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Review

Immune Sensing Mechanisms that Discriminate Self from Altered Self and Foreign Nucleic Acids

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All lifeforms have developed highly sophisticated systems equipped to detect altered self and non-self nucleic acids (NA). In vertebrates, NA-sensing receptors safeguard the integrity of the organism by detecting pathogens, dyshomeostasis and damage, and inducing appropriate responses to eliminate pathogens and reconstitute homeostasis. Effector mechanisms include i) immune signaling, ii) restriction of NA functions such as inhibition of mRNA translation, and iii) cell death pathways. An appropriate effector response is necessary for host defense, but dysregulated NA-sensing can lead to devastating autoimmune and auto-inflammatory disease. Their inherent biochemical similarity renders the reliable distinction between self NA under homeostatic conditions and altered or exogenous NA particularly challenging. In this review, we provide an overview of recent progress in our understanding of the closely coordinated and regulated network of innate immune receptors, restriction factors, and nucleases to effectively respond to pathogens and maintain host integrity.

Nucleic acids (NA) are a common building block of life, yet detection of exogenous genetic material is essential to host defense. The immune sensors employed by our cells to distinguish between self and non-self NA are both effective and ancient, with recent publications revealing that even bacteria utilize sensors surprisingly similar to our own, such as cGAMP synthase (cGAS) and Toll-interleukin Receptor (TIR) domain-containing proteins for anti-phage defense (Cohen et al., 2019; Doron et al., 2018). Although there are also sequence-based, adaptive forms of NA sensing such as CRISPR/Cas and RNA interference (RNAi), which are integral to host defense in other kingdoms and phyla (Berkhout, 2018; Hampton et al., 2020), chordates principally rely on a discreet but powerful system of germline-encoded NA sensors that are activated by molecular hallmarks of non-self or altered self NA (Schlee and Hartmann, 2016). Sensor activation triggers a transcriptional form of host defense, including type-I interferon (IFN-I) release and the autocrine and paracrine induction of interferon-stimulated genes (ISG), known as the antiviral state.

In turn, to avoid immunodetection, pathogens and viruses in particular have engaged in a type of “nucleic acid arms race” to avoid sensing by the host. Pathogens can sequester their NA (e.g., in replication organelles), mask them with characteristics of self (e.g., viral cap-snatching), or even directly disable host signaling. Indeed, recent publications indicate that, while RNAi is present in vertebrate cells and active in embryonic cells (Li et al., 2013a; Maillard et al., 2013), it can be rendered ineffective by the anti-RNAi mechanisms of many viruses (Li et al., 2016; Qiu et al., 2017). Thus, it is tempting to speculate that this disabling of RNAi provided the evolutionary pressure leading to the dominance of the type-I interferon (IFN-I) signaling in vertebrate antiviral defense, a system which is both exceptionally potent and reciprocally antagonistic with RNAi (Maillard et al., 2016; Seo et al., 2013).

It is interesting to note that, although many of the receptors activating type-I IFN signaling are highly conserved evolutionarily, their downstream signaling is unique to chordates. Analogously, caspases and metacaspases are ancient participants in programmed cell death (Bell and Megeney, 2017), yet inflammatory caspase activation by inflammasome proteins, some of which can also be activated directly or indirectly by NA, is unique to vertebrates (Maltez and Miao, 2016). In particular, DNA sensing by inflammasomes is subject to strong evolutionary pressure and divergence even among mammalian species (Brunette et al., 2012; Gaidt et al., 2017). Indeed, the functionalization of CRISPR/Cas systems as a tool for genomic editing have revealed important differences in human and murine NA sensing, including distinct cell subset expression patterns of NA-sensing Toll-Like Receptors (TLRs) or species differences in the structural requirements for the detection of cyclic dinucleotide cGAMP by STING.

In this review, we will focus on the specific molecular mechanisms employed by the nucleic acid sensors of the vertebrate innate immune system to distinguish between physiologically present and pathogen-derived or pathogenically altered NA. We primarily focus on the distinction of self versus non self, and the molecular structure, availability, and localization of NA ligands. Our aim is to provide sufficient background to understand these findings within the broader context of the evolving field of nucleic acid immunity. Special emphasis is placed on what we think is essential information for someone who is new to the field, such as the spectrum of antiviral responses elicited (see Box 1, effector functions), the multiple issues around the first NA ligand “poly(I:C)” (see Box 2, poly(I:C)), or the advantages and disadvantages of using the most common enzymatic method for RNA synthesis, *in vitro* transcription (IVT), and its implications for RIG-I activation (see Box 3, *in-vitro* transcription).



Box 1. Effector Functions of Nucleic Acid Sensing: Signaling, Restriction, and Cell Death

Upon NA binding, immune sensing receptors activate an overwhelmingly transcriptional response. These signaling transduction cascades include nuclear factor- κ B (NF- κ B), interferon regulatory factors (IRF) 3 and 7 and activator protein-1 (AP-1) (Maniatis et al., 1998; Marié et al., 1998), which cooperatively transcribe chemokines and cytokines as well as type-I and, at mucosal barriers, type-III interferons (Onoguchi et al., 2007; Ye et al., 2019). This results in a complexly orchestrated response, including the induction of interferon-stimulated genes (ISG) and the anti-viral state in infected and bystander cells, as well as the activation and the recruitment of innate and adaptive immune cells to the site of infection (González-Navajas et al., 2012).

In contrast, NA restriction factors do not induce transcriptional signaling but rather serve to directly restrict the function of pathogen nucleic acids via their sequestration, destruction, and processing and/or presentation for NA immune sensing receptors. These factors are generally ISGs, and their presence is indicative of the anti-viral state. Restriction mechanisms can be highly specific, such as the sequestration of 2'-O-unmethylated capped RNA by IFIT1 (Daffis et al., 2010) or global, such as protein kinase R activation, which upon binding of long double-stranded(ds)RNA induces the inhibition of cellular transcription and translation, halting the proliferation of cell and virus alike (Levin and London, 1978).

The *ultima ratio* of global pathogen restriction in host defense is cell death, which simultaneously restricts pathogen replication, releases inflammatory mediators, and terminates transcriptional signaling (Maelfait et al., 2020). NA-sensing can trigger a variety of forms of programmed cell death (PCD) from immunologically silent to proinflammatory. Apoptosis can result from overwhelming activation of NA sensors, such as STING (Gulen et al., 2017; Tang et al., 2016) or MAVS (Besch et al., 2009; Kaneda, 2013; Matsushima-Miyagi et al., 2012), particularly in cancer cells. Necroptosis, a lytic proinflammatory form of PCD, can be directly induced by the activation of the ZBP-1 by viral or endogenous Z-form RNA (Maelfait et al., 2017; Thapa et al., 2016) but also indirectly by combined TNF and IFN exposure downstream of STING or MAVS hyperactivation (Brault et al., 2018). Pyroptosis, a rapid, lytic form of PCD accompanied by the release of pyrogenic cytokines, results from inflammasome activation and can be triggered directly by NA-sensing inflammasome proteins or indirectly downstream of NA sensing. In particular, the NLRP3 inflammasome is activated by cellular dyshomeostasis and thus induces pyroptosis secondary to other cell death pathways (de Vasconcelos and Lamkanfi, 2020), as has been described downstream of STING, MAVS, and ZBP-1 activation, as well as viral lytic cell death (da Costa et al., 2019; Franchi et al., 2014; Gaidt et al., 2017; Kuriakose et al., 2016). Since canonical inflammasome assembly induces caspase-1-mediated proteolytic activation of both the pyroptotic effector gasdermin D as well as cytokines from the IL-1 family, it sits at the crossroads of signaling and effector functions (de Vasconcelos and Lamkanfi, 2020). However, it should be noted that this type of signaling is fundamentally different from the IFN and ISG response: inflammasome activation is post-translational, not transcriptional and, theoretically, does not even require a living cell (Franklin et al., 2018). Nonetheless, inflammasome activation is of critical importance to the immune response to many viral infections, since IL-1 family cytokines support immune cell recruitment, NK-cell activation and the formation of anti-viral CD8+ T cell responses (reviewed in Kanneganti, 2010).

Since the type-I IFN response is inherently transcriptional, NA-induced cell death also acts as an important limiter of antiviral responses. Several studies have demonstrated that apoptotic caspases directly inhibit cGAS/STING signaling (Rongvaux et al., 2014; White et al., 2014), with a recent report showing that activated caspase 3 can cleave cGAS, MAVS, and IRF3 (Ning et al., 2019). Similar mechanisms have been demonstrated for pyroptosis. Activated caspase 1 has been reported to cleave cGAS (Wang et al., 2017), and activation of gasdermin D-mediated pyroptosis can also resolve cGAS/STING signaling through K⁺ efflux (Banerjee et al., 2018). Although many recent studies to date have focused on the effect of PCD on cGAS/STING, the reciprocal interplay between NA sensing and cell death is undoubtedly important for all NA-sensing pathways and continues to be the focus of intense research.

Principles of Nucleic Acid Sensing: Localization, Structure, and Availability

Unlike fundamentally exogenous, microbial substances, such as flagellin or LPS, nucleic acids are common to all forms of life, whether pathogen or host. Effective NA sensing thus critically depends on specific and sensitive detection of pathogenic or altered NA among abundant, physiological endogenous molecules. The central principles underlying this distinction are NA (1) structure, (2) localization, and (3) availability (Figure 1).

Structural characteristics of NA include their length, base pairing, secondary structure, 5'- and 3'-termini, and modifications. The localization of NA refers to their cellular and subcellular compartmentalization, monitored by a system of strategically placed NA sensors. Endosomal NA sensors, i.e., the NA-sensing TLRs, are primarily expressed in phagocytic, professional im-

mune cells and are ideally located to sense ligands released by the hydrolytic degradation of pathogens in the endophagosomal compartment (Blasius and Beutler, 2010; Brubaker et al., 2015). This compartmentalization is controlled by trafficking and proteolytic receptor activation in the endosomal compartment, and alterations of receptor localization can lead to fatal autoinflammation (Mouchess et al., 2011). Cytosolic NA sensors, including RIG-I, MDA5, and cGAS, are broadly expressed in nucleated cells where they sense cell-intrinsic infection. Correspondingly, the type-I IFN receptor (IFNAR) is also ubiquitously expressed (González-Navajas et al., 2012), allowing, in theory, any nucleated cell to sense viral infection and enter an antiviral state via autocrine or paracrine IFN. Moreover, recent research has also revealed the importance of nuclear NA sensing (Gentili et al., 2019; Volkman et al., 2019), revising the long-standing

Box 2. Poly I:C

Generated by the annealing of enzymatically generated inosine and cytosine homopolymers (Michelson et al., 1967), polyI:C is by far the most commonly used RNA ligand in research. Clinical applications for polyI:C were explored soon after the discovery that it could produce a robust IFN response (Field et al., 1967; Hilleman, 1970), and clinical trials using polyI:C in tumor immunotherapy continue today (e.g., NCT02166905, NCT03358719). Moreover, over the last 50 years, intense research has focused on characterizing the immune sensor(s) polyI:C activates. This popularity is remarkable given that polyI:C is neither a physiological ligand nor is its mode of action well defined. Rather, its widespread use results from its amenability to enzymatic synthesis (Michelson et al., 1967) and its unique ability to induce a robust type-I IFN response compared to other annealed homopolymers (Field et al., 1967), a feature which remains poorly understood. As a long dsRNA with a 5' diphosphate terminus, polyI:C is known to activate the sensing receptors TLR3, MDA5, and RIG-I (Alexopoulou et al., 2001; Gitlin et al., 2006; Kato et al., 2008; 2006; 2005; Yoneyama et al., 2005), accessory proteins such including LGP2 and members of the DDX and DHX families (reviewed in Oshiumi et al., 2016) as well as the restriction factors PKR and the OAS family (Farrell et al., 1978; Hovanessian et al., 1977; Zilberstein et al., 1978). In theory, any receptor that binds dsRNA could be activated by polyI:C, including further restriction factors, such as ZBP-1 (Z-RNA), although characterizing the specific activity would clearly require multiple gene deletions. This plethora of potential receptors likely contributes to its high toxicity profile, which is reduced in the more selective derivative polyI:C12U (Junt and Barchet, 2015).

