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Sensing and sensibility: the role of non-coding RNAs in autoinflammation and viral infection

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Self RNA sensing by RIG-I like receptors in viral infection and sterile inflammation

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Abstract

The innate immune system uses pattern recognition receptors (PRRs) to survey the intracellular and extracellular environment for signs of infection. Viral infection is detected through presence of viral nucleic acids in infected cells. PRR activation by viral nucleic acids induces expression and secretion of type I interferons (IFN-Is), important mediators of antiviral immunity. RIG-I like receptors (RLRs) are RNA sensors that detect viral RNA in the cytosol and induce an IFN-I response. Viral RNAs contain features that set them apart from host RNAs, allowing RLRs to discriminate between cellular (self) and viral (non-self) RNA. The notion emerged that self RNAs can also engage RLRs and modulate the IFN-I response, indicating that the distinction between self and non-self RNA is not watertight. We review how self RNAs regulate RLR activation and the IFN-I response during viral infection, and how recognition of self RNAs by RLRs is implicated in autoinflammatory disorders and cancer.

Introduction

In all organisms, viral infections need to be detected and combatted quickly and efficiently to ensure survival. In mammals, an antiviral immune response is initiated when viral nucleic acids are detected by DNA sensing receptors such as cyclic GMP-AMP synthase (cGAS), or RNA sensing receptors such as Toll-like receptor 3 (TLR3), TLR7 and RIG-I-like receptors (RLRs). Recognition of viral infection induces the production and secretion of type I interferons (IFN-Is), key cytokines that orchestrate anti-viral immune responses[1].

The RLR family encompasses three members: retinoic acid-inducible gene I (RIG-I), melanoma differentiation-associated protein 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). All RLRs contain an RNA helicase domain and a C-terminal domain (CTD) that are required for RNA recognition 2. The RNA helicase domain has ATPase activity, which is crucial for RLR function. RIG-I and MDA5 also contain two N-terminal caspase activation and recruitment domains (CARDs) that mediate downstream signaling to the adaptor mitochondrial antiviral signaling protein (MAVS; also known as VISA, IPS-1 or Cardif). LGP2 lacks CARDs and instead functions in conjunction with RIG-I and MDA5[1,2].

Recognition of viral RNA by RLRs leads to a series of conformational changes that allow recruitment and prion-like oligomerization of MAVS, creating an interface for the activation of TNF receptor-associated factors (TRAFs), which subsequently activate TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor κB kinase ε (IKKε)[3]. These proteins in turn recruit and phosphorylate the transcription factors interferon regulatory factor (IRF) 3 and IRF7. Alternatively, MAVS-induced activation of the IKK complex, consisting of IKKα, IKKβ and IKKγ, leads to activation of nuclear factor κB (NF-κB) through degradation of its inhibitor, IκB[3]. These transcription factors synergistically induce IFN-I expression (predominantly IFN-α and IFN-β) and other innate response genes (**Fig. 1**)[4]. IFN-Is are secreted and activate the IFN receptor (IFNAR), which is found on nearly all cells, in an autocrine and paracrine manner. IFNAR activation induces JAK-STAT signaling and culminates in the activation of STAT1, STAT2 and IRF9[5]. Together, these transcription factors induce expression of hundreds of interferon-stimulated genes (ISGs), which directly or indirectly restrict viral replication (**Fig. 1**)[6,7]. Most proteins that initiate IFN-I production are IFN-inducible themselves, ensuring a prompt response[6,7]. Besides ISG upregulation, IFN-Is also have an anti-proliferative effect and stimulate adaptive immunity by attracting immune cells and enhancing their function[5].

RLRs recognize both distinct and overlapping sets of viruses. For example, members of the *Orthomyxoviridae* family are predominantly recognized by RIG-I, whereas MDA5 has a strong affinity for *Picornaviridae*. Instead, orthoreovirus and Sendai virus (SeV) are recognized by both RIG-I and MDA5[reviewed in 1]. This preference is dictated by the molecular and structural features of viral RNA, which also form the basis of the ability of RLRs to discriminate between self and non-self RNA[2,8,9].

