNLs.

The NLR family has massively expanded in several plant species. The massive expansions render the NLR family one of the largest and most variable plant protein families (15, 16). This contrasts with the vertebrate *NLR* repertoires, typically comprising ca. 20 members (17–20). Detailed genome-wide surveys, database mining, and degenerate PCR approaches for the species whose genome sequences are currently not available contribute to refine an overview of the NLR repertoires in various plant species (**Table 1**). Most of the plant genomes surveyed so far have a large *NLR* repertoire with up to 459 genes in wine grape (**Table 1**). Interestingly, the bryophyte *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* which represent the ancestral land plant lineages seem to have a relatively small *NLR* repertoire of ∼25 and ∼2 *NLRs* respectively, suggesting that the gene expansion has occurred mainly in flowering plants (**Table 1**; **Figure 1**). It was recently shown that numerous microRNAs target nucleotide sequences encoding conserved motifs of NLRs (e.g., P-loop) in many flowering plants (21). Thus it is hypothesized that such a bulk control of *NLR* transcripts may allow a plant species to maintain large *NLR* repertoires without depletion of functional *NLR* loci (22, 23), since microRNA-mediated transcriptional suppression of *NLR* transcripts could compensate for the fitness costs related to maintenance of *NLRs* (21, 24).

The number of *NLR* genes in flowering plants is largely variable without any clear correlation to the phylogeny, suggesting species-specific mechanisms in *NLR* genes expansion and/or contraction (**Table 1**). This variability can be exemplified by three species in the brassicaceae family: *Arabidopsis thaliana*, *Arabidopsis lyrata*, and *Brassica rapa*, which have 151, 138, and 80 full-length *NLRs,* respectively (**Table 1**). Expansion of *NLR* genes has also occurred in several metazoans such as sea urchin (*Strongylocentrotus purpuratus*) and sea squirt (*Ciona intestinalis*), which possess 206 and 203 *NLRs,* respectively (20, 43, 44). In contrast, the genomes of fruit fly (*Drosophila melanogaster*) and nematode (*Caenorhabditis elegans*) apparently lack *NLRs*, suggesting that *NLRs* have been lost in these invertebrate species (17).

ORIGIN OF NLR BUILDING BLOCKS

Comparison of *NLR* repertoires from higher plants to ancestral taxa common for plants and animals could hint at the time and mechanism which led to the assembly of NLR building blocks into a single multi-domain receptor. Yue et al. (25) conducted a full genome-wide comparison of *NLR* repertoires among 38 model organisms encompassing all the major taxa (6 eubacteria, 6 archaebacteria, 6 protists, 6 fungi, 7 plants, and 7 metazoans). This dataset was further enriched with the genomic and transcriptomic data available for 5,126 species of nine major early plant lineages (chlorokybales, klebsormidiales, zygnematales, coleochaetales, charales, liverworts, bryophytes, hornworts, and lycophytes). The results of this large-scale data mining imply that the core building blocks of NLRs, such as NB-ARC, NACHT, TIR, and LRR, already existed before eukaryotes and prokaryotes

Table 1 | Plant *NLR* gene repertoires identified by genome-wide analyses.

Species	Common name	Genome size (Mbp)	*NLRs*	*TNLs*	*CNLs*	*XNLs*	Reference
Arabidopsis thaliana	Thale cress	125	151	94	55	0	Meyers et al. (18)
Arabidopsis lyrata	Lyre-leaved rock-cress	230	138	103	21	NA	Guo et al. (33)
Brachypodium distachyon	Brachypodium	355	212	0	145	60	Li et al. (34)
Brassica rapa	Mustard	100–145[a] (529)	80	52	28	NA	Mun et al. (35)
Carica papaya	Papaya	372	34	6	4	1	Porter et al. (36)
Chlamydomonas reinhardtii	Chlamydomonas	120	0	0	0	0	Yue et al. (25)
Cucumis sativus	Cucumber	367	53	11	17	2	Wan et al. (37)
Glycine max	Soybean	1115	319	116	20	NA	Kang et al. (38)
Medicago truncatula	Barrel medic	186[a] (500)	270	118	152	0	Ameline-Torregrosa et al. (39)
Oryza sativa	Rice	466	458	0	274	182	Li et al. (34)
Physcomitrella patens	Moss	511	25	8	9	8	Xue et al. (28)
Populus trichocarpa	Poplar	550	317	91	119	34	Kohler et al. (40)
Sorghum bicolor	Sorghum	760	184	0	130	52	Li et al. (34)
Solanum tuberosum	Potato	840	371	55	316	NA	Jupe et al. (41)
Selaginella moellendorffii	Spike moss	100	2	0	NA	NA	Yue et al. (25)
Vitis vinifera	Wine grape	487	459	97	215	147	Yang et al. (42)
Zea mays	Maize	2400	95	0	71	23	Li et al. (34)

The table represents NLR and NLR-like gene numbers corresponding to NB-ARC-LRR-encoding genes. The numbers for TNLs, CNLs, and XNLs correspond to genes encoding either full-length TNLs, CNLs, XNLs, or the NB-ARC-LRR-containing proteins if these can be clearly assigned to one of the NLR types based on their motif composition at the NB-ARC domain. X refers to any N-terminal domain other than TIR or CC. [a]Analyses based on partial genome sequence; the respective complete genome sizes are indicated in brackets.

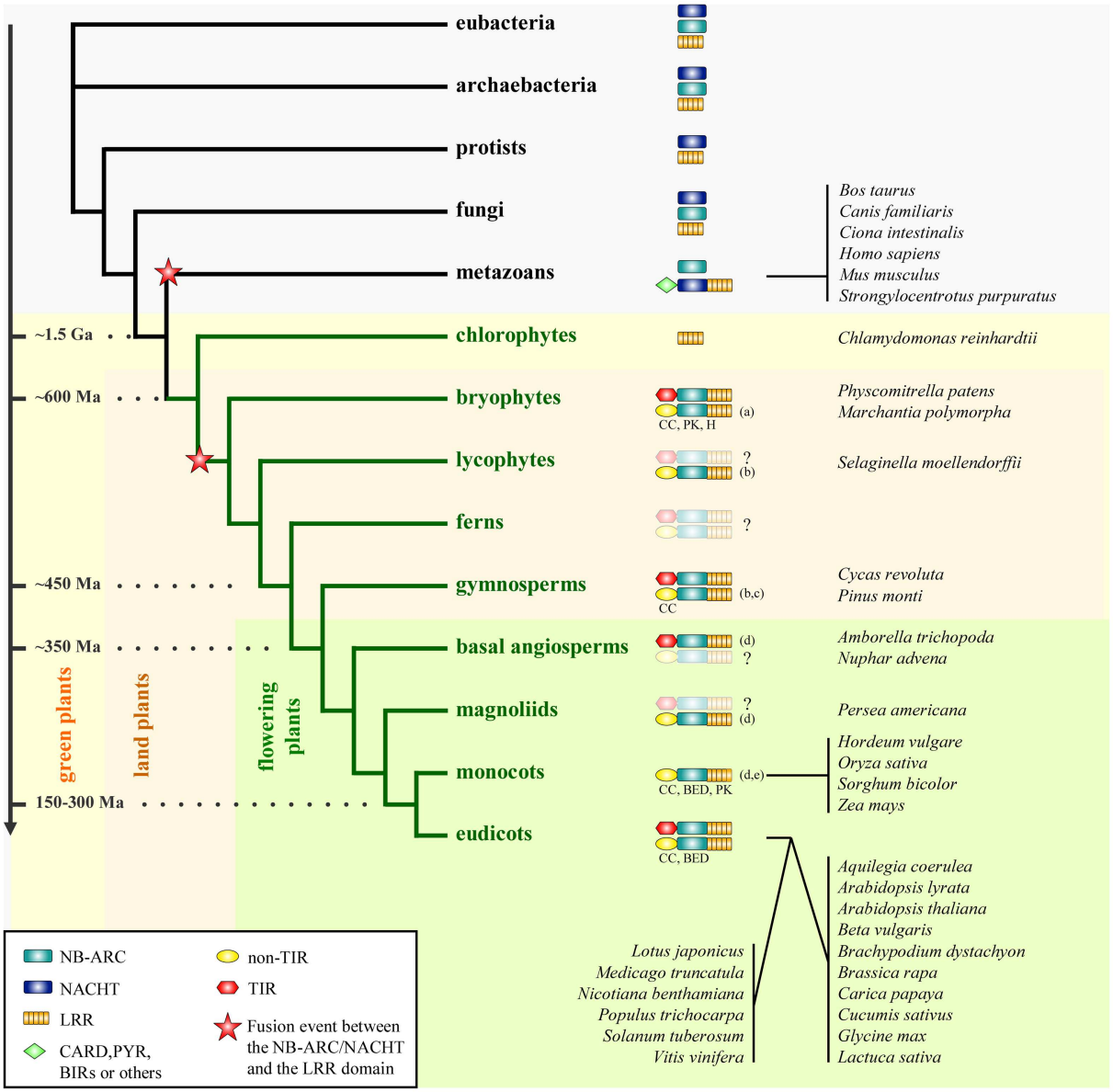

FIGURE 1 | Phylogenetic distribution of the NLR family. The distribution of the individual domains constitutive of NLRs (NB-ARC, NACHT, and LRR) and the different groups of NLRs are mapped on a simplified phylogenetic tree. The fusion events between either the NB-ARC or the NACHT domain and the LRR domain presumably occurred as indicated on the phylogenetic tree. The structural properties of the N-termini of plant NLRs in the non-TIR group are indicated if the information is available (CC, coiled-coil; BED, BED-DNA-binding zinc finger; H, α/β-hydrolase; PK, protein kinase; for more detail, see Atypical Domains Found in the NLR Structure). This figure is adapted from Yue et al. (25), combined with data as indicated below. The divergence dates are adapted from Ref. (26) and (27). Species representative of some taxa are indicated on the right. Ma, million years; Ga, billion years. The question mark (?) indicates that the presence of NLRs is not clearly resolved in given taxa due to lack of data. (a) Xue et al. (28), (b) Kim et al. (29), (c) Heller et al. (30), (d) Tarr and Alexander (31), (e) Faris et al. (32).

diverged, since these constitutive domains are also found in the genomes of eubacteria and archaebacteria surveyed (**Figure 1**).

INDEPENDENT FUSION EVENTS IN THE EARLY HISTORY OF ANIMAL AND PLANT NLRs

The aforementioned study implies that the fusion events between an ancestral NACHT domain and an LRR domain, and between an ancestral NB-ARC domain and an LRR domain occurred independently in the early history of metazoans and plants [Ref. (25); **Figure 1**]. Therefore this further supports the previously proposed idea that plant and animal NLRs are the consequence of a convergent evolution (45–47). Analysis of the phylogeny and motif combinations of the NACHT/NB-ARC domains revealed clear differences between the NACHT and the NB-ARC domains, suggesting either an ancient divergence, or an independent origin of these two domains, which happened before the divergence of eukaryotes, eubacteria, and archaebacteria (25). With the current data, both fusion events could be dated back to a period

coinciding with the appearance of multicellularity (25). In this perspective, plant and animal NLRs provide an interesting example of structural and functional convergence, with a shared ability to discriminate self from non-self and to induce immune responses.

DISTINCT AND ANCIENT EVOLUTIONARY TRACKS FOR TNLs AND nTNLs

Extending the work by Meyers et al. (48), Yue et al. (25) identified the ten most conserved motifs in NACHT and plant NB-ARC domains. This analysis revealed contrasting motif frequencies between animal NLRs and plant NLRs and further discriminates TNLs from nTNLs. This is consistent with the phylogeny based on the NB-ARC domain where plant TNLs and plant nTNLs segregate in two monophyletic clades. This result is also supported with intron phase and position analysis (18). Based on these analyses, both studies revealed a greater diversity in the nTNLs compared to the TNLs. The observed greater diversity could account for an older origin of the nTNL type compared to the TNLs, as proposed previously by Cannon et al. (49) and Meyers et al. (18). Nevertheless, the co-occurrence of TNLs and nTNLs in the bryophyte *P. patens* (25, 28), a representative of one of the most ancient land plant lineages, suggests that both NLR groups appeared in the very early history of land plants (**Figure 1**).