Despite over 50 years of research, many open questions about the molecule's bioactivity remain: why does low-molecular weight polyI:C (< 300bp) preferentially activate RIG-I given that RIG-I senses the 5' diphosphate terminus (Goubau et al., 2014)? Why is polyI:C a robust activator of MDA5 while many other defined, long dsRNAs such as poly(A:U) are not (Colby and Chamberlin, 1969; Pichlmair et al., 2009)? Does the mesh-like structure of poly(I:C) contribute to its immune stimulatory activity, as opposed to just its length (Pichlmair et al., 2009)? Why does the polyI:C derivative poly I:C12U (Ampligen) only activate TLR3 but not RIG-I or MDA-5 (Gowen et al., 2007)?

paradigm that the nuclear compartment is “immune privileged.” Indeed, given that the nucleus is the site the replication of most DNA viruses, as well as retroviral genomic integration, these nuclear sensors are perhaps the last line of defense for our genomic integrity.

NA availability is the net result of the entry or generation of NA structures in a specific compartment versus their sequestration (e.g., by pathogen vacuoles), shielding from receptor binding (e.g., by viral proteins), or degradation by nucleases. Pathogen sequestration can be countered by host proteins, such as interferon-inducible GTPases (Meunier and Broz, 2016), which release ligands into the cytosol. Moreover, host and viral nucleases can act to limit or enhance the availability of potential NA ligands. Indeed, as we and others have shown, the same host nuclease (e.g., DNase 2, RNase T2) can potentially enhance the activity of some receptors while limiting the activity of others, thus acting as a rheostat for NA sensor activation.

Although these principles are generally effective, the inherent risks of this strategy are clear. Inappropriate or uncontrolled sensing of self NA drives autoinflammatory disease, ranging from rare and devastating monogenetic forms of type-I interferonopathy (Rodero and Crow, 2016) to the inevitable senescence of our cells (Glück et al., 2017; Yang et al., 2017). Nonetheless, NA sensing provides a clear evolutionary advantage in that it is essential for host survival, as demonstrated by numerous genetic models of pathogen infection. NA sensors can be categorized into (1) bona fide immune sensing receptors that act to detect pathogenic NA and signal their presence to the host and (2) anti-viral restriction factors that act directly upon viral NA to limit viral replication but are not linked to the transcription of classical immune-related genes such as cytokines (Schlee and Hartmann, 2016).

Endosomal RNA Sensing

The search for immune receptors detecting exogenous RNA has led to the discovery of both endosomal and cytosolic RNA-sensing mechanisms (Figure 2). The transmembrane TLRs TLR3, TLR7, TLR8, and, in lower vertebrates and rodents, TLR13, are all genetically proven endosomal RNA immune sensors (Alexopoulou et al., 2001; Diebold et al., 2004; Heil et al., 2004; Oldenburg et al., 2012). TLR10 has been reported to act as an anti-inflammatory receptor of double-stranded (ds-)RNA, but further studies will be needed to corroborate this finding (Lee et al., 2018).

TLR3 was the first immune sensing receptor of polyI:C discovered (Alexopoulou et al., 2001) and is a sequence-independent sensor of the ribose-phosphate backbone of dsRNA > 35bp (Leonard et al., 2008; Liu et al., 2008) and incomplete dsRNA stem structures of sufficient length within ssRNA molecules (Tatematsu et al., 2013). TLR3 signaling is distinct from other NA-sensing TLRs in several respects: instead of MYD88, TLR3 signals via TRIF to induce IFN- β and NF- κ B signaling (Oshiumi et al., 2003; Yamamoto et al., 2003), with recent research indicating that the nutrient sensor mTORC2 is also critically required (Sato et al., 2018); TLR3 is expressed in non-immune cells, including fibroblasts, endothelial cells, oligodendrocytes, astrocytes, and neurons (Bsibsi et al., 2002; Lafon et al., 2006; Matsu-moto et al., 2002; Zimmer et al., 2011), and it has been reported that, in several of these cell types, TLR3 can also be localized and signal from the cell surface, (Jack et al., 2005; Matsumoto et al., 2002; Pohar et al., 2013) although this would presumably require an acidic environment for dsRNA binding (Liu et al., 2008). Despite the seeming redundancy of dsRNA sensing (see Box 2, polyI:C), humans expressing hypomorphic variants of TLR3 are susceptible to HSV-1 encephalitis (Zhang et al., 2007) and severe influenza pneumonitis (Lim et al., 2019) in

Box 3. *In vitro* Transcription and RIG-I Activation

Phage-encoded DNA-dependent RNA polymerases are commonly used for *in-vitro* transcription (IVT) of DNA into RNA from linear dsDNA or a plasmid DNA template containing the appropriate promoter sequence (Green and Sambrook, 2020). This method allows the rapid, cheap, and efficient generation of long RNA molecules, many of which would be prohibitively expensive to produce synthetically. As such, IVT is a popular, often kit-based, method for producing mRNA and CRISPR guide RNA for use with recombinant Cas Proteins. However, since IVT utilizes promoter-based unprimed RNA polymerization, it creates transcripts with a 5' triphosphate terminus, much like other nascent viral RNA transcripts. We and others used IVT-generated RNA to demonstrate that 5' triphosphate RNA activated RIG-I (Hornung et al., 2006; Pichlmair et al., 2006). However, the RIG-I activating species within the IVT is not its main product, 5' triphosphate ssRNA. 3' end complementarity can prime a fold back reaction resulting in the production of complementary strands and RNA hairpin structures (Cazenave and Uhlenbeck, 1994; Triana-Alonso et al., 1995). As we and others subsequently showed, 5' triphosphate base-paired, blunt-ended RNA, a side product within an IVT, is in fact the true RNA species activating RIG-I (Schlee et al., 2009; Schmidt et al., 2009) reviewed in (Schlee, 2013). Unless modified nucleotides or specific sequences precluding the formation of the complementary strand are used, IVT can potentially generate RIG-I ligands from a variety of templates (Hornung et al., 2006; Kim et al., 2004; Schlee et al., 2009; Wienert et al., 2018). On the one hand, this can lead to unwanted immunostimulatory effects during the generation of mRNA, gRNA, or siRNA. On the other, it means that extreme caution should be used when inferring that a particular sequence or endogenous RNA species activates RIG-I if IVT RNA was used to model its activity. Most endogenous RNA species are processed post-transcriptionally and thus lack accessible 5' triphosphate moieties (Gebhardt et al., 2017; Schlee and Hartmann, 2016), thus using 5' triphosphate IVT RNA is prone to creating immunostimulatory artifacts.

childhood, and Tlr3^{-/-} mice demonstrate a susceptibility to poliovirus (Oshiumi et al., 2011), HSV-1 (Davey et al., 2010), and MCMV (Tabeta et al., 2004). Moreover, perhaps due to its broader expression, TLR3 has a preeminent role in the CNS (Zhang et al., 2013), where it contributes to both protective and deleterious neuroinflammation during host defense (Ménager et al., 2009; Perales-Linares and Navas-Martin, 2013; Sato et al., 2018; Wang et al., 2004).

In contrast, TLR7, TLR8, and TLR13 signal via the adaptor MYD88 and are restrictively expressed in immune cells (Diebold et al., 2004; Heil et al., 2004; Hemmi et al., 2002; Hornung et al., 2002; Shi et al., 2011). TLR7 and TLR8 result from a gene duplication (Roach et al., 2005) and demonstrate numerous structural and functional similarities. Both receptors can be activated by imidazoquinoline compounds and PolyU and GU-rich ssRNA (Judge et al., 2005; Jurk et al., 2002). However, selective ligands have also been reported (Forsbach et al., 2008; Lu et al., 2012; Ostendorf et al., 2020). While TLR7 is functional in both mice and humans, the role of TLR8 in mice remains unclear (Alexopoulou et al., 2012; Heil et al., 2004). In humans, TLR7 and TLR8 demonstrate a differential expression pattern (Hornung et al., 2002). TLR8 is highly expressed on monocytes, conventional DCs and neutrophils, whereas TLR7 is predominantly expressed in plasmacytoid dendritic cells (pDC) and B cells. In human monocytes, TLR8 activation leads to the release of IFN- β and proinflammatory cytokines, including IL12p70, IL6, and TNF (Bergström et al., 2015; Cushing et al., 2017). Very recently, the type-I-interferon-inducible TLR adaptor interacting with SLC15A4 on the lysosome (TASL) has been reported as an additional signaling component linking endolysosomal TLR7 and TLR8 to interferon regulatory factor-5 (IRF5) and IFN- β induction (Heinz et al., 2020). TASL recruits and activates IRF5, and loss of TASL specifically impairs the activation of the IRF pathway without affecting NF- κ B and MAPK signaling, revealing a mechanistic analogy with STING, MAVS, and TRIF. In pDCs, TLR7 activation strongly induces IFN- α via the MYD8-TRAF6-IRF7

pathway (Honda:2004jg. Kawai et al., 2004). TLR7 is also weakly expressed in primary monocytes, where it can also activate downstream signaling (de Marcken et al., 2019; Gantier et al., 2008). The crystal structures of the ligand-bound ectodomains of TLR7 and TLR8 revealed that both bound RNA degradation products (Shibata et al., 2016; Tanji et al., 2015; Zhang et al., 2016). In their first binding pocket, TLR7 binds guanosine and TLR8, uridine, respectively, while the second binds short di- or trinucleotides. Subsequent studies demonstrate that TLR8 activation by RNA ligands critically requires upstream endosomal RNase activity (Greulich et al., 2019; Ostendorf et al., 2020). Since endosomal RNase expression varies substantially between cell types, their activity adds another layer to our understanding of TLR7 and TLR8 specific ligands.

TLR13 is expressed in non-mammalian vertebrates, marsupials, and rodents but not in primates (Hidmark et al., 2012; Oldenburg et al., 2012). Two studies identify the sequence CGGAAAGACC and ACGGAAAGACCCC within 23S rRNA of several species of bacteria as a TLR13-activating motif (Li and Chen, 2012; Oldenburg et al., 2012). The crystal structure of the ectodomain of TLR13 reveals that it senses both RNA sequence and stem-loop conformation (Song et al., 2015). To date, TLR13 is the only completely sequence-specific vertebrate RNA immune sensor identified.