RIG-I preferentially binds to double stranded RNAs (dsRNAs) that contain a 5′-tri- or 5′-biphosphate moiety (5′PP(P))[10–12]. These dsRNA molecules are relatively short (up to 300 nucleotides) and are preferentially blunt-ended[13–15]. RIG-I can also recognize 7-methylguanosine-capped 5′PPP dsRNA molecules, however, further capping by 2′-*O*-methylation of the first two 5′-end nucleotides strongly reduces RIG-I affinity[16]. Similarly, other RNA modifications such as N^6-methyladenosine interfere with RIG-I binding[17–19]. Such modifications may shield self RNA from detection by RIG-I and could be exploited by viruses to evade host immune responses[18–20]. Upon RNA binding, RIG-I changes its molecular structure from an auto-inhibited, 'closed' conformation to a conformation in which RIG-I is tightly bound

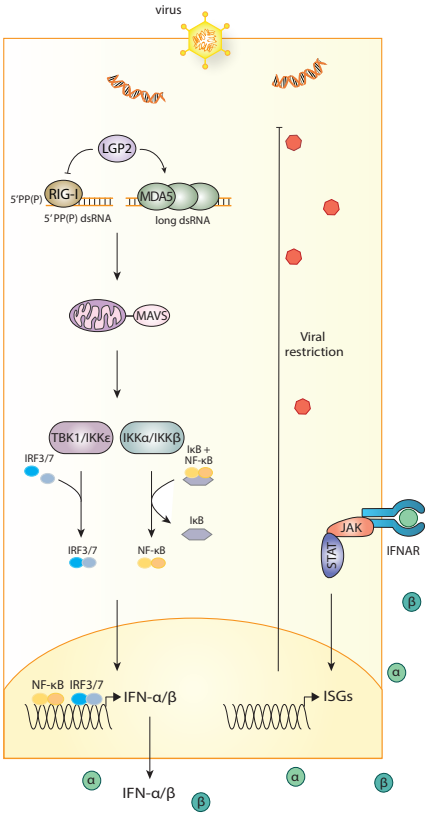

Figure 1. Overview of the RIG-I like receptor pathway. Viral RNA binds and activates RIG-I or MDA5, which subsequently triggers MAVS oligomerization. LGP2 also binds viral RNA and regulates RIG-I and MDA5 activation. Oligomerization of MAVS initiates a signaling cascade, which leads to activation of the transcription factors IRF3, IRF7 and NF-κB. Together, these transcription factors induce IFN-I (mainly IFN-α and IFN-β) expression and secretion, which in turn activates the IFN-I receptor in an autocrine and paracrine manner. The JAK-STAT signaling cascade downstream of the IFN-I receptor culminates in the expression of hundreds of ISGs, which have diverse roles in combatting viral infection.

to the RNA and its CARDs are exposed[21]. After ligand engagement, RIG-I translocates to the interior of the dsRNA in an ATPase-dependent manner, allowing multiple monomers to accumulate and oligomerize on the RNA molecule[22–24]. ATPase activity may also contribute to discrimination between self and non-self RNAs[24]. The two N-terminal CARDs of RIG-I are then modified by K63-linked polyubiquitination, which is essential for RIG-I oligomerization and subsequent recruitment of MAVS[25,26]. Several E3 ligases, such as tripartite motif 25 (TRIM25) and Riplet, may contribute to RIG-I K63-linked polyubiquitination in a highly ordered manner and regulate oligomerization efficiency and the magnitude of the subsequent antiviral response[2,25–28]. K63-linked polyubiquitin chains can also interact with RIG-I CARDs in a non-covalent manner and contribute to MAVS activation as well[29].

