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Central roles of NLRs and inflammasomes in viral infection

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Abstract

The immune response to viral infections is determined by a complex interplay between the pathogen and the host. Innate immune cells are equipped with a set of cytosolic sensors to detect viral infections. Recognition by these sensors induces the production of type I interferons and the assembly of inflammasome complexes that activate caspase-1. Here, I discuss recent progress in our understanding of the central roles of NOD-like receptors (NLRs) and inflammasomes in the immune response during viral infections. This information will improve our understanding of host defence mechanisms against viruses and provide new avenues for interfering in the pathogenesis of infectious diseases.

To detect and respond rapidly to diverse groups of microorganisms, vertebrates have evolved multiple germline-encoded pattern-recognition receptors (PRRs) that detect conserved microbial and viral components known as pathogen-associated molecular patterns (PAMPs)¹. These PAMPs represent molecules that are expressed by the microbes and are usually required for their survival. Bacterial PAMPs include flagellin, the bacterial cell-wall components lipopolysaccharide (LPS), lipoteichoic acid and peptidoglycan, and nucleic acid structures that are unique to bacteria. Viral PAMPs are composed mainly of unique nucleic acids such as double-stranded (ds)RNA, uncapped single-stranded (ss)RNA, and cytosolic DNA but also include several viral fusion glycoproteins (e.g. respiratory syncytial virus (RSV)-F protein and Ebola virus GP1 proteins). Four main classes of PRRs have been described: Toll-like receptors (TLRs), RIG-I (retinoic acid inducible gene-I)-like receptors (RLRs), nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) and the IFI200 family member absent in melanoma 2 (AIM2). Whereas TLRs sample the extracellular space and endosomes, NLRs, RLRs and AIM2 function as pathogen sensors in intracellular compartments².

NLRs comprise a large receptor family characterized by the presence of a conserved NOD motif^{3,4}. Notably, the domain structure of NLRs resembles that of a subset of plant disease-resistance (R) genes, which trigger the hypersensitive response and confer resistance to infection with fungal, viral, parasitic and insect pathogens⁵. The general domain organization of NLRs includes: an amino-terminal effector binding region that consists of protein-protein interaction domains such as the caspase recruitment domain (CARD), pyrin domain (PYD) and baculovirus inhibitor repeat (BIR) domain; an intermediary NOD that is required for nucleotide binding and self-oligomerization; and an array of carboxy-terminal leucine-rich repeat (LRR) motifs presumed to detect conserved microbial patterns and to modulate NLR activity. Bioinformatics studies have shown that there are 22 NLR genes in the human genome and 34 NLR genes in the mouse genome^{3,4}. The current model

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proposes that when a PAMP or DAMP is sensed by the C-terminal LRRs of an NLR, the molecule undergoes conformational rearrangements that trigger oligomerization through the NOD. In turn, NLRs expose the N-terminal effector domains to induce the recruitment and activation of CARD and PYD-containing effector molecules by promoting their proximity and oligomerization. As a result, NLRs are involved in the activation of diverse signalling pathways. For example, the NLR proteins NOD1 and NOD2 interact with the receptor-interacting serine-threonine protein kinase 2 (RIPK2; also known as RICK or RIP2) to induce nuclear factor- κ B (NF- κ B) and mitogen-activated protein kinase (MAPK) signalling. Furthermore, NOD1 and NOD2 have been shown to control the induction of autophagy by recruiting autophagy-related gene 16-like 1 (ATG16L1) to the plasma membrane⁶. NOD2 also associates with mitochondrial antiviral signalling protein (MAVS; also known as VISA, CARDIF or IPS1) for the induction of type I interferons (IFNs) in response to viral infection⁷. Moreover, the NLR family members NLRP1, NLRP3 and NLRC4 assemble large protein complexes known as inflammasomes, which are responsible for the activation of the inflammatory caspase caspase-1 and hence the production of interleukin-1 β (IL-1 β) and IL-18. Finally, NLRX1 and NLRC5 have been shown to inhibit NF- κ B and type I IFN signalling pathways suggesting important roles for these negative regulators in homeostatic control of innate immunity^{8,9}. Clearly, NLRs and inflammasomes participate in a diverse set of innate immune signalling pathways (FIG 1).

Our understanding of the crucial roles that NLRs and inflammasomes have in mounting protective antiviral responses and the molecular mechanisms used by viral pathogens to evade them has progressed remarkably in the past few years. Depending on the nature of the infectious agent, particular NLRs and inflammasome complexes become activated to elicit tailored immune responses. Here, I review recent findings on the roles of the inflammasome regulators NLRP3, RIG-I and AIM2 and the NLRs NOD2, NLRX1 and NLRC5 in activating or inhibiting major antiviral signalling pathways and discuss the crosstalk that exists between these PRRs. Finally, I provide a brief overview of the mechanisms of viral evasion of these pathways.

Inflammasome activation during viral infection

A breakthrough in the identification of the mechanisms controlling the activation of inflammatory caspases came from the identification and characterization of the inflammasome, a large (700 kDa) multi-protein complex that recruits inflammatory caspases and triggers their activation¹⁰. Inflammasomes are often defined by the constituent PRR family member, which functions as a scaffold protein to bring caspase-1 molecules together and induce caspase-1 activation through proximity-induced auto-activation. Currently, three distinct inflammasomes have been shown to be involved in antiviral immunity: the NLRP3 inflammasome^{11–15}, the RIG-I inflammasome¹⁶ and the AIM2 inflammasome^{17–19}. In these complexes, the bipartite, 22-kDa adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) provides a link between NLRP3, RIG-I or AIM2 and the pro-form of the inflammatory caspases through homotypic interactions with N-terminal PYDs and C-terminal CARDs (FIG 2). Activated caspase-1 cleaves pro-IL-1 β and pro-IL-18 into biologically active IL-1 β and IL-18, which upon secretion induce various biological effects associated with infection, inflammation and autoimmune processes^{20,21}. Whereas most cytokines traffic through the Golgi complex before exocytosis, biologically active IL-1 β and IL-18 are secreted through an unconventional and ill-defined mechanism following cleavage of their cytosolic precursors by caspase-1²². IL-1 β participates in the generation of systemic and local responses to infection, injury and immunological challenge by generating fever, activating lymphocytes and promoting leukocyte infiltration at sites of injury or infection²⁰. IL-18 lacks the pyrogenic activity of IL-1 β , but is mainly known as a

factor that induces IFN γ production by activated T cells and natural killer (NK) cells, thereby contributing to T helper 1 (T_H1) cell polarization^{22, 23}.

