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Review

Beyond the inflammasome: regulatory NOD-like receptor modulation of the host immune response following virus exposure

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A complex interaction exists between elements of the host innate immune system and viral pathogens. It is essential that the host mount a robust immune response during viral infection and effectively resolve inflammation once the pathogen has been eliminated. Members of the nucleotide-binding domain leucine-rich repeat [NBD-LRR; known as NOD-like receptor (NLR)] family of cytosolic pattern-recognition receptors are essential components of these immunological processes and have diverse functions in the host antiviral immune response. NLRs can be subgrouped based on their general function. The inflammasome-forming subgroup of NLRs are the best-characterized family members, and several have been found to modulate the maturation of IL-1β and IL-18 following virus exposure. However, the members of the regulatory NLR subgroups are significantly less characterized. These NLRs uniquely function to modulate signalling pathways initiated by other families of pattern-recognition receptors, such as Toll-like receptors and/or Rig-I-like helicase receptors. Regulatory NLRs that augment pro-inflammatory pathways include nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2, which have been shown to form a multiprotein complex termed the NODosome that significantly modulates IFN and NF-κB signalling following viral infection. Conversely, a second subgroup of regulatory NLRs functions to negatively regulate inflammation. These inhibitory NLRs include NLRX1, NLRP12 and NLRC3, which have been shown to interact with TRAF molecules and various kinases to modulate diverse cellular processes. Targeting NLR signalling following infection with a virus represents a novel and promising therapeutic strategy. However, significant effort is still required to translate the current understanding of NLR biology into effective therapies.

Introduction

The host innate immune system is the first line of defence following viral infection. Germline-encoded pattern-recognition receptors (PRRs) sense pathogen-associated molecular patterns (PAMPs) associated with viral infection and are responsible for initiating the biochemical signalling cascades that orchestrate the innate immune response. PRRs are a large group of proteins that include either membrane-bound receptors or cytosolic receptors. The membrane-bound receptors include Toll-like receptors (TLRs) and C-type lectin receptors (CLRs), which are transmembrane proteins found in both plasma and endosomal membranes. The cytosolic receptors include the nucleotide-binding domain leucine-rich repeats [NBD-LRRs; known as NOD-like receptors (NLRs)], Aim2-like receptors and Rig-I-like helicase receptors (RLRs). In addition to these defined sensors, a diverse range of unique cytoplasmic receptors has recently been described that do not fit within currently defined PRR families and which includes unique proteins shown to be relevant during viral infection such as cyclic GMP-AMP synthase (cGAS), stimulator of IFN genes (STING) and DNA-dependent activator of IFN-regulatory factors (DAI) (Broz and Monack, 2013). Together, this repertoire of host PRRs is responsible for driving cellular defence and ultimately facilitating the adaptive immune response following viral infection.

While the role of TLRs, CLRs and RLRs in virus immunity has been relatively well studied, the contribution of NLRs is less defined. There are 22 distinct NLR proteins that have been identified in humans and 34 in mice (Mason *et al.*, 2012). Members of the NLR family are characterized by a tripartite domain structure composed of a variable but limited number of N-terminal domains, which are typically Pyrin or Card domains, a central conserved nucleotide-binding NACHT domain and a C-terminal domain composed of variable numbers of LRRs (Schroder and Tschopp, 2010). The LRR domain is responsible for

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ligand sensing and is thought to maintain an inhibitory conformation until activation (Martinon and Tschopp, 2005). NLR activation results in a protein conformation change allowing the NLR to interact with target proteins to modulate downstream intracellular signalling cascades (Martinon and Tschopp, 2005). The central NACHT domain is necessary for this oligomerization, and the N-terminal domain is important in the recruitment of signalling adaptors and target proteins (Ye and Ting, 2008). The net result of NLR activation is the formation of diverse multiprotein complexes that have the ability to either positively or negatively modulate inflammation.

The NLRs can be divided into three subgroups based on their primary or best-characterized functions. To date, the most thoroughly characterized subgroup of NLRs is associated with the formation of a multiprotein complex termed the inflammasome. Currently, at least eight NLR proteins, including NLRP1, NLRP3, NLRP6, NLRC4 and NLRC5, as well as the PYHIN family member AIM2, have been strongly implicated in inflammasome formation (Agostini *et al.*, 2004; Davis *et al.*, 2011; Hornung *et al.*, 2009; Mariathasan *et al.*, 2004; Martinon *et al.*, 2002; Wlodarska *et al.*, 2014). Inflammasome-forming NLRs recognize specific PAMPs and damage-associated molecular patterns (DAMPs), or sense changes in the intracellular environment following virus infection. Activation of a specific inflammasome-forming NLR results in NLR oligomerization and recruitment of the adaptor protein ASC (Fig. 1). This complex recruits caspase-1, which is maintained in the cell in an inactivated pro-form state. Following inflammasome formation, caspase-1 is cleaved into its activated form, which in turn cleaves pro-IL-1β and pro-IL-18 into their respective mature cytokines. Both of these cytokines are pro-inflammatory mediators and initiate a robust innate immune response following activation and release. In addition to IL-1β and IL-18 generation, NLR inflammasomes are also associated with a unique form of inflammatory cell death, termed pyroptosis (Fig. 1) (Bortoluci and Medzhitov, 2010). Although it has been well studied in the context of bacterial infections, *in vitro* experiments using dsDNA and human immunodeficiency virus type 1 have suggested that pyroptosis can also significantly modulate the host immune response to virus infection (Fernandes-Alnemri *et al.*, 2009; Miao *et al.*, 2010; Silveira and Zamboni, 2010; Steele *et al.*, 2014).

A second subgroup of NLRs includes family members that have been found to function during reproduction and embryogenesis (Van Gorp *et al.*, 2014). There is a paucity of data pertaining to these so-called reproductive NLRs beyond the family members that have been identified primarily through genetic association studies and expression analyses. What is clear about this subgroup is that the primary function of the majority of these NLRs is not associated with IL-1β and IL-18 production, suggesting mechanisms beyond inflammasome formation. These NLRs have been difficult to study due to the lack of orthologues in mice or due to issues associated with embryonic lethality. However, none of the reproductive NLRs has been associated with the modulation of inflammation following virus exposure and they may actually function outside of the immune system. More studies are clearly necessary to better define this NLR subgroup.

The third subgroup of NLRs is defined as regulatory NLRs and these are highly unique among the different families of PRRs. These NLRs can function as either positive or negative regulators of inflammatory signalling cascades. The positive regulatory NLRs, including nucleotide-binding oligomerization domain-containing protein 1 (NOD1) and NOD2, are the best-characterized members of this subgroup. Although NOD1 and NOD2 have predominantly been described in the context of bacteria recognition and signalling, both of these positive regulatory NLRs have also been implicated, either directly or indirectly, as pro-inflammatory mediators of host antiviral immunity. Conversely, it is also essential to attenuate overzealous inflammation following virus clearance to avoid collateral damage and assist in inflammation resolution. In contrast to the inherently pro-inflammatory NLRs, recent studies have identified a group of unique negative regulatory NLRs that appear to attenuate inflammation and augment resolution. These unique NLRs appear to function by attenuating signalling cascades initiated by other families of PRRs, such as TLRs and RLRs (Allen *et al.*, 2011, 2012b; Schneider *et al.*, 2012). This subgroup is currently composed of three members, NLRP12, NLRX1 and NLRC3. Each of these NLRs modulates diverse signalling pathways, including NF-κB and mitogen-activate protein kinase (MAPK) signalling, the type I IFN response, autophagy and the generation of reactive oxygen species (ROS) (Allen *et al.*, 2011, 2012b; Lei *et al.*, 2013; Moore *et al.*, 2008; Tattoli *et al.*, 2008).