Cytosolic RNA Sensing

The observation that the Tlr3^{-/-} mouse still responds to polyI:C provided the first indication of intracellular RNA immune sensing receptors (Diebold et al., 2003). The presence of cytosolic RNA with hallmarks of non self can indicate viral replication (Weber et al., 2006), intracellular bacterial infection (Hagmann et al., 2013; Monroe et al., 2009) or the escape of RNA from the endolysosome via transport or rupture (Nguyen et al., 2017; Watanabe et al., 2011). Two related DExD/H box RNA helicases RIG-I and MDA-5 (Yoneyama et al., 2004), also known as RIG-I Like Receptors (RLRs), sense cytosolic polyI:C and mount a

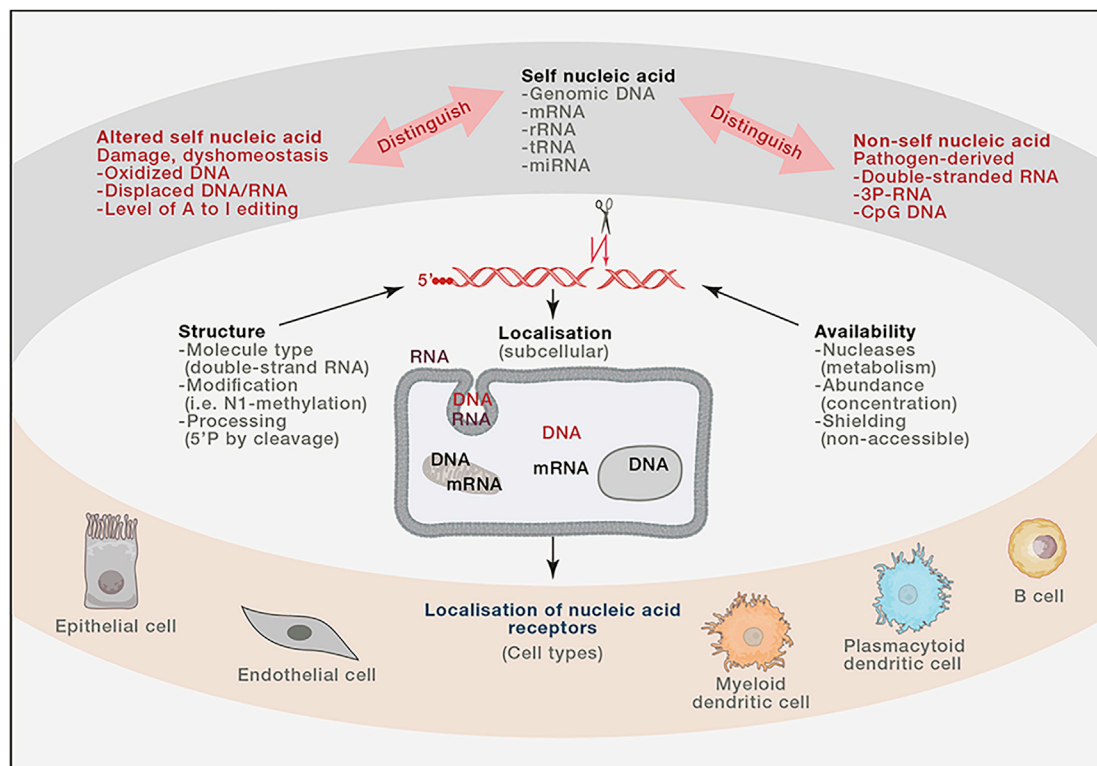


Figure 1. Principles of Self Versus Non-self or Altered-Self Nucleic Acid Recognition

Unlike pathogen-specific molecules such as LPS, nucleic acids in pathogens and the host are biochemically similar. For a reliable distinction of self versus non-self and altered-self nucleic acid recognition, information about the molecular structure, the availability, and the localization is integrated. The localization of nucleic acid receptors on different cell types, immune cells as well as non-immune cells, contribute as well. Lists and cell types depicted are not comprehensive but just represent examples to better illustrate the principles.

type-I IFN response via the mitochondrial adaptor protein MAVS (Kawai et al., 2005; Seth et al., 2005). While RIG-I is activated by shorter (< 300bp, low-molecular weight, LMW) poly I:C molecules, MDA5 requires longer dsRNA (> 300bp, high-molecular weight, HMW) polyI:C (Gitlin et al., 2006; Kato et al., 2005; 2008; 2006; Yoneyama et al., 2005). This overlapping yet differential activity also applies to the sensing of viral replication (Loo et al., 2008; Schlee, 2013). Both sensors contribute to the immune response to dsRNA viruses, such as *reoviridae* (Loo et al., 2008), but RIG-I-mediated sensing dominates the response to many (-) ssRNA viruses such as influenza, which form shorter dsRNA panhandles in their genomes (Rehwinkel et al., 2010; Schlee et al., 2009), whereas MDA5 has a greater role in the host defense against (+) ssRNA viruses, many of which are known to generate large amounts of dsRNA during replication (Weber et al., 2006). Whereas subsequent studies have made substantial advances in characterizing the precise molecular patterns activating RIG-I (see RNA Motifs Distinguishing Self from Non Self), the motifs necessary to activate MDA5 still remain ill defined (Hartmann, 2017).

Both RNA sensors are expressed in almost all primary nucleated cells (Hartmann, 2017; Ida-Hosonuma et al., 2005). Upon activation, RIG-I and MDA-5 oligomerize, thereby inducing the polymerization of MAVS into fibrillar structures (Hou et al., 2011; Xu et al., 2014) and recruiting TRAF2, TRAF6, IKK, and TBK1, which in turn activate NF- κ B and IRF3 and/or IRF7

signaling (Kawai et al., 2005; Seth et al., 2005) and the transcription of proinflammatory cytokines and type-I and type-III IFN. In addition to transcriptional signaling, MAVS complexes can associate with Fas-associated protein with death domain (FADD), Receptor-interacting serine/threonine-protein kinase 1 (RIP1), and caspase 8 (Kawai et al., 2005; Yang et al., 2019). In this context, caspase 8 has been reported to induce apoptosis downstream of MAVS (El Maadidi et al., 2014) but also to terminate signaling without cell death (Rajput et al., 2011; Sears et al., 2011; Yang et al., 2019). Several studies have noted that malignantly transformed cells are more sensitive to RLR-induced cell death, although the precise molecular determinants for this difference remain unknown (Hartmann, 2017; Kumar et al., 2015).

RNA-Sensing Inflammasome Proteins

While it is well established that inflammasome activation is important for antiviral defense (Kanneganti, 2010), the RNA sensors involved are still the subject of intense research. To date, three inflammasome-building NLRs have been reported to participate in cytosolic RNA sensing, with all three requiring accessory RNA-binding proteins. Via the adaptor protein DHX33, NLRP3 is reported to sense viral dsRNA, RNaseL degradation products, and polyI:C, leading to inflammasome formation, caspase-1 cleavage and the release of IL-1 β and IL-18 from in human THP-1 cells and monocyte-derived macrophages (Chakrabarti et al., 2015; Mitoma et al., 2013). Via the RNA

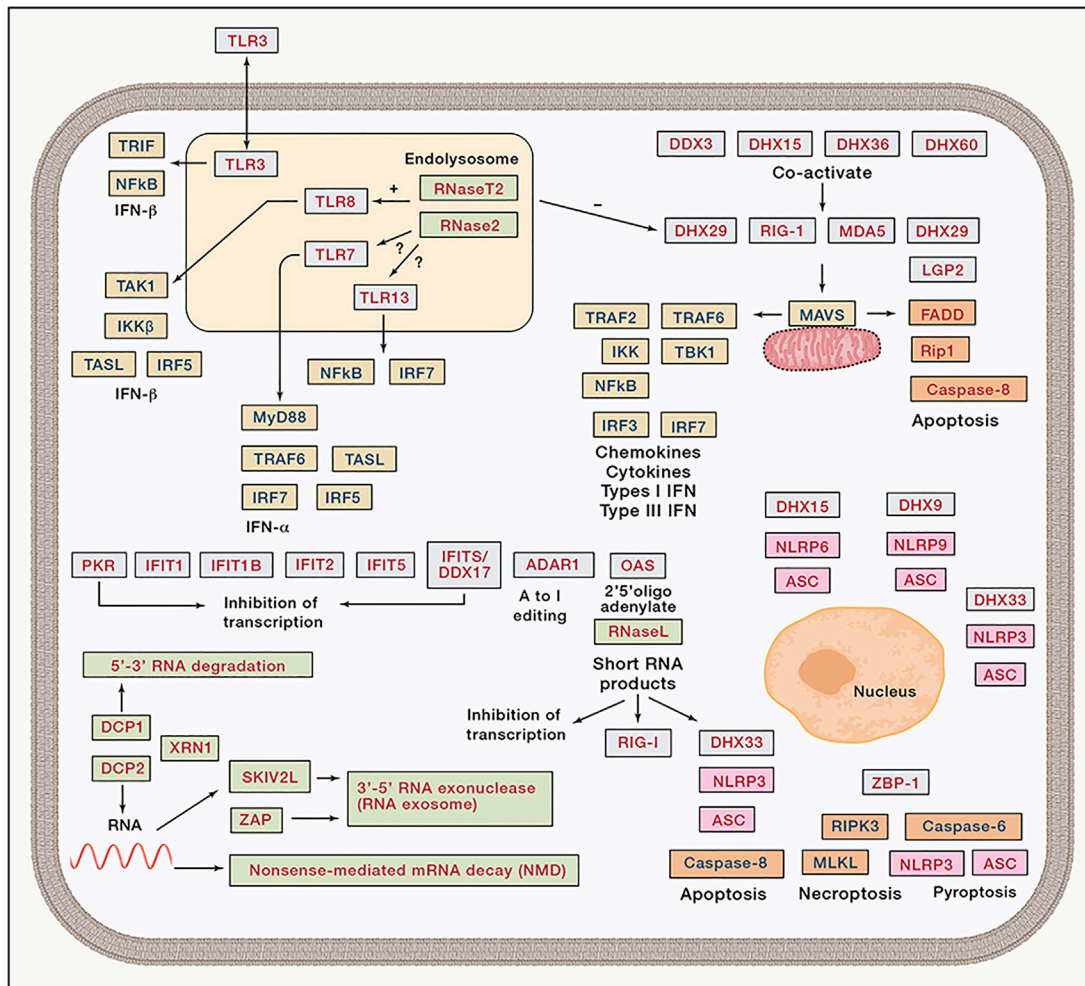


Figure 2. RNA Sensing and Response

Non-comprehensive overview of the most relevant RNA sensing receptors at the relevant localizations, with their downstream signaling molecules and some of the functional outcomes and secondary consequences as applicable (e.g., RIG-I sensing of RNaseL degradation products). RNA sensing receptors are in gray, signaling molecules in yellow, nucleases in green, inflammasome pathways in purple, and cell death pathways in orange. Unlike other TLRs, TLR3 signals from the cell surface and the endolysosome. TLR3 is a sequence-independent sensor of the ribose-phosphate backbone of dsRNA > 35bp and of incomplete dsRNA stem structures of sufficient length within ssRNA molecules. TLR3 signals via TRIF to induce IFN- β and NF- κ B signaling. TLR7 and TLR8 are activated by RNA degradation products, with the first pocket binding guanosine and uridine (TLR7 and TLR8, respectively), and the second pocket binding short di- or trinucleotides. TLR8 activation requires upstream endosomal RNase activity (RNaseT2, RNase2) and, due to homology, RNase activity is likely required for TLR7 as well. TLR7 activation induces IFN- α via the MyD88-TRAF6-IRF7 pathway. TLR8 activation releases IFN- β and proinflammatory cytokines via a TAK1-IKK β -IRF5 pathway. TLR adaptor interacting with SLC15A4 on the lysosome (TASL) was reported as a signaling component linking endolysosomal TLR7 and 8 to IRF5. TLR13 recognizes bacterial 23S rRNA in a sequence-specific manner. Upon activation, the cytosolic immune sensors RIG-I and MDA-5 oligomerize thereby inducing polymerization of MAVS into fibrillar structures leading to the recruitment of TRAF2, TRAF6, IKK, and TBK1, which then activate NF- κ B and IRF3 and/or IRF7 signaling. In addition, MAVS complexes can associate with FADD, RIP1, and caspase 8 and induce apoptosis downstream of MAVS. DDX3, DHX15, DHX36, and DHX60 enhance RIG-I signaling. DHX29 acts as co-receptor for both RIG-I and MDA5. LGP2 supports filament formation by MDA5 but may compete with RIG-I for ligand. Cytosolic RNA sensors with direct anti-viral activity include protein kinase R (PKR) and the 2'-5'-oligoadenylate synthetase system (OAS) which both bind dsRNA > 30bp including poly(I:C). PKR phosphorylates eIF2 α and inhibits cap-dependent translation of viral and host mRNA. OAS induces the formation of 2'-5'-oligoadenylate which acts as a second messenger to activate ribonuclease L (RNase L), which in turn degrades cellular RNA and viral RNA to smaller RNA molecules that can be sensed by RIG-I and DHX33. DHX33 activates the NLRP3 inflammasome, inducing pyroptotic cell death. IFN-induced proteins with tetratricopeptide repeats (IFIT) sequester viral mRNA and block their translation by sensing 5' termini. IFIT1 binds mRNA with a cap0 structure (7mGpppNm), and IFIT1B binds cap0, to a lesser extent cap1 (7mGpppNmNm) structures but not cap2 (7mGpppNmNmNm) structures. IFIT2 binds AU-rich RNA. DDX17 can bind and sequester stem loop structures from some RNA viruses. Adenosine deaminase acting on RNA 1 (ADAR1) catalyzes the C6 deamination of adenosine to inosine in base-paired regions of RNA, and the resulting non-synonymous coding causes amino acid substitutions and potentially renders viral proteins non-functional. The host RNA decay machinery includes nonsense-mediated mRNA decay (NMD), 5'-3' RNA degradation and the 3'-5' RNA exonuclease machinery (RNA exosome). NMD targets mRNA transcripts with a long 3' UTR but also senses viral RNA. The 5'-3' degradation machinery with the decapping enzymes DCP1 and DCP2 and the 5'-3' exonuclease XRN1 (XRN-DCPs) is involved in physiological cellular mRNA turnover and exhibits antiviral activity. The superkiller viralicidal activity 2-like (SKIV2L) and zinc-finger antiviral protein (ZAP) support the binding and transport vRNA to the RNA exosome for degradation. The ISG and viral restriction factor Z-DNA binding protein 1 (ZBP-1) is a death receptor downstream of dsRNA sensing. ZBP-1