Many viruses activate caspase-1 and induce IL-1 β and IL-18 production (Table 1). Recent studies with a mouse-adapted strain of influenza A/PR/8/34 (PR8) virus, these animals demonstrated impaired viral clearance at days 7 and 10 after infection and a 33% increase in mortality compared to wild-type controls in *Il1r1*^{-/-} mice. In addition, neutrophil and lymphocyte recruitment to the infected respiratory tract of *Il1r1*^{-/-} mice was reduced at day 7 after infection and comparable to the wild-type controls by day 10. In addition, there were greatly diminished IgM levels in both serum and lung at days 7 and 10 after infection²⁴. Similar to *Il1r1*^{-/-}, *Il18*^{-/-} mice also had increased mortality, enhanced virus growth, increased neutrophil numbers, and increased nitric oxide production over the first 3 days after respiratory challenge²⁵. However, with a less virulent influenza virus challenge, IL-18 deficiency was associated with decreased cytokine production by CD8⁺ T cells²⁶. In addition to influenza A virus, IL-1 β -deficient mice are also susceptible to herpes simplex virus 1 encephalitis owing to increased viral load, decreased inflammation, including fewer infiltrating immune cells and decreased cytokine and chemokine levels²⁷. Ectromelia virus, an orthopoxvirus, activates caspase-1 in the brain tissue of infected mice and this is thought to have a role in mediating inflammation and cell death²⁸. Further, West Nile virus (WNV) infection causes the migration of resident skin dendritic cells, known as Langerhans cells, to the draining lymph nodes. The migration of Langerhans cells requires IL-1 β , as treatment with an IL-1 β -specific neutralizing antibody decreased Langerhans cell migration and decreased the total number of immune cells recruited to lymph nodes of infected mice²⁹. Hepatitis B virus (HBV) stimulates IL-18 production in human peripheral blood mononuclear cells by means of the histone-like domain of the virus' core antigen (HBVcAg)³⁰. Interestingly, HBV virions deficient in the pre-core antigen (HBVeAg) induced higher levels of IL-18, suggesting a role for HBVeAg in the inhibition of IL-18 signalling. RSV induces the activation of caspase-1 and production of IL-1 β , as well as secretion of IL-1 β from neonatal human monocytes, possibly implicating one or more inflammasomes in the response to RSV infection³¹. The fact that a wide range of viruses and diverse viral components activate caspase-1, IL-1 β and IL-18 shows the potential role of inflammasomes in the recognition of viruses and research in this direction may provide new insights into the activation mechanisms of inflammasomes and their downstream signalling pathways.

NLRP3 inflammasome

The first evidence for the involvement of NLR inflammasomes in viral infection came from a study in which Sendai virus and influenza A virus were shown to stimulate caspase-1 activation and the production of IL-1 β and IL-18³². Subsequent studies have shown that influenza A virus can activate NLRP3 in various cell types *in vitro*, including mouse bone marrow-derived dendritic cells and macrophages, a human nasal airway epithelial cell line and the human monocyte cell line THP-1^{11, 12}. In addition to influenza A virus, infections with modified vaccinia virus Ankara³³ or encephalomyocarditis virus (EMCV)¹⁶ have also been shown to activate the NLRP3 inflammasome. It should be noted, however, that a direct interaction between NLRP3 and viral RNA has not been established. Further, based on the requirement for NLRP3 in IL-1 β production after infection with EMCV but not with VSV suggest existence of NLRP3-dependent or NLRP3-independent mechanisms¹⁶.

The requirement for NLRP3 inflammasome activation *in vivo* during virus infection is best characterized for influenza A virus. Two recent reports demonstrated a role for NLRP3 mediated immune responses to influenza A virus infection *in vivo* resulting in increased mortality^{11, 15}. Deletion of NLRP3, caspase-1 or the adaptor ASC resulted in decreased

infiltration of neutrophils and monocytes to the lungs and decreased IL-1 β , IL-18 and MIP2 levels in the BALF ^{11, 15}. However, IL-6, TNF- α , KC, and IFN- α were also reported to be reduced in NLRP3 deficient mice by one group ¹⁵ and the other group reported differential roles for NLRP3 and ASC in the production of KC and TNF- α respectively ¹¹ suggesting the existence of inflammasome-dependent paracrine and autocrine immune pathways. Similarly, a delay in virus clearance at day 7 post-infection ¹¹ whereas no difference at day 6 post-infection ¹⁵ were reported by these groups which might be due to the differences in the infectious doses (10–1000 PFU) used. Finally, the importance of NLRP3 in the resolution of inflammation and the proper repair of lung damage was reported as NLRP3^{-/-} mice had increased amounts of collagen deposition in the lungs and increased necrosis ¹⁵. These activities might be mediated directly by IL-1 β , as the repair of mechanically injured rat type II alveolar epithelial cell monolayers was promoted by adding IL-1 β to *in vitro* cultures ³⁴. IL-1 β was shown to be the crucial component of edema fluid that stimulated repair in a lung epithelial cell line, which is consistent with reports that IL-1 β can have a pro-fibrotic role in wound healing at particular stages of an infection ³⁵. It should also be noted that both of these papers on NLRP3 observed some slight differences in the severity or magnitude of several outcomes between NLRP3 and ASC or Caspase-1, with NLRP3 at times having the more severe phenotype. This was observed for survival by one group ¹¹ and for collagen deposition by the other ¹⁵ and it was suggested that these results may indicate additional roles for NLRP3 outside of the inflammasome ¹¹. Further, neither of these reports indicated any role for the NLRP3 inflammasome in the generation of the adaptive immune response. In line with above studies, Ichinohe *et. al.*, ¹² also demonstrated a role for the NLRP3 inflammasome *in vitro* as well as in the alveolar space *in vivo*, as BALF had reduced levels of IL-1 β and IL-18 in NLRP3-, ASC- and Caspase-1-deficient mice ¹². Further, at days 8 and 10 post-infection ASC^{-/-} and Caspase-1^{-/-} mice had higher viral titers and were more susceptible to infection with influenza A and had reduced neutrophil and monocyte infiltrates ^{11, 12, 15}. However, this study did not report any difference in cytokines except for IFN- γ in whole lung homogenates for NLRP3^{-/-}, and decreased CD4⁺ and CD8⁺ T cell responses and significantly reduced isotype-specific antibodies in ASC^{-/-} or caspase-1^{-/-} mice and not in NLRP3^{-/-} animals ¹². One possible explanation for the differences found in these reports is the timing of the experiments (days 6, 7, 8, 10 and 11 post-infection), and the infectious doses used (10 PFU-1000 PFU). Together, currently published studies unequivocally establish a role for NLRP3 inflammasome during influenza A infection ^{11, 15, 36} but whether or not NLRP3 is required for adaptive immunity require further validation.

