Holding the inflammatory system in check: NLRs keep it cool

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Abstract

Inflammation is a double-edged sword. While short-lived, acute inflammation is essential for the repair and resolution of infection and damage, uncontrolled and unresolved chronic inflammation is central to several diseases, including cancer, autoimmune diseases, allergy, metabolic disease, and cardiovascular disease. This report aims to review the literature regarding several members of the nucleotide-binding domain, leucine-rich repeat-containing receptor (NLR) family of pattern recognition sensors/receptors that serve as checkpoints for inflammation. Understanding the negative regulation of inflammation is highly relevant to the development of therapeutics for inflammatory as well as infectious diseases.

Introduction

Inflammation (from the Latin, *īnflammō*, "set afire or ignite") is a complex biological response to an injury, pathogen, or irritant. The process of inflammation is initiated by cells that form the innate immune system. These cells include: macrophages, dendritic cells, granulocytes, mast cells, natural killer cells, endothelial cells, and mucosal epithelial cells. To help sense pathogen-associated molecular patterns, and damage-associated molecular patterns, as well as irritants, innate immune cells express germline-encoded receptors called pattern-recognition receptors (PRRs). Following appropriate recognition by PRRs, innate immune cells can phagocytose and eliminate foreign particles, pathogens and debris. Innate cells also release cytokines, chemokines and other molecular mediators that signal to neighboring cells and elicit effector immune responses as needed. Immune cells initiate this response by activating the innate immune pathways, such as nuclear factor-κB (NF-κB), mitogen-activated protein kinase (MAPK), and type-I interferon (IFN) pathways. However, inflammation is a double-edged sword. While acute inflammation is essential for defense against infection, uncontrolled and unresolved chronic inflammation leads to disease. Dysregulated inflammation is central to several diseases including, but not restricted to, autoimmune diseases, allergy, cancer and metabolic disease, and cardiovascular disease. Numerous studies have focused on the discovery and roles of molecular mediators and signaling pathways that initiate or contribute to inflammation, but there are fewer studies on molecular mediators that attenuate or resolve inflammation. This report aims at reviewing the literature regarding key PRRs that belong to the NLR family (also known as NOD-like receptor family), and which hold inflammation in check.

Recent advances

The discovery of gene families encoding PRRs is one of the most important discoveries in immunology. The NLR gene family members are considered to be intracellular PRRs or innate immune sensors [1]. The importance of the NLR family is underlined by its evolutionary conservation from sea urchins to humans. Additionally, NLRs also share structural similarity with nucleotide-binding site-leucine rich-repeat (NBS-LRR) proteins in plants which are responsible for immunity against pathogens of fungal, viral, parasitic, and insect origin. Genetic mutations in several family members are known to cause autoinflammatory diseases in humans. The association of mutations in NLR genes with autoinflammatory diseases has

indicated the important function of these genes in inflammation. For example, mutations in the NLRP3 gene result in three autoinflammatory diseases collectively called the cryopyrin-associated periodic syndromes (CAPS) [2–8]. Also, mutations in NOD2 are associated with susceptibility to Crohn's disease (an inflammatory bowel disease) and Blau syndrome (a granulomatous inflammatory disease) [9,10]. NLR gene mutations are also associated with asthma, vitiligo (a depigmentation skin disease), and urticaria skin rash [11–14].

There are more than 20 distinct NLR-encoding genes that have been identified in humans and more than 30 identified in mice [15]. NLR genes encode cytoplasmic proteins with a tripartite domain structure. This tripartite structure consists of a variable N-terminal effector domain, a central nucleotide-binding domain (NBD), and a variable number of C-terminal leucine-rich repeats (LRRs). The N-terminal domain of NLR proteins comprises a variable number of effector domains that may include combinations of the acidic transactivation domain, Baculo viral inhibitory repeat (BIR)-like domain, caspase recruitment domain, and pyrin domain. Each LRR element is typically 28–29 residues in length and each NLR may contain up to 33 individual LRR elements. NLRs are unique in their capability to sense several structurally diverse pathogen-associated molecular patterns (PAMPs) and danger-associated molecular patterns (DAMPs). The exact mechanism of the sensing of PAMPs and DAMPs remains largely unknown although, increasingly, interactions of NLRs with their cognate or putative ligands have been reported, including the interaction of NAIP5/NLRC4 with flagellin, NOD2 with muramyl dipeptide, and NLRX1 with RNA [16–20]. NLRs are known to play diverse roles, not restricted to their sensing of DAMPs and PAMPs, including contribution to pathways of cell death and regulation of adaptive immunity via regulation of the major histocompatibility complex (MHC). A key function of NLRs is the formation of multiprotein complexes called "inflammasomes" [21]. Frequently, inflammasomes are comprised of an NLR protein, an adaptor protein, ASC (apoptotic speck-containing protein with a CARD (caspase activating and recruiting domain), and procaspase-1. Procaspase-1 undergoes autocatalytic cleavage, and mature caspase-1 cleaves pro-interleukin-1β (pro-IL-1β) and pro-interleukin-18 (pro-IL-18) into their mature active forms. However, in addition to their inflammasome-mediated functions, NLRs also perform non-inflammasome-mediated roles, including the regulation of NF-κB, MAPK activation, cytokine and chemokine production, antimicrobial reactive oxygen species production, IFN production, and ribonuclease L activity. NLRs that serve as checkpoints of immune activation include