Although RIG-I and MDA5 share a similar domain architecture, recognition of non-self RNA by MDA5 is very distinct from that of RIG-I. MDA5 has affinity for dsRNAs that are preferentially longer than 2000 nucleotides, independent of a 5'PPP moiety[15]. Additionally,

complex and branched structures may also increase the stimulatory potential of dsRNA[30]. MDA5 exists in a flexible and 'open' conformation, where its CARDs are not shielded[31]. Upon binding to dsRNA, MDA5 forms filaments along the dsRNA backbone that are stabilized by both protein-protein and protein-RNA interactions[32]. MDA5 filament formation is dynamic, with monomers continuously associating and dissociating. ATP hydrolysis is involved in MDA5 dissociation and occurs equally throughout the filament, but only induces disassembly at the ends[31–34]. Since approximately 10 to 11 MDA5 CARDs need to stably cluster together for subsequent MAVS aggregation, only long MDA5 filaments may be signaling-competent, thus dictating preference for long dsRNA[32,35].

LGP2 binds to termini of dsRNAs, although its exact ligand preference has only been studied in a few instances and remains unclear[36,37]. LGP2 can form short filaments on dsRNA, but lacks signal transduction capacity and instead regulates the activation of RIG-I and MDA5[36,38]. For example, LGP2 enhances MDA5 filament formation by supporting filament nucleation and augmenting MDA5-RNA interactions[39]. LGP2 also increases MDA5 filament quality by inducing generation of more, but shorter MDA5 filaments, which possess greater signaling capacity[38,39]. Additionally, LGP2 inhibits the endoribonuclease Dicer, which cleaves dsRNA substrates into small fragments[40]. Conversely, LGP2 interferes with RIG-I function through various mechanisms such as ligand competition or recruitment of the dsRNA binding protein PACT, which inhibits RIG-I signaling and further potentiates MDA5 signaling[36,41,42].

Many viral ligands that bind and stimulate RLRs were identified through RLR immunoprecipitation and analysis of co-purifying RNAs, but host RNAs have been largely ignored. Recent studies have uncovered that self RNAs can also interact with RLRs and modulate the outcome of RLR activation in different disease contexts. Interestingly, many of these can be classified as RNA polymerase (Pol) III transcripts or retroelements (REs), indicating that in particular these classes of RNA have RLR modulatory capacities. Pol III produces a variety of small non-coding (nc)RNAs such as tRNAs, 5S rRNA, 7SL RNA, vault RNAs and Y RNAs, which all contain a 5'PPP moiety[43]. This moiety is generally removed after synthesis, but failure to do so may yield RIG-I ligands. Pol III transcription was previously implicated in RNA sensing by synthesizing 5'PPP-RNA from AT-rich viral DNA, which subsequently activates RIG-I[44]. Endogenous REs are highly repetitive sequences that are extremely abundant and interspersed throughout the genome. They include three major classes: endogenous retroviruses (ERVs), long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs, which include Alu elements)[reviewed in 4,45,46]. REs originate from ancient retroviral infections of the germline and their subsequent genomic integration. Most REs are incapacitated due to deleterious mutations, recombination events or epigenetic repression and no longer propagate. However, REs can be transcribed as (part of) non-protein coding sequences (in introns, untranslated regions or ncRNAs), as individual Pol III transcripts or be re-expressed in certain diseases following epigenetic derepression[4,45,46]. The repetitive nature of REs and, in some instances, their bidirectional transcription makes them prone to intra- or intermolecular base-pairing and dsRNA formation, allowing detection by RLRs.

Here, we will review how these and other self RNAs regulate RLR function during viral infection and sterile inflammatory responses, as occurs in autoinflammatory diseases and cancer. Mechanistically, self RNAs can engage RLRs through various modes of differential regulation: increased abundance of self RNA, unshielding of RNA through downregulation of RNA-binding proteins (RBPs), altered nucleotide modifications and mislocalization of RNA. We will discuss such mechanistic details by providing several examples of RLR inhibition or activation by self RNAs.