Over the last decade, significant progress has been made in identifying and generally characterizing NLR family members. However, despite this progress, approximately half of the identified NLRs lack significant mechanistic and functional insight (Lupfer and Kanneganti, 2013). Significant questions continue to persist regarding the clinical relevance and therapeutic potential of NLRs in human disease, cell type and temporal control of NLR function, and in mechanisms associated with pathogen recognition and specificity (Lupfer and Kanneganti, 2013). While significant progress has been made in characterizing specific NLRs in bacterial pathogenesis, the role of the NLRs in response to other types of pathogens, such as viruses, is still an emerging area of study. This is especially true for members of the regulatory NLR subgroup. This review focuses on our current understanding related to these unique NLRs and emerging concepts associated with their function in modulating viral immunity.

NOD1 and NOD2 promote inflammation and facilitate host antiviral immune responses

Previous studies have shown that NOD1 and NOD2 are important bacterial sensors that recognize muropeptides

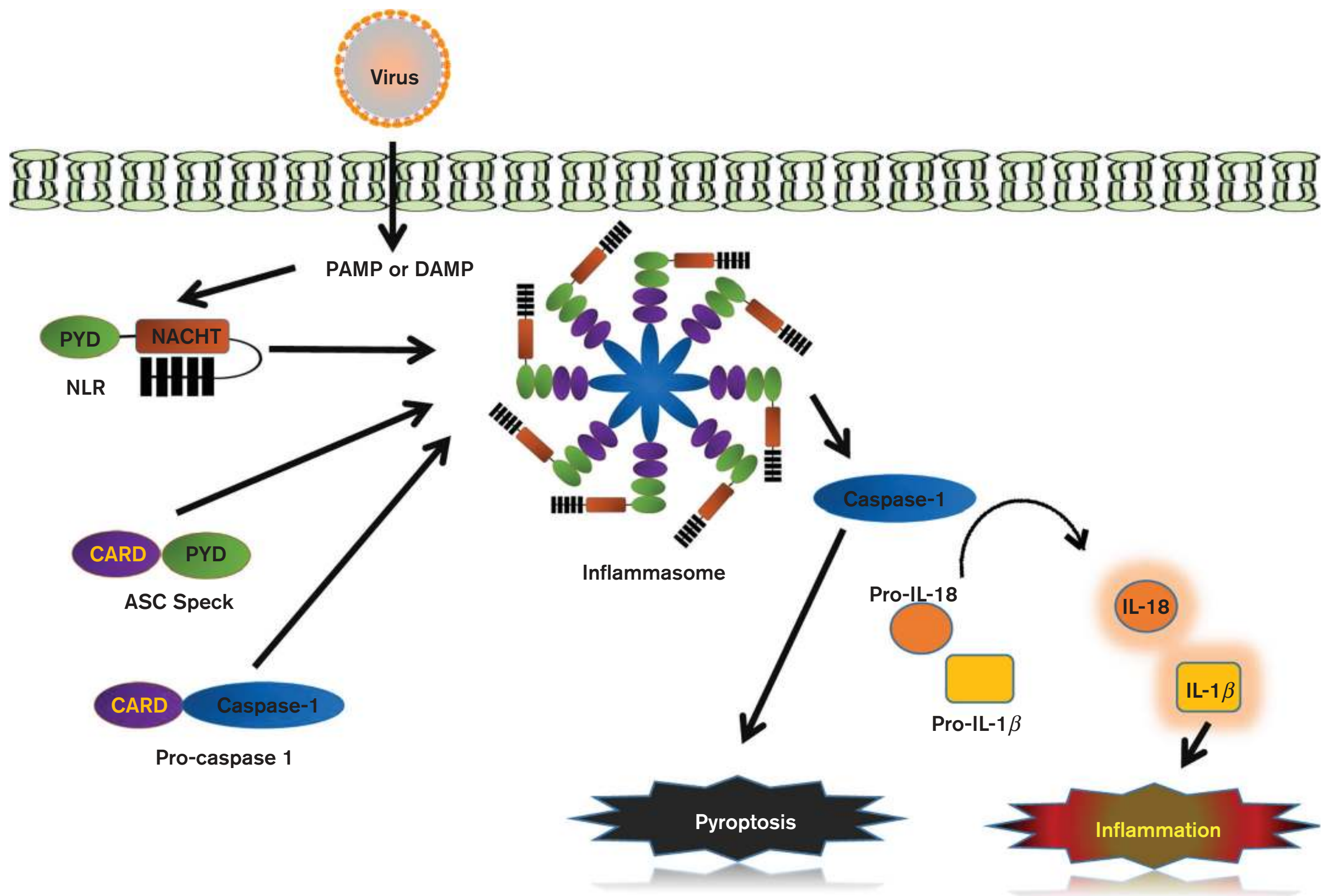

Fig. 1. Schematic of inflammasome formation. Initiation of inflammasome formation occurs following recognition of an NLR by a particular molecular motif (in this image, represented by a virus-associated PAMP or DAMP). Recognition of these motifs then leads to a conformation change in the NLR with subsequent recruitment of the ASC speck (composed of pyrin-containing and CARD domains) and pro-caspase 1. These molecules then oligomerize to form the inflammasome, which then cleaves pro-caspase 1 into its active form. Active caspase-1 can then cleave and activate both pro-IL-1β and pro-IL-18 into their active forms leading to inflammation and/or initiate pyroptosis.

released from the peptidoglycan layer of the bacterial cell membrane. NOD1 is expressed constitutively by various leukocytes and epithelial cells and is upregulated by IFN-γ (Strober *et al.*, 2006). It specifically recognizes γ-D-glutamyl-meso-diaminopimelic acid released from predominantly Gram-negative bacterial organisms, and has been shown in previous studies to recognize *Shigella flexnieri*, *Escherichia coli*, *Chlamydia* spp., *Pseudomonas aeruginosa* and *Helicobacter pylori* (Chamaillard *et al.*, 2003; Girardin *et al.*, 2001; Kim *et al.*, 2004; Opitz *et al.*, 2005; Travassos *et al.*, 2005; Watanabe *et al.*, 2011). NOD2 is also present in various leukocytes and epithelial cells but at very low levels during resting states. Its mRNA and protein levels have been shown to increase greatly when cells are exposed to TNF-α (Rosenstiel *et al.*, 2003). NOD2 recognizes muramyl dipeptide found in both Gram-positive and Gram-negative bacteria, and has been shown in previous studies to recognize *Streptococcus pneumoniae*, *Listeria monocytogenes*, *Salmonella typhimurium* and *Mycobacterium tuberculosis* (Ferwerda *et al.*, 2005; Hisamatsu *et al.*, 2003; Inohara *et al.*, 2003; Kobayashi *et al.*, 2005; Opitz *et al.*, 2004).