NLRX1, NLRP12, NLRC3, NLRC5, NLRP4 and NLRP6, which will be reviewed under three headings: NLRs as checkpoints of immune signals, NLRs as checkpoints of infection, and NLRs as checkpoints of cancer.
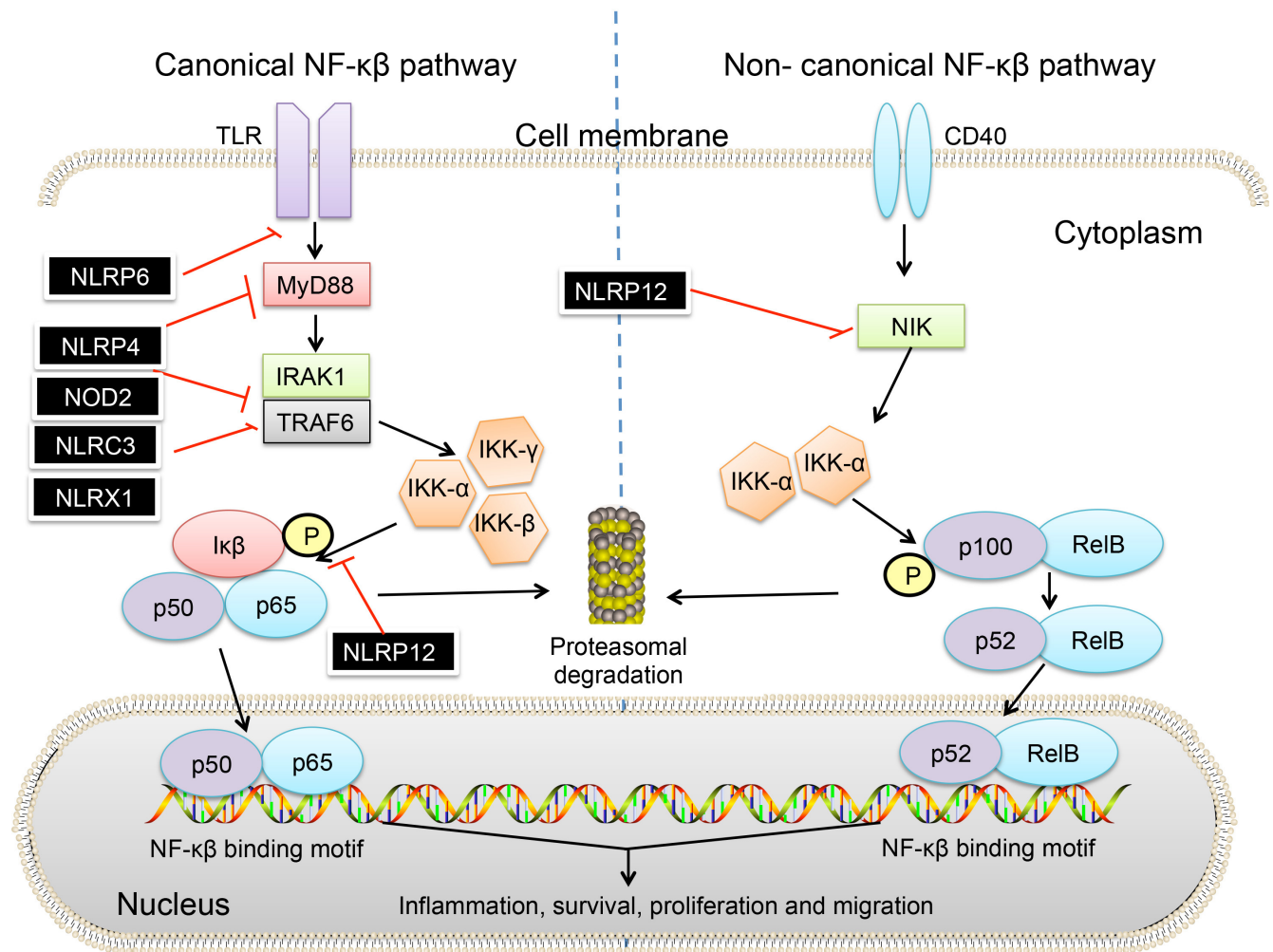
NLRs as checkpoints of immune signals
As mentioned earlier, the NF-κB pathway is central to inflammation. Activation of NF-κB can occur through two distinct mechanisms, referred to as the canonical and non-canonical pathways. Both canonical and non-canonical NF-κB pathway proteins play critical roles in regulating inflammatory and immune responses (Figure 1). NF-κB exists in the cytoplasm in an inactive form bound by the inhibitor IκB. Proteasome-mediated degradation of IκB is linked to its phosphorylation by the IκB kinase (IKK) complex, IKKκ-IKKκ-IKKκ (NEMO). Polyubiquitination and degradation of IκB is initiated by its phosphorylation, which results in the release and nuclear translocation of NF-κB to activate various target inflammatory chemokines, cytokines, and cell surface proteins [22]. While the canonical pathway is required for rapid response following stimulation, the non-canonical pathway exhibits slower kinetics and is dependent on the NF-κB-inducing kinase (NIK) and IKKκ. NIK activates IKKκ to cause phosphorylation of the p100 subunit of NF-κB, resulting in the latter's cleavage into its active p52 form.