RLRs recognize self RNA during viral infection

Self RNAs can bind RLRs during viral infection and either inhibit or potentiate RLR activation and the IFN-I response (**Table 1**). In mouse macrophages, the IFN-I-inducible long non-coding (lnc)RNA lnc-Lsm3b accumulates during late stages of infection with negative-stranded RNA viruses and interacts with RIG-I[47]. Knockdown of lnc-Lsm3b increased IFN-I expression upon infection with vesicular stomatitis virus (VSV) and SeV, indicating that lnc-Lsm3b inhibits RIG-I signaling. Mechanistically, lnc-Lsm3b competes with viral RNA for binding to the RIG-I CTD through its 9 GA-rich motifs, sequestrating and inactivating RIG-I and terminating IFN-I induction (**Fig. 2A**)[47]. Similarly, in human cells, IFN-I-inducible lnc-ATV interacts with and inhibits RIG-I during infection with hepatitis C virus (HCV), Zika virus (ZIKV), Newcastle Disease Virus (NDV) and SeV. Knockdown of lnc-ATV led to an enhanced IFN-I response, whereas overexpression favored viral replication[48].

In contrast, other self RNAs potentiate RLR activation. Upon herpes simplex virus-1

		RNA ID	RLR	Effect on RLR	Context	Differential regulation	Outcome	Source
VIRAL INFECTION		lnc-Lsm3b	RIG-I	Inhibitory	VSV, SeV	Increased abundance	Termination IFN signaling	(47)
		lnc-ATV	RIG-I	Inhibitory	HCV, ZIKV, NDV, SeV	Increased abundance	Pro-viral	(48)
		RNA5SP141	RIG-I	Stimulatory	HSV-1, EBV, IAV	Mislocalization, unshielding, increased abundance	Anti-viral	(49)
		miR-136	RIG-I	Stimulatory	IAV, SeV	Increased abundance	Anti-viral	(50)
		lnc-ITPRIP-1	MDA5	Stimulatory	HCV	Increased abundance	Anti-viral	(51)
		vtRNA	RIG-I	Stimulatory	KSHV	Altered modification	Anti-viral	(52)
		RNY4	RIG-I	Stimulatory	HIV-1	Altered modification	Anti-viral	(53)
STERILE INFLAMMATION	*AUTO-INFLAMMATION*	Alu:Alu hybrids	MDA5	Stimulatory	ADAR1 mutations, MDA5 mutations	Altered modification, Increased affinity for RNA	AGS	(73)
		mt-dsRNA	MDA5	Stimulatory	PNPase mutations	Increased abundance, mislocalization	IE, BSN	(78)
		IRE-1 products?	RIG-I	Stimulatory	SKIV2L mutations	Increased abundance	THES, (SLE)	(79)
	CANCER	U1 (and U2)	RIG-I	Stimulatory	Ionizing radiation therapy	Mislocalization	Anti-tumor	(88)
		RN7SL1	RIG-I	Stimulatory	Stromal Pol III up-regulation in breast cancer	Unshielding, increased abundance	Pro-tumor	(90)
		ERV	MDA5	Stimulatory	DNMTi treatment	Increased abundance	Anti-tumor	(94)

Table 1. Overview of known self-derived RLR ligands and the mechanism by which they are implicated in disease. Abbreviations: BSN, bilateral striatal necrosis; DNMTi, DNA methyltransferase inhibitors; IE, infantile encephalopathy; THES, tricho-hepato-enteric syndrome.

(HSV-1) infection, the Pol III transcript 5S RNA pseudogene 141 (RNA5SP141) is upregulated in HEK293T cells[49]. In uninfected cells, this 5'PPP-bearing self RNA is shielded by two nuclear proteins, TST and MRPL18. HSV-1 downregulates TST and MRPL18 protein levels through host-translational shut-off. This causes unshielding of RNA5SP141 transcripts and alters its localization from the nucleus to the cytosol, where it activates RIG-I and restricts viral replication (**Fig. 2B**). This mechanism was also observed during Epstein-Barr virus (EBV) and – to a lesser extent – influenza A virus (IAV) infection[49]. Of note, the activation of RIG-I by a host RNA during herpesvirus infection equips the RNA sensing machinery with a mechanism to protect against certain DNA viruses. Other stimulatory self RNAs include microRNA 136 (miR-136), which is upregulated upon IAV and SeV infection and activates RIG-I and IFN-inducible lnc-ITPRIP-1, which enhances MDA5 oligomerization along hepatitis C virus (HCV) RNA[50,51].

