Intracellular pattern-recognition receptors

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Abstract

The last ten years of research in the field of innate immunity have been incredibly fertile: the transmembrane Toll-like receptors (TLRs) were discovered as guardians protecting the host against microbial attacks and the emerging pathways characterized in detail. More recently, cytoplasmic sensors were identified, which are capable of detecting not only microbial, but also self molecules. Importantly, while such receptors trigger crucial host responses to microbial insult, over-activity of some of them has been linked to autoinflammatory disorders, hence demonstrating the importance of tightly regulating their actions over time and space. Here, we provide an overview of recent findings covering this area of innate and inflammatory responses that originate from the cytoplasm.

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1. Introduction

Innate immunity is the most ancestral and ubiquitous system of defense against microbial infections. The innate immune system relies on the recognition of conserved microbial molecular signatures, called pathogen associated molecular patterns (PAMPs), thereby allowing for efficient and rapid discrimination between pathogens and self. These patterns represent molecules vital for microbial survival and include lipopolysaccharide (LPS), peptidoglycans (PGNs), or viral and bacterial RNA/DNA. Upon detection of such PAMPs, the innate immune system recruits phagocytic cells, such as monocytes, macrophages, neutrophils, and primary effector cells, such as dendritic cells and natural killer cells, which orchestrate production of antimicrobial peptides and cytokines/chemokines to directly eliminate the microbes (reviewed in [1]). This first arm of defense also activates the adaptive immune system and leads to humoral and cellular responses against the pathogens [2]. In addition to PAMPs, “danger signals”, such as pathogen-associated modifications of host proteins, stress or endogenous cellular products resulting from tissue injury and cell death, can be detected by the innate immune system. The danger signal hypothesis originally proposed by Matzinger, predicted the integration of danger sensing in helping the immune system to discriminate between pathogenic and nonpathogenic microbes [3].

Host organisms have developed a limited number of germline encoded receptors that recognize PAMPs, referred to as pattern-recognition receptors (PRRs) [4]. PRRs in the innate immune system can be in a soluble, membrane-bound or cytosolic form and function at both extracellular and intracellular levels. The best-characterized PRRs are the Toll-like receptors (TLRs), but more recently two other families of signaling PRRs have been identified: the Nod-like receptors (NLRs) and the RIG-like helicases (RLHs).

TLRs are transmembrane proteins localized at the cell surface or within phagosome/endosomes, thereby scanning the extracellular milieu for pathogens [5]. They contain several extracellular leucine-rich repeat (LRRs) domains, involved in direct or indirect pathogen recognition, and an intracellular signal transducing domain, the so-called TIR (Toll/Interleukin-1 Receptor) domain. Activation of TLRs leads to NF-κB-dependent production of innate immune cytokines such as TNFα, IL-6 or IL-12, that contribute to the recruitment of inflammatory cells to the site of infection or inflammation [6].

Studies on TLR signaling pathways and the analysis of key TLR deficient mice revealed that TLRs could not be the only innate immune receptors responsible for cytokine production. Indeed, using computational analysis of the genome, the NLR proteins were identified. NLR proteins are intracellular LRR-containing proteins that resemble plant disease resistance genes. The characterization of these NLRs has greatly advanced in recent years and underlined their essential roles in innate immunity. In addition to the NLRs, another important family of intracellular sensors, i.e. the RIG-like helicases (RLHs) were recently identified. This review will focus on the description of intracellular pathogen and/or danger recognition mediated by the NLR and RLH families of proteins.

2. Intracellular detection of pathogen- and danger-associated molecules

The intracellular NLR protein superfamily regulates innate immunity in response to recognition of various self and non-self molecules. 22 NLRs are described in humans, among which are the NODs (nucleotide-binding and oligomerization domain), Nalps (NACHT-, LRR- and PYD-containing), Iraf (ICE protease activating factor) and Naip (neuronal apoptosis inhibitory protein) [7]. The first NLR proteins identified, Nod1 and Nod2, were shown to detect bacterial PGNs and to activate NF-κB. Structurally similar proteins of the Naip family were further identified and shown to form inflammasomes leading to caspase-1 activation.