ABSENCE OF TNLs IN SEVERAL PLANT SPECIES

Although the origins of TNLs and nTNLs seems to date back to very early land plant lineages, TNLs are known to be absent from monocots [Ref. (25, 50); **Table 1**]. To examine whether the other plant lineages also lack TNLs, Tarr and Alexander (31) retrieved NB-ARC sequences by using degenerate PCR combined with published datasets from diverse plant lineages, since sequences of a motif within the NB-ARC domain can be used to discriminate TNLs and nTNLs (25, 48). This study suggested the presence of TNLs in basal angiosperms and gymnosperms, whereas TNLs seem to be rare in magnoliids (**Figure 1**). In agreement with previous studies, no typical TNLs have been found in monocot species representing three monocot orders (31), supporting the idea that this type of NLR was lost in monocots.

TNLs are also absent from several basal eudicot families/species, such as the Lamiales, the Ranunculacea *Aquilegia coerulea* (51), and the core eudicot *Beta vulgaris* (52). Interestingly, *NRG1* (N Requirement Gene 1) genes encoding members of an atypical CNL group also appear to be absent from the plant species lacking TNLs (51). This intriguing correlation suggests a functional link between NRG1 family and TNLs (51). NRG1 was originally identified with a functional screening of immune components required for the function of N, a TNL (53). It was shown that the ADR1 (Activated Disease Resistance Gene 1) family, a very close homolog of NRG1 family, potentiates salicylic acid signaling pathway (54, 55). Since immunity mediated by many TNLs is conditioned by salicylic acid signaling (56), it is possible that NRG1 has evolved as a regulator of salicylic acid signaling especially for TNL-mediated immunity.

TRACING BACK NLR FUNCTION(S) IN LAND PLANT EVOLUTIONARY HISTORY

When did plant NLRs become immune regulators?

Most of the characterized plant NLRs display a classical resistance (R) gene function consisting of mediating isolate-specific effector recognition and initiating resistance responses. To date all *NLRs* classified as resistance genes belong to the angiosperms (flowering plants), summarized in Plant Resistance Gene Wiki [Ref. (57); http://prgdb.crg.eu], whereas there is no functional data available for the NLRs of other land plant taxa including gymnosperms, ferns, and bryophytes. This might be due to the lack of appropriate pathosystems that allow testing NLR functions in non-angiosperm plants. However, a few studies suggest a link between NLRs and biotic stresses in non-angiosperm plants. For example, a NB-ARC-containing gene of *P. patens* is upregulated upon abscisic acid treatment (58). In higher land plant species, this phytohormone acts in both abiotic and biotic stresses (59). It was also reported that some gymnosperm *NLRs* are differentially regulated upon interaction with microorganisms (30, 60, 61). Although these data are indicative of relatively early occurrence of NLR function in disease resistance in plant lineages, it is necessary to validate immune functions of those genes with appropriate host/pathogen systems.

"Atypical" NLR functions

Recent studies have revealed a role for NLRs apart from the classical R gene function. These "atypical" functions include the conditioning of broad-spectrum resistance, regulatory roles in abiotic stresses, or the role as "helper" NLR for other NLRs.

Among the *NLRs* conferring broad-spectrum resistance, Rice *Panicle blast 1* (*Pb1*) represents a well-characterized example. *Pb1* encodes a CNL (62). Pb1 confers resistance to a broad range of *Magnaporthe grisea* isolates, which contrasts with the isolate-specific resistance mediated by R genes described before. Due to its degenerate domain structure and isolate unspecific resistance phenotype, the immune mechanism mediated by Pb1 is thought to differ from the other "canonical" NLRs (62). It was recently demonstrated that Pb1 physically associates with a transcription factor, OsWRKY45, which is an essential component of the response against *M. grisea* and a prominent regulator of signaling of an important defense phytohormone, salicylic acid, in rice (63, 64). Interestingly, this physical association elevates OsWRKY45 protein amount presumably by preventing the protein degradation from an ubiquitin proteasome system (63). In addition, the successful transfer from maize to rice of Rxo1, a NLR conferring broad-spectrum resistance, suggests that the underlying resistance mechanism seems to be shared among distantly related monocotyledonous species (65).

Arabidopsis ADR1 family (ADR1, ADR1-like1, ADR1-like2) belongs to the RPW8-type of CNLs and is exceptionally conserved among various plant species including monocotyledonous and eudicotyledonous plant species (51). Because of such a high degree of conservation, much attention has been paid to this family, which might represent a conserved and potentially ancestral function. Constitutive expression of ADR1 in Arabidopsis confers drought tolerance (66, 67), indicative of its complex function beyond innate immunity.

Several NLRs are required for the functions of other NLRs. ADR1 family members are also required for PTI and ETI mediated by a distinct set of NLRs, which are dependent on salicylic acid signaling for full immune response (54). Consistent with the immune responses conferred by those NLRs, the ADR1 family is involved in a feedback amplification loop of salicylic acid signaling and its biosynthesis, cooperating with EDS1, an important immune regulator (54, 55). Another example for a helper function

of NLRs is tomato NRC1. NRC1 is required for the immunity conferred by Cf-4, a non-NLR R protein. Silencing of *NRC1* in *N. benthamiana* impairs the hypersensitive response mediated by several other R proteins including two NLRs, Rx, and Mi (68). Because such "helper" NLRs are required for the functions of other NLRs, they might be involved in relaying the signal downstream of the respective innate immune sensors besides a role in defense-phytohormone pathways.

NLR GENE ORGANIZATION AND DYNAMICS IN THE GENOME

NLR repertoires are qualitatively and quantitatively varied among plant species (**Table 1**). This reflects a rapid evolution of the *NLR* family. Here we summarize insights into genomic organization and diversification of plant *NLRs*.

NLRs MAINLY OCCUR IN CLUSTERS

NLRs are distributed unevenly in the genome and show a clear tendency for clustering (18, 19, 39, 41, 69). The size of clusters is rather variable, and the largest clusters contain over 10 *NLRs* in some species (19, 39). In japonica rice, the chromosome 11 alone encodes about a quarter (133 *NLRs*) of total *NLRs* (19). Overall in the rice genome, 51% of the *NLRs* reside in 44 clusters. The proportion of singletons of rice *NLRs* (24.1%) is close to that of *A. thaliana* (26.8%) (18). A similar tendency was observed in *M. truncatula* in which 49.5% of *NLRs* belong to clusters, each comprising of at least 3 *NLRs*, and 39% of *NLRs* belong to two pseudo-clusters on chromosome 3 and 6 if clustering criteria are somewhat relaxed (39). As a comparison, the human genome possesses 22–25 *NLRs* and more than 50% belong to a cluster (70). For example, 14 *NLRs* forming the *NLRP* (Nucleotide-binding oligomerization domain, leucine-rich Repeat, and Pyrin domain containing) family are present on two clusters on chromosome 11 and 19 (71). *NLRP* clusters were also found in mouse (*Mus musculus*), dog (*Canis familiaris*), and cattle (*Bos taurus*) genomes (72, 73). Therefore, clustering is a feature shared by both plant and mammalian *NLRs*.

NLR clusters can be divided into two types depending on the contents of NLRs: (i) homogenous clusters usually contain *NLRs* from the same type (TNL or CNL) (ii) heterogenous clusters contain a mixture of diverse *NLRs*. The former type of cluster is generated by tandem duplication, whereas the latter cluster type is derived from ectopic duplications, transpositions, and/or large-scale segmental duplications with subsequent local rearrangements (74). From an evolutionary perspective, clustering is considered as a reservoir of genetic variation (75). The size of the *NLR* clusters seems to positively correlate with the density of transposable elements on the same chromosome (34, 39). Therefore transposable elements might be involved in *NLR* evolution, possibly by increasing the genomic instability and the probability of recombination.

NLR GENES UNDERGO A FAST EVOLUTIONARY DIVERSIFICATION DRIVEN BY COMBINED GENOMIC REARRANGEMENTS AND POSITIVE DIVERSIFYING SELECTION

The *NLR* gene family has evolved by the conjunction of duplication, unequal crossing over, ectopic recombination, or gene conversion (19, 33, 34, 39, 42, 76, 77). In addition, evidence of positive diversifying selection, an evolutionary force that favors the accumulation of mutations, is often found in *NLRs*. These processes contributed to make the *NLR* family one of the most variable gene families in the plant genomes (15, 16). Here, we further describe *NLR* evolutionary dynamics at three different scales: (i) at a genome-wide level, (ii) at a *NLR* subfamily level, and (iii) at an intragenic level.

(i) Local- and large-scale duplication events are responsible for expansion of *NLR* repertoire, but this process is partially compensated by gene contraction mechanisms (75, 78–80). As an example, *A. thaliana* has experienced two to three times whole genome duplication events, whilst NLR-encoding genes are highly underrepresented (78). These processes result in a high gene turnover, which can continuously refresh *NLR* repertoires while limiting the total number of *NLR* genes, and are together referred to as the "birth and death" process (75). Limiting *NLR* number seems to be biologically relevant, since products of *NLR* genes can come at a fitness cost (24), whereas diversity and novelty of *NLRs* can generate and maintain a broad range of resistance specificities.

(ii) The analysis of a *NLR* subfamily containing multiple *NLR* homologs revealed distinct evolutionary patterns within family members (81). This shows that evolution can shape different homologous *NLRs* in different ways. This aspect is discussed further at the section "Distinct Evolutionary Patterns in *NLR* Genes."

(iii) Different selection mechanisms can be detected at the intragenic level, namely at regions encoding distinct NLR domains. The NB-ARC domain is generally under purifying selection, which disfavors accumulation of non-synonymous mutations, whereas positive diversifying selection is often found at region encoding the LRR domain and sometimes at the other parts of *NLR* (76, 77, 80, 82).

These mechanisms of evolution at various levels contribute to a high degree of inter- and intragenic variation of *NLRs* and account for highly species-specific *NLR* repertoires (25, 34, 76).

SPECIES-SPECIFIC EVOLUTIONARY TRAITS AND POTENTIAL LINKS TO PLANT LIFESTYLES

There are some species-specific features in *NLRs* evolution. For example, a higher *NLR* loss rate has been reported in maize compared to other monocot species (34), a higher degree of *NLR* clustering has been observed in *M. truncatula* (39), and a higher duplication and recombination frequency was found in two perennial woody species, wine grape and poplar (42). The latter result suggests that an increased frequency in duplication and recombination might compensate for the slower evolution rate due to a longer life cycle in some perennial species (42). In a similar manner, *NLRs* in the self-fertilizing species *A. thaliana* tend to evolve faster than in its outcrossing close relative *A. lyrata* (33, 76). Incompatible *NLR* gene interactions in offspring of crosses between particular plant individuals sometimes trigger an autoimmune-like response designated as hybrid necrosis (83). As the occurrence of hybrid necrosis is potentially greater in outcrossing species than in self-fertilizing species, hybrid necrosis might

strongly influence *NLR* evolution in outcrossing species. Taken together, it is tempting to speculate that some factors like life style or reproductive fashion might influence *NLR* evolutionary processes.

DISTINCT EVOLUTIONARY PATTERNS IN *NLR* GENES

The analysis of the *RGC2 NLR* family in diverse lettuce subspecies (*Lactuca* spp.) provided an interesting insight into the evolution of individual *NLR* genes (81). This study identified two distinct evolutionary patterns for *Lactuca NLRs*: "type I" is characterized by a "rapid innovative" mode of evolution consisting of frequent sequence exchanges with other *NLR* loci and diversifying selection, in contrast to "type II" characterized by a "conservative" mode with infrequent sequence exchange and purifying selection. This observation was also confirmed in other species, suggesting that these two mechanisms drive the evolution of a majority of plant *NLRs*. Comparison of *A. thaliana NLR* repertoire with the one of its close relatives *A. lyrata* revealed again these two types of evolutionary patterns, with the type II found in a minority of *NLRs* (<30%) present as singletons or with low copy number variation and the type I found in *NLRs* from multigenic families or clusters (33, 76). Indeed, there is a positive correlation between gene copy number and sequence exchange frequency, and similarly between cluster size and sequence exchange frequency (33, 76). This partially explains why genes in multigenic families or in clusters are more prone to diversification and why singletons are likely to remain as singletons.