NLRP12 (formerly called Monarch, PYPAF7, or CLR19.3) was one of the first NLRs reported to be a negative regulator of inflammation. NLRP12 associates with and induces proteasome-mediated degradation of NIK, leading to the suppression of the non-canonical NF-κB pathway [23]. This suppression causes reduced expression of p52 dependent genes *ccr4*, *cxcl12* and *cxcl13*, which are known to regulate the initial stages of inflammation, including the recruitment of immune cells (Figure 1). Zaki *et al.* have also found that NLRP12 attenuates IκB phosphorylation and degradation, hence attenuating the canonical pathway [24]. Similarly NLRP12 serves as a checkpoint for activated extracellular-signal-regulated kinases (ERK), although the pathway is not clearly identified. Conversely, NLRP12 has been found to activate caspase-1 in response to selected infectious agents, and its impact on infectious diseases will be discussed later.

NLRC3 was also shown to decrease the action of NF-κB, AP-1 and NFAT reporter gene constructs in T cell lines (formerly, CLR16.2) [25]. Protein association studies showed that the association of NLRC3 with TRAF6 and NLRC3 has a functional TRAF-interaction site. This association prevented the K63-activating ubiquitination of TRAF6, thus preventing Toll-like receptor (TLR) signaling (Figure 1). NLRC3 also negatively regulates innate

Figure 1. NLR-mediated suppression of canonical and non-canonical NF-κB signaling
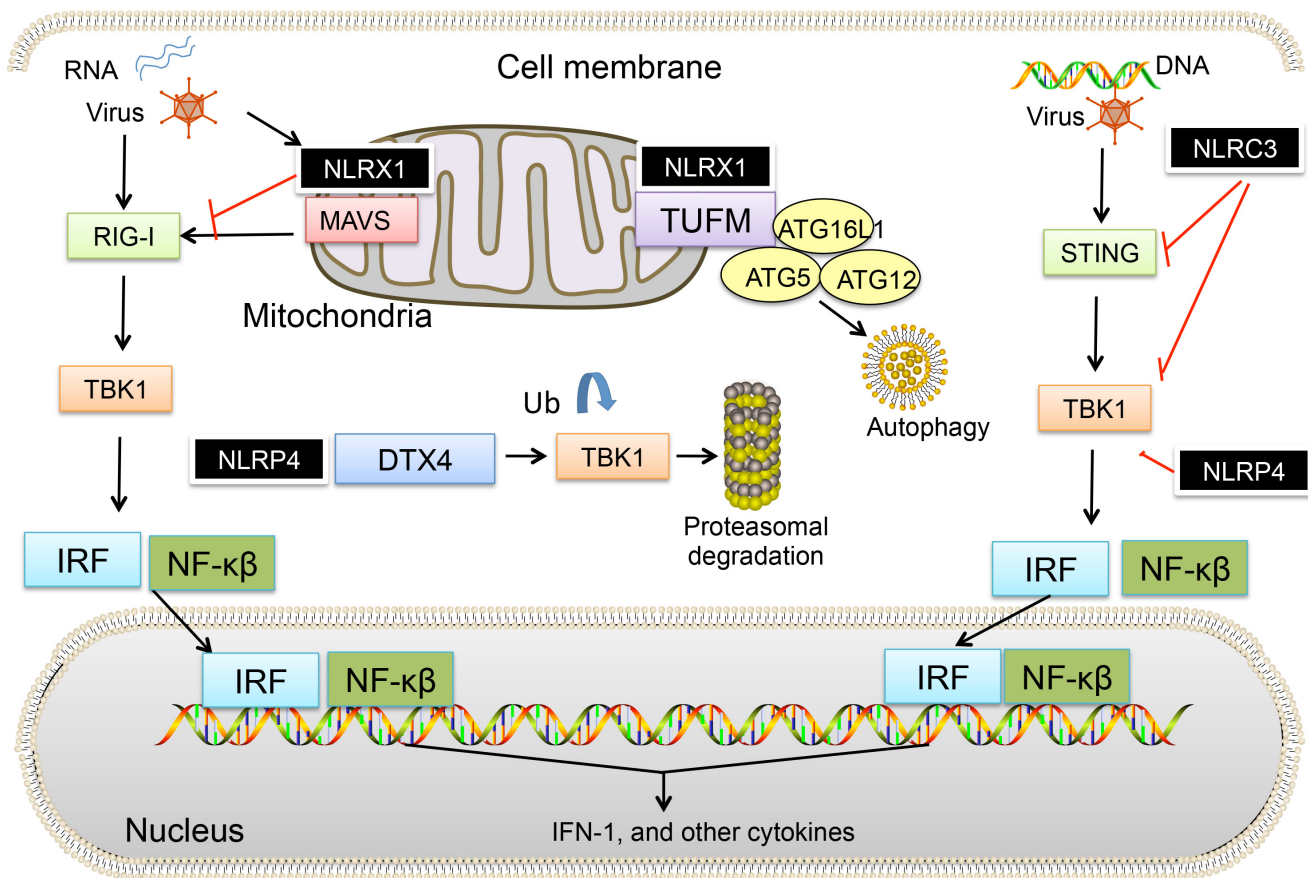