The overall structure of the NLR proteins consists of an N-terminal effector domain followed by a central nucleotide-binding NACHT/NAD domain and a C-terminal LRR domain [8]. The LRR domain is a common motif shared by different cytoplasmic, membrane-bound and extracellular proteins, including the TLRs and plant disease resistance proteins, which is thought to be generally implicated in protein–protein interactions. In TLRs, the LRRs are assumed to be the sensing domain for the PAMP but proof for the hypothesis of direct binding of the ligand to the receptor is mostly still missing. The central NACHT domain is a nucleotide-binding domain implicated in oligomerization of the NLRs, necessary for their signaling abilities. The N-terminal effector domain is required for signal transduction to downstream molecules and consists of a domain of the death fold family. The death domain (DD), the caspase recruitment domain (CARD), the death effector domain (DED) and the pyrin domain (PYD) are all part of this family and have been initially identified as proapoptotic mediators. All these domains can engage other proteins via homotypic interactions. In NODs the effector domain is composed of one (or two) CARD(s), whereas in Nalps the N-terminus of the protein consist of a PYD [7].

2.1. NODs

2.1.1. Nod1 and Nod2 in bacterial detection

Nod1 and Nod2 were the first intracellular PRRs of the NLR superfamily to be identified several years ago. It was shown by Philpott et al. that invasive Shigella flexneri could activate NF-κB in a TLR-independent manner and after intracellular detection of the bacterium in epithelial cells [9]. The PRR responsible for NF-κB activation by Shigella was shown to be Nod1 and the dissection of the signaling pathway leading to production of proinflammatory mediators, such as IL-6 and TNFα, revealed an important role for RIP2, a serine–threonine kinase that is recruited to Nod1 through CARD–CARD interactions [10] (Fig. 1). Another NLR protein, the Nod1 homolog Nod2, is also able to activate NF-κB in a RIP2-dependent manner [11]. The oligomerization of RIP2 leads to activation of the IkB kinase complex, subsequent degradation of the inhibitor IkB and nuclear translocation of the transcription factor NF-κB. RIP2 is also necessary for Nod1-mediated JNK activation after infection with S. flexneri [10]. In epithelial
cells, Nod signaling also leads to production of antibacterial peptides, such as defensins, in addition to cytokines and chemokines that mediate the recruitment of inflammatory cells to the site of infection [12].

Activation of Nod1 and Nod2 occurs after infection with intracellular bacteria, since both proteins recognize muropeptides released from bacterial PGN [13]. PGN is a major constituent of the bacterial cell wall and is essential for bacterial shape and rigidity. Gram-positive and Gram-negative PGN differ in the nature of the third amino acid of the stem peptide linking the sugar chains composed of N-acetylglucosamine and N-acetylmuramylacid. In Gram-positive PGN this amino acid is a lysine, whereas in Gram-negative PGN it is a diaminopimelic acid (DAP). Nod1 is mainly a sensor of Gram-negative bacteria, recognizing a unique muropeptide (GM-TrIDAP), whereas Nod2 is a general bacterial sensor whose activity is mediated by muramyl dipeptide (MDP) recognition [14,15].

A recent study has shown an important role for Nod1 in activating the adaptive immune system [16]. PGN sensing by Nod1 is necessary for priming antigen-specific T cell immunity with a predominant Th2 polarization profile. In conjunction with TLRs, Nod1 signaling is also important for normal Th1, Th2 and Th17 responses. This study provides evidence for an instructive role of the intracellular innate immune system towards adaptive responses.

In addition, a crucial role for Nod2 has been shown in promoting antibacterial Th17 responses [17]. After PGN stimulation, human dendritic cells (DCs) are able to secrete IL-23 and IL-1 leading to IL-17 production in memory human CD4 T cells. This antibacterial IL-23 – IL-1 axis occurs in a PGN – Nod2-dependent manner, since DCs from Crohn’s disease patients are unable to drive a Th17 response in vitro. This study further underlines the importance of NLRs for the elicitation of the adaptive immunity.

2.1.2. Nod2 and inflammatory diseases

Interestingly, polymorphisms in Nod2 have been linked to inflammatory bowel diseases (IBD), and in particular Crohn’s disease [18]. The most common variants affect the LRR domain of the protein or its adjacent region. In vitro studies using these mutations suggest impaired signaling functions of Nod2 resulting in a loss-of-function phenotype, which is in apparent contradiction with the massive inflammation observed in patients [13]. Further studies are still needed to investigate the role of the Nod2 variants in intestinal epithelial cells from patients or in new mouse models. Despite its functional homology to Nod2, a clear link between Nod1 and IBD has not convincingly been established thus far.

Nod2 mutations are also linked to Blau Syndrome, an autosomal-dominant inflammatory disorder characterized by early-onset granulomatous arthritis, uveitis and skin rash [19].

