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Review

PRRs are watching you: Localization of innate sensing and signaling regulators

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ABSTRACT

To prevent the spread of infection, an invading pathogen must first be recognized by the innate immune system. Host pattern recognition receptors detect distinct pathogen-associated molecules and induce the transcription and release of interferon and inflammatory molecules to resolve infection. Unlike infections with pathogens that replicate autonomously from the host, viral infections blur the boundaries of self and non-self. Differentiation of host from virus is achieved by restricting localization of host nucleic acids and by placing pattern recognition receptors in specific subcellular compartments. Within this review, we discuss how several families of pattern recognition receptors act to provide a comprehensive surveillance network that has the potential to induce interferon expression in response to any viral infection.

Contents

Introduction

The production of inflammatory cytokines and interferons (IFNs) is triggered after detection of potentially infectious pathogens and is central to the innate immune response. Type-I IFNs signal in an autocrine and paracrine fashion for the production of hundreds of IFN stimulated genes (ISGs), many of which are still uncharacterized (De Veer et al., 2001; Schoggins et al., 2011). These ISGs play an important role in restricting viral replication and stopping the spread of infection to other cells. Thus, detection of a virus is a vital first step towards immunity.

The innate immune system relies on its ability to recognize general pathogen associated molecular patterns (PAMPs). PAMPs are pathogen-derived molecules unique from the host and difficult to mutate or sequester from detection without a fitness cost (Medzhitov, 2009). These PAMPs are recognized by a limited number of germline encoded pattern recognition receptors (PRRs). The PAMPs detected are produced by a wide variety of pathogens, thus allowing a single PRR to report on many infectious encounters. In the case of viral pathogens, the innate immune system often detects the most intrinsic part of a virus, its nucleic acid

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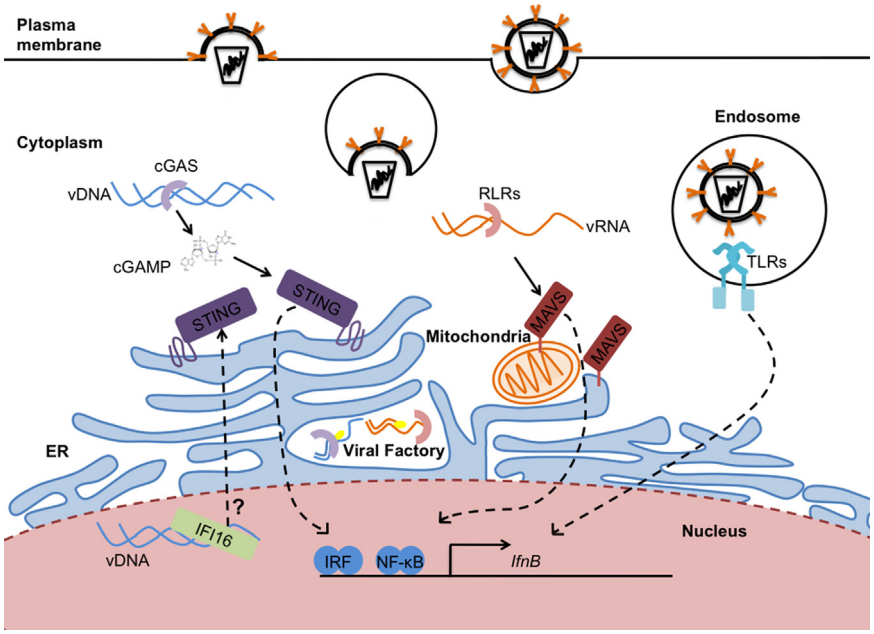

Fig. 1. Multiple organelles initiate signal transduction that lead to type I IFN expression. Viruses can enter most cells through the endocytic pathway or by crossing the plasma membrane. TLRs detect viral genomes in endosomes. Once in the cytoplasm, genomic viral RNA (vRNA) can be detected by the RLRs and activate type-I IFN through MAVS, located on mitochondria and ER membranes. Genomic viral DNA (vDNA) is detected in the cytoplasm by cGAS or in the nucleus by IFI16. Both sensors activate STING to induce type-I IFNs. cGAS and RLRs can also detect viral replication intermediates in viral factories. All pathways activate the IRF and NF-kB transcription factors to stimulate *IFNB* transcription.

genome. Viral genomes exist as RNA or DNA, are single or double-stranded, and can be partitioned into one or more segments.