Vault RNAs (vtRNAs) are cytosolic small ncRNAs that engage RIG-I during Kaposi's sarcoma-associated herpesvirus (KSHV) lytic reactivation[52]. Nascent Pol III transcripts normally contain a 5'PPP moiety that is removed by the nuclear enzyme dual-specificity phosphatase 11 (DUSP11). KSHV lytic reactivation reduces DUSP11 expression, causing RIG-I-mediated recognition of 5'PPP-vtRNAs and an enhanced IFN-I response[52]. Similarly, DUSP11 is downregulated upon human immunodeficiency virus (HIV-1) infection in a VPR-dependent manner causing another ncRNA, Y RNA 4 (RNY4), to activate RIG-I[53]. By modulating DUSP11 levels, host cells have developed an intelligent strategy to enhance RIG-I activation upon viral infection using 5'PPP-containing self RNAs. Whether other RNA phosphatases exist that target Pol III transcripts and function redundantly with DUSP11 is still unclear.

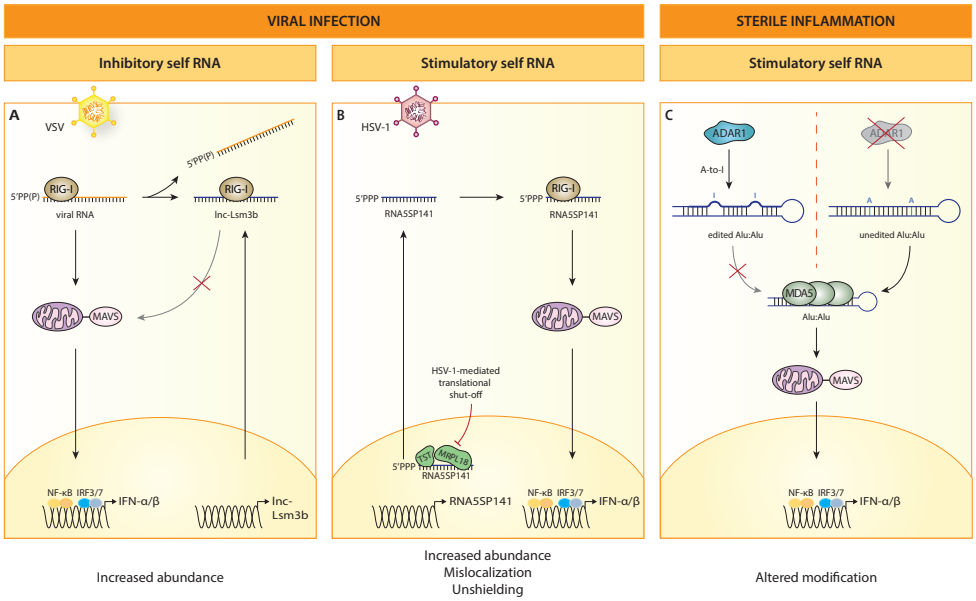

Figure 2. Overview of differentially regulated self RNAs that are recognized by RLRs in viral infection and autoinflammation. A) The IFN-I-inducible lnc-Lsm3b competes with VSV RNA for binding to RIG-I, thereby terminating RIG-I signaling. **B)** HSV-1-mediated host translational shut-off causes unshielding of immunostimulatory RN5SP141, which activates RIG-I and enhances an IFN-I response. **C)** ADAR1 deficiency causes internally base-paired inverted repeat Alu elements to be recognized by MDA5, inducing an IFN-I response and subsequent sterile inflammation.

Other studies have identified a correlation between the presence of immunostimulatory self RNA molecules and RLR activation. For example, IAV infection causes epigenetic derepression of ERV transcription through de-SUMOylation of TRIM28, an epigenetic regulator. The subsequent IFN-I response relies on RIG-I and MDA5 activation[54]. Likewise, encephalomyocarditis virus (EMCV) and SeV infection induce the activation of RNase L, an endoribonuclease that upon infection cleaves self RNAs into small RNA fragments that activate RIG-I and MDA5[55]. It must be noted that a direct interaction between ERVs or RNase L cleavage products and RIG-I or MDA5 was not shown in these studies.