Despite their differences in ligand recognition and response to cytokines, NOD1 and NOD2 are thought to modulate inflammation in a similar manner (Fig. 2). When the appropriate pathogenic stimulus is applied, NOD1 and NOD2 undergo homo-oligomerization and recruit a variety of other molecules to form a multiprotein structure termed the 'NODosome' (Keestra and Bäumler, 2014; Tattoli *et al.*, 2007). The critical protein for the formation of the classical NODosome is RIP2 (receptor-interacting protein 2), which, upon association with NOD1 or NOD2, becomes polyubiquitinated and drives downstream NF-κB activation, MAPK signalling and subsequent pro-inflammatory responses (Hasegawa *et al.*, 2008). In keeping with the theme of NLRs being involved in the

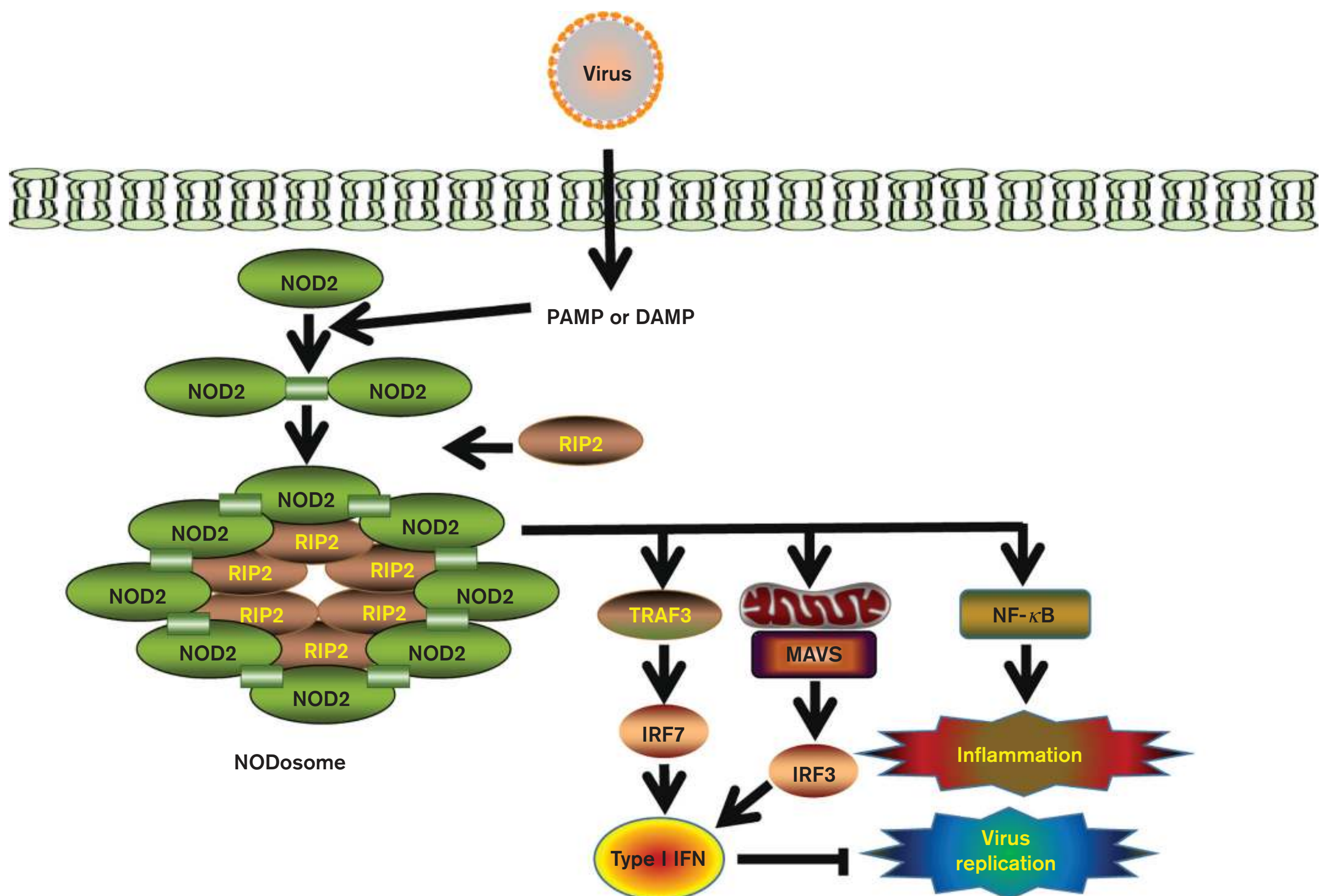

Fig. 2. Positive regulatory NLRs activate inflammatory signalling pathways during viral infection. Positive regulatory NLRs function as promoters of inflammatory responses in the face of PAMP stimulation, with NOD2 being the most classic and well studied in the context of viral infection. Upon stimulation by a virus-associated PAMP or DAMP, NOD2 undergoes homo-oligomerization and recruits receptor-interacting protein 2 (RIP2), resulting in the formation of a large, multiprotein complex termed the NODosome. This complex can interact with several downstream pathways including type I IFN signalling via either TNF receptor-associated factor 3 (TRAF3)/IFN regulatory factor 7 (IRF7) or mitochondrial-associated mitochondrial antiviral signalling (MAVS)/IRF3, or directly by modulating NF-κB signalling, which enhances inflammation aimed at destroying and clearing viral particles.

formation of multiple large-scale functional protein assemblies, several variations of the NODosome have been reported that do not always require RIP2 as a lynchpin. The first of these formations is the interaction reported between NOD2 and the mitochondrial antiviral signalling (MAVS) protein, which results in activation of IFN regulatory factor 3 (IRF3) and type I IFN production (Sabbah *et al.*, 2009). In the context of viral infection, this MAVS–NOD1/2 binding is stimulated by infection with respiratory syncytial virus (RSV) as shown by co-immunoprecipitation studies (Sabbah *et al.*, 2009). It appears to occur at the NBD and LRR domains of NOD1/2 and is independent of RIP2. Similarly, the interactions of NOD1 and NOD2 with the autophagy molecule ATG16L (autophagy related 16-like 1) is another alternative of the NODosome worthy of attention (Hruz and Eckmann, 2011). NOD1/2 recruit ATG16L, which is an essential player in autophagosome formation, to the plasma membrane (Travassos *et al.*, 2010a, b). Indeed, cells containing a mutation in NOD2 lose this ability. Like the NOD/MAVS-mediated IFN response, RIP2 is also dispensable for NOD-dependent autophagy signalling (Travassos *et al.*, 2010b). A third NODosome variation is the interaction of NOD1/2 with the pro-apoptotic protein Bid (Askari *et al.*, 2012; Yeretssian *et al.*, 2011). Although the exact mechanism of this interaction is not known, this has obvious implications in host defence given the intricate relationship of this protein with apoptosis, virus replication and survival. Other proteins such as NF-κB essential modulator (NEMO) (Abbott *et al.*, 2004; Kufer *et al.*, 2008), suppressor of the G2 allele of Skp1 (SGT1) (da Silva Correia *et al.*, 2007) and X-linked inhibitor of apoptosis protein

(XIAP) (Krieg *et al.*, 2009) have also been shown to associate with the NODosome to affect downstream immune signalling.