Nucleic acids are not unique to viruses since they are essential to all cellular life. To differentiate between host and viral nucleic acids, PRRs use two criteria to detect viral nucleic acids specifically. The first criteria is based on the intrinsic ability of PRRs to recognize unusual biochemical features present in viral, but not host, nucleic acids. For example, the PRR retinoic acid-inducible gene 1 (RIG-I) detects RNAs that are tri- (or di-) phosphorylated, and lack a 7-methylguanosine cap (Hornung et al., 2006; Pichlmair et al., 2006; Rehwinkel et al., 2010; Goubau et al., 2014). Host mRNAs are also tri-phosphorylated, but the presence of the cap minimizes recognition by RIG-I. The second criteria that PRRs use to detect viral nucleic acids specifically is based on the ability of the host to restrict its own nucleic acids to specific locations within the cell. For example, host DNA should not be found in the cytoplasm of uninfected cells, and the PRR cyclic guanosine monophosphate-adenosine monophosphate (cGAMP) synthase (cGAS) detects cytoplasmic DNA, which is presumably of viral origin (Sun et al., 2013; Wu et al., 2013).

In this review, we expand on the ideas described above as we describe the PRRs of the innate immune system that function to produce type-I IFNs. We focus on the three major IFN-inducing families of PRRs in mammals, the Toll-like Receptors (TLRs), RIG-I-like Receptors (RLRs) and cGAS (Fig. 1). We highlight how differences in the subcellular localization of host and viral nucleic acids, and the PRRs that detect them, can regulate the ability of the host to activate an IFN response during a viral infection.

Toll-like Receptors

TLRs are PRRs that detect PAMPs derived from various microbes and initiate the transcription of inflammatory cytokines and type-I IFNs. These receptors are transmembrane proteins that contain an extracellular/lumenal domain that consists of a series of leucine rich repeats (LRRs). The LRR-containing domain detects PAMPs either directly or through interactions with accessory proteins that have high affinity PAMP-binding activities. Several TLRs recognize nucleic acids and have an important role in antiviral defense. For example, TLR3 recognizes viral double stranded RNA (Alexopoulou et al., 2001), TLR7 and TLR8 recognize viral single stranded RNA (Diebold et al., 2004; Heil et al., 2004), and TLR9 recognizes DNA containing unmethylated CpG motifs present in numerous viral and non-viral pathogens (Lund et al., 2003; Redecke et al., 2001; Kaisho et al., 2000). In addition, recent work identified murine TLR13 as a nucleic acid sensor, but the ligand is a specific sequence present in bacterial ribosomal RNA (Oldenburg et al., 2012). Thus, while most nucleic acid sensing TLRs have an important role in detecting viruses, at least one (TLR13) has a specific anti-bacterial function.

The nucleic acid sensing TLRs are localized to endolysosomes. During an infection, viral particles are endocytosed. The acidification of endosomes allows for some viruses to uncoat and gain entry into the cytoplasm. However, not all viral particles are successful in the process. In the acidic environment of the lysosome, viral particles are degraded and the genomic nucleic acids are released, allowing for detection by TLRs. Thus, the nucleic acid sensing TLRs are localized to compartments where viral genomes can be exposed.

Binding to nucleic acids promotes interactions between two TLRs, which consequently dimerize the Toll/Interleukin-1 Receptor (TIR) domains present in the cytoplasmic tails of the receptors (Latz et al., 2007). The dimerized TIRs serve as a platform to recruit cytosolic adapter proteins that also contain TIR domains. Two distinct sets of adapters are thought to be recruited to dimerized TLRs. The most common set of adapters, utilized by all TLRs except TLR3, is that of TIRAP and MyD88. The recruitment of these adapters initiates the formation of a large helical oligomer called the myddosome, which is functionally considered a supramolecular organizing center (SMOC) (Kagan et al., 2014). This SMOC mediates kinase- and ubiquitin ligase-dependent events that activate inflammatory transcription factors such as NF-κB and

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IRF7. NF-κB and IRF7 then translocate into the nucleus to promote the expression of IFNs and other inflammatory mediators. The second set of adapters that control TLR signaling consists of TRAM and TRIF, but these adapters are utilized by a small set of TLRs. Whether TRAM and TRIF assemble a SMOC similar to the myddosome that activates inflammatory transcription factors is unclear, although the use of SMOCs as signaling platforms in multiple PRR pathways suggests that this may be the case (Kagan et al., 2014).