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**Fig. 1.** Intracellular sensors initiating innate immunity. Nod-like receptors (NLRs) are characterized by three distinct domains: the ligand-sensing LRRs, the NACHT domain, which is responsible for the capacity of NLRs to oligomerize, and the effector domain, which can be a PYD, CARD or BIR domain. Most NLRs also contain a NACHT-associated domain (NAD). The NODs can trigger activation of NF-κB via RIP2 in response to bacterial infections and detection of PGN. Naip proteins are sensors of a plethora of PAMPs and danger signals and via the adaptor protein ASC can activate proinflammatory caspases leading to the activation of the cytokines IL-1β and IL-18. RIG-like helicases (RLHs) are characterized by two domains: a helicase domain, that can bind nucleic acids, and two CARDs, required for downstream signaling. Signaling pathways leading to IFN production and NF-κB activation occur when RIG-I or Mda5 bind to Cardif (MAVS, IPS-1, VISA).
predominant mutations are localized in the NACHT domain and result in increased basal and MDP-induced transcriptional activity, conferring a gain-of-function phenotype to the protein.

2.2. Nalps

Nalp proteins are characterized by C-terminal LRRs, a central NACHT/NAD domain and an N-terminal effector pyrin domain [20]. In addition to the Nalps, other pyrin domain-containing proteins exist, such as the protein ASC (apoptosis-associated speck-like protein containing a CARD) that is composed of a C-terminal CARD and an N-terminal PYD, or members of the IFI200 family of interferon-inducible proteins whose functions are poorly characterized [21]. In humans there are 14 Nalps described that are predicted to be located in the cytosol and several of them have been studied in detail [20].

The most prominent members are Nalp3 and Nalp1 and it has been shown that these proteins are able to form the so-called 'inflammasome'-complexes [22]. The formation of these multi-protein complexes results in processing and activation of the inflammatory caspase-1 (Fig. 1). Caspase-1 is present in cells as inactive zymogens and undergoes proteolytic processing upon activation. Active caspase-1 is required for the maturation of proinflammatory cytokines such as IL-1β, IL-18 and possibly also IL-33, since caspase-1 deficient mice have a profound defect in the maturation of proIL-1β and proIL-18 [23,24]. IL-18 is mostly responsible for the induction of proinflammatory cytokines, such as IFNγ, and activation of natural killer cells [25]. IL-1β is a potent cytokine implicated in inflammation, fever, induction of sleep and anorexia [26]. Moreover, IL-1β is implicated in tumor angiogenesis and controls invasiveness of different tumors in mice [27]. IL-1β also plays a role in destructive joint and bone diseases, and displays toxicity for the insulin-producing β-islets in the pancreas [28,29]. Hence, IL-1β is an important cytokine with beneficial actions but also potentially dangerous side-effects, so a better understanding of the tight regulation of IL-1β maturation is necessary. Caspase-1 processing is regulated in the inflammasome upon assembly of a platform consisting of a Nalp, the adaptors ASC and/or Cardinal and caspase-1 that will auto-process according to the close-proximity model. The Nalp binds to ASC through PYD–PYD interactions and the CARD domain within ASC recruits caspase-1. The CARD of Nalp1, or alternatively the CARD in Cardinal can recruit another caspase-1 or caspase-5 [20].

2.2.1. Nalp1

Human Nalp1 is a multidomain scaffold protein containing a N-terminal PYD, a central NACHT domain, five tandem LRRs and a C-terminal FIIND (domain with function to find) and CARD. The formation of a molecular complex termed the Nalp1 inflammasome has been described biochemically five years ago and has been shown to be implicated in the processing of caspase-1 and proIL-1β [22]. This inflammasome is composed of Nalp1, ASC, caspase-1 and caspase-5. More recently, an in vitro study using recombinant Nalp1 has shown a direct CARD–CARD interaction between Nalp1 and caspase-1 [30]. The presence of ASC was not necessary but enhanced caspase-1 activation. This study did not address the interaction between Nalp1 and caspase-5 that was originally described to take place through the CARD of Nalp1. In addition this study suggests that bacterial MDP could directly bind to and activate Nalp1. This finding shows for the first time a direct interaction between a PAMP and a NLR. However in vivo a role for MDP detection by Nalp1 has not been addressed. Nalp1 oligomerization and caspase-1 activation would therefore occur via a two-step mechanism requiring MDP and ATP.

In a subsequent study, Reed’s group showed the regulation of the Nalp1 inflammasome by two anti-apoptotic proteins, Bcl-2 and Bcl-XL [31]. Those two proteins bind to Nalp1 and specifically inhibit its function, resulting in reduced caspase-1 activation and IL-1β production. Hence, there seems to be a functional link between host defense and the apoptotic machinery, fine-tuning the mechanisms for cell preservation during stress.