Although classically considered to be receptors involved primarily with bacterial pathogens, NOD1 and NOD2 functions have been expanding to include virus sensing including responsiveness to both viruses themselves and analogues such as poly(I : C) (Kim *et al.*, 2011). The strongest relationship between these regulatory NLRs and host antiviral responses appears to be centred on NOD2. Multiple studies have shown that absence of NOD2 classically results in a failure to produce a robust antiviral response and reduced ability to control virus replication and infection. NOD2 expression is generally increased following virus infection and by type I IFN signalling, as shown in studies involving RSV, influenza A virus (IAV), human cytomegalovirus, mouse norovirus and porcine respiratory and reproductive syndrome virus (PRRSV) (Collins *et al.*, 1990; Jing *et al.*, 2014; Kapoor *et al.*, 2014; Kim *et al.*, 2011; Vissers *et al.*, 2012). As IFN signalling is also a downstream effect of NOD2 activation, this potentially indicates a self-sustaining positive-feedback loop. Although significantly less reported, NOD1 has also been implicated in modulating host–virus interactions and appears to have functions that are analogous to NOD2. While the overarching theme indicates that NOD1 and NOD2 signalling are necessary for specific aspects of the host antiviral immune response, individual studies have provided several unique insights pertaining to the individual function of these regulatory NLRs.

NOD2 has predominantly been investigated following infection with RNA viruses utilizing *Nod2*$^{-/-}$ mice. In general, these animals display reduced IFN responses and increased susceptibility following infection. Recent studies utilizing IAV have revealed that the loss of NOD2 signalling pathways also appears to extend beyond the initial innate immune response (Lupfer *et al.*, 2014). *In vivo* studies have revealed that *Nod2*$^{-/-}$ mice recruit fewer activated leukocytes and increase cell death in the lungs following infection with IAV. Mechanistically, these studies revealed that dendritic cells are exceptionally affected by the loss of NOD2 following IAV exposure, leading to an inability to properly prime the essential CD8^{+} cytotoxic T-cells that are necessary for the host antiviral response (Lupfer *et al.*, 2014). Indeed, the effects of NOD2 on the adaptive immune system have also been implied in other disease processes, including involvement in Th1/Th2 polarization in CD4^{+}T-cells, dendritic cell cross-priming, immunoglobulin production and a proper vaccine response (Asano *et al.*, 2010; Magalhaes *et al.*, 2008; Moreira *et al.*, 2008; Tada *et al.*, 2005; Watanabe *et al.*, 2004).

NOD2 modulates similar positive regulatory responses in models of RSV, where its interaction with MAVS was required for a proper IFN response and where lack of NOD2 resulted in increased lung pathology and viral titre (Sabbah *et al.*, 2009). The necessity for NOD2 in controlling virus replication was also shown *in vitro*, with NOD2-expressing HEK-293 cells displaying decreased viral load. Interestingly, UV-light-exposed RSV failed to activate the NOD2/IRF3 axis, indicating that an intact and active virion is necessary for the response. Furthermore, in these models, both haematopoietic (immune) and non-haematopoietic (e.g. epithelial cells) cell types required NOD2 for IFN production, again extending the role of NOD2 in viral pathogenesis past the innate immune cells themselves. However, the effects of NOD2 in RSV infection appear to be temporal, as knockdown of NOD2 resulted in reduced activation of IRF3 and production of IFN-β earlier in the course of infection rather than later (Sabbah *et al.*, 2009). Similar results were also observed for NOD2 following exposure to vesicular stomatitis virus (VSV) (Sabbah *et al.*, 2009).

Additional effects of NOD2 have been shown in other RSV models, specifically a potential synergy of pathogen sensing within the context of viral infection and secondary bacterial infection. The initial induction of the type I IFN response by RSV and/or poly(I : C) appears to prime NOD2 and increase its sensitivity to muramyl dipeptide exposure, resulting in increased pro-inflammatory responses (Vissers *et al.*, 2012). This appears to be a common phenomenon and extends beyond RSV. For example, in mouse models of murine norovirus infection, a similar synergism was observed when *E. coli* was used as a secondary bacterium (Kim *et al.*, 2011). In this case, *Nod2*$^{-/-}$ mice were significantly more sensitive to the *E. coli* infection and demonstrated increased mortality and TNF-α production. Similar results were obtained in a follow-up model in the above study using intranasal *P. aeruginosa* infection preceded by poly(I : C) exposure. These findings were confirmed *in vitro*, where pre-treatment with poly(I : C) or murine norovirus 1 exposure increased the levels of TNF-α and IL-6 induced by infection with *E. coli* or *P. aeruginosa*. Mechanistically, it appears that exposure to virus or viral analogues and the associated type I IFN production results in upregulation of NOD2 via TIR-domain-containing adapter-inducing IFN-β (TRIF)- and MAVS-dependent pathways, essentially priming the pathway for additional PAMP recognition (Kim *et al.*, 2011).

The NOD2 axis and its associated molecules, such as RIP2, have also been shown to be highly relevant in viruses of veterinary importance, specifically the arterivirus PRRSV (Jing *et al.*, 2014). *In vitro*, knockdown of NOD2 and RIP2 drastically reduced downstream effector pathways, such as NF-κB and MAPK signalling, resulting in the attenuation of pro-inflammatory cytokines. In live piglets, NOD2 and RIP2 were shown to be elevated in alveolar macrophages for up to 10 days after challenge with PRRSV. The exact mechanism is unknown; however, we may theorize that the synergism between NOD2 and TLR3 through the adaptor protein TRIF is associated with these findings. TLR3 has been shown to be an important controller of PRRSV infection, and loss of its synergistic partner, NOD2, may have negative effects on host defence (Sang *et al.*, 2008).