In order to minimize detection of host-encoded nucleic acids, the nucleic acid-sensing TLRs are restricted to endolysosomal compartments by a process mediated by UNC93B1. UNC93B1 is a multipass membrane protein localized to the endoplasmic reticulum (ER) that was identified because the H412R mutation in one of its transmembrane domains abrogates IFN expression elicited by TLR3, 7, and 9 agonists (Tabeta et al., 2006). This protein was originally proposed to not have a function in TLR transport throughout the cell (Tabeta et al., 2006). However, subsequent work demonstrated that bone marrow derived dendritic cells with the H412R mutation in UNC93B1 were defective in trafficking TLR9 to endolysosomes (Kim et al., 2008). UNC93B1 and nucleic acid sensing TLRs bind to each other in the ER, and the H412R mutation ablates those interactions (Brinkmann et al., 2007). UNC93B1 loads the nucleic acid sensing TLRs into COPII vesicles in order to bud from the ER and travel through the Golgi complex, and TLR9 additionally requires the adapter protein-2 complex in order to traffic to endolysosomes (Lee et al., 2013).

In addition to being regulated by UNC93B1 trafficking, TLR3, 7, 8, and 9 need to be cleaved by various proteases such as cathepsins, asparagine endopeptidase, and furin-like proprotein convertases (Ewald et al., 2008, 2011; Park et al., 2008; Sepulveda et al., 2009; Garcia-Cattaneo et al., 2012; Maschalidi et al., 2012; Hipp et al., 2013). The proteolysis occurs as the TLRs are transported through acidic endosomal compartments where these enzymes are active. Although nucleic acids are capable of binding to the unprocessed TLRs, downstream signaling cannot occur until after proteolysis (Ewald et al., 2008). This proteolytic regulation is particularly important to prevent autoimmunity. In the case of systemic lupus erythromatitis, host nucleic acids are immunostimulatory (Leadbetter et al., 2002). Having nucleic acid sensing TLRs mislocalized to the plasma membrane increases the chances of an autoimmune reaction (Barton et al., 2006). By designing TLRs to remain incapable of being activated until they reach their proper destination, the risk of autoimmunity is reduced.

Once in endosomes, nucleic acid sensing TLRs do not remain static. TLR9 can traffic to phagosomes that have been initiated by FcγR signaling irrespective of DNA content (Henault et al., 2012). Upon interaction with CpG DNA, TLR9 initiates a signaling pathway that activates NF-κB-dependent cytokine and IFN expression (Henault et al., 2012). Recent work has indicated that TLR9 activates cytokine expression and IFN expression from distinct endosomal populations, with the latter requiring non-canonical autophagy components to traffic to a distinct vesicle (Henault et al., 2012). Non-canonical autophagy does not form a double membrane. Instead, the autophagy regulator LC3 binds TLR9-containing phagosomes and somehow induces transport to distinct organelles where the IFN-inducing transcription factor IRF7 can become activated (Henault et al., 2012). This requirement for moving TLR9 to induce IFNs is not unique to phagocytosed cargo. Cargo that has been endocytosed instead of phagocytosed also requires movement of TLR9 to induce type-I IFNs (Sasai et al., 2010). However, in the case of endosomes, the movement of TLR9 depends upon activator protein-3 (AP-3) which traffics TLR9 to lysosomal related organelles (LROs) where IRF7 can be activated for IFN induction. Disruption of TLR9 movement in either of these two trafficking pathways by eliminating LC3 or AP-3 results in blocking IFN induction without blocking the production of inflammatory cytokines (Sasai et al., 2010). Although the work published primarily focused on TLR9, some work with TLR3 and 7 ligands in the endocytic pathway suggest that they may be regulated similarly (Sasai et al., 2010).