Although a role of NLRP3 in sensing viruses is proposed, the precise molecular mechanism involved remains poorly understood. Several lines of evidence suggest that the NLRP3 inflammasome might monitor the presence of viral RNA and DNA in intracellular compartments. For example, transfection of human and mouse cell lines with ssRNA or dsRNA analogues such as polyinosinic-polycytidylic acid (poly(I:C)) is sufficient to activate NLRP3 ¹¹. Similarly, purified dsRNA from rotavirus or brome mosaic virus and ssRNA of influenza A virus also activate NLRP3 ^{11, 12, 32}. The NLRP3 pathway has also been implicated in the detection of viral DNA from adenovirus in cell culture ³⁷. Adenovirus vector capsids alone did not activate NLRP3. By contrast, replication-deficient adenovirus vectors comprising an intact genome activated the NLRP3 inflammasome. *In vivo* administration of poly(I:C) or purified influenza A virus ssRNA in mice also resulted in IL-1 β secretion and inflammation through NLRP3 ^{11, 13, 15}.

The discovery that NLRP3 can detect a wide range of divergent PAMPs such as bacterial lipopolysaccharide (LPS), peptidoglycan and bacterial or viral nucleic acids, as well as endogenous danger signals such as monosodium urate (MSU), calcium pyrophosphate dihydrate (CPPD) and ATP, has led to the hypothesis that NLRP3 may sense a common

downstream effect caused by these molecules, rather than the PAMPs themselves. Nicotinamide adenine dinucleotide phosphate (NADPH) oxidase-generated reactive oxygen species (ROS) have been suggested as likely secondary messengers activating the NLRP3 inflammasome^{11,38}. This conclusion was based on pharmacological inhibition of the NADPH oxidase complex in THP-1 cells and peritoneal mouse macrophages, as well as small interfering RNA (siRNA)-mediated downregulation of the NADPH oxidase subunit p22phox³⁸. NLRP3 was also shown to physically interact with a ROS sensing protein TXNIP suggesting a role for NLRP3 inflammasome activation in response to ROS³⁹. Further, a role for ROS during influenza A infection was shown by treating cells in culture or mice infected with influenza A with the ROS inhibitors N-acetyl-L-cysteine (NAC) which resulted in significant decreases in IL-1 β production¹¹. However, NLRP3 activation triggered by silica, MSU crystals or ATP-induced P2X7R activation seems to be normal in macrophages from mice lacking the NADPH oxidase subunit gp91phox⁴⁰. Moreover, the increased ROS production in superoxide dismutase-1 (SOD1)-deficient macrophages was recently found to inhibit rather than promote caspase-1 activation⁴¹. The destabilization and rupture of phago-lysosomal compartments, which then release endogenous mediators into the cytosol was also suggested as the NLRP3 activation mechanism⁴². It is possible that these potential mechanisms of NLRP3 activation may not be mutually exclusive⁴³ and additional studies are required for further validation.

Alternatively, TLRs have been proposed to activate the NLRP3 inflammasome directly⁴⁴. However, most reports indicate that TLR activation or ROS alone is insufficient for NLRP3 inflammasome activation and the production of significant amounts of IL-1 β or IL-18^{45,46}, and so a two-signal hypothesis for inflammasome activation has emerged in which pro-IL-1 β and pro-IL-18 production is regulated independently of caspase-1 activation and only when both signals are received are significant amounts of active IL-1 β or IL-18 produced. In the case of virus infection, dual signalling cues are common. For example, in influenza A virus infection, viral RNA triggers signalling through TLR3 or TLR7⁴⁷⁻⁴⁹ and during virus entry into the host cell cytoplasm endosomes are compromised, resulting in the release of ions and ROS into the cytosol. Recently, the ability of influenza A virus to activate NLRP3 as a result of ion fluxes was found to be mediated by the M2 ion channel of the virus³⁶. After infection, virus-encoded M2 is expressed in the secretory compartment, including the trans-Golgi network. The ion-channel activity of M2 enabled the export of protons from acidified Golgi, and triggered activation of the NLRP3 inflammasome. In this case, M2 expression alone was sufficient to activate the NLRP3 inflammasome³⁶. Vaccinia virus was also shown to require TLR2-dependent production of pro-IL-1 β and endocytic internalization of the virus for robust IL-1 β production by the NLRP3 inflammasome³³. The fact that multiple pathogens and danger signals activate the NLRP3 inflammasome has probably necessitated the evolutionary pressures to develop a precise regulatory mechanism for production of active IL-1 β and IL-18. The requirement for two activating signals, one signal to produce the pro-form of these two cytokines and a second signal to engage their processing by the inflammasome may allow for sensing a wide range of PAMPs and DAMPS and at the same time prevent unwanted inflammation by accidental activation of this pathway.

RIG-I inflammasome

RIG-I is a member of the RLR family. RIG-I has been shown to induce type I IFN signaling through the mitochondrial adaptor protein MAVS, followed by TRAF3/6, IKK ϵ and IRF3/7 signaling^{50,51}. Several studies have examined the role of RIG-I during RNA virus infections *in vitro*, including Newcastle disease virus (NDV), vesicular stomatitis virus (VSV), Sendai virus, hepatitis C virus (HCV), Japanese encephalitis virus (JEV), influenza A virus, rabies virus, measles virus, and RSV⁵²⁻⁵⁹ (Table 1). *In vivo*, RIG-I-deficient mice

have only been studied in the context of JEV infection and were found to be highly susceptible to infection with this virus⁵³. However, due to the difficulty in generating RIG-I^{-/-} mice many other viruses have yet to be examined *in vivo*. Another member of the RLR family is MDA5. This RNA sensing cytosolic PRR has also been shown to recognize RNA virus infection and some debate has occurred over the last few years concerning exactly what RNA species RIG-I and MDA5 recognize. Recent work identified full-length virus genomes as the major RIG-I agonist in cells infected with influenza virus and Sendai virus⁶⁰. These studies also suggested that nongenomic viral transcripts, short replication intermediates and cleaved self-RNA do not contribute substantially to type I IFN induction in cells infected with negative-strand RNA viruses. Rather, ssRNA viral genomes bearing 5'-triphosphates constitute the natural RIG-I agonists that trigger cell-intrinsic innate immune responses during viral infection. In case of MDA5, the RNA species appears to be specific dsRNA intermediates produced during positive-strand RNA virus infections, particularly by members of the picornavirus family like encephalomyocarditis virus^{53, 61} but also by flaviviruses like West Nile virus⁶² and Dengue virus⁶³.