Additionally, some differences might exist in the evolutionary pattern depending on the *NLR* type considered (*TNLs* or *CNLs*) although these do not show clear common trends (42, 76). For example, *TNLs* are characterized by a higher number of introns while *CNLs* are often encoded by single exons (18). Introns might give more flexibility in the recombination events. *TNLs* are therefore more prone to structure diversification via domain reshuffling.

STRUCTURAL DIVERSITY OF PLANT NLRs: AN IMPLICATION FOR THEIR DIVERSIFIED FUNCTIONS?

Beside NLRs with the conventional structure like CNL or TNL, plant genomes encode a significant number of NLRs and NLR-like proteins displaying unconventional domain composition and/or atypical domain arrangements (18, 25, 28, 39, 42). In the following paragraphs we will review the structural diversity of NLRs and NLR-like proteins in various plant species.

The "Rosetta Stone Hypothesis" proposes that when two proteins that are separate in some species are fused in another species, their fusion likely reflects a previously hidden interaction between the two seemingly non-related proteins (84). Arabidopsis RRS1 is a TNL which contains an additional WRKY domain (85). Consistent with the "Rosetta Stone Hypothesis," a functional and physical interaction between a NLR and a WRKY transcription factor has been demonstrated in barley (86). Furthermore co-expression of individual NLR domains (i.e., N-terminal, NB-ARC, and LRR domains) can often reconstitute the full-length protein function (87–89). This suggests that the domains found in NLRs were originally separated and have then been assembled into a single multi-domain receptor during evolution. Based on the "Rosetta Stone Hypothesis," comparison of domain structures among NLRs and NLR-relatives in various land plants and their ancestral taxa might help to detect hidden (immune) components and mechanisms constructing NLR functions.

TANDEM ASSEMBLY OF NLR DOMAINS

In contrast to the "typical" domain arrangements such as TNL and CNL [TIR (T), CC (C), NB-ARC (N), and LRR (L)], many "atypical" domain arrangements of plant NLRs have been reported. Some examples are TNTNL and TTNL in Arabidopsis (18), TNLT, TTNL, TNTNL, and NTNL in *M. truncatula* (39), TNLT, TNLN, TNLTN, TNLTNL, CNNL, CNLNL, and TCNL (a possible mixture of TNL and CNL) in wine grape and TNLT, TNLN, TNLTN, CNNL, TCNL in poplar (42, 90). The functional analysis of RPP2a (a TNTNL) suggests that these atypical NLRs can indeed function in disease resistance and are not just inactive chimeras (18, 91).

Tandem assemblies of the same domains are reminiscent of homotypic dimerization (oligomerization) that have been reported for several plant NLRs (89, 92–95). Apart from the TCNL arrangement with yet unidentified functions found in poplar and wine grape (42, 90), chimeras between CNLs and TNLs appear to be rare. On one hand, this might result from infrequent recombination events between *CNLs* and *TNLs* or from negative selection acting on the resulting chimeras. On the other hand, it might suggest that physical interaction between CNLs and TNLs is not functionally relevant. At least, the CNLs in monocots and some other particular plant species can function in the absence of TNLs (see Absence of TNLs in Several Plant Species). However a paradox would be the fact that some TNL functions are dependent on ADR1 family and also likely on NRG1 which both belong to the CNL type of NLRs (53, 54). Thus there might be a molecular constraint that makes fusion of two types of NLRs difficult. Alternatively, functions of TNLs might not require direct interactions with ADR1/NRG1 family.

"TRUNCATED" FORMS OF NLRs

NLRs are modular proteins and therefore the reverse implication of the "Rosetta Stone Hypothesis" would suggest that separated modules or "truncated" versions of NLR could still be functional proteins. Below we discuss the phylogenetic and functional analyses, which support this hypothesis.

The genome-wide survey of Arabidopsis genes encoding either TIR- or NB-ARC-LRR-containing proteins has revealed that a significant proportion (∼28%) of those proteins are truncated forms of NLR (18). These truncated forms lack either an N-terminal domain, or the C-terminal region including the LRR with a variable part of the NB-ARC domain. *A. thaliana* genome encodes 20 TNs and 27 TXs (X indicates a domain other than CC, TIR, NB-ARC, or LRR). According to the phylogenetic analysis of the TIR-encoding genes in Arabidopsis, some large families of TNs and TXs share a common origin with TNLs, but diversified independently from the TNL family (96).

Similar truncated forms were identified in numerous other plant species including gymnosperm species, wine grape, poplar, and rice (42, 96). Phylogenetic analyses suggest that some TXs and some TNs might have orthologs in other species (42, 96, 97).

A particular family composed of atypical XTNXs was identified in Arabidopsis. BLAST searches revealed 35 homologs for these XTNXs in rice, grape, soybean, poplar, sorghum, physcomitrella, castor bean, maize, cassava, cucumis, papaya, and mimulus. These homologs have a high identity percentage. Therefore, this XTNX family seems to be highly conserved among land plants, including monocots, basal angiosperms, and magnoliids (98).

Although the function of these TN, TX, and XTNX proteins remains unclear, their diversification and conservation would suggest that at least some of these proteins do have important functions. Yet some studies on Arabidopsis TXs and TNs suggest possible roles in immunity and beyond. Arabidopsis *CHS1* encodes a TN protein which confers cold resistance by limiting chloroplast damage and cell death at low temperature. CHS1 function is achieved by regulating a PAD4-EDS1-dependent and SA-independent resistance pathway like many other TNLs (99). In several cases like CHS1, TNs appear to lack a functional NB-ARC domain (96, 99). A systematic overexpression analysis of Arabidopsis TXs and TNs in tobacco or Arabidopsis suggests that at least some TXs and TNs might function in disease resistance (98). Interestingly some TNs and TXs were shown to interact with other NLRs and/or pathogen effectors in yeast-two-hybrid assay (98).

Arabidopsis RPW8.1 and RPW8.2 (named together RPW8) possess a putative N-terminal transmembrane domain and a CC motif. This CC motif displays a high similarity with the CC found at the N-termini of a group of CNLs, sometimes referred to as RPW8-type CNLs (51, 100). RPW8 confers broad-spectrum powdery mildew resistance in Arabidopsis. RPW8 requires the phytohormone salicylic acid, EDS1, NPR1, and PAD4 for its function, suggesting that RPW8 signaling might integrate downstream components required for TNLs or basal immunity (101). RPW8 probably does not represent an ancestral function of NLRs, since RPW8 has evolved recently in Arabidopsis (102). As mentioned before, RPW8-type CNLs include ADR1 family which also displays atypical functions in and beyond innate immunity (51, 66, 67).

Truncated NLR forms can be produced by alternative splicing of full-length *NLR* transcripts. This phenomenon has already been described for diverse NLRs like L6 and N (103, 104), and those variants appear to be required for fine-tuning of the function of those NLRs (105). *RLM3* predominantly encodes a TX protein due to alternative splicing. The truncated RLM3 confers broad-spectrum resistance to necrotrophic fungal pathogens, a pathogen type that kills its host to acquire nutrients (106). Therefore, RLM3 exemplifies that, in some cases, the truncated form can be the active form.

VARIABILITY AT THE CENTRAL NB-ARC DOMAIN: NLRs LACKING A CONVENTIONAL NUCLEOTIDE-BINDING MOTIF

Binding of ADP/ATP at the central domain (i.e., NB-ARC domain) is pivotal for plant NLR function. It has been proposed that perception of the cognate effector induces an initial conformational change of the receptor, leading to an exchange of ADP by ATP at the NB-ARC domain. The ATP binding is expected to induce subsequent conformational changes of the NLR for signal initiation (9). This model is drawn by an auto-active phenotype and loss-of-function phenotype of plant NLRs carrying non-ATP-hydrolyzing mutations and non-ATP/ADP-binding mutations at the NB-ARC domain, respectively (9, 107). However, it becomes evident that several plant NLRs confer pathogen resistance without the conventional nucleotide-binding motif (i.e., P-loop motif). For example, Rice *Pb1* encodes an unconventional NLR protein that contains two N-terminal CC domains (with a degenerate EDVID-motif) and a degenerate NB domain that completely lacks the P-loop motif (62). Interestingly, many of the NLR or NLR-like proteins which do not require a functional NB-ARC domain have non-canonical functions. For example, Pb1 confers broad-spectrum resistance to rice blast (62). The ADR1 family, as described earlier in this review, seems to have a regulatory role in biotic and abiotic stress signaling (51, 54, 55).

Altogether, these data suggest that a subset of NLRs might use an unconventional activation mechanism. Some of them also have an atypical function, suggesting that along the diversification process, some functional innovations might have arisen in these NLR families.

ATYPICAL DOMAINS FOUND IN THE NLR STRUCTURE

The study of NLRs and NLR-like proteins in various plant species has revealed that some NLRs consist of domain combinations different from the classical TNL or CNL structures. Other additional domains and other N-terminal domains have been reported. We believe that these findings might help uncovering hidden interactions and mechanisms involved in NLR function.

In the indirect recognition mode, the NLR detects effector-induced modifications of a plant protein, which is designated as "guardee," a protein targeted by an effector, or "decoy," a protein that mimics the target of an effector but does not have a clear biological function. It has been reported that different NLRs could monitor a guardee/decoy to detect different effector activities when effectors target the same guardee/decoy (5–8). In light of the "Rosetta stone hypothesis," it seems plausible that a fusion event has occurred between the NLR and its cognate decoy or guardee protein. Rice RGA5 can directly bind its two cognate effectors via a non-LRR C-terminal domain. The corresponding 70 amino acids have features like a heavy metal-associated domain related to the yeast copper binding protein ATX1 (RATX1 domain) (10). Therefore RGA5 might illustrate such a fusion event between NLR and its cognate decoy or guardee. A similar RATX1 domain was found in the N-terminal domain of rice Pik-1, where it also likely contributes to effector binding (108). Therefore additional domains fused to the core NLR structure might contribute to different functions (effector recognition, NLR regulation, downstream signaling), independent of their position in the NLR backbone.

A mutation in the WRKY domain of RRS1 impairs DNA-binding and induces constitutive defense activation (109). Interestingly, the CNL MLA interacts with WRKY1/2 which also act as negative regulators of disease resistance (86). However OsWRKY45 interacting with Pb1 is a positive regulator of the Pb1-mediated immunity (63). These examples suggest diverse roles of WRKY transcription factors in plant NLR functions.

A negative regulatory role was found for the C-terminal LIM domain (named after Lin11, Isl-1, and Mec-3) of CHS3/DAR4 (110, 111). Other domains or structures have been identified at the C-terminus of some NLRs, like the Zn-metallopeptidase domain

(18) or the Exo70 subunit of exocyst complex (112), but their functions remain unknown.

The N-terminal part of NLRs is typically considered as a signaling module, although it sometimes also contributes to effector recognition (1, 113), because expression of the N-terminal TIR or CC domain alone is able to trigger host cell death (51, 94, 95, 114). A variety of N-terminal domains other than TIR or CC have been identified, which are often restricted to certain taxa. CNLs in *Solanaceae* often possess an extended N-terminus. This extended N-terminus frequently contains a homologous domain, called the solanaceae domain (SD) (115). The SD domain is present in Mi-1.1, Mi-1.2, Rpi-blb2, Hero, and Prf (116). The SD domain does not resemble any known protein motif therefore its function is difficult to predict. A function of the SD domain was reported in Mi-1.2. In this case, different parts of the SD domain act as either positive or negative regulator of Mi-1.2 function (116).

More interestingly, some atypical N-termini show similarities to known structures: 6 NLRs of *P. patens* have a protein kinase (PK) domain [Ref. (28, 117); **Figure 1**], several NLRs of *Marchantia polymorpha* have a α/β-hydrolase domain (28), 37 NLRs of *Populus trichocarpa* have a BED-DNA-binding zinc-finger domain (42, 90). A similar zinc-finger, DNA-binding domain was found in Xa1 and in two other rice NLRs (97). The most striking example might be WRKY19/MEKK4 in *A. thaliana,* which consists of a TNL fused with a WRKY domain at its N-terminus and a MAPKKK domain at its C-terminus (WRKY-TNL-MAPKKK) (18). In addition to the known interaction between WRKYs and NLRs, these fusion events are also consistent with the reported MAPK cascade requirement for NLR function (118). Unfortunately, apart from Xa1, these atypical NLRs have not been functionally characterized (97). PK, MAPKKK, α/β-hydrolase, BED, and WRKY might represent some modules required for NLR function, either in *cis* or in *trans*. Future studies will be needed to confirm the functional link between NLR function and these modules. So far, the BED-NLRs of *P. patens* are reminiscent of the interaction of Prf with Pto kinase in tomato (115). The presence of BED and WRKY domains also suggests a possible direct role of some NLRs in transcription regulation.