NOD2 has also been shown to modulate host innate immune responses to DNA viruses, although there have been significantly fewer studies compared with those on RNA viruses. This has been best illustrated by the ability of NOD2 to curtail the replication of human cytomegalovirus (HCMV). *In vitro*, infection by both laboratory strains and human isolates of HCMV resulted in NOD2 upregulation (Kapoor *et al.*, 2014). These effects were amplified in overexpression studies where NOD2 restricted virus replication and enhanced antiviral and pro-inflammatory cytokine production; these effects were lost when NOD2 was knocked down using a lentiviral system (Kapoor *et al.*, 2014). The exact PAMP or DAMP associated with the DNA virus that NOD2 recognizes is currently unknown. However, it appears the HCMV-encoded glycoprotein B, which is necessary for cell entry and cell-to-cell transmission, is not required for NOD2 induction (Kapoor *et al.*, 2014). It is also worth noting that a replication-competent virion is also necessary for NOD2 activation, similar to RSV. NOD2 recognition appears to have some level of specificity, as no such activation was observed in cells administered herpes simplex viruses (HSV) (Kapoor *et al.*, 2014). However, vaccinia virus, another DNA virus, failed to activate IRF3 in NOD2-expressing cells (Sabbah *et al.*, 2009). Interestingly, patients with inflammatory bowel disease have been reported to develop HCMV-induced colitis, typically associated with immunosuppressive therapy (Kim *et al.*, 2010; Matsuoka *et al.*, 2007). This is an interesting correlation given NOD2's well-characterized loss-of-function mutation that is associated with inflammatory bowel disease in human subpopulations (Ogura *et al.*, 2001; Hugot *et al.*, 2001). Based on *in vivo* and *in vitro* studies, NOD2 appears to be activated by nucleic acids, particularly viral ssRNA, activating downstream pathways in a manner similar to Rig-like helicases (Sabbah *et al.*, 2009). Indeed, immunoprecipitation studies have shown an association of NOD2 with viral RNA using primers for RSV nucleocapsid protein, but not with control RNA. Given this interaction, it is possible that nucleic acid itself is also its target for DNA viruses. However, the exact mechanism and binding sites of the nucleic acids within NOD2 are still under debate, although the nucleotide-binding domain has been suggested (Mo *et al.*, 2012).

The role of NOD2 in DNA virus pathology has not been studied to the extent of RNA viruses, so full characterization of the signalling differences remains incomplete. However, based on the limited data available, it appears that the interaction of NOD2 and DNA viruses may be more pathogen specific than its relationship with RNA viruses/viral RNA. It may not be a difference of physical interaction and downstream pathway activation but rather a difference in sensing ability and specificity. More work will need to be done in this area to examine the full repertoire of the interactions of NOD2 with DNA viruses.

NOD1 has been studied considerably less than NOD2 within the context of viral infections, but seems to perform similar functions and have similar outcomes when eliminated or stimulated. For example, mouse norovirus infection and/or type I IFN signalling upregulates NOD1 (Kim *et al.*, 2011), similar to NOD2. Subsequent *in vitro* mechanistic studies have shown that the effects of NOD1 on IFN production appear to be independent of IRF3, based on a lack of ssRNA-induced IRF3 activation and IFN-β signalling (Sabbah *et al.*, 2009). Indeed, the interactions of NOD1 with IRF7 via the RICK/TRAF3/TBK1/IKKε cascade appears to be the major mechanism of NOD1 IFN stimulation, which previously has been shown in *Helicobacter pylori* studies (Watanabe *et al.*, 2010, 2011). In addition to its effects on innate immune signalling, NOD1 has also been suggested to play a significant role in linking the innate and adaptive immune responses following virus exposure. This was suggested based on findings following RSV infection in one of the few studies to specifically evaluate NOD1 in antiviral immunity (Senft *et al.*, 2010). In this study, RSV was found to inhibit IFN-β- and IFN-γ-activated transcription in alveolar macrophages, including attenuation of NOD1 and class II transactivator (CIITA; also known as NLRA) transactivation (Senft *et al.*, 2010). The downregulation of IFN-induced NOD1 expression occurred via inhibition of tyrosine kinase 2 and STAT1 (signal transducer and activator of transcription 1) phosphorylation (Senft *et al.*, 2010). Despite the findings from these prior studies, additional insight is still necessary to better characterize the function of NOD1 during host antiviral immune responses.

NLRX1 negatively regulates diverse aspects of host antiviral immunity to attenuate overzealous inflammation following virus infection

NLRX1 is a regulatory NLR that has been identified as being involved with a variety of cellular functions (Fig. 3). While some ligands have been identified, it is highly likely that the full repertoire of viral PAMPs that signal through NLRX1 have yet to be fully elucidated. It is considered unique among the NLR family as it has been found to be associated with the mitochondria in multiple studies (Arnoult *et al.*, 2009; Moore *et al.*, 2008; Tattoli *et al.*, 2008). Initially, Moore *et al.* (2008) showed that NLRX1 is most likely associated with the mitochondrial outer membrane (Moore *et al.*, 2008). Subsequent evaluation indicated that NLRX1 inhibits type I IFN production through its interaction with MAVS and attenuation of the RIG-I and melanoma differentiation-associated protein 5 (MDA-5) signalling pathways (Moore *et al.*, 2008). However, the localization data conflict with other studies that suggest NLRX1 is associated with the inner mitochondrial membrane and matrix (Arnoult *et al.*, 2009; Tattoli *et al.*, 2008). While the exact localization of NLRX1 around or within the mitochondria is still an area of debate, it is possible that NLRX1 behaves similarly to