TLR3, 7, 8 and 9 all induce type-I IFNs in plasmacytoid dendritic cells where they are expressed, but the signaling molecules downstream differ between TLR3 and the others. TLR7, 8 and 9 utilize MyD88, and were recently found to also depend on TIRAP for their signaling functions (Bonham et al., 2014). The requirement of TIRAP for signaling from TLR7 and TLR9 was originally missed because experimental conditions utilizing synthetic nucleic acids rather than viruses masked the requirement for TIRAP (Yamamoto et al., 2002; Horng et al., 2002). TIRAP has a lipid-binding domain that interacts promiscuously with acidic phosphoinositides, allowing it to survey the plasma and endosomal membranes for TLRs bound to ligand. TLR activation causes TIRAP to oligomerize with MyD88 and IRAK kinases, thus creating a SMOC called the myddosome that promotes the expression of inflammatory cytokines and IFNs. In contrast, TLR3 engages only the cytosolic adapter TRIF to induce cytokine and IFN expression (Fitzgerald et al., 2003; Yamamoto et al., 2003). It is unclear why TLR3 evolved to utilize TRIF instead of MyD88 to induce IFNs. One possibility is that TLR3 resides in an endosomal population that differs from TLR7 and TLR9, and thus may have access to different sets of adapter proteins. In support of this idea, TLR3, TLR7, and TLR9 traffic to endosomes differently. TLR7 proceeds directly from the trans-Golgi network to endosomes, whereas TLR3 is delivered to the plasma membrane before reaching endosomes (Lee et al., 2013; Pohar et al., 2013). However, TLR9, which signals via MyD88, can also traffic to the plasma membrane before its proteolytic processing in endosomes (Lee et al., 2013). Additional studies that identify the precise endosomal populations inhabited by nucleic acid sensing TLRs would greatly benefit this discussion.

In addition to the nucleic acid sensing TLRs, two TLRs localized to the plasma membrane sense viral pathogens. Although TLR2 and TLR4 are better known to recognize bacterial lipoproteins and lipopolysaccharides, respectively, these PRRs are also activated by viral ligands. TLR4 can initiate TRIF-dependent signaling after binding the glycoprotein of vesicular stomatitis virus (Georgel et al., 2007). In a subset of inflammatory monocytes, TLR2 is activated by mouse cytomegalovirus and vaccinia virus (Barbalat et al., 2009). TLR2 is activated by additional viruses in other cell types (Hoffmann et al., 2009; Michaud et al., 2010). Although these PRRs are located at the plasma membrane, IFN-inducing signal transduction occurs from endosomal compartments (Barbalat et al., 2009; Dietrich et al., 2010). Whether TLR2 and TLR4 recognize common molecular motifs among the viruses is currently unknown.

RIG-I-like Receptors

TLRs are limited in their ability to detect viruses because they only survey the extracellular space and are expressed in a limited number of cell types. In contrast, the RLRs, RIG-I and melanoma differentiation-associated protein 5 (MDA5), are expressed in almost all cells. RIG-I and MDA5 are cytoplasmic RNA helicases that recognize non-self RNA motifs. RIG-I binds 5′ tri- and diphosphates present on short double stranded (ds) RNA whereas MDA5 binds long dsRNA molecules (Hornung et al., 2006; Pichlmair et al., 2006; Goubau et al., 2014; Kato et al., 2008, 2006; Gitlin et al., 2006; Wang et al., 2010; Lu et al., 2010; Luo et al., 2011). RIG-I and MDA5 activate IRF3 and NF-κB through the adapter, mitochondrial antiviral signaling protein (MAVS). RIG-I and MDA5 form filaments upon RNA binding which oligomerizes the RLR caspase recruitment domain (CARD), and enhances

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interactions with the MAVS CARD (Peisley et al., 2013, 2011; Wu et al., 2013). The interaction between RLRs and MAVS CARDs leads to the formation of a SMOC with prion-like properties on the surface of mitochondria. The MAVS SMOC then functions to activate transcription factors that induce type-I IFNs (Hou et al., 2011).