Nalp1b, one of three Nalp1 paralogues in mice, has been implicated in the susceptibility to cell death induced by lethal toxin (LT) from Bacillus anthracis [32]. LT is believed to be responsible for causing death in systemic anthrax infections. LT-induced macrophage death requires caspase-1, suggesting that Nalp1b directly or indirectly activates caspase-1 in response to LT. Further studies will be needed to elucidate the mechanisms of Nalp1b activation and the role of the inflammasome in anthrax pathology.

In humans, Nalp1 has recently been associated with vitiligo, a skin disorder characterized by depigmentation due to loss of melanocytes, generally without signs of inflammation, as well as autoimmune and autoinflammatory disorders. A recent study has suggested an association with Nalp1 in the case of familial vitiligo [33]. The functional significance of these findings is still unknown.

2.2.2. Nalp3

Similarly to Nalp1, Nalp3 can also form an inflammasome that is composed of Nalp3, the adaptors ASC and Cardinal, and caspase-1, which is responsible for the processing of proIL-1β and proIL-18 [34]. Cardinal is a protein with similar domains to the C-terminal extension in Nalp1, containing both a FIIND and a CARD. The respective CARD–CARD interactions between ASC/caspase-1 and Cardinal/caspase-1 allow activation of caspase-1 due to spatial apposition of two caspase molecules. This Nalp3 inflammasome has been shown to have high proIL-1β processing activity and a similar inflammasome can be formed by Nalp2. Formation of mature IL-1β requires at least a two-step activation mechanism: first, transcriptional upregulation of proIL-1β mRNA and translation of the precursor protein is regulated by TLR signaling, and a second signal, leading to activation of caspase-1, processing of proIL-1β to mature IL-1β and secretion of the cytokine. This second signal can be sensed by the Nalp3 inflammasome. Enormous progress has been made in the last few years in the identification of signals able to activate the Nalp3 inflammasome and a plethora of potential activators has been identified. It became clear that Nalp3 cannot only detect pathogen-derived molecules, but also endogenous danger signals produced by the host cells. Among the PAMPs described to activate Nalp3, there are such diverse molecules as MDP, bacterial toxins (Nigericin, aerolysin) and whole bacteria...
(Listeria monocytogenes, Staphylococcus aureus), but also viral and bacterial RNA [35–40]. On the other hand Nalp3 can sense danger signals such as ATP or gout-associated uric acid crystals (MSU), but also chemical skin irritants (such as TNCB or TNP-CL) and UVB irradiation [36,40–43]. It is somewhat difficult to imagine that all these structurally different molecules can separately activate Nalp3 by binding to the leucine-rich repeats that could represent the sensing moiety of the protein. It has been shown that potassium efflux is crucial for Nalp3 signaling, as all Nalp3 activators tested (as well as the Nalp1 activator lethal toxin) can be blocked by inhibiting K⁺ efflux from the cells [44,45]. Ipaf inflammasome activation (see below) however is independent of potassium efflux, indicating a certain specificity in the activation mechanisms for different inflammasomes.

The importance of the Nalp3 inflammasome was dramatically underscored by the discovery of missense mutations in the NACHT domain of Nalp3. These mutations, resulting in a constitutively active Nalp3, are involved in three autoinflammatory disorders, namely Muckle Wells syndrome (MWS), familial cold autoinflammatory syndrome (FCAS) and chronic infantile neurological cutaneous and articular syndrome (CINCA) [46–48]. In these patients IL-1β is spontaneously processed and secreted, leading to recurrent episodes of fever, skin rashes and inflammation.

2.2.3. Other Nalps

In addition to Nalps1–3, 11 other Nalps (4–14) have been identified. Little is known about their function, but expression profiles revealed that several Nalps are expressed in the reproductive organs, suggesting a potential role in development. Mutations in Nalp7 have been shown to cause recurrent hydatidiform mole and reproductive failure in humans [49]. Hydatidiform mole is an abnormal pregnancy with no embryo and cystic degeneration of placental villi. Mutated Nalp7 is also responsible for recurrent spontaneous abortions and intrauterine growth retardation.

Finally, Nalp12 and Nalp6 have been suggested to play a role in immunity by activating NF-κB signaling and caspase-1-dependent cytokine processing [50,51]. Nalp12 function is still a matter of debate, since contradictory reports also exist, probably due to overexpression studies in a cell culture system [52]. More recently, Nalp12 has been shown to suppress the non-canonical NF-κB pathway by inducing degradation of NIK (NF-κB inducing kinase) [52]. The capacity of Nalp12 to form an “inflammasome” still awaits experimental proof.