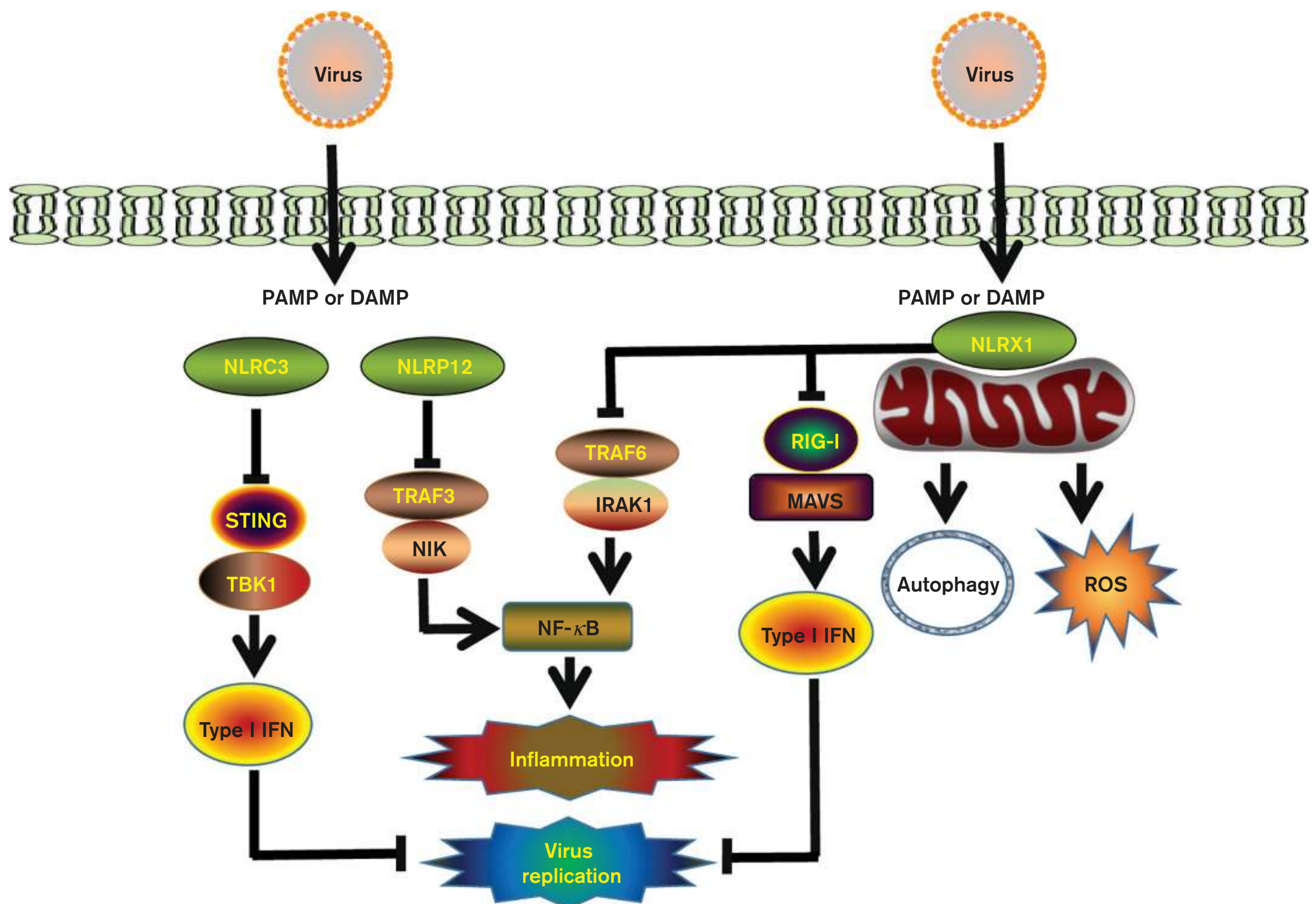

Fig. 3. Negative regulatory NLR functions during viral infection. Three negative regulatory NLRs, NLRC3, NLRP12 and NLRX1, have been described. Upon stimulation with virus-associated PAMPs or DAMPs, NLRC3 blocks the association and migration of the STING/TGF-β-activated kinase 1 (TAK1) complex, resulting in downregulation of IFN production responsible for keeping virus replication in check. NLRX1 is uniquely localized at the mitochondria, which facilitates its inhibitory association with MAVS and retinoic acid-inducible gene I (RIG-I). This results in dampening of IFN production. NLRX1 also interacts directly with TRAF6 to act as an inhibitor of NF-κB signalling. Additional functions of NLRX1, such as modulation of autophagy and ROS, also have implications in host defence against viral pathogens. NLRP12 is capable of suppressing elements of the NF-κB pathway and may have a role in regulation of the host immune response following viral infection. IRAK1, IL-1 receptor-associated kinase 1; NIK, NF-κB-inducing kinase; TBK1, TANK-binding kinase 1.

other NLR family members that shuttle from the cytosol to the mitochondrial matrix. Indeed, the ability to shuttle appears to be a common feature among NLR family members. For example, CIITA (NLRA) and NLRC5 have the ability to shuttle from the cytosol to the nucleus to modulate the host-antivirus response or following stimulation with virus-associated PAMPs (Benko *et al.*, 2010; Meissner *et al.*, 2010; Orlandi *et al.*, 2011; Tosi *et al.*, 2011). Likewise, both NOD1 and NOD2 have been shown to shuttle from the cytosol to the plasma membrane, where membrane association has been linked to function (Kufer *et al.*, 2008). Thus, it is possible that all of the initial findings associated with mitochondrial localization are feasible and may actually reveal a more complex function for NLRX1 than previously recognized.

In addition to modulating type I IFN production, NLRX1 has been shown to play a role in a variety of additional cellular processes, including NF-κB signalling and ROS production. The identification of its role in NF-κB signalling was found when *Nlrx1*$^{-/-}$ macrophages exposed to lipopolysaccharide showed significantly higher amounts of IL-6 compared with WT cells (Allen *et al.*, 2011). Using both overexpression and endogenous systems, these studies showed that NLRX1 can negatively regulate NF-κB signalling through its interactions with TRAF6 (Allen *et al.*, 2011). Subsequent studies provided additional mechanistic insight that revealed, upon stimulation, that NLRX1 dissociates from TRAF6 to interact with the IκB kinase (IKK) complex and downregulate NF-κB signalling (Xia *et al.*, 2011). While NLRX1 appears to negatively

regulate type I IFN and NF-κB signalling, it also seems to augment ROS production through interactions with the mitochondrial protein ubiquinol–cytochrome *c* reductase core protein II (UQCRC2; Arnoult *et al.*, 2009). Functionally, HeLa cells transfected with NLRX1 significantly increased ROS production induced by TNF-α, *Shigella* sp., poly(I : C), *Chlamydia trachomatis* and TNF-α/cycloheximide (Abdul-Sater *et al.*, 2010; Singh *et al.*, 2015; Tattoli *et al.*, 2008). In addition to these functions, NLRX1 is broadly expressed in a variety of cell types and probably regulates immune signalling through a cell type-, temporal- and stimuli-specific mechanism.

Within the context of viral infection, NLRX1 appears to attenuate overzealous inflammation and facilitate inflammation resolution. This is highly advantageous for host survival, but can have implications in the replication and persistence of viruses. Dampening of both IFN and the associated pro-inflammatory mediator IL-6 via NLRX1 has been shown in the context of many different viral pathogens including simian virus 5, Sendai virus (SeV), VSV and IAV (Allen *et al.*, 2011; Moore *et al.*, 2008). During infection of *Nlrx1* $^{-/-}$ mice with IAV, there is significant upregulation of IFN-β, STAT2 and IL-6, underlining the role of NLRX1 as a negative regulator of inflammation (Allen *et al.*, 2011). These mice also displayed increased lung pathology and clinical disease, although viral clearance was enhanced (Allen *et al.*, 2011). Interestingly, one of the most prevalent phenotypes observed in the *Nlrx1* $^{-/-}$ mice following IAV infection appeared to be increased airway epithelial cell damage and denuding of the airway (Allen *et al.*, 2011). This is consistent with recent studies evaluating the role of NLRX1 in chronic obstructive pulmonary disease, where cigarette smoke induced significantly more airway remodelling, protease production, cell death and alveolar destruction in the lungs of *Nlrx1* $^{-/-}$ mice through a MAVS-dependent mechanism (Kang *et al.*, 2015). These observations could be due to direct effects of NLRX1 deletion on the airway epithelial and stromal cells or indirectly associated with increased IFN-β in the local microenvironment following infection or damage. Further independent studies have confirmed many aspects of these findings and have indicated that NLRX1 can recognize and bind to specific parts of IAV, including the PB1-F2 protein and viral RNA (Hong *et al.*, 2012; Jaworska *et al.*, 2014). The binding of the viral RNA is not specific to IAV, as subsequent studies have shown that NLRX1 is also capable of binding rhinovirus (RV) RNA and poly(I : C) (Unger *et al.*, 2014). This recent study suggests that both RV RNA and poly(I : C) interact with NLRX1 in the cytoplasm and at the apical surface, and translocate to the mitochondria following RV infection (Unger *et al.*, 2014). NLRX1 is one of the best-characterized NLRs in terms of PAMP recognition, and the C terminus appears to form a specific binding site for viral RNA (Hong *et al.*, 2012). However, there is still a paucity of information on the exact mechanism of viral recognition, and consensus regarding target specificity is still emerging. Mechanistically, NLRX1 acts as a negative regulator of RIG-1/MAVS signalling (Allen *et al.*, 2011), a well-studied and critical pathway in viral recognition, similar to the aforementioned NOD2–MAVS interaction (Sabbah *et al.*, 2009). However, the difference in mechanism of action is clear: NOD2–MAVS interactions exacerbate inflammatory processes, whereas NLRX1–MAVS interactions form an inhibitory complex. This interesting push-and-pull relationship between positive and negative regulatory NLRs, using MAVS as the keystone, exemplifies the many interactions this family of proteins has not only with other signalling molecules but also with each other. The effects of NLRX1 on IFN production also take a slightly different downstream turn, with a lack of direct interaction with IRF3 as compared with NOD2 (Allen *et al.*, 2011), indicating a potentially unique pathway. The current hypothesis suggests that NLRX1 binds directly with MAVS, competing with RIG-I, which results in downstream NF-κB and IRF3 suppression (Moore *et al.*, 2008). NLRX1 is also able to curtail NF-κB signalling in a MAVS-independent fashion through TRAF6 signalling (Allen *et al.*, 2011; Xia *et al.*, 2011). Given the diverse effects of NLRX1 on other proteins, there are likely to be many additional undiscovered indirect mechanisms through which this highly unique protein exerts its negative regulatory effects.