CONSERVATION OF NLR-MEDIATED IMMUNITY IN PLANTS

In addition to the aforementioned mechanisms, plant-pathogen arms race also accounts for highly species-specific *NLR* repertoires. Pathogens have evolved effectors either to increase virulence or to escape detection by the cognate NLR; in turn, plants further evolved NLRs to detect the novel effectors (119). These iterative cycles of effector and receptor adaptations drive co-evolution of many plant NLRs with pathogen effectors, thereby driving species-specific evolution of each NLR-mediated innate immune mechanisms (1). Since interfamily transfer of *NLRs* previously failed to produce stable transgenic plants with expected disease resistance, the proposed restricted taxonomic functionality of individual NLRs has been considered as a major barrier to explore *NLR* genes in unrelated plant species (120). Interfamily transfer of NLR function was shown in a few cases by co-expression of an NLR, its cognate effector and the effector target (121). However, these data are often based on transient gene expression with strong promoters and use host cell death as proxy for NLR activity. Since NLR-mediated host cell death responses can be uncoupled from NLR-mediated pathogen growth restriction in several cases (1, 122), it was unclear if plant NLRs also confer disease resistance in stable transgenic plants in phylogenetically distant species.

Recently it was shown that a subset of plant NLRs confers disease resistance across different taxonomic classes (123, 124). Our group demonstrated that a CNL designated as MLA1 (Mildew A 1) from the monocotyledonous plant barley (*Hordeum vulgare,* Poaceae) functions in the eudicot plant thale cress (*A. thaliana*: Brassicaceae) against barley powdery mildew *Blumeria graminis* f. sp. *hordei* (*Bgh*) (123). The MLA1-triggered immunity including host cell death response and disease resistance is fully retained in Arabidopsis mutant plants that are simultaneously impaired in the well-characterized defense-phytohormone pathways (ethylene, jasmonic acid, and salicylic acid). These data suggest the existence of an evolutionarily conserved and phytohormone-independent CNL-mediated immune mechanism. Similar to MLA1, co-acting Arabidopsis TNL pair, RPS4 (Resistance to *Pseudomonas Syringae 4*) and RRS1 (Resistance to *Ralstonia Solanacearum* 1) also confers resistance in cucumber (Cucurbitaceae), *N. benthamiana*, and tomato (Solanaceae) (124). Additionally the Arabidopsis *RPW8.1* and *RPW8.2* encoding truncated CNL-like proteins, confer resistance to powdery mildews in *N. tabacum* and *N. benthamiana* as in Arabidopsis (125). These results strongly imply that a subset of plant NLRs, despite their evolutionary separation, still follows a common principle in innate immunity.

Large-scale yeast-two-hybrid assays revealed that independently evolved effectors from different pathogen kingdoms (Gram-negative bacterium *Pseudomonas syringae* and obligate biotrophic oomycete *Hyaloperonospora arabidopsidis*) physically associate with the same host (Arabidopsis) proteins positioning at intersections of the host protein interaction network (126). Those proteins are designated "cellular hubs" and most of the tested hubs exhibit immune functions (126). Since the pair of RRS1-RPS4 detects three independently evolved effectors from different pathogen species (127), RRS1-RPS4 might monitor modification of a cellular hub targeted by three different effectors, enabling indirect detection. In this case, the expected cellular hub should be conserved in cucumber, *N. benthamiana,* tomato, and Arabidopsis. Indeed, such a conserved protein, EDS1, has been shown to be the target of two unrelated Pseudomonas effectors, suggesting that EDS1 might be a cellular hub guarded by RRS1-RPS4 (128, 129). Alternatively, RRS1-RPS4 might detect three cognate effectors by direct interaction as demonstrated with the co-acting rice RGA4-RGA5 (R-gene analog 4 and 5) pair, of which RGA5 physically interacts with two sequence-unrelated effectors of the rice blast fungus, *Magnaporthe oryzae* (10). At least for MLA, domain swap experiments between different MLA receptors that detect genetically diverse *Bgh* effectors, imply that recognition specificity is determined by the LRR domain (130). In addition, sequence comparison of ∼20 different MLA receptors possessing different recognition specificities revealed that diversified selection sites are predominately accumulated at the surface of the concave side of a hypothetical model of the MLA LRR structure, indicative of a direct receptor-effector interaction at the LRR domain (82, 123). Although two cognate effectors for RRS1-RPS4 have been isolated from *Pseudomonas syringae* and *Ralstonia solanacearum*,

the effector of *Colletotrichum higginsianum* remains to be isolated (124, 131, 132). In addition, the cognate effector for MLA1 has not been isolated, yet. To examine how RRS1-RPS4 and MLA1 detect the cognate effectors (i.e., indirect or direct) in their native plant species and heterologous species will most likely require the identification of these effectors.

The existence of evolutionarily conserved immune mechanisms, especially downstream signaling mechanisms mediated by plant NLRs prompts a new question: how could a "conserved mechanism" have been retained during evolution despite the presumed emergence of pathogen counter arsenals that intercept this conserved signaling? It is unlikely that plant NLRs rely on a single conserved immune signaling pathway, which could be easily disarmed by pathogens. In an attempt to solve this paradox, we proposed that a single NLR could mediate immune responses via multiple signaling pathways (123), since it is difficult for pathogens to evolve an effector which simultaneously hampers multiple signaling pathways. Plants deploy NLRs at various sub-cellular locations for perception of effectors and/or initiation of immune signaling (see the review by Qi and Innes in the same issue). Thus it is tempting to speculate that entry nodes for NLR-signaling might exist at various sub-cellular locations in plants. Existence of multiple immune targets downstream of a single plant NLR (i.e., entry nodes for signaling pathways) would contribute to the robustness against rapidly evolving pathogens. This might also contribute to the conservation of plant NLR-signaling mechanism across plant species (123, 124), since a "foreign" NLR transferred with transgenic technology could have higher chances to find an entry node for downstream signaling in different plant species. Collectively, NLRs can be exploited for disease resistance breeding in a much wider range of plant species than previously thought.

HIJACKING OF PLANT NLR-MEDIATED IMMUNITY BY PATHOGENS

Transferring NLRs into different plant species might be a causal agent of unexpected disease, since some pathogens hijack plant NLR-mediated immunity for their proliferation. Based on nutrition modes, plant pathogens are classified into biotrophs, necrotrophs, and their intermediate, hemibiotrophs (133, 134). Biotrophic pathogens rely on living host cells for nutrition, whereas necrotrophic pathogens actively kill host cells to acquire nutrients. Hemibiotrophic pathogens are initially biotrophic and shift later to necrotrophy. Similar to biotrophic pathogens, many necrotrophic pathogens have a narrow host range infecting only one or few related plant species [summarized in Ref. (134)]. In addition to lytic enzymes and secondary metabolites, necrotrophic pathogens secrete toxins, which function as effectors to promote host cell death response. These toxins are often host-plant species-specific, thus called host-selective toxins and mediate effector-triggered susceptibility (ETS), which mirrors ETI to some extent (134).

It has been implicated that susceptibility to necrotrophic pathogens or sensitivity to their host-selective toxins is associated with *NLR* loci in diverse plant species such as Arabidopsis (135), sorghum (136), and wheat (32). These NLRs are likely maintained for resistance to other pathogens but targeted by virulent necrotrophs (137, 138). The ETS caused by the pathogenic fungus *Cochliobolus victoriae* in Arabidopsis is conditioned by a CNL, LOV1 (Locus orchestrating victorin effects 1). LOV1 is activated upon direct binding of its cognate toxin, called victorin, to a host thioredoxin related to immunity (138). Since Arabidopsis, barley, bean, Brachypodium, oats, and rice are sensitive to victorin (137, 138), the underlying principle for victorin sensitivity is expected to be conserved across plant species. However it is likely that different NLRs other than LOV1 homologs monitor the victorin action in the respective plant species, since analysis of cereal DNA databases failed to detect obvious *LOV1*-like genes (137).

Resistance to host specific necrotrophs is mediated by PTI, detoxification of toxins, loss of toxin recognitions, or restricting toxin-mediated cell death response (139). Plant NLRs seem to play minor roles in resistance to necrotrophic pathogens. However Arabidopsis *RLM3* locus, which encodes a truncated TNL lacking NB and LRR domains, confers resistance to a broad range of necrotrophs by unknown mechanisms (106).

NLR-mediated susceptibility is also observed in animal-pathogen interactions. In mouse, an NLR designated NOD2 (nucleotide-binding oligomerization domain-containing protein 2) mediates susceptibility to *Yersinia pseudotuberculosis*, a gut-living bacterial pathogen that disrupts the interstitial barrier to invade host cells (140). Similar to plant pathogens, *Y. pseudotuberculosis* delivers a set of effectors through the type III secretion system for virulence. Among the effectors, YopJ, an acetyl-transferase, mediates the intestinal barrier dysfunction by redirecting NOD2 signaling. YopJ acetylates RICK (Rip-like interacting caspase-like apoptosis-regulatory PK), an immediate downstream target of NOD2, resulting in reduced binding affinity of RICK to NOD2. As a consequence, NOD2 is able to form a complex with caspase-1 other than RICK, resulting in higher IL-1β production. This appears to increase the intestinal permeability for the bacterial invasion (140). Consistently, Crohn's disease-associated NOD2 mutations found in ∼20% of healthy white individuals are likely maintained to protect the host from systemic infection by common enteric bacteria (141). Similar to *Y. pseudotuberculosis*, *Salmonella enterica* subspecies trigger host immune responses (i.e., inflammation) to obtain a niche in the already established gut microbial community (142), suggesting that induction of inflammatory responses might be a common strategy for pathogenesis of enteric bacteria.

Thus host immune response is sometimes beneficial for pathogens in plants and animals. Plant pathogens might also exploit host immune mechanisms to compete with host associating microorganisms. Plants and animals deploy an array of NLRs to fight against pathogens, whilst deployment of NLRs must be tightly balanced. Otherwise, these could be exploited by pathogens. Such a constraint might also contribute to shaping the current repertoires of NLRs in plants and animals.

PLANT NLRs REGULATING TRANSCRIPTION

Apart from the host cell death response, NLR action is often associated with transcriptional changes. Here we review the emerging picture how NLRs actively participate in transcriptional regulation in plants.

It has been shown that transcriptional differences in resistant vs. susceptible interactions are rather quantitative than qualitative in several cases. This implies that NLRs amplify or sustain defense-related gene expression mediated by pattern-recognition receptors

(123, 143–147). Transcriptome analysis comparing gene expression mediated by a TNL and a CNL, each recognizing different effectors from the same pathogen, identified a common set of target genes. This indicates that the underlying mechanism for transcriptional regulation might be shared by both types of NLRs (148). Recent studies start to unravel how NLR action is converted to transcriptional reprograming.

Recognition of the cognate effectors by plasma membrane-associated CNLs RPS2 (Resistance to *Pseudomonas Syringae* 2) and RPM1 (Resistance to *Pseudomonas Syringae* pv *Maculicola* 1) results in transcriptional reprograming (144, 149), indicating a mechanism that relays signals from the plasma membrane to the nucleus. To uncouple ETI from PTI with a synchronized homogeneous cell population, Gao et al. (150) used an Arabidopsis protoplast system, in which the cognate effectors for RPS2 or RPM1 are expressed under an inducible promoter. Genome-wide transcriptome analysis with the protoplast system identified *WRKY46* as an early marker gene shared in RPS2- and RPM1-mediated signaling. Since chemical inhibitors affecting various Ca^{2+} channels suppressed the effector-mediated *WRKY46* promoter activation, potential involvement of Ca^{2+}-dependent protein kinases (CPKs) were examined. A genetic and biochemical screen identified a group of Ca^{2+}-dependent PKs (CPK 4, 5, 6, and 11), acting as signaling mediators between the NLRs and the transcription factors WRKY8, WRKY28, and WRKY48. Those WRKYs are proposed to regulate gene expression downstream of RPS2 and RPM1. Notably, another group of CPKs (CPK1 and 2) appears to be involved in host cell death response rather than transcriptional reprograming, suggesting the existence of a bifurcated CPK-dependent signaling pathway mediating distinctive NLR-triggered immunity outputs (i.e., cell death and transcriptional reprograming). However, it still remains unclear how RPS2 and RPM1 activate the set of CPKs. So far, a direct interaction between the CPKs and RPS2 or RPM1 was not detected (150). Potential players in the RPS2 or RPM1-CPK signaling cascade might be CNGCs (cyclic nucleotide-gated ion channels), a family of putative Ca^{2+} channels, some of which are involved in plant immunity (151, 152). However, the mechanistic link between NLRs and CNGCs remains unknown.