2.3. Ipaf, Naip

Ipaf and Naip are two members of the NLR family that have been linked to intracellular detection of bacteria. Both proteins bear a C-terminal LRR domain and a central NACHT domain. For the N-terminus part, Ipaf contains a CARD, while Naip presents 3 BIR (baculovirus IAP repeat) domains [7]. Ipaf oligomerization provides a platform for the recruitment of caspase-1 by homotypic interaction of the CARD domains [53]. Activated caspase-1 then leads to the processing of IL-1β. Salmonella typhimurium and Legionella pneumophila infection of macrophages results in Ipaf-dependent activation of caspase-1, which leads to IL-1β maturation and caspase-1-dependent cell death [36,54–57]. The bacterial structure recognized by Ipaf has been shown to be flagellin, which must be delivered to the cytosol to activate Ipaf. Salmonella and Legionella mutants that do not express flagellin are unable to activate caspase-1. Both bacteria can deliver virulence factors to the host cytosol during infection by the means of type III (Salmonella) or type IV (Legionella) secretion systems and it is thought that bacterial flagellin can gain access to the cytosol through this kind of apparatus. In addition to Ipaf, murine Naip5 (one of seven paralogue genes) has been shown to be required for the intracellular response to flagellin [58–61]. Hence, mice that do not express Naip5 are susceptible to infection with L. pneumophila and several studies have shown that Naip5 is necessary for Legionella growth restriction, but independently of caspase-1 activation. These data would suggest that Ipaf and Naip5 are both required for an efficient response against Legionella but act in parallel pathways. The intracellular detection of flagellin leading to caspase-1 activation is independent of TLR5, a known PRR expressed on the cell surface also recognizing bacterial flagellin. Although the TLR5 LRR probably directly binds to flagellin, a direct interaction has not been shown for Ipaf. In contrast to Nalp3-dependent caspase-1 activation, Ipaf signaling does not depend on potassium efflux [44]. However, other modes of activation are also possible for Ipaf, since S. flexneri triggers Ipaf-dependent caspase-1 activation independently of flagellin [62]. Maybe a more general function of the bacterial type III secretion system and/or delivery of other bacterial effectors could explain activation of the Ipaf inflammasome. Similarly to the K⁺ efflux which is a common event to all Nalp3 activators, Ipaf activation could be achieved by a change in the ionic composition or a modification of a host protein triggered by the bacterial type III or IV secretion system. The role of the adaptor ASC in the Ipaf inflammasome is not entirely clear. ASC-deficient macrophages have only partially reduced abilities to secrete IL-1β in response to Salmonella together with delayed cell death [36]. However ASC has been described to interact with caspase-1 and with Ipaf [63]. ASC could stabilize the Ipaf inflammasome and enhance signaling to caspase-1. Therefore, in the absence of ASC, the Ipaf inflammasome would be weakened but not abolished.

There are other bacteria that activate caspase-1, such as Francisella tularensis, but in a Nalp3- and Ipaf-independent manner [64]. Thus far, only ASC and caspase-1 have been linked to F. tularensis induced cytokine secretion and cell death. The Francisella sensor still awaits identification.

2.4. NLRs and nucleic acid sensing

As described above, there have been reports showing that Nalp3 can sense nucleic acids of bacterial and viral origin. Nunez and colleagues observed that bacterial RNA, as well as the TLR7 ligands R837/R848, could induce IL-1β and IL-18 secretion from macrophages in a Nalp3-dependent manner [38]. In addition they also showed that polyIC, RNA of viral origin and Sendai or influenza virus infections, are able to activate the Nalp3 inflammasome [39]. Viral double stranded RNA also

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triggers the production of type-I IFNs, TNFα and IL-6 through TLR3 and RIG-I stimulation (see in Section 3). There is little evidence for the role of IL-1 to fight off viral infections. IL-18 however is able, in synergy with IL-12, to activate NK cells and cytotoxic T cells important for viral clearance. Moreover, viruses are able to interfere with the host immune system by expressing proteins that block innate signaling pathways. A poxvirus PYD-only protein has been described recently that is able to interact with ASC and inhibits caspase-1 activation by the virus [65]. Most recently, a study has addressed IL-1beta production by adenovirus infections in macrophages (Muruve DA, Pétrilli V, Zaiss AK, White LR, Clark SA, Ross PJ, Parks RJ and Tschopp J). The inflammasome recognizes cytosolic microbial and host DNA and triggers an innate immune response, Nature, in press). Activation of the Nalp3 inflammasome is achieved through cytoplasmic recognition of the adenoviral DNA, leading to IL-1beta secretion. These results suggest a general implication of the inflammasome in DNA virus infections. However, IL-1 production most likely reflects a general inflammatory state triggered by adenoviruses, rather than a direct antiviral activity. In addition, bacterial and mammalian DNA can also trigger caspase-1 activation and lead to IL-1 mediated inflammation.