Nuclear factor κB (NF-κB) exists in the cytoplasm in an inactive form bound by the inhibitor IκB. Proteasome-mediated degradation of IκB is linked to its phosphorylation by the inhibitor of NF-κB kinase (IKK) complex. This complex consists of IKKα, IKKβ, and IKKγ (also known as NEMO; NF-κB essential modulator). Polyubiquitination and degradation of IκB initiated by its phosphorylation results in the release and nuclear translocation of NF-κB to activate various genes for inflammatory chemokines, cytokines, and cell surface proteins. While the canonical pathway is required for rapid response following stimulation, the non-canonical pathway exhibits slower kinetics and is solely dependent on the NF-κB-inducing kinase (NIK) and IKKα, not the trimeric IKK complex. NIK associates with the p100 subunit (NF-κB2) and induces its cleavage to its active form, p52. Tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6) activates the canonical NF-κB signaling and TNF receptor-associated factor 3 (TRAF3) attenuates the non-canonical NF-κB signaling. NLRP6, NLRP4, NOD2, NLRC3, and NLRX1 have all been reported to suppress canonical NF-κB signaling, while NLRP12 inhibits both canonical and non-canonical NF-κB signaling pathways, the latter through degradation of NIK and the former through inhibition of IκB phosphorylation. NLRX1 and NLRC3 associate with and inhibit TRAF6 and the IKK complex, resulting in the attenuation of canonical NF-κB signaling following Toll-like receptor (TLR) stimulation. NLRP4 also activates the E3 ubiquitin ligase, DTX4, causing ubiquitination of TANK-binding kinase 1 (TBK1), leading to the latter's degradation and reduced type I IFN.

immune signaling by the stimulator of interferon genes (STING, also MITA, MYPS, or ERIS) which is a pivotal intracellular DNA as well as a cyclic dinucleotide sensor [26]. STING induces type I interferon through its interaction with TANK-binding kinase 1 (TBK1). By using recombinant purified proteins and/or cell lysates, NLRC3 was shown to directly associate with STING and TBK1 thus

preventing their interaction (Figure 2), leading to reduced IFN and other cytokine production. Interestingly, the zebrafish ortholog of an NLRC3-like protein showed that it suppresses inflammatory activation of microglia in a cell-autonomous fashion. Equally significant, mutation of this protein resulted in an overt inflammatory response that reduced microglial migration to the central nervous system.

Figure 2. NLR-mediated suppression of type I IFN signal

NLRX1 has a unique mitochondrial localization. It associates with mitochondrial antiviral signaling (MAVS) protein and prevents its interaction with retinoic acid inducible gene-I (RIG-I) during viral infections. This interaction leads to reduced activation of IRF and consequently reduced interferon (IFN) and cytokines, such as IL-6. Stimulator of IFN genes (STING) induces type I IFN through its interaction with TANK-binding kinase I (TBK1). NLRC3 directly associates with STING and TBK1 thus preventing their interaction. This leads to reduced activation of IRF and reduced IFN signaling. Conversely, NLRX1 causes autophagy by associating with the protein TUFM and the mitochondrial immune signaling complex that consists of ATG5-ATG12-ATG16L1. This complex is critical to virus-induced autophagy and thus suppresses IFN signaling. NLRP4 induces ubiquitination of TBK1 by the E3 ubiquitin ligase DTX4. Post ubiquitination TBK1 is targeted for proteasomal degradation, thus reducing IRF activation and IFN signaling.