Signaling relay via a mediator such as CPK might be one mechanism by which membrane-associated NLRs regulate transcriptional reprograming. However, recent work indicates that some soluble NLRs participate in an even shorter signaling pathway. Localization into the nucleus has been shown for several NLRs. When excluded from the nucleus by fusion with a nuclear exclusion signal, immunity mediated by the nucleo-cytoplasmic barley MLA10 (CNL) is compromised (86). Similarly, nuclear exclusion of the nucleo-cytoplasmic N (TNL) resulted in compromised immunity in *N. benthamiana* (153). Disruption of the nuclear localization sequence of Arabidopsis RPS4 (TNL) resulted in impaired immunity toward *Pst* DC3000 expressing its cognate effector (154). Together, these data point toward a nuclear function of a subset of NLRs.

Recent studies have started to elucidate the activity of nuclear-localizing NLRs. Following up on the demonstration that barley MLA10 interacts with HvWRKY1 and HvWRKY2, negative regulators of immunity, Chang et al. (155) elucidated the mechanism by which this interaction results in immunity. They demonstrated that the CC domain of barley MLA10 interacts not only with the aforementioned repressors but also with the transcriptional factor HvMYB6, a positive regulator of immunity. Strikingly, only the active form of MLA10 is able to bind HvMYB6, which is sequestered by HvWRKY1 in the absence of the activated MLA10. The interaction through the MLA CC domain prevents WRKY1 from interacting with HvMYB6, thereby allowing HvMYB6 binding to the corresponding *cis*-element. The MLA10-HvMYB6 complex, in turn, greatly enhances transcription downstream of the *cis*-element compared to HvMYB6 alone in a transient assay. While this interaction greatly adds to our understanding of MLA function in barley, it cannot explain the conserved function of MLA1 in Arabidopsis (123), since HvMYB6 is a highly monocot-specific transcription factor (155).

Pb1, a rice CNL, has also recently been shown to interact with the transcription factor OsWRKY45, likely leading to transcriptional reprograming. However, in contrast to the MLA-HvMYB6 interaction, the transcriptional activity is regulated via OsWRKY45 abundance, since Pb1 protects OsWRKY45 from degradation upon pathogen attack (63).

A third example aiding in our understanding of NLR nuclear activity is the interaction of N with the transcription factor SPL6 (SQUAMOSA PROMOTER BINDING PROTEIN-LIKE 6) in *N. benthamiana* (156). The association of N and SPL6 at subnuclear bodies occurs only in the presence of the cognate effector. A genetic requirement for SPL6 was shown in *N. benthamiana* for N-mediated disease resistance as well as in *A. thaliana* for RPS4-mediated immunity. A number of RPS4-mediated defense responsive genes are differentially regulated upon *AtSPL6* silencing (156).

Close re-examination of yeast-two-hybrid data generated by Mukhtar et al. (126) provides further support of NLR-transcription factor interaction as a more common mechanism of NLR actions. Mukhtar et al. (126) tested interactions using as bait N-terminal domains of Arabidopsis CNLs and TNLs, which have previously been demonstrated to function as minimal signaling domains in some cases (94, 95), and as prey full-length constructs of ∼8,000 immune-related genes including transcriptional regulators. Strikingly, of those NLRs showing interactions, the majority interacted with one or more transcriptional regulators. Furthermore, these interactions could be found for both CNLs and TNLs. Interaction between transcriptional regulators and NLRs has already been demonstrated too, for example the interaction of the transcriptional co-repressor TPR1 (Topless-related 1) with the Arabidopsis TNL SNC1 (157).

Taken together, these studies draw an emerging picture in which nuclear localized NLRs mediate transcriptional reprograming via interaction with transcription factors in various plants species. Interaction with transcriptional regulators appears not to be limited to one subclass only or to just a few specialized NLRs. Instead, this type of interactions might be a more common phenomenon, implying a possible general mechanism of direct regulation of transcriptional reprograming via plant NLRs. Transcriptional regulation via NLRs also occurs in animals. Two well documented NLRs, CIITA and NLRC5, both regulate a set of genes, MHC class I and class II genes, by recognizing specific *cis*-elements and recruiting a group of transcriptional regulators (158, 159). The protein

complex formed is known as enhanceosome (160, 161). It remains to be proven whether NLRs in plants also form such large order complexes or modulate transcription by interacting with only a few transcriptional regulators at a time.

STRUCTURAL INSIGHT INTO AUTO-INHIBITION MECHANISM OF NLRs

Very recently the first crystal structure of an NLR monomer (mouse NLRC4) in its inactive state was resolved (162). The structure revealed the presence of multiple "security locks," coordinated by several and distinctive intra-domain interactions to keep the receptor in an inactive state. These locks prevent the receptor from homo-oligomerization driven by associations through the central domain. The observed intra-domain interactions cluster in close proximity of the potential ligand-binding pocket, which is primarily shaped by the LRR domain together with the other domains (162). Thus, it is proposed that ligand-binding at the pocket could release the multiple locks all at once, enabling a subsequent conformational change of the receptor (e.g., ADP-ATP exchange, oligomerization). Interestingly, the structure and the experimental evidence suggest that ADP-binding at the P-loop motif also contributes to auto-inhibition of the receptor. However, the inhibition mechanism seems to be distinctive from that mediated by the other intra-domain interactions, since the position of ADP in the crystal is distant from the pocket (162). Unlike animal NLRs, plant NLRs lack the HD2 sub-domain (also known as ARC3 sub-domain) in the central NB-ARC domain (14), and general applicability of the central domain mediated homo-oligomerization of plant NLRs upon receptor activation is unclear.

The LRR domain of plant NLRs is also involved in forming "security locks" by cooperating with the other domains in the absence of pathogens (93, 163–166). A structure-function analysis combined with docking simulations of structural models of the NB-ARC and the LRR domains identified regions that determine intra-domain interactions in two CNLs, Rx1 and Gpa2 (166). At least in the case of these two highly homologous CNLs, the association between the N-terminal repeats of the LRR domain and a small region of the ARC2 domain are sufficient to keep these NLRs in an inactive state, whilst the rest of C-terminal repeats of the LRR domain act as the major determinant of the effector recognitions (166). Thus it is proposed that detection of the cognate effectors at the C-terminal repeats of the LRR domain disrupts the intra-domain interaction to activate the receptor (166).

CONCLUSION AND PERSPECTIVE

Over the past few decades, many NLRs and NLR-like proteins were isolated from plants and animals and their functions have been extensively studied. The development of new technologies has further accelerated research on NLR biology. For example, deep sequencing technology offers more opportunities to conduct comparative genome-wide analyses of *NLRs* in various species. Whole-transcriptome analysis at single transcript level combined with ChIP-seq analysis (chromatin immunoprecipitation followed by sequencing) allows to uncover underlying mechanisms for NLR functions in the nucleus. Furthermore, structural biology provides in-depth understanding of mechanistic insights into NLR actions. Nevertheless, a balanced combination of those technologies and "classical" genetics and biochemical studies are important to unravel the principle of NLR functions.

As we discussed above, a plant NLR might initiate downstream signaling by connecting to multiple signaling targets rather than through a single evolutionarily conserved target. Despite a lack of direct experimental evidence to date, putative compartment-specific activities of plant NLRs, particularly in the cytoplasm and nucleus (129, 167), suggest that a single NLR interacts with structurally different downstream components to initiate immune responses in different compartments. Thus, it might be possible that a second, third, or even more downstream signaling layers exist for a given NLR, including several interacting components that might constitute "as a whole" the downstream innate immune mechanism. Finally, we imagine that comprehensive knowledge of NLR actions would allow the design of synthetic NLRs in order to control pathogens and manipulate NLR functions even beyond innate immunity.

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Recent advances in plant NLR structure, function, localization, and signaling

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Nucleotide-binding domain leucine-rich repeat (NLR) proteins play a central role in the innate immune systems of plants and vertebrates. In plants, NLR proteins function as intracellular receptors that detect pathogen effector proteins directly, or indirectly by recognizing effector-induced modifications to other host proteins. NLR activation triggers a suite of defense responses associated with programed cell death (PCD). The molecular mechanisms underlying NLR activation, and how activation is translated into defense responses, have been particularly challenging to elucidate in plants. Recent reports, however, are beginning to shed some light. It is becoming clear that plant NLR proteins are targeted to diverse sub-cellular locations, likely depending on the locations where the effectors are detected. These reports also indicate that some NLRs re-localize following effector detection, while others do not, and such relocalization may reflect differences in signaling pathways. There have also been recent advances in understanding the structure of plant NLR proteins, with crystal structures now available for the N-terminal domains of two well-studied NLRs, a coiled-coil (CC) domain and a Toll-interleukin Receptor (TIR). Significant improvements in molecular modeling have enabled more informed structure-function studies, illuminating roles of intra- and inter-molecular interactions in NLR activation regulation. Several independent studies also suggest that intracellular trafficking is involved in NLR-mediated resistance. Lastly, progress is being made on identifying transcriptional regulatory complexes activated by NLRs. Current models for how plant NLR proteins are activated and how they induce defenses are discussed, with an emphasis on what remains to be determined.

Keywords: plant innate immunity, leucine-rich repeats, disease resistance, hypersensitive response, *Pseudomonas syringae*, pathogen effectors

INTRODUCTION

Plants do not have an adaptive immune system like that found in vertebrate animals. Instead, plants depend solely on an innate immune system that bears intriguing similarities to animal innate immune systems, but is likely independently evolved [see review by Jacob et al. (1)]. Plant innate immunity is a two-tier resistance system (2). The first tier consists of plasma-membrane (PM) localized pattern recognition receptors (PRRs) that mediate detection of conserved microbial molecules referred to as pathogen associated molecular patterns (PAMPs). This type of resistance is known as PAMP triggered immunity (PTI). Most plant PRRs are transmembrane receptor kinases, with the majority containing extracellular leucine-rich repeats (LRR), thus have functional and structural similarity to the Toll-like Receptors of animals. The second tier system consists of intracellular receptors that detect the presence of pathogen proteins inside the host cell. Pathogen proteins that get inside host cells are commonly referred to as effector proteins, thus this second tier is usually referred to as effector triggered immunity (ETI).

Effector triggered immunity is mostly mediated by nucleotide-binding leucine-rich repeat (NLR) proteins. Plant NLR proteins usually contain a C-terminal LRR domain and a central NB-ARC domain (nucleotide-binding adaptor shared by Apaf-1, Resistance proteins, and CED-4) (3).The NB-ARC proteins form a subclass in the STAND super family (signal transduction ATPases with numerous domains) and function as molecular switches regulating many processes, including immunity and apoptosis (4, 5). Plant NLRs are roughly divided into two groups, depending on their N-terminal structures, CNL (CC-NB-LRR) with an N-terminal coiled-coil domain and TNL (TIR-NB-LRR) with an N-terminal Toll/interleukin-1 receptor domain (TIR) (6). Plant NLR proteins recognize the presence of pathogens either directly by binding to pathogen effectors, or indirectly by sensing effector-induced modification of other host proteins. The activation of ETI usually results in localized cell death at the infection site, which is referred to as a hypersensitive response (HR). The HR is commonly used as a read-out for the activation of NLR proteins in plants. The first NLR proteins, N and RPS2, were cloned in 1994 based on their ability to confer resistance to specific diseases in plants (7–9). However, the molecular mechanisms that control NLR activation and signaling remain poorly understood.

Here, we focus on the advances made in the last 2 years toward understanding how plant NLRs are activated and how signaling

is initiated and transduced. We highlight the compartmentalization of plant NLRs, intra-/inter-molecular interactions before and after activation, and structural and genetic insights into NLR downstream signaling.