2.5. Therapeutics

Based on the knowledge acquired in basic research, the administration of the IL-1β receptor antagonist IL-1ra (Anakinra) to patients with autoinflammatory diseases (MWS, CINCA, FCAS) was initiated and has lead to a rapid and dramatic decrease of disease symptoms [47,66]. Treatment with Anakinra has also proven efficacy in patients suffering from gout or pseudogout, two inflammatory diseases due to microcrystal deposition in the joints, as suggested by the discovery that MSU crystals are responsible for IL-1β secretion in macrophages. A pilot open-labeled study treating 10 patients, with documented acute gouty attacks that could not tolerate or had failed standard therapies, revealed a very rapid and efficient response in those patients [67].

3. Activation of antiviral pathways upon recognition of nucleic acids

Intracellular recognition of pathogens is crucial in the case of viruses that hide inside the cell to escape efficient immune responses and recognition of the innate immune system in the first place. Viral nucleic acids, which are accessible in the host cell cytoplasm during viral replication, are sensed by several detection systems. Here, we will begin by a description of RNA-mediated antiviral responses, whose signaling pathways and core components have been identified and characterized during recent years. Then, we will approach host responses to DNA, in which signaling machineries remain to be discovered to a large extent.

3.1. Cytoplasmic RNA sensing and production of antiviral type-I interferon

While several Toll-like receptors (notably TLR3, TLR7 and TLR8) detect extra-cytoplasmic RNA molecules, a few cytoplasmic proteins also have the capacity to recognize viral RNA species and mount antiviral responses. Some well-studied examples include the interferon (IFN)-inducible protein kinase R (PKR) and 2′−5′ oligoadenylate synthetase (OAS). Both molecules are activated by dsRNA and trigger antiviral responses, PKR by inhibiting protein translation in general, and OAS by activating RNaseL which cleaves cytoplastmic (host and virus-derived) ssRNA. Importantly, PKR, OAS and RNaseL are all IFN-inducible, hence are not the primary trigger but amplifiers of an antiviral response loop. Upon a viral infection, type-I IFN, which includes IFN-α and IFN-β, is induced rapidly. The crucial transcription factors that increase IFN expression levels are interferon regulatory factor (IRF) family members, notably IRF3 and IRF7 (referred collectively as IRF hereafter) [68]. IRF activation during a viral infection is critical for the efficiency of the host response, as exemplified in mice lacking these transcription factors [69,70], and also in the myriad of viral strategies that aim at impairing their activation [71]. Despite IRFs, the IFN-β promoter also contains βB and AP-1 sites, conferring sensitiveness to NF-κB-β and ATF2-c-Jun activities, respectively [72]. However, in contrast to IRF, the relative contribution of these transcription factors to IFN-β induction might be weaker [73]. The activation of IRF transcription factors depends on the βB kinase (IKKβ)-related cytoplasmic kinases TBK1 (NAK, T2K) and I KKc (IKKi), which directly phosphorylate IRF, hence allowing homodimerization and nuclear entry [74,75]. Both kinases, and also the “conventional” IKKs (IKKα and IKKβ) share, in addition to their similar kinase domain, a ubiquitin-like domain (ULD). In TBK1 and IKKi, this domain critically controls kinase activity and IRF phosphorylation, acting as a physical bridge that recruits the kinase domain to the IRF transcription factor [76]. Other molecules that have been recently characterized in the IRF activation pathway include TANK, TRAF3 and NEMO (IKKγ) [77–81], and that others, yet-to-be identified proteins will increase this list in the near future is a likely possibility.