This is indicative of conservation of the immunosuppressive functions of NLRs across species [27]. Furthermore, the report showed that the lack of NLRC3 reduced microglial presence.

NLRP4 (formerly, PAN2) was first cloned and characterized as a negative regulator of NF-κB [28]. Introduction of exogenous NLRP4-encoding plasmid into HEK293 cells with a NF-κB-dependent reporter gene plasmid showed NLRP4 to be a potent inhibitor of NF-κB induction by tumor necrosis factor (TNF)-α and, to a lesser extent, by IL-1β. These results were confirmed by an electrophoretic mobility shift assay that showed reduced binding of NF-κB to DNA in the presence of NLRP4. Importantly, NLRP4

overexpression markedly reduced NF-κB activity induced by intracellular adapter proteins (TRAF2, TRAF6, and MyD88) and kinases (RIP and IRAK2) that functionally connect TNF and IL-1R receptors to NF-κB responses (Figure 1).

The function of NLRC5 has been studied by multiple laboratories, but findings other than its role in MHC class I regulation have not been consistent. Meissner *et al.* first showed positive regulation of MHC class I by NLRC5 in culture, and this function of NLRC5 as a transcriptional transactivator has now been verified by multiple analyses of different gene deletion mice [29–37]. However, other findings are not consistent.

Benko *et al.* found that overexpression of NLRC5 in a mouse macrophage cell line led to reduced levels of NF-κB and IFN response elements in reporter assays, and knockdown of NLRC5 led to an enhanced expression of cytokines, such as IL-1β, IL-6 and enhanced CD40, TNF, and class I MHC in response to lipopolysaccharide or IFN stimulation [38]. Cui *et al.* showed endogenous interaction of NLRC5 with IκB kinases (IKK) α/β to reduce the phosphorylation of IKKs [39]. They also showed that a knockdown of NLRC5 resulted in elevated IFN response to the antivesicular stomatitis virus and found that NLRC5 interacts with retinoic acid inducible gene-I (RIG-I) and MDA5 but not with mitochondrial antiviral signaling (MAVS) protein, resulting in inhibition of RIG-I-like receptor-mediated type I interferon responses [39]. In contrast, Kuenzel *et al.* and Neercinx *et al.* reported that NLRC5 mediated positive regulation of cytokine response to viruses and nucleic acids [40,41]. However, Robbins *et al.* and Kumar *et al.* have instead reported normal responses to lipopolysaccharide, RNA viruses, DNA viruses and stimulatory DNA in *Nlrc5*-deficient mice [30,42]. Reconciling these different findings has been difficult.

Finally, NLRC5 is also reported to cause inflammasome activation in human monocytic cell lines and primary monocytes. NLRC5 gene knockdown in THP1 monocytic cell line and primary human blood monocytic cells led to reduced inflammasome activation in response to activators that also activated the NLRP3 inflammasome [43,44]. Furthermore, NLRC5 associates with NLRP3 and can reconstitute an inflammasome activation assay, an ultimate assay of inflammasome function [43]. A recent report on the role of NLRC5 in rhinovirus in human cells showed nearly identical findings for this protein in inflammasome activation [44] and this finding is supported by one strain of *Nlrc5* $^{-/-}$ mice [42] but not by others. The underlying reason for these different findings regarding the role of NLRC5 in inflammasome response is unclear, while it is important to keep in mind that different gene deletion strategies were employed and that there might be species-specific differences when comparing data generated in human cells vs. mice. Species differences have been shown previously, as numerous differences among mice and human TLRs have been noted throughout the literature.

NLRX1 is another protein that attenuates adaptive immunity. It has been reported that it attenuates type I IFN and inflammatory cytokine responses in infections, and these data will be described below. A study of *Nlrx1*-deficient mice showed that these mice exhibit enhanced clinical symptoms in a Th1 and Th17-mediated disease

model of multiple sclerosis, experimental allergic encephalitis [45]. Consistent with its role as a checkpoint against overzealous inflammation, the gene-deficient mice exhibit enhanced Th1, Th17 cellular infiltrate into the spinal cord and enhanced inflammatory cytokines/chemokines produced by both macrophages and microglia. Thus, NLRX1 impacts both innate and adaptive immunity.