(legend continued on next page)

helicase DHX9, the previously uncharacterized Nlrp9b (human homolog NLRP9) forms an inflammasome in intestinal epithelial cells in response to rotavirus, leading to the maturation of IL-18 and the activation of gasdermin D-mediated pyroptosis (Zhu et al., 2017). Via the RNA-binding accessory protein DHX15, NLRP6 has been reported to sense dsRNA in intestinal epithelial cells (Wang et al., 2015). However, this does not result in caspase-1 activation but rather MAVS-dependent type-I and III IFN induction. This function of NLRP6 is reportedly restricted to the gastrointestinal tract, as *Nlrp6*^{-/-} mice demonstrate increased mortality and viremia after oral but not systemic viral infection. Since NLRP6 can form an inflammasome (Elinav et al., 2011; Shen et al., 2019), how DHX15 induces NLRP6-MAVS interaction rather than oligomerization with the inflammasome adaptor ASC remains unclear.

RNA Restriction Factors and RNases

Other DExD/H-box proteins have been reported to act as accessory proteins to cytosolic RNA signaling (Oshiumi et al., 2016). DDX3, DHX15, DHX36, and DDX60 have been reported to enhance RIG-I signaling (Miyashita et al., 2011; Oshiumi et al., 2010; Patabhi et al., 2019; Yoo et al., 2014), whereas DHX29 acts as a co-receptor for both RIG-I (Sugimoto et al., 2014) and MDA5 (Zhu et al., 2018). The individual contributions of these proteins to RNA sensing remain unclear and occasionally contradictory. In one study, DDX3 has been found to induce MAVS activation independently from RIG-I and MDA-5 (Gringhuis et al., 2017); in another, it has been found to support RIG-I signaling (Oshiumi et al., 2010), and, in another, it was even found to inhibit IFN-I release (Loureiro et al., 2018). Likewise, the role of DDX60 is also controversial, with one group finding that it promotes RLR signaling and another reporting that it has no role (Goubau et al., 2015; Miyashita et al., 2011). Future studies will be needed to resolve these differences.

LGP2, a DExD/H-box protein from the RLR family, contains the RLR c-terminal domain (CTD) and helicase domains but lacks the CARD necessary for downstream signaling (Schlee, 2013). Accumulating evidence indicates that LGP2 acts (1) as a structural accessory protein that supports filament building and activation of MDA5 (Bruns et al., 2014; Deddouche et al., 2014; Satoh et al., 2010) and (2) as an interferon-inducible inhibitor of RNAi (Takahashi et al., 2018b; van der Veen et al., 2018). LGP2 has been reported to competitively bind dsRNA and inhibit the RNA silencing enhancer, TAR-RNA binding protein (TRBP) (Takahashi et al., 2018b; 2018a), an accessory protein of Dicer. This mechanism also highlights the inherent antagonism between Dicer-mediated cleavage of dsRNA during RNAi and dsRNA sensing by the innate immune system.

In addition to the DExD/H-box protein, Zinc-finger protein ZCCHC3 has been reported to bind viral dsRNA and act as a cofactor for RIG-I and MDA5, and *Zcchc3*^{-/-} mice are more susceptible to RNA virus infection (Lian et al., 2018b). The same group has also reported in parallel that ZCCHC3 acts as a

cofactor for dsDNA sensing and that *Zcchc3*^{-/-} mice are also more susceptible to lethal herpes simplex virus type 1 or vaccinia virus infection (Lian et al., 2018a). Here, further studies will be necessary to determine how ZCCHC3 fulfills such diverse roles in host sensing.

Protein kinase R (PKR) and the 2'-5'-oligoadenylate synthetase system (OAS) are the first RNA sensors discovered in the cytosol. Both sense dsRNA > 30bp and are activated by poly(I):C (see Box 2, poly(I):C). PKR phosphorylates eIF2 α and thus inhibits cap-dependent translation of viral and host mRNA (Levin and London, 1978). OAS binding of dsRNA leads to the production of 2'-5'-oligoadenylate (Hovanessian et al., 1977; Zilberstein et al., 1978), a second messenger activating latent ribonuclease L (RNase L) (Zhou et al., 1993). RNaseL, in turn, degrades cellular and viral RNA. Global RNase-L degradation does not only prevent viral replication but also generates smaller RNA molecules sensed by RLRs (Malathi et al., 2007, 2010) and DHX33-mediated activation of NLRP3, inducing pyroptotic cell death (Mitoma et al., 2013). In cells without NLRP3, RNase L can also trigger apoptosis to prevent viral propagation (Castelli et al., 1997).

The host RNA decay machinery also contributes to antiviral defense, and the role of nonsense-mediated mRNA decay (NMD), 5'-3' RNA degradation and the 3'-5' RNA exonuclease machinery (RNA exosome) are all the subject of current, intense research. To guard the cell against aberrant self mRNA, NMD targets mRNA transcripts with a long 3' UTR, indicative of a mutation leading to a premature stop codon (Molleston and Cherry, 2017). However, NMD also forms an important intrinsic barrier to viral infection (Balistreri et al., 2014; Fontaine et al., 2018; Li et al., 2019). Conceivably, NMD senses viral RNA via several complementary mechanisms, including the unusual translation dynamics of polycistronic viral transcripts, instability due to non-standard codon usage (Hia et al., 2019) and targeting by NMD-associated proteins (Li et al., 2019). The 5'-3' degradation machinery, comprising the decapping enzymes DCP1 and DCP2 and the 5'-3' exonuclease XRN1 (XRN-DCPs), is primarily involved in physiological cellular mRNA turnover. However, XRN1 is also specifically targeted by and inhibited by flaviviruses (Molleston and Cherry, 2017), demonstrative of antiviral activity. One recent study demonstrates that XRN1-DCPs specifically colocalize with the RNA of Newcastle disease virus and encephalomyocarditis virus (EMCV) and repress viral replication (Ng et al., 2020), although it is still unknown how XRN1-DCPs are recruited to viral RNA.

Recruitment to the RNA exosome is better understood. Superkiller viralicidal activity 2-like (SKIV2L) (Aly et al., 2016) and zinc-finger antiviral protein (ZAP) (Gao et al., 2002; Guo et al., 2007) support the binding and transport viral RNA to the RNA exosome for degradation. How SKIV2L specifically targets viral RNA is still unknown. However, recent studies have demonstrated that ZAP directly senses HIV-1 RNA via its relative abundance in CG dinucleotides (Ficarelli et al., 2020; Takata et al., 2017), thus revealing

activates multiple programmed cell death pathways, including pyroptosis, apoptosis, and necroptosis, termed PAN-optosis. Upon dsRNA binding, ZBP-1 interacts with RIPK3 supported by caspase-6, resulting in MLKL activation and necroptosis, in caspase-8-induced apoptosis and in NLRP3 dependent inflammasome activation and pyroptosis. Three inflammasome-building NLRs participate in cytosolic RNA sensing: DHX33 via NLRP3, the RNA helicase DHX9 via Nlrp9b (human homolog NLRP9), and the RNA-binding accessory protein DHX15, besides its supporting function for RIG-I-like helicases, via NLRP6.

a sequence-dependent mechanism for self versus non-self discrimination. Of note, the RNA exosome is also involved in the degradation of immunostimulatory endogenous RNA transcripts, and hypomorphic variants of SKIV2L induce type-I interferonopathy that is dependent on RIG-I (Eckard et al., 2014).

We and others recently demonstrated that RNaseT2 contributes to the activation of TLR8 (Greulich et al., 2019; Ostendorf et al., 2020). However, hypomorphic variants of RNaseT2 are associated with a rare form of proinflammatory cystic leukoencephalopathy (Henneke et al., 2009), likely resulting from cytosolic sensor activation by accumulating RNA (Haud et al., 2011). Our work also demonstrates that RNase2, a member of the RNaseA family only expressed in apes and old-world monkeys, synergistically participates in uridine release from ssRNA to activate TLR8 in monocytes (Ostendorf et al., 2020). Antimicrobial functions have also been attributed to other members of the RNaseA family (Lu et al., 2018).

Members of the IFN-induced protein with tetratricopeptide repeats (IFIT) family specifically sequester viral mRNA and thus block their translation by sensing 5' termini with markers on non self. IFIT1 binds mRNA with a cap0 structure (7mGpppNN), and IFIT1B binds cap0, to a lesser extent cap1 (7mGpppNmN) structures but not cap2 (7mGpppNmNm) structures. IFIT5 binds uncapped 5' triphosphate RNA (Abbas et al., 2013; Habjan et al., 2013; Kumar et al., 2014), and IFIT2 binds AU-rich RNA (Yang et al., 2012). In addition to the IFITs, DDX17 can bind and sequester stem loop structures from the rift valley fever virus in *Drosophila* and humans (Moy et al., 2014), and it seems highly likely that other DEX/H box RNA helicases can perform similar functions.

Adenosine deaminase acting on RNA 1 (ADAR1) catalyzes the C6 adenosine-to-inosine deamination of base-paired regions of RNA (George et al., 2014). Due to their C6 carbonyl group, these new inosines are decoded non-synonymously as guanosines, causing amino acid substitutions and potentially rendering viral proteins non-functional (Samuel, 2011). In addition, the introduction of less stable I-U wobble pairs changes base-pairing dynamics (Špačková and Réblová, 2018). These alterations of the secondary structure affect both viral regulation and host innate immune sensing, acting in a manner that can be pro- or antiviral (Samuel, 2019; 2011).