What are the upstream events that activate IRF and promote an antiviral response? In addition to TLRs3, -7, -8 and -9, a cytoplasmic viral RNA sensor was characterized in 2004, the RNA helicase RIG-I [82]. This remarkable study not only identified a new antiviral receptor in a new pathway, it also opened an entire area of research, which focused on identifying components that are functionally linked to RIG-I. RIG-I is a DExD/H box RNA helicase that contains two CARDs at the N-terminus. While the CARDs transmit downstream signaling, the RNA helicase domain interacts with viral RNA, more particularly with 5′ triphosphate RNA [83,84]. Hence, these findings imply that viruses that present, at one moment during their replication cycle, uncapped RNA (ss or ds) bearing 5′ tri-phosphates are likely to be recognized by the RIG-I-dependent defense system. Another RIG-like helicase, named MDA5 or Helicard, also defends the host against viral infections. The domain organization is conserved in the RIG-I parologue, which shows an overall amino acid identity of 35%. In contrast to RIG-I, MDA5 does not recognize 5′ tri-phosphate RNA species [83,84] (the true MDA5ligand(s) is still to be discovered) and it protects the host against a different spectrum of viruses [85–87]. Despite this major difference in virus discrimination, at least two functional similarities link
MDA5 and RIG-I. First, both helicases can become activated by cellular RNA produced by the catalytic activity of RNAseL, hence showing that the RLH family can sense the presence of non-self but also self-RNA [88]. Second, downstream signaling by RLH is transmitted through a common adaptor. Both RIG-I and MDA5 utilize their CARDs to bind to the CARD of the adaptor molecule Cardif (MAVS, IPS-1, VISA) [89–92] (Fig. 1), a protein that contains a C-terminal transmembrane region that targets it to the outer mitochondrial membrane [91]. Interestingly, TRIM25-mediated ubiquitination of RIG-I at Lys 172 (adjacent to the second CARD) is required for Cardif recruitment and downstream signaling, a phenomenon not observed with MDA5 [93]. Once activated, Cardif most likely acts as a platform to recruit downstream signaling molecules, such as TBK1, IKKε, TRAF3, RIP1, FADD and likely other unidentified interaction partners [80,89–92]. Although how exactly the platform is orchestrated during a response to viral infections is still to be defined, it is known that Cardif requires mitochondrial localization to transmit antiviral signaling [91]. A third RIG-like helicase (RLH) family member, LGP2, which lacks the CARDs present in both RIG-I and MDA5, may act as a regulatory molecule, although its mechanism of action is still obscure [94–98]. Other molecules have been proposed to act as negative regulators, possibly participating in the fine-tuning of antiviral activities. These include SIKE and Pin1, among others [99,100].

Because RLH signaling constitutes an important arm of innate immune defense, it is not surprising that viruses have established efficient strategies that derail these pathways. A striking illustration is Cardif, which is cleaved and inactivated by NS3-4A and 3ABC proteases from hepatitis C and hepatitis A viruses, respectively [90,101]. In this respect, it will be interesting in the future to see if other viruses also target Cardif for cleavage as a mean to evade innate immune responses. Importantly, such findings may help to design inhibitory molecules that aim at preventing virus-mediated Cardif inactivation. Noteworthy, BILN2061, a small molecule compound that blocks NS3-4A activity, has proven striking anti-HCV effects in humans [102]. Hence, BILN2061 or other NS3-4A inhibitors are promising anti-HCV molecules, because the protease activity of NS3-4A is required for both the maturation of HCV proteins and for Cardif inhibition.

### 3.2. Cytoplasmic DNA sensing and production of antiviral type-I interferon

DNA is, like RNA, able to trigger antiviral-like innate immune responses, which include production of type-I IFN. In this case, it is interesting to note that, when present in the cytoplasm, not only viral and bacterial, but also self-DNA can trigger antiviral-like responses, hence suggesting that the host might not be able to fully discriminate between self- and non-self-DNA [103–105]. Hence, prevention from immune responses to self-DNA might reside in the physical confinement of DNA within the nucleus and mitochondria. Interestingly, these conditions are not always maintained, for example during expulsion of nuclei from erythroid precursor cells, and in apoptotic cells. Indeed, in both cases macrophages engulf such materials, and ingested DNA is degraded by DNaseII, a phenomenon that prevents innate immune reactions. In DNaseII-deficient fetuses, the ingested DNA accumulates within macrophages in the liver, and causes an immune response that is deleterious to the developing embryo. Disruption of the type-I IFN receptor prevents the embryonic death, indicating that the lethality is caused by type-I IFN signaling [106]. Furthermore, this anti-self-DNA response is TLR-independent [104]. These results have raised the following questions: what is/are the receptors and adaptors that detect undigested DNA? What is the point of convergence between RLH signaling and anti-DNA responses? In this respect, it is interesting to note that the effector molecules TBK1 and IKKε were shown to participate in anti-DNA responses [103]. In contrast, the upstream adaptor Cardif is dispensable, as inferred from results obtained through analysis of cells derived from Cardif-deficient mice [107,108]. However, a few studies have suggested that human Cardif might participate in signaling responses to DNA species [109,110]. Undoubtedly, more studies are required to fully determine the contribution, if any, of Cardif to anti-DNA responses, and to understand possible species-specific functions of this protein. Recently, DNA-dependent activator of IFN-γ-regulatory factors (DAI, also known as DLM-1 or ZBP1) was reported as a candidate innate immune receptor for cytoplasmic DNA [111]. DAI fulfills the requirements for acting as such a receptor. It binds dsDNA, triggers induction of type-I IFN and, when knocked-down by RNA interference strongly impaired DNA-induced type-I IFN responses. Interestingly, as anticipated [103–105] DAI does not discriminate between dsDNA sources, as it is activated by viral, bacterial and mammalian DNA [111]. The future generation of mice deficient in DAI should permit us to clearly establish if this molecule is the long-sought (and perhaps the sole) cytoplasmic innate immune receptor for dsDNA. Furthermore, biochemical analyses, such as DAI pull-down experiments, should enable us to identify DAI-specific adaptor(s) that transmits responses to the IRF kinases, TBK1 and IKKε.