Finally, while NOD2 is frequently considered an activator of immune signaling (this topic has been extensively reviewed), there is a body of work indicating that it attenuates immune signals and inflammation [46]. Activation of NOD2 in human dendritic cells by its ligand (muramyl dipeptide), reduces TLR-mediated inflammatory responses, and this is related to increased IRF-4 which inhibits the activating K-63 ubiquitination of TRAF6 and RICK, leading to downregulated NF-κB [47]. This is consistent with the earlier finding of Watanabe *et al.* that the activation of NOD2 by its ligand protected mice against experimental colitis in an IRF4-dependent fashion [48]. Rosenzweig *et al.* and Petnicki-Ocwieja *et al.* have also found that NOD2 deficiency results in increased susceptibility to inflammatory conditions, such as uveitis, arthritis and carditis [49,50]. Also, a report by Borm *et al.* showed that, while NOD2 functions as a negative regulator of TLR2 signaling in mice, the NOD2 effect on TLR2-mediated cytokine responses in humans is dependent on activation dose as well as on NOD2 genotype [51].

NLRs as checkpoints in inflammation caused by infection
NLRs have been cited as intracellular sensors for diverse PAMPs, including bacterial cell wall components, lipopolysaccharide, lipoproteins, type 3 secretory molecules, flagellin and bacterial and viral nucleic acids [52–57]. Most of the initial studies showed an NLR-dependent positive regulation of inflammatory responses following infection. However, there are increasing examples of attenuation of these pathways by NLRs. TNFR and TLR signals are reduced in human monocytic cell lines, accompanied by a drop in inflammatory cytokines and NF-κB activation [58]. Further, there is a bidirectional regulation in that lipopolysaccharide (TLR4 ligand), peptidoglycan (TLR2 ligand), and/or bacteria have been found to reduce the expression levels of NLRP12 and NLRC3 (Figure 1). A recent study by Zaki *et al.* confirmed these data by using *Nlrp12*$^{-/-}$ mice to find that ablation of the gene increased IkBα and ERK phosphorylation in macrophages and increased proinflammatory and antimicrobial peptides, rendering the host resistant to *Salmonella* [59]. However, this may be pathogen specific, in that *Nlrp12*$^{-/-}$ mice exhibited normal host response to other bacteria [60]. This might also point to the existence

of compensatory pathways *in vivo* that can take over the role of NLRP12 in its absence. By contrast, Ataide *et al.* and Anand *et al.* found that NLRP12 activates caspase-1-mediated inflammatory response during infections with *Yersinia pestis* (causative agent of plague) and *Plasmodium* (causative agent of malaria), pointing to different functions for this protein [61,62]

Similarly, NLRP6 (formerly, PYPAF5) was found to play a role in impeding clearance of both Gram-positive and negative bacterial infections [63]. Utilizing *Nlrp6*- deficient mice, Anand *et al.* demonstrated increased resistance of these mice to *Listeria monocytogenes, Salmonella typhimurium and Escherichia coli*. Utilizing bone marrow chimeras, Anand *et al.* showed that both *Nlrp6*$^{-/-}$ *mice* transplanted with wildtype bone marrow and wildtype mice transplanted with *Nlrp6*$^{-/-}$ bone marrow showed an intermediate level of protection against *L. monocytogenes*, suggesting that both hematopoietic and non-hematopoietic cells contribute to NLRP6-mediated bacterial clearance. To unveil the underlying molecular mechanism, they utilized macrophages cultured from *Nlrp6*$^{-/-}$ mice and showed that these cells had enhanced the activation of MAPK and canonical NF-κB upon TLR, but not cytosolic NOD1/2 ligation. As a result, infected cells exhibited increased IκBα and ERK phosphorylation and produced elevated levels of NF-κB- and MAPK-dependent cytokines and chemokines (Figure 1). However, Normand *et al.*, Chen *et al.*, and Elinav *et al.* have found an enhancing role for *Nlrp6* in inflammasome activation in models of colonic inflammation, since the inflammasome product, IL-18, is reduced in *Nlrp6*$^{-/-}$ mice. A caveat is that none of these reports directly measured caspase-1, the key biomarker for inflammasome activation [64-66]. However, in an earlier study, Grenier *et al.* utilized an overexpression system to show that co-expression of ASC with NLRP6 led to enhanced caspase-1 activation [67]. Data with gene deletion cells or mice should bolster this finding. Similar to other NLRs, NLRP6 may mediate distinct functions, both as an activator of IL-18 production and an attenuator of the NF-κB and MAPK pathways.