Until recently, RIG-I was thought to be solely involved in activation of IFN α/β genes through recruitment of the adaptor MAVS and the transcription factors IRF3 and IRF7². Two recent reports, however, show that RIG-I can also trigger IL-1 β production and/or IL-18 production through the caspase-1 and -3 activation^{16, 64}. RIG-I activation by transfected 5'-triphosphate RNA or VSV infection resulted in the production of pro-IL-1 β through a MAVS-CARD9-NF- κ B signalling pathway. In response to the same stimuli, RIG-I also directly activated the inflammasome pathway by recruiting caspase-1 through the adaptor ASC. This pathway is not universal for all RLRs as MDA5 does not interact with ASC. These findings indicate that RIG-I might be able to mediate crosstalk between the type I IFN and inflammasome pathways. With regard to this crosstalk, RIG-I may also be able to single handedly mediate the dual signaling discussed above for the NLRP3 inflammasome as RIG-I mediated MAVS signaling can induce NF- κ B activation and pro-IL1 β production as well as form an inflammasome and activate caspase-1. However, this possibility will require additional research.

AIM2 inflammasome

AIM2 belongs to a family of proteins that includes at least six members in mice (IFI202, IFI203, IFI204, IFI205, Pyhin1 and AIM2) and four members in humans (IFI16, MNDA, IFIX and AIM2). These IFI200 family members (also known as HIN200 proteins) are IFN-inducible proteins with a 200-amino acid repeat at the C-terminus known as the HIN domain and an N-terminal PYD. Four groups recently identified AIM2 as a cytosolic dsDNA sensor that binds cytosolic dsDNA with its C-terminal HIN domain^{17-19, 65-67}. Similar to NLRP3, the AIM2 N-terminal PYD motif is capable of recruiting ASC and caspase-1. Cytosolic dsDNA from both viruses and bacteria can serve as ligands to activate the AIM2 inflammasome^{19, 37}. These observations were recently confirmed using AIM2-deficient mice^{65, 68}. Macrophages from AIM2 deficient mice were found to be defective in caspase-1 activation and induction of pyroptotic cell death in response to liposome-delivered cytosolic DNA. Furthermore, AIM2 was also shown to be required for the activation of caspase-1 in response to infection with vaccinia virus and mouse cytomegalovirus (mCMV)^{65, 68} (Table 1). These cell culture experiments were confirmed by testing AIM2^{-/-} mice with mCMV⁶⁵. AIM2^{-/-} mice were defective in IL-18 and IFN- γ production and their NK cells especially produced less IFN- γ upon restimulation compared to littermate controls⁶⁵. Because activation of the AIM2 inflammasome not only leads to the generation of pro-inflammatory cytokines, but also to the elimination of infected cells through pyroptosis, AIM2 is likely to be important for the efficient clearance of viral infections. Indeed, AIM2^{-/-} mice had higher mCMV titers than control mice likely as a result of decreased pyroptosis and NK cell

activity⁶⁵, although the contribution of both these factors remains to be examined independently.

Thus, studies in AIM2-deficient macrophages provided conclusive genetic evidence for a crucial role of the AIM2 inflammasome in recognition of cytosolic DNA and infection with vaccinia virus and mCMV. Further, AIM2 was suggested to have a crucial role in mediating the immune response against additional viruses that produce cytosolic DNA during their lifecycle (such as adenovirus and perhaps ssRNA retroviruses, which reverse transcribe their RNA into double-stranded DNA in the cytoplasm). Intriguingly, this same paper reported that the dsDNA virus herpes simplex virus-1 (HSV-1) did not require AIM2 for inflammasome activation⁶⁵. These results may indicate that AIM2 is unable to recognize certain DNA viruses or that some viruses have evolved the ability to inhibit AIM2 signaling or block AIM2 mediated recognition of their viral genomic DNA. Additional research will be needed to determine if all families of DNA viruses are recognized and what factors control AIM2 mediated recognition and signaling.

NLRs in type I IFN responses

Although most NLRs are thought to be involved in either NF- κ B and MAPK signalling or inflammasome activation and IL-1 β and IL-18 production, recent studies have revealed a new and unexpected role for NLRs in type I IFN signalling. Type I IFNs were originally described as antiviral agents and comprise multiple IFN- α molecules (13 subtypes in humans) and IFN- β ^{69, 70}. IFN- β expression is required to exert positive-feedback control over the induction of IFN- α genes⁷¹ and to transduce intracellular signals through the common receptor IFNAR1–IFNAR2, leading to further enhancement of type I IFN gene induction through a complex of STAT1/2 and IRF9 (also called Interferon-stimulated gene factor 3 (ISGF3))⁷¹. Thus, type I IFNs are key components of the antiviral innate immune system and might be responsible for driving broad cellular antimicrobial activity in general. Below is a brief description of the new roles of NLRs in type I IFN responses.

NOD2

The NLR family member NOD2 is known to sense bacterial molecules that are produced during the synthesis and/or degradation of peptidoglycan, allowing recruitment of downstream adaptor molecule RIPK2 through homotypic CARD interactions. This CARD-containing serine-threonine kinase directly binds and promotes K63-linked polyubiquitylation of the regulator inhibitor of NF- κ B kinase- γ (IKK γ) and activation of the kinase TAK1^{72, 73}, which are prerequisite for activation of the IKK complex. IKK activation results in degradation of the NF- κ B inhibitor I κ B α and the subsequent translocation of NF- κ B to the nucleus, where transcription of NF- κ B-dependent target genes, such as those encoding IL-6 and TNF occurs. In addition to activation of the NF- κ B pathway, NOD2 stimulation results in activation of the MAPKs p38, ERK and JNK^{74, 75}. The CARD-containing adaptor protein CARD9 was found to be important for the activation of p38 and JNK downstream of NOD2, although it was dispensable for NF- κ B activation⁷⁶. Nevertheless, the NF- κ B and MAPK pathways are thought to cooperate to upregulate the expression of proinflammatory molecules such as IL-1 β , TNF and IL-6 that stimulate both innate and adaptive immune responses. In addition, NOD2 has been shown to induce a type I interferon response to the intracellular bacterium mycobacterium tuberculosis (MTB)⁷⁷. In this context it was shown that recognition of unique peptidoglycans from MTB by NOD2 resulted in activation of a RIPK2 and IRF5 mediated signaling cascade with type I IFN production.