In addition to the aforementioned cell death pathways, the ISG and viral restriction factor Z-DNA binding protein 1 (ZBP-1) is a dedicated death receptor downstream of dsRNA sensing. ZBP-1 was originally described as a dsDNA sensor capable of inducing type-I IFN. However, subsequent studies have demonstrated that ZBP-1 is a dsRNA sensor that activates multiple PCD pathways, including pyroptosis, apoptosis and necroptosis, termed PAN-optosis (Malireddi et al., 2019). Upon dsRNA binding, ZBP-1 enters a homotypic RHIM-RHIM interaction with the kinase RIPK3 supported by caspase-6 (Zheng et al., 2020). This can result in MLKL activation and necroptosis (Maelfait et al., 2017; Thapa et al., 2016), caspase-8-induced apoptosis (Nogusa et al., 2016; Thapa et al., 2016) and, in NLRP3 expressing cells, inflammasome activation and pyroptosis (Kuriakose et al., 2016). How these normally hierarchical forms of PCD are coordinated after ZBP-1 activation is not yet understood (Maelfait et al., 2020). Nonetheless, ZBP-1-mediated PCD has been shown to be important to controlling murine

influenza A virus (IAV) infection. Moreover, human H1N1 pandemic IAV but not seasonal IAV has been demonstrated to suppress RIPK3-mediated necroptosis, indicating that inhibiting this cell death pathway also contributes to viral virulence in humans (Hartmann et al., 2017).

RNA Motifs Distinguishing Self from Non Self

Cytosolic long dsRNA is considered a hallmark molecular pattern of viral infection: it is generally absent from the cytosol of uninfected cells, yet it forms the genome of dsRNA viruses and is generated during the replication of ssRNA and DNA viruses (Son et al., 2015; Weber et al., 2006). However, recent research indicates that the absence of endogenous cytosolic dsRNA is not coincidental. Our genome contains a large number of complementarily inverted mobile elements, particularly *Alu* elements, which could potentially form long dsRNA (Reich and Bass, 2019), and their overwhelming absence in the cytosol results from both strong purifying transcriptomic selection (Barak et al., 2020) and A-to-I editing of the remaining transcripts by ADAR1 (Ahmad et al., 2018; Chung et al., 2018). In addition, mitochondria utilize an RNA helicase, SUV3, and polynucleotide phosphorylase (PNPase) to actively eliminate dsRNA species resulting from bidirectional transcription thus preventing innate immune activation (Dhir et al., 2018; Pajak et al., 2019). The importance of this sophisticated protective machinery becomes evident when it breaks down: hypomorphic variants of ADAR1 and hypermorphic variants of the dsRNA sensor MDA5 cause devastating forms of type-I interferonopathy (Rodero and Crow, 2016) resulting from the sensing of *Alu* elements (Ahmad et al., 2018; Chung et al., 2018).

However, the contribution of ADAR1 to antiviral defense is less clear. A-to-I conversion destroys the coding potential of viral RNA, thus inhibiting viral replication, yet it also destabilizes long dsRNA structures indicative of replicating pathogens (Samuel, 2011). The same reciprocal antagonism in dsRNA recognition occurs between the RNAi system and RNA sensing receptors. Dicer-mediated processing of dsRNA interferes with viral replication yet destroys the very structure which immune sensors recognize.

The critical importance of dsRNA as a molecular pattern is underscored by the convergent evolution of immune sensors and restriction factors it activates, making dsRNA a “promiscuous” ligand with diverse downstream effects (Hartmann, 2017) (see Box 2, poly I:C). Unfortunately, the precise requirements for the dsRNA length of many of these sensors remains unknown. To date, we are unaware of any publications detailing a minimal ligand for the 8 DDX and DHX proteins detailed in this review, ADAR1, LGP2, or ZBP-1. We hope future studies will characterize the precise nature of the dsRNA activating these sensors. Based on our knowledge to date, cytosolic dsRNA sensing via its ribose-phosphate backbone requires dsRNA > 30bp, including PKR, OAS, and MDA5 (> 300bp for polyI:C). Detection of dsRNA < 30bp requires amenable 5' termini (see below), which contain further information on the origin of dsRNA (5' phosphorylation, capping, cap structure, etc.). This cutoff strongly suggests the presence of physiological endogenous dsRNA < 30bp in our cytosol. Nonetheless, MDA5 ligands, while intensely researched, remain ill defined (Hartmann, 2017). The length requirement of > 300bp has been

determined for polyI:C (Kato et al., 2008) but remains unknown for viral and endogenous ligands (Schlee and Hartmann, 2016). Indeed, while most inverted *Alu* elements form dsRNA of approximately 300bp, they are only weakly stimulatory for non-AGS MDA5 variants when compared to polyI:C (Ahmad et al., 2018).

Endosomal dsRNA also represents an optimal recognition motif for viral infection. As it is more resistant to endosomal RNases than ssRNA (Ostendorf et al., 2020), dsRNA provides an important indication of viral infection in efferocytosed cells. Here, it is interesting to note that the dsRNA length required for TLR3 dimerization falls from > 90bp in the endosome to 39–48bp in late endolysosome (Leonard et al., 2008), at lower pH and after longer RNase exposure. Thus, endosomal sensing seemingly results from a combined calculus of length and RNase resistance. The precise length requirement at the cell surface is unclear, but presumably > 90bp. Of note, TLR3 can also sense dsRNA structures within long > 600 nt ssRNA of poliovirus (Tatematsu et al., 2013), which would be indicative of a likely bulge tolerance between the two parts of the TLR3 dimer. However, reports of TLR3 activation with shorter RNA, e.g., siRNA < 30bp, are limited to mouse TLR3 (Weber et al., 2012), and one study on the activation of TLR3 by mRNA is based on *in vitro* transcription (Karikó et al., 2004), which may have led to the generation of longer dsRNA as well as RIG-I ligands (see Box 3, *in-vitro* transcription). Nonetheless, certain endogenous RNA species, such as unedited *Alu* elements capable of activating MDA5, may also activate TLR3.

The 5' terminus contains a wealth of information about RNA origin. In higher eukaryotes, endogenous mRNA receives a m7GpppN cap, further modified by 2'O-methylations to m7GpppNm (cap 1) and m7GpppNmNm (cap 2). The capping process controls gene expression by modulating nuclear export, splicing, protein translation and mRNA turnover (Dimitrova et al., 2019). Of note, 2'O-methylation coevolved with the IFN system and is even used to specifically regulate ISG expression (Williams et al., 2020). In addition to the extensive modification of mRNA, small nuclear RNAs (snRNAs) receive methylguanosine caps which are hypermethylated into trimethylguanosine in the cytoplasm, and ribosomal RNA (rRNA) and transfer RNA (tRNA) are both generated via processing of precursors which creates 5' monophosphate (5'P) termini and are extensively 2'O-methylated. Thus, capping, N1 and N2 2'O-methylation of capped RNA, and the presence of 5'P termini can all be viewed as hallmarks of self.

The cytosolic dsRNA sensor RIG-I can be activated by dsRNA > 18-19 bp but critically requires that the 5' terminus is compatible with its C-terminal domain (CTD). Strikingly, the CTD binding cleft accommodates RNA bearing signs of “non self”: it binds 5' triphosphate (5'PPP) (Hornung et al., 2006; Schlee et al., 2009) and 5' diphosphate (5'PP) (Goubau et al., 2014) and, to a lesser extent 5'OH termini (Binder et al., 2011; Marques et al., 2006), but not 5'P (Ren et al., 2019). Moreover, while a cap0 structure reduces RIG-I activity, 2'O-methylation (cap1, cap2) abrogates it completely (Schuberth-Wagner et al., 2015). Detection of cap0 RNA allows RIG-I to sense viruses that code their own capping enzymes but do not have methyltransferase activity, such as Sindbis virus (Hefti et al., 1975). Moreover, the discrimination between 5'P and 5'OH for RIG-I is of particular importance for the dsRNA products of RNase ac-

tivity. The RNases processing tRNA and rRNA create 5'P RNA, as does Dicer. In contrast, RNaseL products have 5'OH ends and can thus activate RIG-I (Malathi et al., 2007). The RIG-I response to 5'PPP dsRNA is strongly reduced by 3'overhangs and abrogated by 5'overhangs at the 5'PPP end of the molecule (Schlee et al., 2009). Blunt dsRNA occurs naturally in the panhandle genome of (-) ssRNA viruses. However, rRNA, tRNA, and dicer products all contain 5' or 3' overhangs, a further preclusion to RIG-I activation. Sensing of the 5' terminus is also a strategy followed by members of the IFIT family in their sensing of ssRNA. IFIT1 sequesters ssRNA or dsRNA with a 5' overhang of > 5nt that have a 5'PPP or a cap0 but not a cap1 structure. IFIT1B sequesters 5'PPP, cap0 >> cap1 but not cap2, and IFIT5 senses 5'PPP ssRNA or dsRNA with an overhang of at least 3nt (Abbas et al., 2013).

In order to escape immunodetection, a number of viruses conceal the 5' termini of their RNA or disguise themselves with molecular markers of self. Some (-) ssRNA viruses, including hantavirus and bunyavirus, process their 5' termini to 5'P, avoiding detecting by RIG-I and the IFITs. Arenaviruses use prime-and-realign mechanisms to generate RNA with 5' overhangs, which allows for IFIT binding but may even act as a competitive inhibitor of RIG-I activity (Marq et al., 2011). Others code for their own 2'O-methyltransferases (Daffis et al., 2010), and some even perform “cap snatching,” removing the host 5' G7m cap along with 10–14nt and integrating it into their own mRNA. Given the exquisite sensitivity of 5' terminus sensing, it is hard to imagine the presence of many endogenous ligands capable of activating RIG-I. However, hypomorphic variants of the SKIV2L subunit of the RNA exosome drive type-I IFN mediated autoinflammation via RIG-I (Eckard et al., 2014), which can recognize RNA generated by inositol-requiring enzyme 1 (IRE-1) during the unfolded protein response. (See Figure 2). However, to date the identity and structure of these transcripts remain unknown.

Sequence-Specific RNA Sensing and Sensing of RNA Degradation Products

Another strategy to discriminate self from host is the sensing sequence-specific motifs. In the cytosol, IFIT2 senses AU-rich RNA (Yang et al., 2012), although the precise function of this sensing is unclear, as AU-rich elements also occur in our transcriptomes (Elliott and Ladomery, 2017). ZAP, which ferries RNA to the RNA exosome, binds CG-rich motifs in RNA (Takata et al., 2017), a strategy enabled by the CG-suppression of the vertebrate genome (Karlin and Mrázek, 1997) that may explain why some viruses actively avoid CG motifs (Schlee and Hartmann, 2016). In the endosome, TLR13 senses a genuinely sequence-specific motif: A(CGGAAGACC)CC within 23S rRNA. It is not clear why such a sequence-specific receptor evolved, but there are different possible explanations for its disappearance in higher mammals. Oldenburg et al. (2012) has demonstrated that a known methylation coded by an erythromycin-resistance gene leading to CGGmAAAGACC could abrogate TLR13 activity. This gene is of ancient origin in bacteria and could explain a loss of utility for TLR13. While Li and Chen (2012) demonstrate that this methylation has no effect, they find that this base within 23S rRNA is exceptionally sequence-specific, i.e., that ACGGBAAGACCCC (B = not A) could no longer activate TLR13. While it is remarkable that no further study has resolved this controversy, it seems clear

that TLR13 is an exceptionally mutation- and/or modification-sensitive receptor, which may account for its evolutionary loss.