DIVERSE COMPARTMENTALIZATION OF PLANT NLRs

The activation of NLR proteins is commonly associated with significant transcriptional reprograming. Consistent with this observation, several plant NLRs have been shown to accumulate in the nucleus upon effector-induced activation (10–13)(**Figure 1A**). For example, in the presence of the cognate powdery mildew effector AvrA10, the barley CNL, MLA10, translocates into the nucleus and interacts with both WRKY transcriptional repressors and MYB6, a transcriptional activator, to activate defense responses (10, 14). Similarly, nuclear accumulation of the *Arabidopsis* TNL, RPS4, is required for RPS4-mediated resistance in the presence of its cognate effector AvrRps4 (11, 15, 16). However, a number of recent studies have demonstrated that coordinated nucleo-cytoplasmic trafficking of plant NLRs is required for the full activation of defense responses, suggesting that a single NLR protein may activate distinct signaling pathways in the cytoplasm and nucleus. For example, the RPS4 protein of *Arabidopsis*, a TNL that mediates recognition of the effector protein AvrRps4 from *P. syringae*, appears to localize to both the nucleus and cytoplasm and activate different pathways in each. Forced nuclear accumulation of AvrRps4 is sufficient to activate RPS4-mediated bacterial growth inhibition, but blocks RPS4-mediated HR (16). On the other hand, sequestration of AvrRps4 in the cytosol using a nuclear export signal significantly impairs RPS4-mediated resistance but only moderately reduces RPS4-mediated HR. Therefore, nucleo-cytoplasmic partitioning of plant NLR proteins seems to be a regulatory mechanism for differential activation of downstream signaling. These studies also point out that activation of host cell death (HR) can be separated from activation of resistance.

Not all plant NLRs require nuclear localization for activation of resistance, and in fact, this may be the exception rather than the rule. The CNL protein, Rx, which mediates recognition of the Potato Virus X coat protein (CP), localizes to both the nucleus and cytosol (17, 18). Sequestration of Rx in the nucleus impairs its function, but forced cytosolic accumulation enhances Rx function (19). Moreover, Rx is not activated in the presence of forced nuclear PVX CP accumulation (20). Taken together, these results suggest that both pathogen recognition and resistance signaling by Rx need to take place in the cytoplasm. Thus, nuclear accumulation of Rx may represent a form of negative regulation. Alternatively, but not exclusively, Rx may have to traffic to the nucleus to form a functional complex and then back to the cytosol, where it surveys the presence of the cognate virus CP.

In contrast to Rx, RPS4, and MLA10, the CNL proteins RPS5 and RPM1 from *Arabidopsis* require PM localization to function (**Figure 1B**). This requirement likely reflects the localization of the pathogen effector proteins detected by each. RPS5 detects the *P. syringae* effector AvrPphB, which is a cysteine protease that targets the protein kinase PBS1.(21). AvrPphB autoprocesses upon entry into host cells, revealing an N-terminal motif that becomes myristoylated by host cell enzymes, which then targets AvrPphB to the PM (22). RPS5 is also acylated on its N-terminus and localizes to

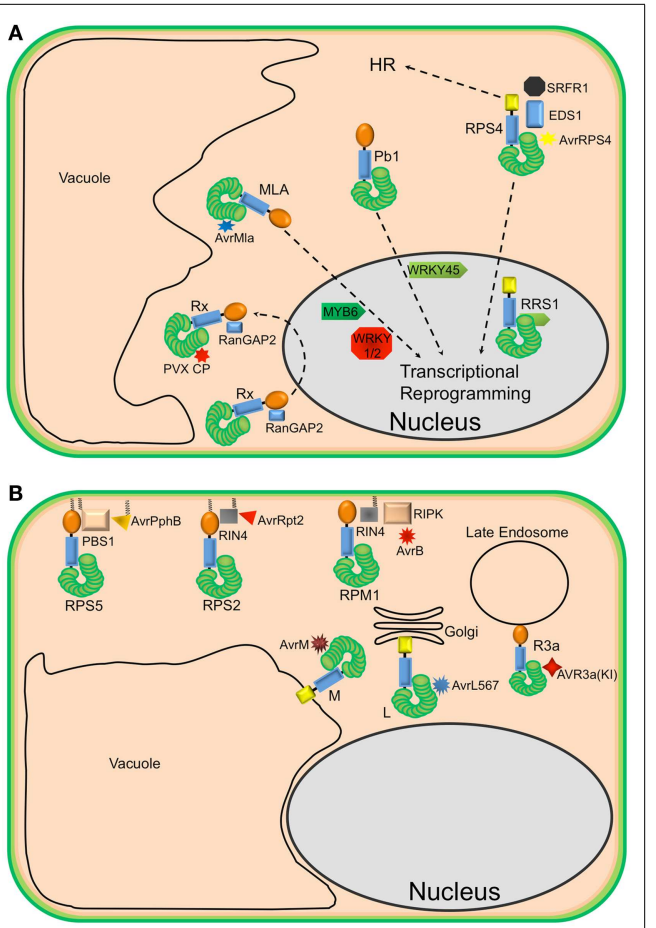

FIGURE 1 | Diverse localization of plant NLR proteins.
(A) Nuclear-localized plant NLRs. The barley MLA proteins reside in the cytoplasm but, in the presence of corresponding AvrMla effectors, translocate into the nucleus where they interact with both WRKY transcription repressors and MYB6, a transcriptional activator, to activate defense responses. Similarly, the rice CNL Pb1 also accumulates in the nucleus where it interacts with and stabilizes rice WRKY45 to activate defense responses. RPS4 also translocates into the nucleus, upon recognition of AvrRps4, to activate defense responses in conjunction with RRS1, an atypical TNL in *Arabidopsis* that contains a WRKY domain. At the same time, a subset of RPS4 complexes stays in the cytoplasm to activate HR. The potato CNL, Rx, interacts with the cytosolic Ran GTPase Activating Protein 2 (RanGAP2) and actively shuttles between the nucleus and the cytoplasm. However, the recognition of PVX CP and activation of signaling seem to occur in the cytoplasm. **(B)** Endomembrane associated plant NLRs and their corresponding "guardees" and pathogen effectors. RPS5 (an NLR), PBS1 (guardee), and AvrPphB (*P. syringae* effector) localize to the plasma membrane (PM). This is mediated by N-terminal acylation (myristoylation and/or palmitoylation). Similarly, RPS2 (an NLR) is PM-associated via a predicted N-terminal palmitoylation signal while RIN4 (guardee) localizes to the PM via a C-terminal prenylation or palmitoylation signal. RPM1 (NLR) also localizes to the PM, but lacks a predicted acylation signal. These three well-studied *Arabidopsis* NLR proteins are activated on the PM and initiate signaling on the PM. Relocalization following activation does not appear to occur. The flax rust resistance proteins L6 and M are respectively targeted to the Golgi apparatus and vacuolar membrane. Re-directing L6 to the vacuolar membrane, however, does not affect its function. The potato resistance protein, R3a, relocalizes from the cytoplasm to late endosomes in the presence of its corresponding effector AVR3a(KI), which also relocalizes to late endosomes in the presence of R3a.

the PM (23). Mutation of the predicted acylation sites of RPS5 (Glycine 2 and Cysteine 4) disrupts RPS5-mediated HR and PM localization (23). Although RPM1 does not possess predicted acylation motifs at its N-terminus, it also localizes to the PM, where its corresponding effector AvrB and co-activators RIPK and RIN4 also localize (24–26). An auto-active RPM1 mutant, T166E, also localizes to the PM, indicating that RPM1 does not move following activation. Furthermore, sequestration of RPM1 on the PM does not affect RPM1-mediated resistance (25). Together, these observations indicate that activation of RPS5 and RPM1 and subsequent signaling occurs on the PM.

Plant NLR proteins have also been localized to other endomembrane locations. For example, the flax rust resistance proteins L6 and M localize to the Golgi apparatus and the tonoplast, respectively (27). Swapping the N-terminal sequences between L6 and M swapped their localization, indicating that the localization signals reside at the N-termini of these proteins, which are predicted to function as signal anchor sequences. Signal anchors are hydrophobic N-terminal sequences that direct nascent polypeptides to the endoplasmic reticulum, but unlike signal peptides, are not removed by a signal peptidase. Interestingly, changing the localization of L6 from the Golgi to the tonoplast did not affect its ability to detect its corresponding effector protein, nor activate resistance. Deletion of the signal anchor sequence, however, destabilized L6 protein accumulation, rendering it non-functional (27). A similar loss of protein stability was observed for RPS5 when its acylation motif was mutated (23), suggesting that at least a subset of NLR proteins require membrane localization for function and stability.

Plant NLRs can also move between the cytosol and endomembrane system. The potato resistance protein, R3a, relocates from the cytoplasm to endosomal compartments in the presence of the *Phytophthora infestans* effector AVR3a(KI) but not an unrecognized derivative AVR3a(EM) (28). Moreover, AVR3a(KI), but not AVR3a(EM), relocalizes to endosomes in the presence of R3a. Treatment with inhibitors of endocytic trafficking affects both the relocalization of R3a and its function. These observations suggest that the recognition of AVR3a(KI) by R3a and signal initiation occur in endocytic vesicles.

MOLECULAR INTERACTIONS DURING PLANT NLR ACTIVATION AND SIGNALING

The LRR domains are the most polymorphic part of plant NLR proteins, which likely reflects their role in effector recognition. Direct interaction between NLR proteins and pathogen effectors has been demonstrated for only a subset of plant NLRs, however. The best characterized of these is the flax L protein, in which allele specific interactions between L and its corresponding fungal effector AvrL567 have been demonstrated for the C-terminal LRR regions using yeast-two-hybrid analysis (29, 30). More recently, race-specific interactions between the *Arabidopsis* RPP1 LRR domain and the oomycete ATR1 effector have been demonstrated using co-immunoprecipitation analysis (31). However, race-specific physical interactions have also been shown between the coiled-coil (CC) domains of a rice NLR, Pik, and corresponding Avr-Pik effectors from the fungus *Magnaporthe oryzae* (32).

In addition to its role in effector recognition, the LRR domain also plays an important role in keeping NLR proteins in the "off" state. Studies of Bs2, RPS5, and Rx have demonstrated that the LRR domain physically associates with the NB-ARC domain (33–35). Furthermore, deletion of the LRR domain typically results in auto-activation (20, 35). A recent study on RPS5 established that only the first four LRRs are required to inhibit this auto-activation (23). Auto-activation has also been reported for the potato NLR Rx when its CC-NB-ARC region was co-expressed with RanGAP2 in tobacco plants (20). Auto-activation is also frequently observed when LRR domains are swapped between NLR proteins, suggesting that the LRR and NB-ARC domains co-evolve with each other (23, 36, 37). Consistent with this hypothesis, a highly acidic loop region in the Rx ARC2 domain has been shown to associate with basic patches in the N-terminal end of the Rx LRR domain (37).

The recently solved crystal structure of the mouse NLRC4 protein [NLR family, Caspase activation and recruitment domain (CARD) containing 4] provides additional insights into the physical interactions between the NB-ARC domain and the LRR domain that function to inhibit NLR auto-activation (38). NLRC4 displays an inverted "question-mark" structure, where the N-terminal region of the horse-shoe shaped LRR interacts with the NB subdomain of the NB-ARC. This interaction sterically restricts the accessibility of the side of the NB that is required for oligomerization. Deletion of the LRR domain, or point mutations in the NB/LRR interaction surface, result in constitutive activation of NLRC4 (38). The N-terminal region of the NLRC4 LRR domain also interacts with the ARC3 subdomain (also known as the helix domain 2), with this interaction playing an important role in the overall positioning of the LRR domain relative to the NB-ARC. Plant NLRs, however, do not contain an ARC3 subdomain (39), making it difficult to predict whether the LRRs of plant NLRs will be similarly positioned.