Autophagy is also a critical pathway in the context of viral pathogenesis, with its upregulation being associated with virus clearance. Intracytoplasmic virions can be captured within the autophagy pathway and transferred to lysosomes for eventual breakdown and/or pattern recognition resulting in the activation of innate and adaptive immune responses (Shoji-Kawata and Levine, 2009). NLRX1's promotion and regulation of autophagy has been reported in several instances within the context of virus exposure (Lei *et al.*, 2012, 2013). These studies revealed that NLRX1 is capable of augmenting autophagy pathways by associating with the Tu translation elongation factor (TUFM) protein (Lei *et al.*, 2012). TUFM is a molecule that not only potently suppresses RIG-I signalling, but is also associated with the autophagy complex ATG12–ATG5–ATG16L1. NLRX1 and TUFM appear to act together to keep type I IFN production in check and also prevent decreases in autophagy (Lei *et al.*, 2012, 2013). The ATG12–ATG5 complex can also interact directly with MAVS to inhibit type I IFN. For example, its absence has been shown to lead to accumulation of MAVS on the mitochondria and elevation of type I IFN (Lei *et al.*, 2013). Thus, while NLRX1 seems to enhance autophagy, this may actually augment its negative regulation of type I IFN.

The enhancement of ROS production by NLRX1 also has significant implications in virus sensing. Historically, ROS are important in viral infection in several different ways. Viruses often increase ROS production in host cells and inhibit antioxidant enzymes, such as superoxide dismutase (Schwarz, 1996). This ROS production can also result in downstream activation of NF-κB and other inflammatory mediators. Oxidative stress, although damaging to the host, may also serve as a mechanism to suppress virus replication via inflammation and destruction

of infected cells. While the suppressive effects of NLRX1 on inflammation are well established, it appears that NLRX1 may act as a dual-edged sword by promoting ROS in an effort to enhance virus clearance. Generation of ROS can result in downstream apoptosis of cells, which is another cellular defence mechanism associated with NLRX1 function. In terms of viral infection, NLRX1 appears to function to suppress mitochondrial-induced apoptosis in IAV-infected macrophages, resulting in increased cell survival (Jaworska *et al.*, 2014). In contrast, another study showed that NLRX1 actually promotes intrinsic apoptotic signals such as endoplasmic reticulum stress (Soares *et al.*, 2014). Intriguingly, a recent study revealed that NLRX1 is required for RV-mediated epithelial barrier disruption (Unger *et al.*, 2014). When NLRX1 was silenced in polarized airway epithelial cells, RV-induced ROS generation was eliminated and transepithelial resistance was reduced (Unger *et al.*, 2014). Furthermore, NLRX1 was shown to interact with either RV RNA or poly(I : C) and stimulate mitochondrial ROS production, and was also found to be essential for RV-induced NOX-1 expression in polarized airway epithelial cells (Unger *et al.*, 2014). Together, these data raise interesting questions as to the specificity of NLRX1's involvement in oxidative stress and the resulting apoptosis of cells, and whether its effect may potentially be variable depending on the instigating signal.

All of these functions for NLRX1 are not without controversy, both in terms of general mechanism and virus signalling. Several studies have suggested that NLRX1 may function as a promoter rather than an inhibitor of IFN and NF-κB signalling or may have no direct role at all depending on the virus, the experimental conditions and the *Nrlx1*$^{-/-}$ animals being evaluated (Jaworska *et al.*, 2014; Soares *et al.*, 2013; Tattoli *et al.*, 2008). However, it is highly likely that these confounding data reflect a greater level of complexity associated with NLRX1 function. It is possible that NLRX1 acts as a regulator rather than a simple attenuator of these pro-inflammatory signalling pathways. Likewise, it is possible that the effects of NLRX1 on viral infection are exquisitely sensitive to a variety of factors including viral species and/or strain, infection conditions, environment and a host of other factors that have yet to be elucidated. Indeed, future studies are likely to reveal that the regulation of NLRX1 must be tightly controlled in a cell type-, temporal- and stimuli-specific manner to maintain immune system homeostasis following virus exposure.

NLRP12 attenuates non-canonical NF-κB signalling with potential ramifications on host–virus interactions

Unlike NLRX1, which was only relatively recently characterized, NLRP12 was one of the first NLRs to be identified as a negative regulator of inflammation and has been previously referenced as Monarch-1 or PYPAF7 (Tuncer *et al.*, 2014). NLRP12 functions as a negative regulator of inflammation by modulating elements of the NF-κB signalling pathway (Fig. 3) (Allen *et al.*, 2012a, b; Arthur *et al.*, 2007; Lei *et al.*, 2012, 2013; Moore *et al.*, 2008; Tsuchiya *et al.*, 2010; Wagner *et al.*, 2009; Xia *et al.*, 2011; Zaki *et al.*, 2014). This hypothesis is supported by recent *in vivo* studies utilizing *Nlrp12*$^{-/-}$ mice in models of acute colitis and colitis-associated tumorigenesis. Collectively, these studies showed that, when compared with WT mice, *Nlrp12*$^{-/-}$ mice exhibited increased susceptibility to acute colitis as well as to colitis-associated tumorigenesis, and had increased pro-inflammatory cytokine production and increased NF-κB-dependent signalling molecules (Allen *et al.*, 2012b; Zaki *et al.*, 2011). These data support early *in vitro* findings indicating that NLRP12 negatively regulates non-canonical NF-κB signalling and the related chemokines CXCL12 and CXCL13 (Lich *et al.*, 2007).