RNA viruses enter the cytoplasm after breaching plasma or endosomal membranes. Unlike many DNA viruses, RNA viruses must rely on their own enzymes for genome replication. Replication intermediates formed by these enzymes contain uncapped RNA or have dsRNA character and can be recognized by RLRs. In order to catalyze RNA replication, RNA viruses may set up organelle-like replication complexes in the cytoplasm. These viral "factories" are composed of cellular membranes, usually ER-derived, but examples can be found for viruses utilizing the Golgi and lysosomal compartment as well (Den Boon et al., 2004). Mitochondria are also recruited to sites of RNA virus replication presumably to take advantage of mitochondrial ATP production during RNA replication (Novoa et al., 2005).

Intriguingly, MAVS is situated at the same membranes that many RNA viruses exploit for their replication, namely the mitochondria, peroxisomes and mitochondrial-associated ER membranes (MAM), which are tethered to mitochondria and peroxisomes by the protein mitofusin 2 (MFN2) (De Brito and Scorrano 2008). MAVS on the mitochondria and peroxisomes induce different types of IFN. Mitochondrial MAVS induces both type-I and type-III, whereas peroxisomal MAVS induces type-III IFN (Odendall et al., 2014; Dixit et al., 2010). It has been proposed that the MAM coordinates MAVS-dependent responses from mitochondria and peroxisomes (Horner et al., 2011). Viral antagonists that target MAVS can lead to a profound loss of signaling while only inactivating a small pool of MAVS in a cell (Horner et al., 2011; Meylan et al., 2005; Li et al., 2005). These observations suggest that the distribution of MAVS on multiple organelles is not redundant, but is rather a functionally important aspect of the RLR network that promotes antiviral immunity (Horner et al., 2011; Li et al., 2005).

STING pathway

While RLRs and MAVS produce IFN in response to viral RNA, a parallel pathway detects intracellular viral DNA. Stimulator of IFN genes (STING) is an ER-localized protein that coordinates the type-I IFN response to viral DNA (Ishikawa et al., 2009; Ishikawa and Barber 2008). While many DNA sensors have been proposed to activate STING (Goubau et al., 2013), only cGAS has a clear mechanism of STING activation. cGAS is a cytoplasmic protein that recognizes B-form DNA. Upon binding to these ligands, cGAS synthesizes the cyclic dinucleotide cGAMP, which binds and activates STING to promote IFN gene expression (Sun et al., 2013; Wu et al., 2013). Intermediates of the retroviral replication cycle, namely RNA:DNA hybrids, have also been proposed to activate cGAS. In vitro, cGAS can synthesize cGAMP in response to synthetic RNA:DNA ligands (Jakobsen et al., 2013; Mankan et al., 2014; Rigby et al., 2014), but it is unclear if cGAS-dependent signaling can occur and if cGAS is activated in response to natural RNA:DNA ligands. Some bacteria also synthesize cyclic dinucleotides through the actions of a prokaryotic cGAS-like enzyme. These bacterial dinucleotides can activate STING (Burdette et al., 2011). While both cGAMP and bacterial dinucleotides bind STING and activate type-I IFN, cGAMP binds STING at a higher affinity due to its noncanonical 2′-5′ phosphodiester linkage (Diner et al., 2013; Ablasser et al., 2013; Zhang et al., 2013; Gao et al., 2013).

In order to induce IFNs, STING must traffic from the ER to vesicles (Saitoh et al., 2009; Konno et al., 2013). This movement appears to be orchestrated by proteins involved in the autophagy pathway (Saitoh et al., 2009; Konno et al., 2013). Upon binding dinucleotides, STING moves from the ER to the Golgi via a mechanism dependent on VPS34, a Class III PI3K that regulates many pathways including endocytic trafficking (Konno et al., 2013; Jaber et al., 2012). STING then leaves the Golgi and associates with TBK1 on vesicles to activate IRF3 and NF-κB (Ishikawa and Barber 2008). The specific identity of these IFN-inducing vesicles remains undefined. After ATG9 depletion, STING and TBK-1 still traffic to vesicles, but more type-I IFN is induced than wild-type cells, implying that ATG9 or autophagy negatively regulates STING activation of TBK-1 (Saitoh et al., 2009). ULK1, another kinase involved in autophagy, phosphorylates human and mouse STING at serine 366 and 365 respectively to negatively regulate cGAMP-induced STING signaling (Konno et al., 2013). Knockdown of ULK1 leads to loss of phosphorylation of serine 366/365, which ultimately prevents STING degradation and sustains IFN expression (Konno et al., 2013). STING is likely degraded by autophagy in the autolysosome because after activation, STING associates with LC3 and p62/SQSTM1, proteins that target cargo for autophagy (Saitoh et al., 2009). In addition, treatment of cells with chloroquine, which inhibits acidification of endolysosomes, also prevents STING degradation (Konno et al., 2013).