An open question in plant NLR studies is which domain(s) of plant NLRs is/are directly responsible for downstream signaling. In mammalian cells, the NLR activation usually results in the recruitment and activation of pro-caspase-1 through homotypic interaction with the N-terminal CARD (40). This leads to the formation of inflammasomes, which is linked to pyroptosis. By analogy, it is reasonable to assume that activation of plant NLRs exposes the N-terminal domain for downstream signaling. Indeed, overexpression of the N-terminal CC or TIR domains from two plant NLRs causes effector-independent HR, supporting a signaling role (41, 42). Crystal structures of the CC domain of the barley MLA10 CNL and the TIR domain of the flax L6 TNL indicate that homodimerization is necessary for downstream signaling activity (41, 42). In both studies, mutations at the dimer interface disrupted dimerization and signaling activity. However, mutations in the BB loop and αC helix of the L6 TIR domain did not affect homodimerization, but did disable downstream signaling, indicating the presence of discrete interfaces for self-association and engaging other unidentified signaling molecules (41). In addition, the presence of the L6 NB-ARC domain inhibited the dimerization of the L6 TIR and prevented signaling.

The above studies on L6 and MLA10 provide strong support for the CC and TIR domains functioning as the sole domains engaging downstream signaling components. However, conflicting data have been obtained from studies on the potato Rx and *Arabidopsis* RPS5 proteins. In the case of Rx, overexpression of the NB subdomain alone was found to be sufficient for inducing cell death, suggesting that this domain plays a roll in engaging downstream signaling components (43). For RPS5, overexpression of the CC or NB-ARC domains alone did not induce cell death, while overexpression of a CC-NB-ARC construct did, suggesting that the two domains function together to engage downstream components (35). It is not yet clear whether these conflicting data reflect fundamental differences between NLR proteins in terms of their signaling mechanisms, or are due to differences in how the experiments were conducted (e.g., different levels of overexpression, different epitope tags, etc.).

In addition to signaling, the N-terminal domains of plant NLRs may also function in effector recognition. For example, many effector targets, such as Pto, RIN4, PBS1, and NRIP, are found to interact with the N-terminal domains of their corresponding NLRs (44). Thus, the N-terminal domains of these NLRs may be responsible for directly monitoring effector-induced modifications of these target proteins or, alternatively, place their LRR domains in appropriate proximity for optimal surveillance. As mentioned above, race-specific interactions are reported to occur between the CC domains of the rice NLR, Pik, and corresponding Avr-Pik effectors (32). Similarly, L6 and L7 from the flax L locus recognize different effectors, but their amino acid sequences differ only in the N-terminal TIR domains (45).

Although direct association with pathogen effector proteins has been documented for some plant NLRs, many appear to detect pathogen effectors indirectly via sensing effector-induced modifications of other host proteins (46, 47). As mentioned above, the *Arabidopsis* CNL RPS5 detects the presence of the cysteine protease effector AvrPphB by monitoring the integrity of PBS1. In addition, insertion of seven amino acids at the AvrPphB cleavage site of PBS1 activates RPS5 as strongly as PBS1 cleavage, suggesting that RPS5 senses subtle conformational changes in PBS1 associated with its cleavage (48). Sensing of these structural changes by RPS5 is likely mediated by the LRR domain, as partial deletions as small as one LRR abolished activation by PBS1 cleavage, but did not abolish auto-activation by mutations in the NB-ARC domain (23). As a second example of indirect recognition, the *Arabidopsis* CNL, RPM1, detects modification of the *Arabidopsis* RIN4 protein induced by the *P. syringae* effector proteins AvrB and AvrRpm1. Current data indicate that AvrB physically associates with the *Arabidopsis* protein kinase RIPK, which then phosphorylates RIN4 (26). The phosphorylation of specific RIN4 residues then leads to the activation of RPM1 (24, 26). AvrRpm1 appears to induce modification of other RIN4 residues, but the specific residue(s) modified, and whether it is by phosphorylation, is not yet clear. A third example of indirect recognition of pathogen effectors is recognition of the *P. syringae* effector AvrRpt2 (a cysteine protease) by *Arabidopsis* RPS2. In this example, RPS2 is activated by the degradation of RIN4 following cleavage by AvrRpt2 (49). Thus RIN4 is required to keep RPS2 in an off state, and *rin4* null mutations are lethal in *Arabidopsis* if *RPS2* is functional.

NUCLEOTIDE BINDING AND OLIGOMERIZATION

The NB-ARC domain is conserved among plant and animal NLRs, and in the animal literature is often referred to as the nucleotide binding and oligomerization domain (NOD). NB-ARC domains form a deep nucleotide-binding pocket. In the "off" state, the NB-ARC domain adopts a "closed" structure where ADP is preferentially bound and coordinates intramolecular interactions to stabilize this structure (38, 50, 51). Activation is thought to require release of the ADP to be replaced by ATP and adoption of an "open" structure. This structural change is then thought to promote homo-oligomerization via the NB-ARC domain, which in turn enables the N-terminal domains to engage in downstream signaling. Thus, the NB-ARC domain is thought to function as a molecular switch that determines the "on" and "off" state of NLR signaling with ADP bound form for "off" and ATP bound form for "on." Due to difficulties in purifying soluble plant NLR proteins, however, this long-standing model was not tested until recently. Biochemical studies using the CC-NB-ARC region of tomato I-2 and Mi-1 demonstrated that ATP is bound by these proteins (52). This binding activity depends on a functional P-loop (Phosphate-binding loop), also known as the Walker A motif, which is a glycine-rich flexible loop containing a highly conserved lysine residue that interacts with the phosphates of the nucleotide and with a magnesium cation that coordinates the β- and γ-phosphates (53). In addition, these proteins display ATPase activity. A follow-up study reported that mutations within the NB-ARC domain that cause an auto-activation phenotype impair the ATPase activity of I-2, supporting the model that the ATP bound form represents the "on" state (54). The first direct experimental evidence that a full-length plant NLR protein preferentially binds ADP in its resting state was reported in 2011. In that study, the barley CNL, MLA27, co-purified specifically with ADP but not ATP (42). Also in 2011, the flax rust resistance protein M was found to co-purify with ADP, while an auto-active mutant form (D555V in the conserved MHD motif) co-purified with more ATP than ADP (55), supporting the model that nucleotide exchange is required for switching from the off state to the "on" state.

Apaf-1 and CED-4 are known to form oligomers through NB-ARC-mediated interaction (51, 56). The crystal structure of the mouse NLRC4 protein mentioned above revealed that ADP coordinates interactions between the central NB subdomain and the ARC2 subdomain (also called the winged-helix domain) to stabilize a closed conformation. A second interaction surface between the ARC3 subdomain and the NB masks an α-helix of the NB subdomain that participates in oligomerization. This α-helix is part of a conserved structure within the STAND family of ATPases (38). Specific point mutations within these interaction surfaces also result in an auto-active phenotype. A structure for NLRC4 in an active confirmation is not yet available, but it is predicted that ligand binding leads to the disengagement of the ARC2, ARC3, and LRR domains from the NB simultaneously, allowing the oligomerization of NLRC4 mediated by the NB subdomain.

There are several reports that indicate plant NLR proteins can also self-associate. For example, tomato Prf forms a dimer, which then incorporates into a complex containing two accessory

molecules of the Pto protein kinase (57, 58). Similarly, co-immunoprecipitation assays have demonstrated that *Arabidopsis* RPS5 self-associates prior to activation (35). Oligomer formation has also been reported for the tobacco TNL protein, N, but only its N-terminal TIR domain has been associated with oligomerization (59). The crystal structures of the MLA10 CC and L6 TIR domains revealed that both form homodimers (41, 42), and the MLA1 protein (allelic to MLA10) was observed to self-associate *in planta* (42). Whether the NB-ARC domain also plays a role in plant NLR oligomerization remains unclear, however. So far, only the RPS5 NB-ARC domain is known to self-associate, and this was shown under conditions of transient overexpression (35). In contrast, the L6 NB-ARC domain was shown to inhibit the homodimerization of its TIR domain and the activation of defense responses (41). ATP binding seems to be a must, however, for oligomerization because P-loop mutations disrupted the formation of N oligomers (59).

In addition to self-association, different NLRs may interact with each other to form heterodimers or hetero-oligomers. Interaction between NLRs has been reported between mouse NLRC4 and NAIP2, and between NLRC4 and NAIP5 (60, 61). NLRC4-containing oligomers assemble in response to two distinct PAMPs, flagellin and PrgJ, a component of type III secretion systems (40, 62). However, NLRC4 does not directly interact with flagellin or PrgJ. Instead, these PAMPs bind to NAIP2 and NAIP5, respectively, which then bind to NLRC4 (60, 61), triggering formation of a functional NLRC4 inflammasome. Like NAIP2 and NAIP5, some plant NLRs appear to require a second NLR for signaling. The *Arabidopsis* NLR RPS4 requires a second NLR, RRS1, to recognize the AvrRps4 effector protein from *P. syringae* (63, 64). Interestingly, RRS1 is encoded by a gene immediately adjacent to *RPS4*, in a head-to-head orientation (63, 64). This pair of NLRs is also involved in recognition of the PopP2 effector protein from the bacterial pathogen *Ralstonia solanacearum* and an unidentified effector(s) from the fungus *Colletotrichum higginsianum*. It is not yet known whether RRS1 and RPS4 physically associate, but both at least partially localize to the nucleus in the presence of effectors (11, 65).

A second example of a "helper" NLR is the *Arabidopsis* ADR1 (Activated Disease Resistance 1) family, which contributes to defense responses activated by *Arabidopsis* RPS2 (a CNL), RPP2, and RPP4 (TNLs) (66). There are three copies of ADR1 in the *Arabidopsis* genome and all three must be knocked out to affect RPS2 signaling. Interestingly, ADR1 does not rely on an intact P-loop motif for this function, suggesting that ATP binding is not required for signaling. ADR1 family members are also required for basal resistance and PTI, suggesting that this family of CNLs may function more generally in regulating defense responses, rather than functioning specifically in effector detection. Consistent with this, attempts at showing direct physical interactions between ADR1 and other NLRs have not been successful (66). In addition, mutations in ADR1 family members suppress runaway cell death triggered by loss of the *LSD1* gene, and autoactivating mutations in ADR1-L2 cause large increases in the defense hormone salicylic acid (67). Together, these data suggest that ADR1 may be part of an amplification loop that leads to elevated levels of salicylic acid during defense responses.

An ADR1 homolog has also been described in tobacco, and has been named NRG1. The NRG1 protein is required for resistance mediated by the TNL protein N, which mediates recognition of tobacco mosaic virus (TMV) (68). Consistent with their proposed role in signaling, transient expression of the CC domains of NRG1 and ADR1 induces HR in tobacco plants (69). NRG1 and ADR1 belong to an ancient clade of CNLs that is unusually conserved relative to other plant NLR proteins. Phylogenetic analyses have revealed the correlated absence of both NRG1 homologs and TIR-NB-LRR-encoding genes from the dicot *Aquilegia caerulea* and the dicotyledonous order Lamiales, as well as from the grass family (Poaceae), suggesting that the TNL family may be dependent on ADR1 family members for activating resistance (69). Since grasses contain numerous CNL family members, this functional requirement appears not to be true for CNLs in general. Indeed, resistance mediated by the *Arabidopsis* RPM1 protein is not affected by loss of ADR1 function (66).

DOWNSTREAM SIGNAL TRANSDUCTION AND DEFENSE ACTIVATION

It has been almost 20 years since the cloning of the first plant NLR gene. During this period, major advances have been made in our understanding of NLR structure, activation, and localization. However, little is known about the signal transduction steps following plant NLR activation. Forward genetic screens have been mostly unsuccessful at identifying downstream components, likely due to redundancy of signaling pathways. One exception was the identification of EDS1 (enhanced disease susceptibility 1) in *Arabidopsis*, which is required for resistance mediated by TNLs but not CNLs (70, 71). EDS1 has recently been shown to form protein complexes with the *Arabidopsis* TNLs RPS4, RPS6, and SNC1 (72). These complexes also contain an unrelated protein named SRFR1, which was identified in a screen for mutations that restored resistance to *rps4* mutant *Arabidopsis* (73, 74). Furthermore, the bacterial effectors recognized by RPS4 and RPS6 (AvrRps4 and HopA1) bind to EDS1 and disrupt EDS1-SRFR1 interactions (72). This study suggests that EDS1 may be a "guardee" of RPS4 and RPS6 (and possibly other TNLs) and that these TNLs are activated by effector mediated disruption of the EDS1-SRFR1 complex. More recent work, however, found that the C-terminal half of AvrRps4, which is necessary and sufficient for activating RPS4, does not interact with EDS1 in co-immunoprecipitation or yeast two-hybrid assays (75). This finding suggests that physical association between AvrRps4 and EDS1 is not required for activation of RPS4, thus the molecular mechanism underlying AvrRps4 recognition remains unclear.