Recent studies have examined the role of NOD2 in response to ssRNA using a transfection assay designed to detect activation of the transcription factor IRF3, which is involved in the

production of type I IFNs⁷. Both synthetic ssRNA and viral ssRNA genomes were found to activate IRF3 in a NOD2 and MAVS-dependent manner⁷. The important physiological function of NOD2 in antiviral defence was evident from decreased type I IFN responses, and increased RSV pathogenesis, lung disease and virus susceptibility of *Nod2*-deficient mice. Similarly, macrophages and mice lacking NOD2 had reduced IRF3 phosphorylation and diminished production of type I interferons in response to influenza A and parainfluenza viruses⁷. Furthermore, NOD2^{-/-} cells were deficient in their ability to inhibit VSV replication⁷. This work suggested that NOD2 functions as a viral PRR that is important for the production of type I IFNs in response to RNA viruses and suggests that, similar to RIG-I, NOD2 can trigger multiple signalling pathways in response to cytoplasmic pathogens. Furthermore, RSV infection was shown to result in NOD2 relocalization to mitochondria, where it binds to MAVS⁷. It is therefore likely that NOD2 binding to peptidoglycan induces recruitment and activation of RIPK2 signalling, whereas binding of NOD2 to ssRNA results in mitochondrial relocalization and activation of MAVS-dependent pathways (FIG 1). It should be noted, however, that NOD2 was not specifically shown to bind ssRNA and only to be required for ssRNA induction of type I interferons. However, how NOD2 is capable of recognizing the presence of both bacterial peptidoglycan and viral ssRNA and the biochemical mechanism that links MAVS to IRF3 activation during NOD2 signaling require further analysis.

Another novel role for NOD2 during virus infection was recently implicated when NOD2 was shown to interact with 2'-5'-oligoadenylate synthetase type 2 (OAS2)⁷⁸. This interaction was shown to positively regulate the enzymatic activity of OAS2 in response to poly I:C, which resulted in increased degradation of RNAs by RNase L. OAS2 is required for the activation of RNase L which degrades viral and cellular RNA and inhibits virus replication⁷⁹. At the same time, RNase L provides a feedback mechanism for type I IFN production by generating new PAMPS for RIG-I and MDA5⁸⁰. These reports further implicate NOD2 in the efficient induction of type I IFNs in response to viral infection.

NLRX1

NLRX1 is the only NLR known so far to localize to mitochondria. Indeed, this NLR family member contains a mitochondrial targeting sequence in its N-terminus^{8,9,81}. Overexpression of NLRX1 resulted in inhibition of RIG-I signalling induced by synthetic RIG-I ligands and viruses such as Sendai virus or Sindbis virus. Depletion of NLRX1 using specific siRNA amplified virus-induced type I IFN production and decreased viral replication. However, the precise subcellular localization of NLRX1 in mitochondria remains to be determined. In one study, NLRX1 was found in close proximity to the antiviral RLR adaptor protein MAVS, which is known to be associated with the mitochondrial outer membrane. Physical association between MAVS and NLRX1 prevented viral RNA-induced type I IFN responses and NF- κ B activation, suggesting that NLRX1 is a negative regulator of RLR signalling⁹. A second study contested the localization of NLRX1 to the outer mitochondrial membrane and showed that NLRX1 fully translocates to the mitochondrial matrix⁸. Accordingly, NLRX1 was found to amplify NF- κ B and JNK signalling by augmenting ROS production in mitochondria in response to poly I:C treatment⁸¹. Although the mechanism of NLRX1 function is unclear and its role in antiviral signaling is under debate, the mitochondrial localization of NLRX1 seems to be essential for regulating its activity. Further studies will be required to resolve the precise localization of NLRX1 in mitochondria, and whether NLRX1 exerts a pro- or anti-inflammatory effect or if the role of NLRX1 is context dependant. The generation of NLRX1-deficient mice may prove instrumental in determining the physiological role of this unique NLR family member.

NLRC5

Similar to NLRX1, NLRC5 is an atypical NLR family member in that it contains a C-terminal effector domain that is predicted to adopt a death-domain (DD) fold without obvious homology to the CARD and PYD motifs found in most NLRs⁸². Furthermore, its LRR domain is the longest among NLRs and comprises over 1000 residues. It is thought to organize into a helical structure rather than the 'horseshoe' fold that is typical for most LRR domains⁸³. One study recently reported that the protein is primordially expressed in hematopoietic tissues and cells of the myeloid and lymphoid lineages⁸³, whereas another study suggested a ubiquitous expression pattern with lowest expression levels found in lymphoid tissue and leukocytes⁸⁴.

NLRC5 was recently shown to be specifically upregulated by IFN γ and was implicated in modulating IFN-dependent gene transcription⁸³⁻⁸⁶. This suggests an important role in antiviral immune responses, although the precise molecular mechanism remains enigmatic. Knockdown of endogenous NLRC5 in THP-1 cells and in primary human dermal fibroblasts impaired type I IFN responses as shown by decreased IFN- β and CCL5 release, and decreased type I IFN- β and IP-10 mRNA induction, after Sendai virus infection and poly(I:C) stimulation⁸³. In agreement, NLRC5 knockdown diminished the induction of IFN- α in human foreskin fibroblast cells stimulated with poly(I:C) or infected with CMV⁸⁴. In contrast to these two reports, it was recently suggested that NLRC5 is a negative regulator of NF- κ B and type I IFN signalling during viral infection⁸⁷. These results were comparable to another recent study which showed that siRNA knock down of NLRP5 in RAW264.7 mouse macrophage cell line resulted in increased NF- κ B and IFN signaling and IL-6, IL-1 β , and TNF- α production⁸⁵. Further, an inhibitory role for NLRC5 in MHC I and CD40 expression was also demonstrated. However, another study outlined NLRC5 as a positive regulator of MHC I expression along with other genes involved in antigen presentation⁸⁶. Although above studies are contradictory, the ability of IFN- γ in upregulating NLRC5 expression is consistent. Thus, the generation of NLRC5-deficient mice will undoubtedly contribute to resolving these apparent discrepancies and to characterizing the physiological role of this NLR in antiviral immune signalling.

NLRs and inflammasomes recognize specific nonself-motifs in viral products, the viral replication program (a cytosolic phase of genome amplification and/or mRNA metabolism), viral protein expression or the ion-channel activity of viruses. After virus infection, multiple signalling pathways are activated simultaneously or sequentially to eliminate the invading pathogen and to prevent the establishment of a persistent infection. This activation of distinct and shared signalling pathways leads to pathway crosstalk as described above and eventually determines the specific immune response directed at clearing the virus. As the host has evolved factors and strategies for detecting and responding to viral infection, viruses have developed countermeasures to inhibit these processes. Owing to genomic constraints, many pathogenic viruses focus their disruptive efforts on host targets that are key players in the antiviral response. Thus, common host molecules that connect virus recognition to downstream effectors and inflammatory cytokine expression are usually targeted by viral immune evasion proteins. An overview of the mechanisms by which viruses block NLRs and inflammasomes is provided below.