### 3.3. Targeting antiviral components to treat human diseases?

Innate antiviral signaling is, like most intracellular signaling pathways, governed by phosphorylation events, which are crucially regulated by IKKε, TBK1, and possibly other kinase proteins. Kinases are interesting targets for therapies, since small molecule-mediated inhibition can be designed to block their activity in a selective way. Hence, the development of such compounds might influence the outcome of RLH or DAI signaling. However, in which circumstances would it be important to attenuate antiviral activities? One possibility is to prevent spontaneous activation of these pathways that may lead to chronic inflammatory reactions in the absence of any viral infection. Although it is currently unknown if this happens in humans, future investigations will tell if polymorphisms of some components of these pathways may confer different basal activities among the human population, and may be associated with auto-inflammatory diseases, as discussed above for NLR proteins.

Would the targeting of RLH core components, such as IKKε and/or TBK1, also be beneficial in the treatment of other diseases? In this respect, recent studies have demonstrated that
IKKε might play a crucial role in breast cancer, as its gene was found amplified in several breast cancer cell lines and tissue samples [112]. Importantly, it was determined that the pron oncogenic role attributed to IKKε does not depend on its capacity to activate IRF, but NF-κB. Furthermore, in tumor cells, TBK1 was found associated with RalB GTPase in an anti apoptotic complex [113]. Hence, these examples and also others (that cannot be mentioned here due to space limitation) document the potential interest in targeting TBK1 and IKKε activities for anti-tumor therapies. Future studies should help to evaluate if indeed these kinases are realistic targets for drug development.

4. Discussion and perspectives

Enormous progress has been made in recent years in the characterization of NLR signaling. Still, most of the NLR family members have no assigned function and their potential role in innate immunity or other cellular processes remains elusive. Future studies are needed to further elucidate the signaling pathways implicated and the exact activation mechanisms of Nod and Nalp proteins. In this way, new therapeutic targets can be identified and used for drug development. One possibility would be the elaboration of new mouse models. The future generation of reporter mice should enable us to temporally and spatially follow in vivo which pathway becomes activated upon challenge with a defined stimulus, being a PAMP or a danger signal. An excellent example of such an approach in viral infection settings has been reported recently [114]. In that study, knockin mice that express GFP under the control of the endogenous Ifi200 promoter were generated. Using this reporter mouse model the authors could establish that RLH signaling plays a prominent role in alveolar macrophages and conventional dendritic cells of the lungs upon intranasal infection with several viruses. These two cell types rapidly mounted an antiviral status, dritic cells of the lungs upon intranasal infection with several knockin mice that express GFP under the control of the endog enous Ifi200 promoter might still be susceptible to TLR-dependent antiviral responses. This impor viruses that derail the RLH surveillance system might still be Fus in case of viral escape. Interestingly, pDCs utilize TLR but might preferentially serve as a backup for production of type-I IFNs. These two cell types rapidly mounted an antiviral status, dendritic cells of the lungs upon intranasal infection with several knockin mice that express GFP under the control of the endogenous Ifi200 promoter might still be susceptible to TLR-dependent antiviral responses. Future studies are needed to further elucidate the signaling pathways implicated and the exact activation mechanisms of Nod and Nalp proteins. In this way, new therapeutic targets can be identified and used for drug development. One possibility would be the elaboration of new mouse models. The future generation of reporter mice should enable us to temporally and spatially follow in vivo which pathway becomes activated upon challenge with a defined stimulus, being a PAMP or a danger signal. An excellent example of such an approach in viral infection settings has been reported recently [114]. In that study, knockin mice that express GFP under the control of the endogenous Ifi200 promoter were generated. Using this reporter mouse model the authors could establish that RLH signaling plays a prominent role in alveolar macrophages and conventional dendritic cells of the lungs upon intranasal infection with several viruses. These two cell types rapidly mounted an antiviral status, whereas it was inferred that plasmacytoid dendritic cells (pDCs) might preferentially serve as a backup for production of type-I IFN in case of viral escape. Interestingly, pDCs utilize TLR but not RLH signaling to mount antiviral responses, indicating that viruses that derail the RLH surveillance system might still be susceptible to TLR-dependent antiviral responses. This important report illustrates the power of reporter mice that enable us to address different issues in vivo and ex vivo.

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