Human TLR8 has been suggested as an evolutionary replacement of murine TLR13 (Krüger et al., 2015). While TLR8 is not active in mice, in humans, it is essential for recognition of bacterial RNA (Eigenbrod and Dalpke, 2015). However, this TLR8-TLR13 equivalency only applies to pathogens containing the TLR13 consensus sequence. TLR8 also senses plasmid RNA in humans, (Coch et al., 2019), which activates TLR7, not TLR13 in mice. Indeed, how TLR8 senses non-self RNA is an unresolved question. Two studies have made clear that TLR8 senses RNase degradation products rather than ssRNA (Greulich et al., 2019; Ostendorf et al., 2020). In line with previous reports, it is clear that the degraded ssRNA must contain uridine since free uridine is required for the first binding pocket (Tanji et al., 2015). One study has postulated that UURU is a minimal motif for TLR8 activation (Greulich et al., 2019), in line with its known stimulatory activity (Forsbach et al., 2008). However, under uridine supplementation, the di- or trinucleotide RNA for the second binding pocket of TLR8 does not require uridine for activity (Ostendorf et al., 2020; Shibata et al., 2016). Thus, the precise requirements for the second binding pocket and the true nature of any minimal TLR8 ligand remain unclear. Moreover, given the broad number of conceivable di- or trinucleotide ligands for the second binding pocket, it seems unlikely that such motifs are not contained in self RNA, or that they could all be rendered inactive by modifications such as pseudouridine or 2'-O-methylation (Freund et al., 2019). In addition, it is unclear which inhibitory modifications affect TLR8 activation and which inhibit upstream RNases. 2'-O-methylation is a known inhibitor of TLR8 (and TLR7) activity (Freund et al., 2019) but also of upstream RNaseT2 and RNase2 (Ostendorf et al., 2020). Conceivably, self RNA does not avoid immunorecognition in the classical sense but rather contains inhibitory motifs perhaps similar to those synthetically designed for TLR7 and TLR8 (Schmitt et al., 2017) using 2'-O-methylation, but further studies will be necessary to determine if these sequences have natural counterparts in our self RNA.

Endosomal Sensing of DNA

TLR9 was the first immune NA sensor identified (Hemmi et al., 2000) and is the only endolysosomal DNA receptor. Despite extensive transient trafficking, the signaling competent form of TLR9 is exclusively localized to the endolysosome, and mislocalization of TLR9 to the cell surface induces strong autoinflammation (Mouchess et al., 2011). The availability of TLR9 ligands in the endolysosome is tightly regulated by DNases (See [Nucleases and the Sensing of Self versus Non-self DNA](#)). TLR9 preferentially detects ssDNA containing unmethylated cytosine-phosphate-guanine (CpG) motifs which are less frequent in eukaryotic self DNA (methylation of the C5 carbon of cytosine) compared to bacterial DNA (Coch et al., 2009; Hartmann and Krieg, 2000; Krieg et al., 1995), with a recent study identifying highly conserved stimulatory CpG-DNA fragments in multiple 16S and 23S rDNA sequences in bacterial genomes (Liu et al., 2020). Moreover, a recent structural study reports that TLR9 has two binding sites, one binding ssDNA with an unmethylated CpG motif and the second binding short ssDNA carrying a 5' hydroxyl (Ohto et al., 2018). The second site prefers 5' hydroxyl

ssDNA with a cytosine at the second position from the 5' end (5'-xCx DNA). Combined CpG and 5'-xCx DNA binding cooperatively promotes TLR9 dimerization and activation. In line with this, previously established potent TLR9 ligands such as ODN2006 contain both structural elements, unmethylated CpG motifs and a cytosine at the second position from the 5' end (Hartmann et al., 2000).

Previous work on stimulatory TLR9 ligands reveal important species-specific differences. In recent years, the sequence specificity of DNA ligands for both humans and mice has been further investigated (Pohar et al., 2017a, 2017b). In these studies, the minimal DNA sequence for human TLR9 has been defined as TCGT6-10 CGT9-19 (21 to 35-mer), and the 5'-TCG was found to be essential for activation, consistent with the two binding sites identified by Ohto et al. (2018). Interestingly, mouse TLR9 has a much lower requirement for the 5'-xCx sequence than human TLR9 (Ishida et al., 2018; Pohar et al., 2017b). In fact, mouse TLR9 can be dimerized by CpG DNA alone due to TLR9-CpG DNA interactions unique to mice (Ishida et al., 2018). Not only ligand specificity but also the cellular expression pattern differs between murine and human TLR9. Whereas, in the mouse, TLR9 is widely expressed in immune cells, including myeloid cells, in humans, it is restricted to B cells and pDC (Barchet et al., 2008), and their downstream immune effects differ accordingly. Notably, TASL was recently identified as an additional signaling protein linking TLR9 to IRF5 (Heinz et al., 2020).

Cytosolic Sensing of DNA

The discovery that dsDNA induces type-I IFN came 40 years after dsRNA (Ishii et al., 2006; Stetson and Medzhitov, 2006). Moreover, the first pathway identified involved RNA but not DNA sensing (Ablasser et al., 2009; Chiu et al., 2009). Like poly(I:C), poly(dA:dT) was used as a standard DNA stimulus due to its strong IFN-I activation and its amenability to enzymatic synthesis. However, poly(dA:dT) provides a template for transcription of AU-rich RNA by the pol III pathway. These polyUA transcripts bear 5' triphosphate and fold into dsRNA, thus acting as RIG-I ligands and inducing type-I IFN independently of a true DNA sensor.

At the end of 2012, the cGAMP synthase (cGAS)-Stimulator of Interferon Genes (STING) pathway was identified as the principle cytosolic dsDNA sensor inducing type-I IFN (Sun et al., 2013; Wu et al., 2013). A number of other candidate receptors have previously been proposed and may have accessory and/or cell-type-specific functions (see [Figure 3](#) and [Box 4](#), cytosolic DNA and Type-I IFN). The carboxyl terminus of cGAS binds to the ribose-phosphate backbone of dsDNA in a sequence-independent manner, inducing dimerization and formation of the cyclic dinucleotide (CDN) c[G(2'-5')pA(3'-5')p] (2'3'-cGAMP). 2'3'-cGAMP then acts as a second messenger (Ablasser et al., 2013a; Gao et al., 2013a), activating STING, followed by TBK1 and IRF3 phosphorylation. The downstream sensor STING itself is also a cytosolic receptor of the bacterial CDN c[G(3'-5')pA(3'-5')p] (3'3'-cGAMP), c[G(3'-5')pG(3'-5')p] (c[di-GMP]) and c[A(3'-5')pA(3'-5')p] (c[di-AMP]) (Burdette et al., 2011; Gao et al., 2013b). Notably, murine STING was far more reactive to bacterial CDN than human STING (Ablasser et al., 2013a; Gao et al., 2013b), with hSTING^{H232} showing near-perfect selectivity for 2'3'-cGAMP (Gao et al., 2013b). Nevertheless, several natural

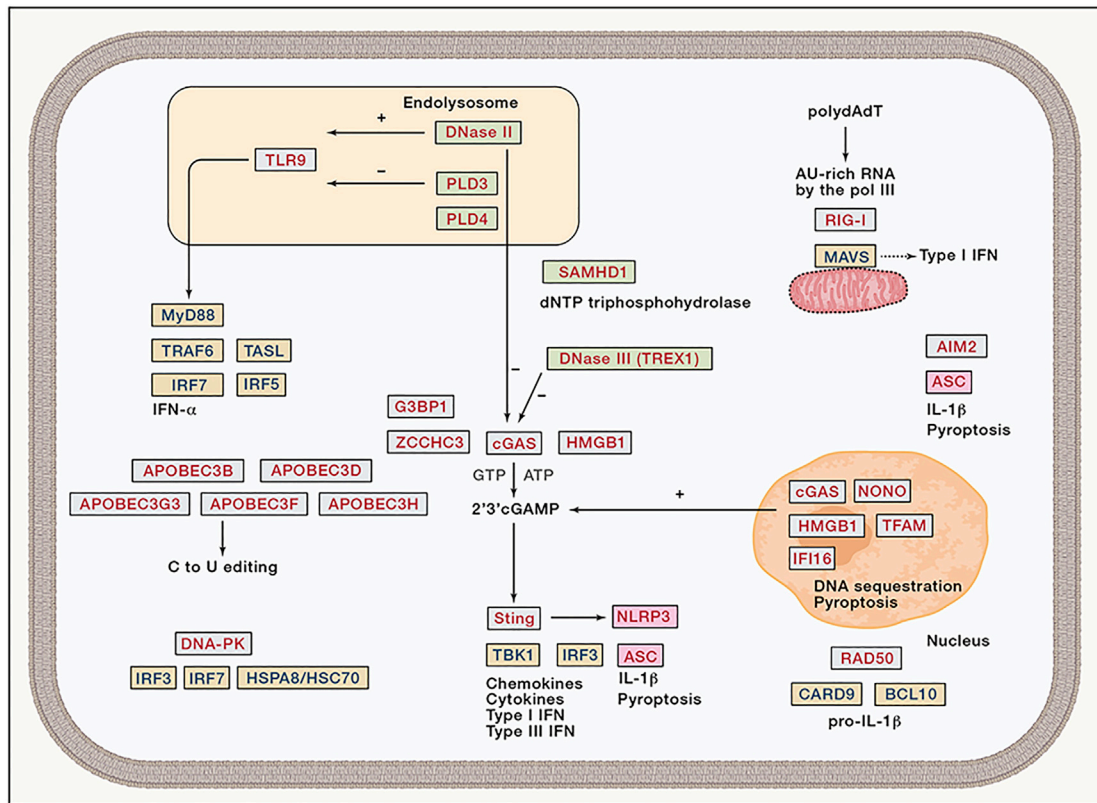


Figure 3. DNA Sensing and Response

Non-comprehensive overview of the most relevant DNA-sensing receptors at the relevant localization, their downstream signaling molecules, and the functional outcome and secondary consequences as applicable (e.g., STING sensing of cGAS product 2'3'-cGAMP). DNA-sensing receptors are in gray, signaling molecules in yellow, nucleases in green, and inflammasome pathways is purple. The signaling competent form of TLR9 is exclusively localized to the endolysosome and preferentially detects ssDNA containing unmethylated cytosine-phosphate-guanine (CpG) motifs which are less frequent in eukaryotic self DNA compared to bacterial DNA. TLR9 has two binding sites, one site binding ssDNA with an unmethylated CpG motif and the second site binding short ssDNA carrying a 5' hydroxyl, both cooperatively promoting TLR9 dimerization and signaling, including the recently identified signaling component TASL linking signaling to downstream IRF5. TLR9 ligands in the endolysosome are tightly regulated by endolysosomal DNase II, PLD3, and PLD4 which coordinately degrade single- and double-stranded DNA. Cytosolic DNA sensing includes the RNA sensor RIG-I which detects RNA transcribed from poly(dA:dT) by pol III. The cGAMP synthase (cGAS)/Stimulator of Interferon Genes (STING) pathway is the principle cytosolic dsDNA sensor pathway, activating a type-I IFN response, and via STING a NLRP3 dependent inflammasome response. Availability of cytosolic DNA for this pathway is regulated by DNase III (TREX1). Accessory proteins for cGAS are HMGB1, the GTPase-activating protein SH3 domain-binding protein 1 (G3BP1), TFAM, and CCHC-type zinc-finger protein 3 (ZCCHC3) which bind, bend, and stabilize dsDNA in a manner amenable to the nucleation of cGAS dimers along the dsDNA strand. cGAS is also localized in the nucleus where it senses foreign but not self DNA. Here, non-POU domain-containing octamer binding protein (NONO), but also HMGB1 and TFAM, assist in binding of DNA to cGAS. IFI16 is a nuclear restriction factor, binding, and silencing viral or transfected DNA. The DNA damage protein RAD50 upon stimulation with dsDNA induces proIL1β via the CARD9-BCL10 pathway. In the human system, a STING-independent DNA sensing pathway via the DNA damage response protein DNA-dependent protein kinase (DNA-PK) senses linear DNA, and signals via IRF3/IRF7 and HSPA8/HSC70. Absent in Melanoma (AIM2) is the principle cytosolic dsDNA sensor responsible for inflammasome activation. Several family members of the Apolipoprotein B editing complex (APOBEC) act as restriction factors by performing C-to-U editing on first(minus) strand cDNA, resulting in G-to-A mutations in the plus strand. The sterile alpha-motif (SAM) and histidine-aspartate (HD) domain-containing protein 1 (SAMHD1) is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase removing triphosphate moieties from dNTPs thereby reducing the intracellular dNTP concentration and inhibiting reverse transcription.

polymorphisms in human STING influence the sensitivity to 2'3'-cGAMP and 3'3'-CDN (Yi et al., 2013), and the most common STING variant, hSTING^{R232} shows a moderate selectivity for 2'3'-cGAMP (Gao et al., 2013b). Further studies will be needed to elucidate the relevance of human STING as a direct CDN sensor in host-pathogen interaction.