Although NLRP12 has not been studied specifically within the context of viral infection, several theories may be formulated based on its modes of action. The non-canonical NF-κB pathway, which results in the production of specific, chronic chemokines as opposed to acute inflammatory responses, can readily be activated by inducers of RIG-I/MAVS such as RSV (Liu *et al.*, 2008) and has been shown to be a critical negative regulator of type I IFN production (Jin *et al.*, 2014). It appears that RNA viruses, such as VSV or SeV, stimulate this NF-κB pathway via upregulation of NF-κB-inducing kinase (NIK) and induction of TRAF3 degradation. NIK itself is a central molecule in the non-canonical pathway, responsible for IKKα activation, phosphorylation of p100 and release of RelB–p52 dimers into the nucleus to promote gene transcription. Mice deficient in the NIK-encoding gene, *Map3k14*, displayed increased survivability and decreased viral load upon exposure to VSV infection as opposed to WT counterparts, and increased IFN-α and IFN-β. In addition to active promoters, non-canonical NF-κB signalling also regulates epigenetic changes, in particular histone modifications, to the *Ifnb* promoter (Jin *et al.*, 2014). Given the involvement of non-canonical NF-κB signalling in viral pathogenesis and considering NLRP12's negative regulation of this pathway, NLRP12 activation may result in the suppression of IFN-related genes, similar to NLRX1.

NLRP12 also acts as a negative regulator of the canonical NF-κB pathway (Zaki *et al.*, 2011). Thus, concurrent canonical NF-κB suppression may also result in NLRP12 expression curtailing the production of a range of pro-inflammatory cytokines following virus infection. Likewise, NLRP12 has also been implicated in the suppression of extracellular signal-regulated kinase (ERK) pathways (Allen *et al.*, 2012b). Upregulation of the ERK pathways has been associated with various viral diseases resulting in a variety of effects including altered cellular inflammation profiles, enhanced infectivity and increased virus replication (Anders *et al.*, 2003; Lee *et al.*, 2008;

Pleschka *et al.*, 2001; Popik *et al.*, 1998; Yang and Gabuzda, 1999; Zhao *et al.*, 2005, 2013, 2006).

NLRC3 negatively regulates canonical NF-κB signalling during virus infection

NLRC3 is one of the most recently characterized and least studied NLRs. It was initially identified for its role in modulating T-cell signalling (Conti *et al.*, 2005). However, a recent study provided data indicating that NLRC3 acts as a negative regulator of inflammation through its interactions with TRAF6 (Fig. 3). In this study, an NF-κB luciferase reporter vector was transfected into HEK293T cells, and NLRC3 was shown to attenuate signalling (Schneider *et al.*, 2012). Further mechanistic studies revealed that NLRC3 inhibited NF-κB signalling when activated by MyD88 and TRAF6, but not by p65 or IKKα (Schneider *et al.*, 2012). The findings were subsequently confirmed by co-immunoprecipitation studies. These studies further indicated that NLRC3 reduces the overall ubiquitination of TRAF6. However, this decrease is associated with K-63 ubiquitin chains, which are typically recognized as activating signals, resulting in attenuation of signalling. These results were confirmed by exposing peritoneal macrophages from *Nlrc3*$^{-/-}$ mice to lipopolysaccharide and revealing increased levels of TNF, IL-6, IL-1β and IL-12. In a model of septic shock, *Nlrc3*$^{-/-}$ mice had increased disease severity and circulating levels of TNF and IL-6 (Schneider *et al.*, 2012).

NLRC3 is another negative regulatory NLR that has been implicated in virus sensing, although it has not been studied to the extent of NLRX1. Upon introduction of cytosolic dsDNA, cyclic di-GMP and DNA viruses, NLRC3 acts to suppress IFN responses (Zhang *et al.*, 2014). Indeed, cells lacking NLRC3, when challenged with HSV, display elevated phospho-TANK-binding kinase 1 (TBK1) and phospho-IRF3, resulting in increased signalling pathway activation (Zhang *et al.*, 2014). *In vivo*, *Nlrc3*$^{-/-}$ mice challenged with HSV had increased IFN-β, TNF and IL-6, and lower overall mortality/morbidity associated with reduced virus replication (Zhang *et al.*, 2014). Although NLRC3's classic mode of action appears to be the suppression of inflammation via TRAF6 and NF-κB signalling, these data suggest a unique signalling pathway for NLRC3 specifically in the context of viral infection, as TRAF6 is not required for HSV-1-induced type I IFN activation (Zhang *et al.*, 2014).

This alternative pathway has been determined to be via a negative regulatory interaction between NLRC3 and STING (Mangan and Latz, 2014; Zhang *et al.*, 2014). STING itself is an intracellular DNA-sensing protein that induces downstream IFN responses by interacting with TBK1 (Burdette *et al.*, 2011). Upon stimulation with intracellular pathogens such as HSV, STING relocalizes from the endoplasmic reticulum to perinuclear vesicles, resulting in IRF3 activation and pro-inflammatory cytokine production (Ishikawa and Barber, 2008; Ishikawa *et al.*, 2009). Mice lacking STING are unable to control HSV-1 infection and replication due to lack of an IFN response. NLRC3 appears to have several ways of curtailing the STING/TBK1 axis including directly associating with STING, preventing STING trafficking to the perinuclear vesicular regions and by binding to STING's partner, TBK1 (Zhang *et al.*, 2014). NLRC3's suppression of NF-κB also appears to be via STING. The kinase-containing N terminus of TBK1 has been shown to be a binding partner for NLRC3, with the likely partner domain being NLRC3's NBD, although additional interactions at the LRR domain have not been ruled out. The interaction of NLRC3 at such a critical junction in virus sensing presents this protein as an extremely powerful modulator of the antiviral response. Additionally, the multiple signalling pathways that NLRC3 appears to control based on stimulus (i.e. lipopolysaccharide versus viral) further underlie the multifunctionality of the NLR family with regard to PAMP recognition and downstream signalling effects.

One of the most intriguing findings associated with the characterization of NLRC3 has been the conceptualization of the 'TRAFasome' (Zhang *et al.*, 2014). The negative regulatory NLR proteins appear to be intricately linked to TRAF signalling. NLRC3 and NLRX1 appear to be associated with TRAF6, and NLRP12 appears to have an affinity for TRAF3 (Allen *et al.*, 2011, 2012b; Zhang *et al.*, 2014). Similar to the inflammasome and NODosome, the current hypothesis suggests that these NLR–TRAF complexes provide multiple signalling avenues to allow these regulatory NLRs a mechanism to balance positive and negative inflammatory responses depending on specific microenvironmental conditions, the instigating stimulus and other cell-specific factors. Certainly more insight is necessary pertaining to TRAFasome function, composition and mechanism, especially as it pertains to virus immunity. However, this is yet another example of the unique mechanisms employed by members of this novel family of PRRs to modulate the host immune response following pathogen exposure.

Concluding remarks

Over the last decade, the in-depth functional characterization of the regulatory NLR family members has significantly expanded our understanding of this unique class of PRRs. It is now evident that NLR function extends well beyond the formation of an inflammasome and the simple post-translational processing of IL-1β and IL-18. The recent discovery of NLRs that negatively regulate the function of other families of PRRs emphasizes the point that a large number of NLR proteins identified in humans and other mammalian species have yet to be significantly characterized. In the context of host–virus interactions, future studies addressing issues associated with ligand binding, cell type and temporal regulatory mechanisms, and with viral specificity, are all direly needed. Likewise,

additional mechanistic insight associated with the formation and function of multiprotein complexes in regulating the host immune response will significantly improve our overall understanding of host antiviral immunity.

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