NLRX1 has been found to negatively regulate the host inflammatory immune response during viral infection and TLR treatment [68]. NLRX1 has a unique mitochondrial localization. Mitochondria play central roles in the energy metabolism of a cell (ATP generation and oxidative phosphorylation), reactive oxygen species generation, programmed cell death, autophagy and innate antiviral response. Similar to NLRP12, NLRX1 negatively regulates NF-κB signaling induced by TLR ligands in macrophages and macrophage cell lines [69,70]. After receiving an activating signal, NLRX1 associates with TRAF6 and IκB kinase (IKK). On activation, NLRX1 is ubiquitinated and

it disassociates from TRAF6 to bind the IKK complex, which inhibits subsequent canonical NF-κB activation. In fibroblasts but not macrophages, NLRX1 negatively regulates type-I interferon (IFN-I) signaling by inhibiting the interaction between the PRR, RIG-I, and the MAVS protein following virus exposure [70] (Figure 2). In support of a role for NLRX1 in RNA recognition, structural analysis indicates binding of the C-terminus with RNA [20]. However, this negative regulatory response has not been observed by others using different gene deletion mice, and these differences remain unresolved [71,72]. It is possible that these differences are due to the different gene targeting strategies used, hence it is important to perform side-by-side comparisons of these gene deletion mice to unveil the underlying reasons for these discrepancies.

NLRX1 also contributes in a positive fashion to reactive oxygen species (ROS) induction and autophagy activation. Autophagy and ROS are functionally linked and regulate each other under many circumstances [73]. Autophagy is a process by which cellular components in addition to invading pathogens (bacteria, viruses) are sequestered inside double membrane vesicles and then delivered to the lysosome for degradation. NLRX1 associates with the mitochondrial protein TUFM, which in turn recruits the ATG5-ATG12-ATG16L1 complex. Consistent with their common function, both NLRX1 and TUFM augment autophagy but suppress IFN signaling [74]. Interestingly, NLRX1 is found to reduce RIG-I function, which is consistent with multiple reports documenting the inverse relationship between RIG-I/interferon induction and autophagy [75,76]. NLRX1 is also required for ROS induction in response to pathogens [77,78], but the link between NLRX1-induced autophagy and ROS has thus far not been investigated.

NLRP4 is another NLR that has functions in both the regulation of type I IFN and autophagy. Cui *et al.* showed that NLRP4 plays a role in attenuating the type I IFN response to DNA and RNA in HEK293T cells [79]. Specifically, it targets activated TBK1 for degradation through K48-linked ubiquitination of TBK1 by the E3 ubiquitin ligase DTX4, and this is dependent on Lys670 present in TBK. These results suggest that Lys670 in TBK1 is essential for NLRP4-DTX4-mediated K48-linked ubiquitination-associated degradation of activated TBK1, leading to the negative regulation of type I interferon signaling (Figure 2) [79]. Another report by Jounai *et al.* showed that NLRP4 most strongly associates with Beclin1, an early regulator of autophagy. Using over-expressed and RNA interference approaches, the authors found that NLRP4 attenuates autophagy. During infection

with Group A Streptococcus, NLRP4 dissociates from Beclin1, allowing autophagy to proceed [80].

NLRs as checkpoints of cancer

NLRs have been investigated in a limited number of cancer models; the mouse model of colitis-associated colorectal cancer (CAC) is one of the most widely used models. In this model, cancer is induced by administration of azoxymethane and dextran sodium sulfate (AOM-DSS) to mice. NLRP3 serves as a checkpoint for the prevention of CAC development in this model [81,82]. $Nlrp3^{-/-}$ mice had increased polyp numbers and size and worsened pathology compared to wildtype mice. This phenotype was also seen in $Asc^{-/-}$ and $caspase\text{-}1^{-/-}$ mice, indicating that the NLRP3 inflammasome is important in suppressing CAC development [81-83]. Importantly, it was shown that the presence of NLRP3 in hematopoietic cells was necessary for the tumor-suppressing effect in response to AOM-DSS challenge. Mechanistically, IL-18 levels were dramatically reduced in the colon of $Nlrp3^{-/-}$ and $caspase\text{-}1^{-/-}$ mice. Treatment of $caspase\text{-}1^{-/-}$ mice with recombinant IL-18 led to a reduction in disease, demonstrating a crucial role of IL-18 in protection against CAC development [84,85].