Regardless of whether EDS1 is a true target of AvrRps4, it is clear that EDS1 and SRFR1 represent a signaling complex that is employed by multiple TNLs. SRFR1 contains a tetratricopeptide repeat domain and displays similarities to transcriptional repressors in *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (74). Consistent with SRFR1 possibly functioning as a transcriptional repressor, two independent studies reported that loss of SRFR1 function activates the expression of SNC1, an *Arabidopsis* TNL, resulting in constitutive defense responses (76, 77). Furthermore, bifluorescence complementation analyses

showed that SRFR1 interacts with RPS4 and SNC1 in the nucleus (72), suggesting that TNLs may directly regulate SRFR1 activity.

Other transcriptional regulators have also been shown to directly interact with TNLs. For example, Topless-related 1 (TPR1) interacts with SNC1, a TNL protein, and knocking out TPR1 compromises immunity mediated by SNC1 (78). Significantly, TPR1 represses the expression of two well-known negative regulators of immunity, Defense no Death 1 (DND1) and Defense no Death 2 (DND2). Therefore, the SNC1-mediated immune responses are activated by TPR1 through its repression of negative regulators. SPL6, a squamosa promoter binding protein (SBP)-domain transcription factor interacts with the N protein of tobacco within distinct nuclear compartments (79). The *Arabidopsis* ortholog of SPL6 is required for the RPS4-mediated resistance, indicating that this transcription factor plays a conserved role in activating TNL-mediated defenses. Also, as described above, the CNL protein MLA 10 translocates into the nucleus upon activation and interacts with both WRKY transcriptional repressors and MYB6, a transcriptional activator, to activate defense responses (10, 14). Most recently, the rice CNL Pb1, which confers resistance to rice blast (*Magnaporthe oryzae*), was shown to interact with the WRKY45 transcription factor in the nucleus (80). This interaction is mediated by the CC domain of Pb1, and mutations in the CC domain that disrupt the interaction compromise Pb1-mediated resistance. Thus both CNLs and TNLs have the capacity to impact gene expression by direct interaction with transcriptional repressors and activators, making these NLR signal transduction pathways quite short.

It is unlikely, however, that all NLR proteins regulate gene expression by direct interaction with transcription factors. As described above, the CNL proteins RPS2, RPS5, and RPM1 are localized to the PM. Activation of defenses by PM-localized NLRs appears to require an influx of extracellular Ca^{2+}, as cell death induced by RPS2 and RPM1 can be eliminated by the calcium channel blocker $LaCl_3$ (81). Recent reverse genetic studies indicate that RPS2- and RPM1-mediated resistance is at least partially dependent on calcium dependent protein kinases (CPKs) (82, 83), with different CPKs being involved in different aspects of resistance (83). Specifically CPK1 and CPK2 contribute to HR development, while CPK4/5/6/11 all contribute to transcriptional reprograming by phosphorylating the transcription factors WRKY8/28/48. Additionally, CPK1/2/4/11 also contribute to production of reactive oxygen species via phosphorylation of PM-associated NADPH oxidases. Based on these observations, the authors proposed a model in which NLR activation triggers a sustained influx of calcium, which then triggers multiple CPK signaling pathways that lead to ROS production, defense gene activation, and cell death. In addition to cytoplasmic calcium signaling, RPS2 and RPM1 activation has been shown to elicit specific Ca^{2+} signatures inside chloroplasts (84). These calcium transients are dependent on a chloroplast-localized protein named CAS for calcium-sensing receptor. Mutations in the *CAS* gene compromise both PTI and HR development during ETI. This study thus provides a possible link between NLR activation and chloroplast functions such as the production of the defense-related hormones jasmonic acid and salicylic acid.

Although it is clear that different plant NLRs employ different signaling pathways, these signaling pathways appear to be broadly conserved across plant species, as evidenced by functional transfer of NLRs between species. For example, the *RRS1-RPS4* gene pair described above has been functionally transferred from *Arabidopsis* to five different plant species from three different families (*Brassica rapa* and *Brassica napus* (Brassicaceae); *Nicotiana benthamiana* and *Solanum lycopersicum* (tomato) (Solanaceae), and *Cucumis sativus* (cucumber, Cucurbitaceae) (85). In addition, cell death can be activated in *N. benthamiana* and/or *N. tabacum* (tobacco) by transient expression of several different TNL and CNL proteins from diverse plant species, including *Arabidopsis*, flax, and barley (35, 86–88). Particularly noteworthy is the recent demonstration that the MLA1 protein from barely can function in transgenic *Arabidopsis* to confer resistance against the barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (89). Interestingly, this resistance remains effective in *Arabidopsis* mutants defective in ethylene, jasmonic acid, and salicylic acid signaling, indicating the presence of a hormone independent NLR-mediated defense mechanism that is conserved between barley (a monocot and member of the grass family) and *Arabidopsis* (a dicot and member of the mustard family).

The HR is usually associated with NLR-activated immunity in plants. However, the HR can be genetically uncoupled from restriction of pathogen growth, at least in the case of resistance to *P. syringae* (16, 90). In addition, it remains unclear how cell death is executed, or indeed, whether different classes of NLRs share the same cell death pathway. For the PM-localized NLRs, RPM1, and RPS2, cell death is preceded by fusion of the vacuolar membrane with the PM, resulting in release of vacuolar proteins to the apoplast (extracellular space) (91). The resulting extracellular fluid possesses both antibacterial activity and cell death-inducing activity. This membrane fusion process depends on the activity of the proteasome subunit PBA1, suggesting that there may be an "HR inhibitor" protein that must be degraded to enable HR activation.

Plants lack canonical caspase proteases that are associated with apoptosis in mammalian cells. However, they do contain proteins with weak structural similarities to caspases called metacaspases that have recently been implicated in regulating HR cell death (90). Knockout of the *Arabidopsis* metacaspase AtMC1 reduces, but does not eliminate, RPM1-mediated HR, but has no effect on RPM1-mediated growth restriction of *P. syringae.* Conversely, knockout of a second *Arabidopsis* metacaspase AtMC2, enhances RPM1-mediated HR, but again has no effect on restricting bacterial growth. These observations suggest that metacaspases play an accessory role in regulating HR, but are not a central trigger.

A second type of protease associated with HR regulation in plants has recently been identified and named phytaspase (90, 92). Phytaspases are structurally unrelated to animal caspases, but like caspases, catalyze cleavage following aspartate residues. RNAi-mediated silencing of phytaspase in tobacco reduced N-gene mediated HR triggered by TMV infection and reduced resistance to TMV, indicating that phytaspases may play a central role in resistance mediated by N, a TNL family member (90, 92). Interestingly, tobacco phytaspase is constitutively expressed and secreted to the extracellular space, but during the HR, partially relocalizes to the

cytoplasm (90, 92), raising the possibility that it is actively transported back into the cell during the HR, where it must cleave specific substrates to activate cell death. Although phytaspase has also been purified from rice, there are not yet any reports on whether it is required for NLR-mediated resistance in other plant species.

Several recent studies indicate that secretion may play an important role in NLR-mediated defense. For example, *Arabidopsis* AtMIN7, an ADP ribosylation factor-guanine nucleotide exchange factor (ARF-GEF) protein, has recently been shown to be required for RPS2- and RPS5-mediated resistance, but not for HR cell death (93). ARF-GEF proteins regulate the activity of small GTPases involved in endomembrane trafficking. AtMIN7 is a target of the *P. syringae* effector, HopM1, which promotes proteasome-dependent degradation of AtMIN7 (94). Activation of RPS2 and RPS5 somehow prevents HopM1-mediated degradation of AtMIN7 (93). Consistent with AtMIN7 playing a role in endomembrane trafficking, confocal microscopy showed that MIN7 and HopM1 localize to the *trans*-Golgi network/early endosomes. Further evidence that endomembrane trafficking/secretion plays a role in RPS2-mediated resistance comes from quantitative proteomic analysis of PMs following RPS2 activation (95). In this study, a transgenic *Arabidopsis* line expressing a dexamethasone-inducible AvrRpt2 gene was used to activate RPS2. Comparison of activated to unactivated samples uncovered 235 proteins that were significantly up-regulated. This set of up-regulated proteins was highly enriched in proteins involved in endocytosis and exocytosis, including Syntaxin of plants 122 (SYP122) and *N*-ethylmaleimide-sensitive factor vesicle fusing ATPase, and soluble *N*-ethylmaleimide-sensitive factor adaptor protein 33 (SNAP33). RPS2 has also been shown to upregulate production of miR393b, a microRNA that targets at least three different genes likely involved in endomembrane trafficking (MEMB12, a golgi-localized SNARE protein; VPS54, homologous to a yeast protein involved in retrograde transport from late endosomes to the Golgi, and EXO70H3, a subunit of the exocyst complex thought to be required for exocytosis (96). Knockout of MEMB12 enhances secretion of the defense protein PR-1 that is induced by RPS2 activation. Thus MEMB12 appears to function as negative regulator of exocytosis, with RPS2 inducing production of a miRNA that inhibits translation of the MEMB12 protein. Lower MEMB12 protein levels then enable an increase in defense protein secretion. Consistent with this model, the MEMB12 knockout line displays enhanced basal resistance in the absence of RPS2 activation (96). The endomembrane trafficking system is thus emerging as important arm of the NLR-mediated defense system that is also targeted by pathogen effectors.

PROSPECTIVE

As should be apparent from the discussion above, plant NLRs have evolved diverse mechanisms for recognizing pathogens and diverse mechanisms for activating resistance. However, a feature that is likely shared among all "sensor" NLRs in plants (as opposed to "helper" NLRs) is the dual role of the LRR domain in keeping the NLR in the "off state" in the absence of pathogen, and promoting the switch to the "on state" in the presence of pathogen (via binding to effectors or effector-modified host proteins). We have very little insight, however, into how the LRR domain accomplishes either of these roles. The recent structure of mouse NLRC4 indicates that in animal NLRs, the LRR folds back across NB-ARC domain with the N-terminal portion of the LRR making multiple contacts with the NB and ARC3 domains, effectively placing a lid over the ADP/ATP binding pocket. The absence of the ARC3 domain in plant NLRs makes it a certainty that the contacts between the LRR and NB-ARC will differ from NLRC4, but based on the locations of autoactivating mutations and on deletion analyses, the general structure is likely to be similar, with just the N-terminal portion of the LRR (approximately four repeats) required to form the lid (23). The C-terminal portion of the LRR appears to be where specificity for effector recognition generally lies, but how effector binding alters NB-ARC:LRR interaction is unknown. What remains a holy grail for the field, in both plants and animals, is obtaining the structure of an NLR complexed with its activating protein. The insolubility of NLRs when overexpressed in bacteria or insect cells has been a major barrier to progress on this front. Surmounting this barrier for plant NLRs may require purification from plant systems in which the necessary chaperones should be present.

A second holy grail is identifying the immediate downstream interacting proteins for PM associated CNLs. Although several transcription factors have now been identified that interact with nuclear-localized TNLs and CNLs, we still lack good candidates for downstream signaling proteins for NLRs that signal from the PM such as RPM1, RPS2, and RPS5. Proteomic approaches hold some promise for shedding light on this unknown (95, 97), but face the additional challenge of rapid turnover of NLR proteins following activation. The finding that extracellular calcium influx is required for RPM1- and RPS2-mediated HR suggests that there may be a fairly direct link between NLR activation and calcium channels (81), which merits further exploration.

A third holy grail is a better understanding of how cell death is executed during NLR-mediated HR. Although cell death is apparently not required for resistance, at least to *P. syringae*, the HR is still a hallmark of NLR activation. One study has implicated fusion of the vacuolar and PM as the primary event leading to cell death (91). If this is true, the question becomes how NLR activation triggers such membrane fusion events. More generally, accumulating data have implicated endomembrane trafficking as playing a central role in NLR-mediated resistance, presumably to increase secretion of antimicrobial compounds. How does NLR activation regulate this process?

In summary, although much has been learned in the nearly 20 years since the first NLR was identified, major questions remain. Providing answers to these questions will require both creativity and improvements in technology, but will no doubt come.

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