Altogether, self RNA recognition by RNA sensors during viral infection is increasingly appreciated as a strategy to modulate the IFN-I response to enhance antiviral defense during early stages of infection, detect DNA viruses or retroviruses or terminate the IFN-I response during late stages of infection and prevent IFN-I-associated pathology.

Recognition of self RNAs by RLRs in sterile inflammation: autoinflammatory diseases

The above studies illustrate how discrimination between self and non-self RNA becomes blurred during viral infection. Discrepancies in self and non-self RNA recognition can also trigger unwanted RLR activation in absence of infection, such as in autoinflammatory and autoimmune diseases (**Table 1**). Autoinflammatory type I interferonopathies comprise a spectrum of rare, severe monogenic diseases defined by an enhanced production of IFN-Is in absence of infection[56]. Most of them share clinical features such as intracranial calcification and skin inflammation[57]. A number of clinical trials are under way to test the effectiveness of compounds that block the IFN-I pathway in these diseases[58].

With nucleic acid sensing being an important catalyst for IFN-I production, it is not surprising that many identified mutations linked to interferonopathies involve genes implicated in nucleic acid sensing. Amongst these are a number of gain of function (GoF) mutations in RLRs that cause Aicardi-Goutières syndrome (AGS), Singleton-Merten syndrome (SMS) or monogenic systemic lupus erythematosus (SLE)[59–61]. Genome-wide association studies have also identified genetic linkages between polymorphisms in *IFIH1*, the gene encoding MDA5, and classical autoimmune diseases[56,62]. For some polymorphisms, the mechanism behind this association has been clarified, with GoF mutations in *IFIH1* generally increasing susceptibility to autoimmune diseases (e.g. SLE, type I diabetes)[63–66] and loss of function (LoF) mutations protecting against such conditions (e.g. type I diabetes, psoriasis)[67–69] as well as increasing susceptibility to viral infection[70].

Mechanistically, many GoF mutations in MDA5 (for example G495R) increase its affinity for (self) RNA, while such mutations in RIG-I cause constitutive activation of this receptor[60,61,65,71,72]. Thus, besides alterations in self RNA abundance, shielding, nucleotide modifications or localization, mutations in RLRs can lead to accidental activation by self RNAs.

Endogenous stimulatory ligands for MDA5^{G495R} and other GoF mutants have been identified using an *in vitro* RNase protection assay[73]. Recombinant MDA5^{G495R} forms filaments along its preferred RNA ligands when combined with a mixture of cytosolic RNAs. Filament formation protects these RNA ligands from degradation by RNases, allowing their purification and identification by deep sequencing. Using this assay, MDA5^{G495R} was found to associate with Alu:Alu hybrids, duplex RNA structures formed by inverted repeat Alu elements, leading to aberrant MDA5 activation and diseases such as AGS[73].

Alus are frequently modified post-transcriptionally by the RNA editing enzyme adenosine

deaminase acting on RNA (ADAR1), which converts adenosines into inosines (A-to-I editing) and thereby destabilizes base-paired regions within RNA duplexes[74]. ADAR1 knockout or knock-in of an editing-deficient ADAR1 mutant results in embryonic lethality in mice due to aberrant IFN-I production. This phenotype is rescued by simultaneous deletion of MDA5 or MAVS, but not RIG-I, indicating that ADAR1 editing prevents activation of the MDA5 pathway (**Fig. 2C**)[75–77]. Loss of function mutations in ADAR1 also cause interferonopathies such as AGS and monogenic SLE[59]. Consistently, when recombinant wild type MDA5 was combined with cytosolic extracts of ADAR1-deficient HEK293T cells *in vitro*, MDA5 associated with unedited Alu:Alu hybrids and protected these from endonucleolytic cleavage by RNases[73].