Hu *et al.* reported the role of NLRC4 in the mouse model of CAC [86]. They showed that, while both $Nlrc4^{-/-}$ and $caspase\text{-}1^{-/-}$ mice exhibited increased tumor formation compared to wildtype mice, the role of NLRC4 was restricted to tumor and not to colitis, indicating that inflammation might not be the driving force for tumorigenesis in these mice. Hu *et al.* followed this study with an investigation of NLRC4 and Caspase-1 in colonic epithelial cells. Utilizing bone marrow chimera experiments they showed that $Nlrc4^{-/-}$ mice receiving a wildtype bone marrow transplant had similar tumor numbers to $Nlrc4^{-/-}$ mice receiving $Nlrc4^{-/-}$ bone marrow in the CAC model, but exhibited significantly higher tumor numbers than wildtype mice receiving either wildtype or $Nlrc4^{-/-}$ bone marrow. This observation supports the conclusion that NLRC4 suppresses CAC via its role in non-hematopoietic cells. An alternative hypothesis for the role of NLRC4 in CAC involves the role of commensal microbiota. It was reported that NLRC4-deficient mice have alterations in commensal microbiota populations when compared to their wildtype counterparts. When $Nlrc4^{-/-}$ mice were co-housed with wildtype mice so that both strains shared commensal microbiota, they showed no difference in CAC [87]. This study indicates that NLRC4 in non-hematopoietic cells may have a role in regulating commensal microbiota.

NLRP12 also serves as a checkpoint protein in colon inflammation and colorectal tumorigenesis [22,24].

$Nlrp12$-deficient mice showed an increased susceptibility to colitis and colorectal tumorigenesis, and this was accompanied by increased NF-κB and ERK activation. While one report implicates the NF-κB canonical pathway downstream of NLRP12, another report provides evidence of significantly elevated non-canonical NF-κB signaling. The increase in tumorigenesis was associated with increased NIK-regulated genes ($Cxcl12$ and $Cxcl13$) that have been associated with multiple solid cancers in the colons of $Nlrp12^{-/-}$ animals. Bone marrow chimera analyses indicated that a hematopoietic as well as a non-hematopoietic source of NLRP12 provided important checks against CAC. Together, these data implicate NLRP12 as a critical checkpoint during inflammation and tumorigenesis in colon cancer.

Conclusion

Future directions

Overall, this review presents mounting evidence that NLRs can serve as checkpoint proteins important in preventing overzealous responses. This is relevant for inflammation, infection, and other diseases of high impact, such as cancer and metabolic diseases. There are several key questions that remain at the forefront of this vibrant field of research regarding checkpoint NLRs.

Identifying what stimulates the checkpoint function of NLRs

Progress has been made in identifying how NLRs sense PAMPs and DAMPs. How these pathways are utilized by checkpoint NLRs has not yet been studied.

Resolving multiple functions performed by a single NLR protein

Many NLRs have exhibited multiple functions. Resolving how NLRs can participate in functions that activate or attenuate inflammation is necessary in order to understand their overall contribution to immunity.

Identifying the role of NLRs in heterogeneous cellular and tissue sites

Most of the research on NLR function has focused on their role in the immune system. Studies exploring their role in multiple tissue and cell types, which involve better mouse models, should provide a more comprehensive picture of their physiological roles as activators and/or attenuators of inflammation.

Resolving how the subcellular localization of NLR proteins impacts their functions

The dynamic subcellular localization of certain NLR proteins indicates that this might also apply to other NLRs. How the subcellular localization affects the function of NLRs as activators or attenuators of inflammation under different milieu will be important in order to understand how the checkpoint functions of NLRs are achieved.

Understanding how alternate forms of NLRs produced by post-transcriptional or post-translational modifications can exert distinct functions

NLRs are known to produce difference splice forms, and some are subjected to cleavage and post-translational modifications. Assessing the function of different forms of NLRs will be important in understanding their diversified roles.

Challenges

Considering the intense pace of research in the past decade regarding the underlying mechanisms of action of NLRs in inflammation, PAMP or DAMP recognition, signaling pathways, and disease relevance, the most important challenge for the future is to utilize this knowledge for development of therapeutics for diseases and to improve patient outcomes.

Abbreviations

ASC, apoptotic speck-containing protein with a CARD; CAC, colitis-associated colorectal cancer; ERK, extracellular-signal-regulated kinases; DAMPs, danger-associated molecular pattern; IFN, interferon; IKK, NF-κB kinase; LRR, leucine-rich repeat; MAPK, mitogen-activated protein kinase; MAVS, mitochondrial antiviral signaling; MHC, major histocompatibility complex; NBD, nucleotide-binding domain, leucine-rich repeat containing receptor; NBS, nucleotide-binding site; NF-κB, nuclear factor κB; NIK, NF-κB-inducing kinase; NLR, nucleotide-binding domain, leucine-rich repeat-containing receptor; PAMP, pathogen-associated molecular pattern; PRRs, pattern recognition receptors; RIG-I, retinoic acid inducible gene-I; ROS, reactive oxygen species; STING, stimulator of interferon genes; TBK1, TANK-binding kinase 1; TRAF, TNF receptor associated factor; TNFα, tumor necrosis factor-α; TLR, Toll-like receptor.

Disclosures

The authors declare that they have no disclosures.

Acknowledgments

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