Virus immune evasion of NLRs and inflammasomes

In the evolutionary arms race between a pathogen and their host, the host has selected for various mechanisms to detect the presence of viruses and closely regulates the production of master cytokines such as IL-1 β and IL-18 by the inflammasome. In response, viruses have undergone selective pressure to evade the inflammasome. Several viruses are known to encode proteins that interfere with inflammasome signalling (Table 2). Inflammasome

signalling is interrupted at the level of the adaptor protein ASC or caspase-1 itself, as both these molecules are downstream of multiple NLRs, as well as AIM2 and RIG-1 inflammasomes.

The large genomes of poxviruses encode multiple inhibitors that interfere with innate and adaptive immunity⁸⁸. Bioinformatics studies show that many poxviruses encode a putative PYD-containing protein that is hypothesized to interact with ASC; however, the myxoma virus M13L-PYD and Shope Fibroma virus S013L proteins are the only examples that have been studied in detail⁸⁹⁻⁹¹. M13L-PYD is required for the pathogenesis of myxoma virus and deletion of this protein results in severe virus attenuation *in vivo*, as evidenced by a decrease in viremia due to inefficient replication in lymphocytes and leukocytes and increased inflammation at the initial site of infection, which lead to more rapid resolution of disease. Infection of cells *in vitro* with myxoma virus lacking M13L-PYD leads to increased activation of caspase-1 and thereby increased levels of IL-1 β and IL-18, which indicates that M13L-PYD has an immune-suppressive activity⁹¹. M13L-PYD and S013L associate with ASC through direct PYD-PYD interactions. Expression of either M13L-PYD or S013L alone inhibits caspase-1 activation and IL-1 β maturation downstream of the NLRP3 inflammasome in cell culture^{90,91}. Poxvirus PYD-containing proteins may therefore inhibit inflammasome activation at the level of the adaptor protein ASC and prevent PRRs from activating caspase-1 in virus-infected cells. In addition, ASC might have several caspase-1-independent immune functions, such as T cell and lymph node cell activation. It is therefore possible that poxvirus PYD-containing proteins block inflammation by inhibiting these processes as well.

Poxviruses also encode various serpin-like protease inhibitors that inhibit caspase-1. For example, CrmA (also known as SPI-2) of cowpox virus is a well-known caspase-1 inhibitor. Deletion of CrmA decreases the number and severity of pox lesions on the allantoic membrane when the virus is grown in chicken eggs^{92,93}. Infecting the respiratory tract of mice with cowpox virus or rabbit poxvirus mutants that lack CrmA results in decreased inflammation and viral loads compared with wild-type virus^{94,95}. After intradermal inoculation with cowpox virus, a more rapid virus clearance and more robust inflammatory response is observed with virus lacking CrmA⁹⁶. It is interesting to note that no differences in IL-1 β levels were observed in the lungs of C57BL/6 mice infected with wild-type cowpox virus versus mutant virus lacking CrmA⁹⁶ even though the studies *in vitro* indicate that CrmA can inhibit IL-1 β maturation and secretion.

Other poxviruses also encode SPI-2 homologues, such as Serp2 in myxoma virus⁹⁷. Deletion of myxoma virus Serp2 results in severe attenuation of the virus in rabbits⁹⁸. Some vaccinia viruses also encode a SPI-2 protein. However, deletion of vaccinia virus SPI-2 had no effect on IL-1 β -induced fever responses seen in infected mice and vaccinia virus SPI-2 mutants were not attenuated^{94,95}. Instead, fever reduction and attenuated weight loss in vaccinia virus-infected mice were dependent on vaccinia virus-encoded IL-1 β scavenger receptor (vIL-1 β R)⁹⁴. Several poxviruses also encode an IL-18-binding protein that blocks IL-18-induced signalling⁹⁹. It is becoming clear that poxviruses evolved a variety of inflammasome inhibitors and molecules interfering with downstream signalling of the inflammasome products IL-1 β and IL-18. However, viral inflammasome inhibitors are not limited to poxviruses. Influenza A/PR/8/34 H1N1 virus NS1 (PR/8 NS1) protein prevents caspase-1 activation and IL-1 β maturation¹⁰⁰. The N-terminus of PR/8 NS1 was also suggested to interfere with inflammasome activation, although the details remain unclear. Influenza A/PR/8/34 H1N1 virus lacking the N-terminus of NS1 is attenuated in cell culture and induces higher levels of IL-1 β and cell death. Interestingly, inhibition of the dsRNA kinase PKR also decreases IL-1 β maturation in PR/8 NS1-mutant influenza virus. Although these results do not provide a clear inhibitory mechanism for PR/8 NS1, they do show the

importance of this protein for virus replication. However, the ability of NS1 to block caspase-1 activation seems to be strain specific as NS1 from highly pathogenic H5N1 bird flu activates caspases and induces apoptosis¹⁰¹. One possible explanation for the ability of H5N1 virus to grow despite activating caspases is that it downregulates the NLRP3 and IL-1 β expression levels by 24h post infection¹⁰² and therefore H5N1 virus may not require inhibition of caspase-1 to suppress inflammation. By contrast, PR/8 influenza virus has been shown to upregulate inflammasome components¹¹ and may therefore require NS1-mediated inhibition of caspase-1 to prevent inflammation and to replicate successfully.

It is likely that additional viral evasion pathways exist that target inflammasome components. Therapeutic interventions that are designed to target the virus–host interface of such immune evasion strategies to restore the functions of PRRs and their signalling pathways will offer the potential to restore innate immune induction and inflammatory programs that initiate immunity for the control of virus infection.

Concluding remarks

Host proteins involved in the recognition of virus infection have only recently been identified and the initial characterization of their roles in this intricate system is just now starting to be understood. Identification of the NLR and RLR family of sensors within the past several years has provided critically important and necessary information concerning cellular recognition of RNA viruses. It is evident from the information presented here that NLRs and inflammasomes are necessary for the innate inflammatory control of virus infection, as well as healing responses. In addition, the presence of multiple viruses that encode inflammasome inhibitory proteins is consistent with the importance of this pathway for inhibiting virus replication. However, our knowledge of inflammasome biology is still in its infancy and many questions remain to be answered. Specifically, how are these pathways regulated? Is there crosstalk with other PRRs recognizing the same pathogen? What is the physiological significance of each of these systems and which viral triggers are responsible for their activation? As research continues into the role of NLRs and inflammasomes in antiviral immunity it is likely that the list of PRRs involved and viruses that activate and suppress NLR and inflammasome signalling will continue to grow.