STING is not the only pathway member to be regulated by autophagy. When cGAS is bound to DNA in the cytoplasm, Beclin-1 binds cGAS and inhibits its enzymatic activity (Liang et al., 2014). Upon cGAS binding, Beclin-1 releases the negative regulator of autophagy, Rubicon, which allows for autolysosome maturation. This autophagic response may be directly antiviral as opposed to negatively regulating the cGAS/STING pathway. Knockdown of Beclin-1 or cGAS reduces LC3 association and clearance of foreign DNA.

Although the identification of cGAS has contributed to our understanding of innate immune sensing, a major conundrum still exists in our understanding of how viral DNA is detected. Unlike RNA viruses, many DNA viruses replicate in the nucleus of a cell. One notable exception to this observation is poxvirus replication, which occurs in the cytoplasm (Cairns, 1960). Without a lifecycle in the cytoplasm, how are DNA viruses detected? One hypothesis is that cGAS may bind incoming DNA viral genomes as they traverse the cytoplasm. However, many DNA virus genomes are protected by a viral capsid or travel to the nucleus through membrane bound compartments (Whittaker et al., 2000; Tsai and Qian, 2010), so this mechanism of sensing may only occur during an abortive infection.

Before the discovery of cGAS, gamma-IFN-inducible protein 16 (IFI16) was identified as a nuclear DNA sensor necessary for the induction of IFN during herpes simplex virus-1 (HSV-1) infection (Unterholzner et al., 2010; Orzalli et al., 2012). IFI16 can traffic between the cytoplasm and nucleus, but at steady-state IFI16 localizes to the nucleus, allowing IFI16 to bind HSV-1 DNA and activate IRF3 (Li et al., 2012). IFI16 also induces IFN expression in response to HIV-1 single-stranded DNA and double-stranded DNA replication intermediates (Jakobsen et al., 2013). Although IFI16-induced IFN is STING-dependent, no direct activation of the STING pathway by IFI16 has been demonstrated. Interestingly, IFN induction during HSV-1 and HIV-1 infection is also dependent on STING and cGAS (Sun et al., 2013; Gao et al., 2013). Given the dual dependence on cGAS and IFI16, the sensors could potentially cooperate to detect viral DNA where IFI16 could bind DNA in the nucleus, traffic to the cytoplasm, and subsequently interact with cGAS to activate signaling through STING.

cGAS may also have a role in controlling RNA virus replication. cGAS-deficient mice display enhanced mortality to lethal challenges of the RNA virus West Nile virus (WNV) compared to infections in wild-type mice (Schoggins et al., 2014). Additionally, WNV infection of cGAS-deficient macrophages leads to modestly

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increased viral titers compared to wild-type controls. This control of viral replication is not due to direct recognition of RNA viruses by cGAS because this enzyme is not activated by double-stranded or single-stranded RNA (Sun et al., 2013; Zhang et al., 2014; Civril et al., 2013). Rather, the role of cGAS in controlling RNA virus infections may be linked to the observation that cGAS deficient cells exhibit decreased basal levels of ISGs. Low basal ISGs may allow for a viral replication advantage from which the host cannot recover, even if a robust IFN response is mounted later in infection.

Conclusion

Identifying innate immune sensors has been crucial to the understanding of how a host defends itself from viral infection. However, identification is insufficient, and these sensors must be placed in a cell biological context. As such, the subcellular location of these signaling pathways plays a major role in their function. The localization of PRRs helps differentiate between host and foreign nucleic acids, and the localization of downstream signaling molecules can also have major effects on the signaling outcomes that result. One theme slowly developing is that innate immune sensing is not static and the movement of the sensors and their immediate downstream effectors are of consequence. Understanding how this dynamic behavior affects the function of sensors and downstream signaling proteins will be important for our understanding of how the type-I IFN response is achieved.

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