The role of the PYHIN proteins IFI16 and p204 in STING activation has been controversial (see Box 4, cytosolic DNA and Type-I IFN). Human IFI16 and its reported ortholog, murine p204, are coded by the AIM2-like receptor (ALR) locus, an evolutionary hotbed (see DNA-Induced Inflammasome Activation). An initial publication has demonstrated that RNAi targeting human

IFI16 and murine p204 reduces IFN-I induction from human THP-1 cells, murine embryonic fibroblasts, and RAW264.7 macrophages, respectively (Unterholzner et al., 2010). However, the importance of p204 has been challenged by subsequent studies (Brunette et al., 2012; Gray et al., 2016), causing some confusion in the field. Further studies using genome editing in human cells could provide clear evidence for the role of IFI16 in IFN-induction in keratinocytes and macrophages, both in supporting cGAS signaling and cGAS-independent DNA sensing (Almine et al., 2017; Jönsson et al., 2017). However, due to the genetic diversity of the ALR locus, it is possible that this role in DNA sensing is unique to primates.

Box 4. Cytosolic DNA and Type-I IFN

A large number of receptors have been implicated in the immune sensing of cytosolic DNA, including DHX9, DHX36, DDX41, DAI (ZBP-1), Mre11, DNA-PK/Ku70/Ku80, the ALRs PYHIN1, IFI16(p204), and the nucleotidyltransferase cGAS (Diner et al., 2015b; Unterholzner, 2013). The type-I IFN response to cytosolic DNA was described relatively late in the history of the field (Ishii et al., 2006; Stetson and Medzhitov, 2006). Here, the two principle ligands used were an annealed, synthetic, mixed-sequence 45-mer designed to avoid CpG motifs, known as interferon-stimulatory DNA (ISD) (Stetson and Medzhitov, 2006) and the dsDNA sequence poly(dA-dT):poly(dT-dA), known as poly(dA:dT) synthesized in an enzymatic reaction (Ishii et al., 2006). Soon after, it was established that stimulator of interferon genes (STING) was involved DNA-mediated activation of IRF3 (Ishikawa and Barber, 2008; Zhong et al., 2008), and all of the putative receptors, except for DHX9 and DHX36, were reported to act upstream of STING in some manner. While it is entirely possible that redundancies in function, species or cell-specific effects or other differences can account for the plethora of DNA sensors postulated, these somewhat contradictory studies still pose a conundrum for the field. Moreover, two groups also found that poly(dA:dT) also activated RIG-I after its transcription into RNA by RNase polymerase III (Ablasser et al., 2009; Chiu et al., 2009), opening the question whether other DNA ligands may be indirectly sensed by other pathways. With the discovery of cGAS (Sun et al., 2013; Wu et al., 2013), it became clear that cGAS-STING was the principle pathway of cytosolic DNA sensing in mammalian cells. However, the advent of CRISPR-Cas9 genome editing in 2013 has also provided important genetic proof of the function of putative human DNA receptors: human IFI16 has been shown to act as an important DNA sensor upstream of STING (Almine et al., 2017; Jonsson et al., 2017). However, one study removed the entire ALR locus in mice (AIM2 + 12 paralogs), without affecting the IFN response to cytosolic DNA (Gray et al., 2016), effectively dispelling any notion that an ALR is required for DNA-induced IFN in mice. Of note, a very recent publication has proposed a further cytosolic DNA sensing pathway which is active in human but not murine cells, DNA-PK (Burleigh et al., 2020). However, in contrast to previous reports in murine cells, this study demonstrates that human DNA-PK acts independently of STING. Altogether, there seem to be important differences in mammalian DNA signaling, and we hope that further studies will genetically investigate putative DNA sensing pathways in humans (and other non-murine species) and clear up the controversies remaining in the field.

In addition, a recent publication has identified a STING-independent DNA sensing pathway (SIDSP) in human but not murine cells via DNA-dependent protein kinase (DNA-PK) (Burleigh et al., 2020), a protein previously associated with STING-dependent responses (see Box 4, cytosolic DNA and Type-I IFN). Although DNA-PK is important for sensing DNA damage, SIDSP does not occur downstream DNA damage but requires the introduction of foreign DNA. In contrast to cGAS, DNA-PK requires linear ligands, suggesting that it senses dsDNA termini. Its signaling pathway requires IRF3 and/or IRF7 but not TBK1 and possibly the DNA-PK dependent phosphorylation of HSPA8 and HSC70. Of note, a much earlier study has observed that DNA-PK could directly phosphorylate IRF3 (Karpova et al., 2002). To date, it remains unclear whether the STING-dependent DNA-PK pathway in mice is related to SIDSP in humans or what would be the basis for a species-specific requirement for STING activity.

Another DNA damage protein that has been implicated in DNA sensing is RAD50. Upon stimulation with dsRNA, RAD50 has been reported to induce proIL1 β mRNA via the CARD9-BCL10 pathway, thus providing proIL1 β for concurrent DNA-induced inflammasome activation (see DNA-Induced Inflammasome Activation) (Roth et al., 2014).

Nuclear DNA Sensing

Cytosolic mislocalization of dsDNA was long thought to be required for its sensing (Schlee and Hartmann, 2016). However, many DNA viruses replicate in the nucleus (Schmid et al., 2014), and it is also the site of retroviral integration into genomic DNA (Volkman and Stetson, 2014). Thus, despite the wealth of nuclear self DNA, host defense in this compartment would be advanta-

geous. Several recent publications have demonstrated that cGAS also localizes to the nucleus where it senses foreign but not self DNA (Gentili et al., 2019; Volkman et al., 2019) as well as inhibiting homologous recombination and DNA repair (Jiang et al., 2019; Liu et al., 2018), processes requiring direct interaction with nuclear self DNA. One study has observed nuclear “tethering” of cGAS via its NTase domain preventing activation by self DNA, but how this tethering is regulated or relaxed to allow cGAS to perform its various nuclear functions, such as the sensing of HIV-2 (Lahaye et al., 2018), remains unclear.

IFI16 is also reported to participate in nuclear retroviral sensing as an inflammasome-forming protein (see next section). Furthermore, several reports provide evidence that IFI16 works as a nuclear restriction factor, efficiently binding and silencing viral or transfected DNA (Johnson et al., 2014; Orzalli et al., 2013). Likewise, another ALR PYHIN1(IFIX) is reported to restrict herpesvirus in the nucleus (Diner et al., 2015a). Future studies will be necessary to characterize how these and other DNA sensors act in the nuclear compartment.

DNA-Induced Inflammasome Activation

Three DNA-sensing inflammasomes have been described to date (see Figure 3): the Absent in Melanoma 2 (AIM2), IFI16 inflammasomes, and NLRP3 activation secondary to STING-mediated cell lysis. AIM2 and IFI16 are both ALRs (Brunette et al., 2012; Unterholzner et al., 2010), which can bind DNA via their HIN200 domain(s) and engage in homotypic pyrin-pyrim domain interaction with the inflammasome adaptor protein ASC. The ALR locus is genetically diverse: unique to mammals, it contains 1 gene in marsupials and cows, 5 in humans, 4 in rats, and 13 in mice, and is absent in monotremes and bats (Ahn et al.,

2016; Brunette et al., 2012; Cridland et al., 2012; Khare et al., 2014). AIM2 is the best conserved gene in this locus and the principle cytosolic dsDNA sensor for inflammasome formation in mice (Bürckstümmer et al., 2009; Fernandes-Alnemri et al., 2009; Hornung et al., 2009). However, AIM2 is a pseudogene in several mammalian species, including cows and dogs (Cridland et al., 2012). Moreover, in human monocytes and the monocytic model cell line, transdifferentiated BLaER1, DNA-induced inflammasome activation is dependent on NLRP3, not AIM2 (Gaidt et al., 2017, 2018), and direct STING activation can also activate NLRP3 in monocytes. Here, STING activation leads to a lytic form of cell death and K⁺ efflux in turn activating NLRP3. However, whether this form of STING-mediated cell death occurs in other cells is unknown. In cells connected by gap junctions, this seems implausible since bystander STING activation would theoretically induce a wave of cellular lysis and pyroptosis. Moreover, AIM2 acts as a DNA-sensing inflammasome protein in other human myeloid cell types, including THP-1 cells (Gaidt et al., 2017) and human macrophages (Su et al., 2018).

Human IFI16 forms a nuclear inflammasome in T cells, sensing HIV-1 cDNA and inducing pyroptosis (Monroe et al., 2014). IFI16 also senses Kaposi Sarcoma-Associated Herpesvirus (KSHV) in primary human endothelial cells (HMVEC) and the monocytic cell line THP-1 (Kerur et al., 2011; Singh et al., 2013). Since pyroptosis is the predominant form of PCD (95%) of quiescent CD4⁺ T cell during HIV-1 infection, IFI16-mediated pyroptosis may be of particular pathophysiological relevance for this disease (Doitsh et al., 2014). Although p204 has been reported to the murine ortholog IFI16, we are unaware of a report of p204 directly building an inflammasome, and the recent publication of the *Ifi204*^{-/-} mouse indicates a distinct role in LPS sensing (Yi et al., 2018).

Although it is conceivable that other ALRs in the human and murine locus containing HIN200 and pyrin domains could build inflammasomes, none have been reported to date. Indeed, we are only beginning to understand the function of other ALRs. The human ALR Pyrin-only protein 3 does not contain a HIN200 domain and is reported to act as an inflammasome inhibitor (Khare et al., 2014). Human PYHIN1 was reported to act as an IFN-I-inducing DNA sensor (see Box 4, cytosolic DNA and Type-I IFN) but has also recently been shown to control TNF and IL-6 release downstream of STING activation (Massa et al., 2020). Better understanding of the mammalian ALRs is not only important to our own antiviral defense but also that of livestock and important mammalian viral reservoirs, such as bats for paramyxoviruses and coronaviruses (Drexler et al., 2012; Li et al., 2005).

Antiviral Restriction Factors, Accessory Proteins, and DNases

Accessory proteins: cGAS activation requires oligomerization (Li et al., 2013b), even forming large protein foci through liquid-phase separation (Du and Chen, 2018), as previously described for P-bodies and stress granules (Shin and Brangwynne, 2017). Several accessory proteins have been reported that could contribute to lowering the activation energy for phase transition (see Figure 3). The DNA structural proteins, HMGB1 and TFAM, have been reported to bind, bend, and stabilize dsDNA facilitating the nucleation of cGAS dimers along the dsDNA strand (Andreeva et al., 2017). Other identified cofactors include

CCHC-type zinc-finger protein 3 (ZCCHC3) (Lian et al., 2018a), reported by the same group to act as an accessory protein to the RLRs (Lian et al., 2018b), and GTPase-activating protein SH3 domain-binding protein 1 (G3BP1) (Liu et al., 2019). For nuclear cGAS activation, the host protein non-POU domain-containing octamer binding protein (NONO) supports the binding of HIV-2 DNA to cGAS (Lahaye et al., 2018). Theoretically, these different cofactors could influence the activation state and multimeric structure of cGAS.

There are multiple reports of IFI16 acting as a viral restriction factor by suppressing viral promoters and interfering with replication (Lo Cigno et al., 2015; Gariano et al., 2012; Johnson et al., 2014). Nuclear IFI16 has been reported to bind and sequester unchromatinized DNA and promote its integration into heterochromatin, thus restricting HSV-1 infection (Johnson et al., 2014; Orzalli et al., 2013), with one recent publication showing that it forms filamentous structures with viral DNA, thus blocking the activity of HSV-1 RNA pol II (Merkl and Knipe, 2019).