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Glossary

Pattern-recognition receptors (PRRs)	Receptors that bind to molecular patterns found in pathogens, but not mammalian cells. Examples include the mannose receptor, which binds to terminally mannosylated and polymannosylated compounds, and Toll-like receptors, which are activated by various microbial products, such as bacterial lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNA
Pathogen-associated molecular patterns (PAMPs)	A molecular pattern that is found in pathogens but not mammalian cells. Examples include terminally mannosylated and polymannosylated compounds, which bind the mannose receptor, and various microbial products, such as bacterial

	lipopolysaccharides, hypomethylated DNA, flagellin and double-stranded RNA, which bind Toll-like receptors
NOD-like receptors (NLRs)	A large family of cytosolic PRRs that resembles a subset of plant disease-resistance (R) genes, which are involved in the hypersensitive response against virulent plant pathogens. There are 23 NLR genes in the human genome and at least 34 in the mouse genome. NLRs are involved in innate immune sensing and in the regulation of inflammatory and cell death responses
Nucleotide-binding oligomerization-domain (NOD)	Members of the NLR family and plant disease-resistance (R) gene products contain a central domain known as a NOD
Hypersensitive response	A form of programmed cell death. The hypersensitive response is a marker of disease resistance in plants and is characterized by rapid localized pathogen-induced cell death and restriction of further pathogen growth
Autophagy	An evolutionarily conserved process in which acidic double-membrane vacuoles sequester intracellular contents (such as damaged organelles and macromolecules) and target them for degradation, through fusion to secondary lysosomes
Inflammasome	A large multiprotein complex containing certain NLR, RLR and IFI200 proteins, the adaptor protein apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and pro-caspase 1. The assembly of the inflammasome leads to the activation of caspase 1, which cleaves pro-IL-1 β and pro-IL-18 to generate the active pro-inflammatory cytokines
Caspases	Caspases are evolutionarily conserved cysteine proteases that are synthesized as inactive zymogens. Upon proteolytic activation, they initiate or execute cellular programs, leading to inflammation or cell death. Caspases are categorized as either proinflammatory or proapoptotic, depending on their participation in these cellular programs. The inflammatory caspases are caspase-1, caspase-11 and caspase-12 in mouse and caspase-1, caspase-4, and caspase-5 in human
Danger signal	Signal released by injured or damaged tissues that trigger an innate immune response
Pyroptosis	A form of cell death associated with antimicrobial responses during inflammation and dependent on the activation of an inflammasome and inflammatory caspases such as caspase-1
Small interfering RNA	Synthetic double-stranded RNA molecules of 19–23 nucleotides, which are used to ‘knockdown’ (silence the expression of) a specific gene. This is known as RNA interference and is mediated by the sequence-specific degradation of mRNA
K63-linked polyubiquitylation	Ubiquitin is a highly conserved 76kDa protein that is ligated to many proteins in both monomeric and polymeric forms. The two most common ubiquitin polymer chains are linked through an isopeptide bond between glycine-76 and either lysine-48 (K-48) or lysine-63 (K-63). K-63-linked polyubiquitination results in

signals related to intracellular trafficking, cell signalling, ribosomal biogenesis and DNA damage repair. There are also reports that K63-linked polyubiquitin is involved in proteasome-independent proteolysis through autophagy

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Online Summary

- Inflammasomes and nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) mediate the recognition of viruses by detecting evolutionary conserved motifs in their genomic RNA or DNA. In addition to viral RNA, NLRP3 senses a myriad of pathogen-associated molecular patterns (PAMPs) through incompletely characterized mechanisms, such as ion flux caused by viral ion channels or multiple coordinated signals.
- Inflammasomes have a crucial role in the immune response to viral infections. NLR proteins were previously thought to be the only mediators of inflammasome activation. However, recent research has shown that members of additional cytoplasmic pattern recognition receptor (PRR) families such as retinoic acid inducible gene_1 (RIG-I) and absent in melanoma 2 (AIM2) can engage the inflammasome in virus-infected cells.
- NOD2, which was originally reported to activate mitogen-activated protein kinase and nuclear factor- κ B signalling through the kinase receptor interacting serine-threonine protein kinase 2 (RIPK2) in response to bacterial peptidoglycan, has been shown to also signal through mitochondrial antiviral signalling protein (MAVS) and induce the production of type I interferons (IFNs) in response to viral ssRNA.
- NLR proteins also have regulatory functions during virus infection. NLRX1 negatively regulates type I IFN production mediated by the RIG-I–MAVS–IFN response factor 3 (IRF3) signalling cascade.
- Crosstalk between innate immune signalling pathways has become increasingly more evident. RIG-I was originally discovered as a mediator of type I IFN production but is now recognized as a potential inflammasome activator.
- Several viruses encode inhibitors of inflammasome and MAVS signalling pathways, thus suggesting a key role for these pathways in controlling virus replication and the requirement for viruses to interfere with their activation in order to successfully infect a host.

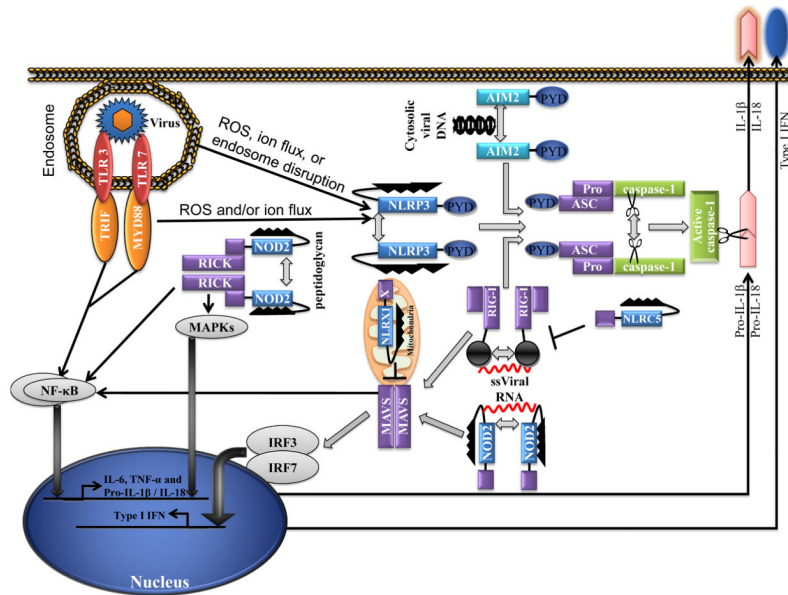


Figure 1. Intracellular sensors in innate immunity to viruses

Viral pathogen-associated molecular patterns (PAMPs) activate nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs) and inflammasomes to initiate signalling cascades that lead to the production of proinflammatory cytokines, thereby amplifying antiviral resistance. In the presence of viral PAMPs, NLRP3 and absent in melanoma 2 (AIM2) oligomerize and recruit ASC, the adapter protein engaged by inflammasomes through their pyrin (PYD) domains. The CARD domain within ASC binds the CARD domain of caspase-1, leading to caspase 1 activation and the production of interleukin-1 β (IL-1 β) and IL-18. Retinoic acid inducible gene-I (RIG-I) contains an RNA helicase domain and N-terminal CARD domains. The helicase senses the 5' triphosphate moiety of RNA viruses and then signals via CARD-CARD interactions with the mitochondrial localized adapter molecule MAVS to the downstream IKK-related kinase, TBK1, which subsequently phosphorylates and activates IRF3 to turn on transcription of type I interferon (IFN α/β) genes. RIG-I also regulates IL-1 β production transcriptionally and post-translationally following recognition of 5'-triphosphate dsRNA. Whereas RIG-I triggered transcription of pro-IL-1 β is NF- κ B-dependent and mediated by a MAVS, the caspase-1 inflammasome formation and IL-1 β /IL-18 production by RIG-I involves ASC. The NLRs NOD2, NLRX1 and NLRC5 associate with MAVS. Whereas NOD2 mediates the induction of IFN α/β , NLRX1 and NLRC5 inhibit RIG-I-MAVS and thus negatively regulate type I IFN signalling. Both MAVS and caspase-1 signalling are blocked by influenza NS1 protein.

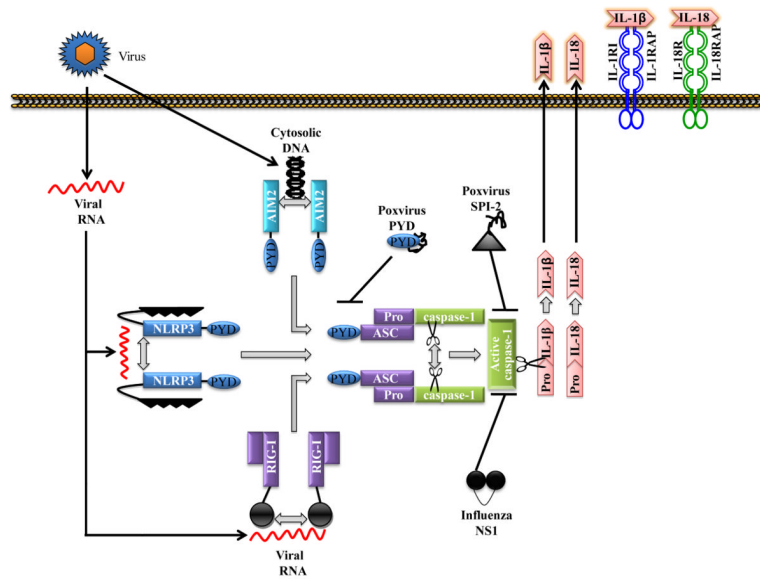


Figure 2. Inflammasome pathways

The pattern recognition receptors (PRRs) nucleotide oligomerization and binding domain (NOD)-like receptor protein 3 (NLRP3), absent in melanoma 2 (AIM2) and retinoic acid inducible gene-I (RIG-I) assemble caspase-1-activating inflammasome complexes in response to microbial pathogen-associated molecular patterns (PAMPs). The NLRP3 inflammasome recognizes multiple PAMPs including viral RNA in combination with ATP, ion flux or reactive oxygen species. By contrast, AIM2 and RIG-I directly bind to dsDNA and ssRNA viral genomes, respectively. All three types of inflammasome induce caspase-1 activation and interleukin-1 β (IL-1 β) and IL-18 production. The adaptor protein apoptosis-associated speck-like protein containing a CARD (ASC) is essential in all inflammasome complexes to bridge the interaction between upstream PRRs and inflammatory caspases through its N-terminal pyrin domain (PYD) and C-terminal caspase-recruitment domain (CARD) motifs, respectively. To interfere with inflammasome signalling, the large genomes of poxviruses encode multiple inhibitors such as PYD and SPI-2 that inhibit ASC and caspase-1, respectively.

Table 1

NLRs and inflammasomes involved in the recognition of virus infection

Gene (alternate names)	Signalling pathways during viral infection	Viruses recognized	Viral PAMP recognition	Cytokines	References
NLRP3	Inflammasome	Influenza virus, Sendai virus, adenovirus, EMCV	RNA	IL-1 β and IL-18	11, 12, 15, 32, 33, 36, 37
RIG-I	MAVS, Inflammasome	Vesicular stomatitis virus, hepatitis C virus, Japanese encephalitis virus, Rabies virus, measles virus, respiratory syncytial virus, influenza virus, Sendai virus	5'-triphosphate ssRNA	IFN α /IFN β , IL-1 β and IL-18	52-64
AIM2	Inflammasome	Vaccinia virus, m cytomegalovirus	Cytosolic DNA	IL-1 β and IL-18	17-19
NOD2 (NLR2)	MAVS OAS2	Respiratory syncytial virus, influenza A virus, parainfluenza virus-3, vesicular stomatitis virus	ssRNA	IFN α and IFN β	7
NLRX1	MAVS	Sendai virus, Sindbis virus	Exact sensing capacity is unknown	Negatively regulates IFN α and IFN β production	9, 81
NLRC5	RIG-I, MDA5, NF- κ B	Sendai virus, vesicular stomatitis virus, cytomegalovirus	PolyI:C	IFN α , IFN β , CCL3, CCL5, IL-6, TNF α , IP10	82-86

Table 2

Virus encoded inhibitors and the inflammasome targets

Inhibitor classes	Virus	gene	Cellular target	References
PYD homologues	Myxoma virus Shope Fibroma virus	M13L-PYD S013L	Inhibits inflammasome formation by binding ASC; inhibits subsequent caspase-1, IL-1 β and IL-18 activation	89–91
SPI-2 homologues	Cowpox virus Rabbit pox virus Myxoma virus Vaccinia virus	CrmA CrmA Serp2 SPI-2	Protease inhibitor that directly blocks caspase- 1, IL-1 β and IL-18 activation	92–99
NS1	Influenza A virus	NS1	Inhibits caspase-1 and IL-1 β activation; also inhibits RIG-I	100–102