The Apolipoprotein B editing complex (APOBEC) family was discovered via a C-to-U base modification in apolipoprotein B (apoB) mRNA leading to a premature stop codon (Arias et al., 2012). The seven proteins (A–G) of the rapidly evolving primate APOBEC3 (or A3) subfamily are ISGs countering retroviruses and endogenous retroelements (Ito et al., 2020). Several APOBEC3s act as restriction factors for HIV-1, performing C-to-U editing on first(minus) strand cDNA that result in plus-strand G-to-A mutations. APOBEC3 mutates preferentially TGG motifs, converting them to a stop codon (TAG) and is targeted by the HIV accessory-protein Vif (along with APOBEC3D, F, and H). APOBEC3 proteins are also active against other viruses, including parvovirus, HBV, HTLV, and RSV (Arias et al., 2012). However, the drawbacks of this editing strategy include evolution of more virulent viral strains and genomic mutagenesis, which for APOBEC3B has been linked to several forms of cancer (Burns et al., 2013).

The sterile alpha-motif (SAM) and histidine-aspartate (HD) domain-containing protein 1 (SAMHD1) is a deoxynucleoside triphosphate (dNTP) triphosphohydrolase that removes triphosphosphate moieties from dNTPs (Goldstone et al., 2011). SAMHD1 was initially identified as a myeloid-cell specific HIV-1 restriction factor and target of the lentivirus auxiliary protein Vpx (Laguette et al., 2011). SAMHD1 decreases the concentration of intracellular dNTPs in myeloid cells to inhibit reverse transcription (Goldstone et al., 2011), and also inhibits the replication of DNA viruses, such as HSV-1 (Hollenbaugh et al., 2013). Hypomorphic variants of SAMHD1 induce type-I interferonopathy in patients and mice, which is dependent on the cGAS-STING pathway (Maelfait et al., 2016).

Similarly, hypomorphic variants of the principal cytosolic nuclease in mammalian cells, DNase three-prime repair exonuclease 1 (TREX1 or DNase III), are linked to type-I interferonopathy as well as familial chilblain lupus (FCL) and systemic lupus erythematosus (SLE) (Rodero and Crow, 2016). TREX1 degrades ss- and dsDNA (Grieves et al., 2015), and TREX1-deficiency leads to cytosolic accumulation of DNA ligands (Stetson et al., 2008) and activation of the cGAS-STING pathway (Ablasser et al., 2014; Gehrke et al., 2013). *Trex1*^{-/-} mice develop systemic inflammation (Morita et al., 2004) ameliorated by simultaneous

deficiency in STING or cGAS (Gray et al., 2015). Moreover, modifications such as UV-light exposure render DNA TREX1-resistant and potentiate cGAS activation (Gehrke et al., 2013).

DNase II is the predominant endolysosomal endonuclease and also degrades ss- and dsDNA (Drew, 1984). DNase II deficiency leads to dsDNA accumulation which enters the cytoplasm, causing overwhelming inflammation (Kawane et al., 2006; Yoshida et al., 2005). Patients with expressing hypomorphic DNase II present also present with type-I interferonopathy (Rodero et al., 2017), and DNase II deficiency is embryonically lethal but can be rescued by IFNAR deficiency (Kawane et al., 2006). Of note, cGAS-STING and AIM2 both contribute to the phenotype of DNase II deficiency (Ahn et al., 2012; Baum et al., 2017; Jakobs et al., 2015).

Recently, two further endosomal nucleases have been discovered, Phospholipase D3 (PLD3) and D4 PLD4. Unlike DNase II, PLD3 and PLD4 are exonucleases which degrade ssDNA from the 5' terminus (Gavin et al., 2018). This discovery has its origins in genome-wide association studies linking PLD3 to Alzheimer disease and PLD4 to rheumatoid arthritis and systemic sclerosis. PLD4-deficient mice demonstrate elevations in IFN- γ and splenomegaly. PLD3 and PLD4 activity requires a 5'OH terminus and the absence of secondary structure, both features of DNase II cleavage products, enabling it to act cooperatively with DNase II (see next section).

Nucleases and the Sensing of Self Versus Non-self DNA

Initially, sensing of dsDNA was thought to rely entirely on subcellular localization (see Figures 1 and 3). While DNA does not undergo the same exquisite processing as RNA, nuclear DNA sensing clearly depends on hallmarks of self and non self, and DNA modifications can strongly affect the availability of cytosolic and endosomal DNA.

DNA sensing is tightly controlled by its availability. Cytosolic dsDNA must contend with the high-affinity 3'-exonuclease TREX1. Similarly, activation of TLR9 is antagonized by the 5'-exonuclease activity of PLD3/4 in the endosome. Exonuclease activity is an elegant strategy for controlled sensor activity: (1) the NA ligand is degraded base by base, allowing for a gradual removal of ligands, (2) whereas inhibiting endonucleases requires extensive modification to a substrate, exonucleases can be blocked by a simple modification on the 5' or 3' terminus, which can, in turn, be removed by an endonuclease. Cytosolic dsDNA becomes potently stimulatory with modifications that inhibit TREX degradation, e.g., 8'OH guanosine after UV irradiation (Gehrke et al., 2013). Endosomal PLD3 and 4 only degrades ssDNA and is inhibited by a 5'P-terminus (Gavin et al., 2018). DNase I, TREX1, and caspase-activated DNase perform 5'P-3'OH cleavage of phosphodiester bonds. Thus, incoming endosomal DNA will be predominantly (1) double-stranded and (2) have a 5'P terminus, rendering it inaccessible to PLD3 and 4. In contrast, DNase II is a 5'OH-3'P endonuclease with activity on ss- and dsDNA and is thus critically required to render DNA accessible to PLD3 and PLD4. Furthermore, phosphorothioate (PTO) modification also inhibits PLD3 and 4 activity 20- to 50-fold. PTO-linkage has been employed for decades to potentiate stimulatory oligonucleotides and also occurs naturally in bacterial DNA, rendering it a better stimulus for TLR9. However, single PTO modifications do not inhibit the endonuclease DNase II. In parallel to the role of

RNase T2 and RNase 2 for TLR8, DNase II does not inhibit TLR9 activity. Rather, it is critically required for the generation of TLR9 ligands from complex natural ligands (Chan et al., 2015; Pawaria et al., 2015) by releasing ssDNA from genomic dsDNA and generating 5'OH termini for the second binding pocket (Ohto et al., 2018).

As with dsRNA, length is a key determinant of whether dsDNA is stimulatory. TREX1 is non-processive, so longer dsDNA will have a longer half-life in the cytosol (Höss et al., 1999). Longer dsDNA is more easily bent into structures amenable to dimer formation and cGAS-dependent phase transformation (Andreeva et al., 2017; Du and Chen, 2018; Hooy and Sohn, 2018). For IFI16 and AIM2, longer naked dsDNA stretches are more amenable to the oligomerization of adjacent pyrin domains, a process which supports HIN200-dsDNA binding and stability (Morrone et al., 2015, 2014), provides a platform for the pyrin-pyrin interaction with ASC during inflammasome activity (Matyszewski et al., 2018), and effectively sequesters and neutralizes long dsDNA molecules. The influence of dsDNA length on receptor oligomerization, signal strength, and duration may account for the distinct signaling outcomes, e.g., restriction, cytokine induction, cell death, observed with different stimuli and cell types. For humans, cGAS require dsDNA of at least 45bp and AIM2 requires 80bp, whereas murine cGAS sensing can occur with shorter sequences (≥ 25 bp) (Fernandes-Alnemri et al., 2009; Jin et al., 2012; Luecke et al., 2017). At the structural level, 39bp was shown to be the minimal length for the assembly of two human cGAS dimers (Andreeva et al., 2017). Whereas IFI16 is reported to require at least 60–70bp for IFN activation (Unterholzner et al., 2010), we are not aware of a study determining the minimal length necessary for inflammasome activation and other IFI16 effector functions.

Another important aspect of dsDNA sensing is packaging. An essential distinction between self and non self in the nucleus is the tight winding of chromosomal DNA (Gentili et al., 2019; Volkman et al., 2019), which allows cGAS binding but not activation. This seems difficult to reconcile *a priori* with the activation of cytosolic cGAS by micronuclei (Harding et al., 2017; Mackenzie et al., 2017). Possibly, micronuclei contain unwound regions or other features amenable to cGAS sensing, or cGAS activation has different requirements in the cytosol. Understanding this process is of great importance to studies of cGAS-mediated cellular senescence.

A notable exception to the length requirement is cGAS activation by G-rich Y-form DNA, base-paired DNA ≥ 12 bp containing an unpaired G in adjacent ssDNA (Herzner et al., 2015). This mechanism could explain various forms of ssDNA sensing attributed to cGAS, including secondary-structured ssDNA originating from retroviral genomes (Coquel et al., 2018; Herzner et al., 2015). Although direct sensing of Y-DNA was demonstrated in a cell-free system (Herzner et al., 2015), an accessory protein may contribute *in cellulo* as has been observed for many other ligands. Regardless, Y-DNA-mediated cGAS activation provides evidence of 5'- and 3'-terminal sensing (here, of guanines) of cytosolic dsDNA. Another recent example is the STING-independent DNA-PK pathway, which fitting its function as a sensor of DNA damage and due to its requirement for linear dsDNA, seems interacts with dsDNA termini, although the precise structural features required remain unknown (Burleigh et al., 2020).

STING can be activated cell intrinsically and in bystander cells by endogenous 2'3'cGAMP (Ablasser et al., 2013b; Gao et al., 2013a). 2'3'cGAMP is targeted by hydrolysing poxins expressed by members of the *Poxviridae*, such as vaccinia virus (Eaglesham et al., 2019). Clearly, inhibiting cGAS-STING signaling is of advantage to DNA viruses. In contrast, certain human STING alleles have evolved to tolerate bacterial CDN while retaining sensitivity for endogenous cGAMP. This intentional desensitization to non self is highly unusual and thus likely indicative of a strong evolutionary advantage for not avoiding IFN induction in response to certain bacteria. Here, it should be noted that the role of IFN in bacterial defense remains rather unclear and differs starkly between bacterial species (Boxx and Cheng, 2016).

There is abundant self ss- and dsDNA in the endolysosome of phagocytes, requiring a strategy beyond sensing of the ribose-phosphate backbone. To avoid unnecessary activation: (1) TLR9 signaling is strictly contained within the endolysosomal compartment via an intricate system of trafficking and proteolytic activation, (2) TLR9 activation is regulated by the activity of endosomal DNases (see previous section), and (3) TLR9 senses unmethylated CpG dinucleotides within ssRNA. CpG motifs are suppressed in eukaryotic DNA (Krieg, 2002), since their methylation can drive their hydrolytic deamination (Shen et al., 1994). However, unmethylated CpG-rich DNA can be found in bacteria, viruses, and fungal pathogens.

Concluding Remarks

The tremendous progress outlined above suggests a tight functional integration of innate immune responses and cell-autonomous defense mechanisms in response to exogenous NA. In this context, NA-related research to date has followed three, almost completely separate, principle research directions: immune sensing of NA, antiviral restriction factors, and NA metabolism. We now understand that those three principles are all integral parts of a tightly controlled NA defense system. Impairment or failure of this system caused by dysregulation, genetic alterations, or pathogen challenge causes erroneous detection of self NA resulting in autoinflammation. Therefore, an improved understanding of the intricate mechanisms that govern the distinction of endogenous and exogenous NA as summarized in this review will guide better diagnostic procedures and treatments for inflammatory and infectious diseases. In particular, this insight allows for targeted activation of functionally distinct NA-sensing pathways in order to elicit immune response pathways that have not been previously accessible for treatment.

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DECLARATION OF INTERESTS

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