Mislocalization of mitochondrial dsRNA (mt-dsRNA) has also been linked to irregularities in IFN-I production in patients carrying biallelic hypomorphic mutations in the *PNPT1* gene with clinical manifestations such as deafness and hypotonia[78]. Mitochondrial DNA allows for bidirectional transcription, and the resultant heavy and light transcripts can overlap to form long dsRNAs. Stimulation of MDA5 by mt-dsRNA is avoided by physical separation and swift degradation of the light transcript by the mitochondrial RNA degradosome. Loss of the mtRNA helicase SUV3 and the phosphorylase PNPase, key components of the mitochondrial RNA degradosome, induce accumulation of dsRNA in the cytosol triggering an MDA5-mediated IFN-I response. However, only deficiency in PNPase leads to spontaneous release of mt-dsRNA into the cytosol. Cells from patients with mutations in *PNPT1*, the gene encoding PNPase, show accumulation of mt-dsRNA in both the mitochondrial compartment and the cytosol as well as an IFN-I transcriptional signature resembling that of interferonopathies[78].

Finally, knockdown of the RNA exosome subunit SKIV2L leads to an accumulation of immunostimulatory RNAs that activate RIG-I[79]. Consistently, SKIV2L deficiency in patients causes a strong IFN-I signature in blood matching that of AGS patients[79]. In addition, a previous study also linked a *SKIV2L* single nucleotide polymorphism to SLE[80]. The source of the RIG-I activating ligands in SKIV2L deficiency may involve RNA cleavage products generated by the endoribonuclease IRE-1, which is activated during the unfolded protein response in the endoplasmic reticulum[79].

Taken together, these examples illustrate how inadvertent activation of RLRs by self RNAs can induce an unwanted IFN-I response, causing pathology and disease. Understanding the precise mechanisms by which self RNAs cause disease will aid the development of targeted therapies that avoid the detrimental side effects associated with an overall blockade of the IFN-I pathway.

Recognition of self RNAs by RLRs in sterile inflammation: cancer

The IFN-I response in cancer

In contrast to autoinflammatory diseases, IFN-Is can have protective effects within the sterile tumor microenvironment and correlate with a favorable prognosis[reviewed in 81,82]. Both tumor cells and immune cells can produce and respond to IFN-Is. Besides their direct anti-proliferative effects, IFN-I-driven inflammation can change an immune-deprived 'cold' tumor into an immune-infiltrated 'hot' tumor by recruiting and activating various immune cells, such as dendritic cells and cytolytic T lymphocytes[81,82]. IFN-Is can also increase the effectiveness of chemo-, radio- and immunotherapies (discussed below)[81,82]. Of note, chronic IFN-I signaling can also diminish responsiveness to immunotherapy, e.g. by enhancing the expression of T cell inhibitory receptors, including PD-L1[83,84].

DNA sensors that are activated by self-derived DNA or synthetic agonists have an

important role in intra-tumoral IFN-I production[85]. Likewise, treatment with artificial RIG-I agonists increases anti-tumor immunity and responsiveness to immunotherapy in mice[86,87]. However, much remains to be learned about the precise nature of self RNA species that induce an IFN-I signature in tumors, but in a few instances the identity of such ligands has been clarified (**Table 1**).

RIG-I-associated small RNAs in cancer

Deep sequencing of RNA species bound to RIG-I in HEK293 and HCT116 cells exposed to radiation therapy revealed enrichment of the small nuclear RNAs U1 and U2[88]. These RNAs translocate from the nucleus to the cytosol to bind and activate RIG-I. Furthermore, signaling via RIG-I and MAVS (but not MDA5) mediates an IFN-I response and cell death in irradiated cancer cells and mice[88]. LGP2, instead, diminishes irradiation-induced IFN-I production and cell death, perhaps through inhibition of RIG-I activation[88,89].

In a breast cancer co-culture model, Pol III-transcribed 5'PPP ncRNAs and REs were transferred via RNA-containing exosomes (exoRNA) from stromal cells to tumor cells and induced a RIG-I-mediated IFN-I response[90]. Amongst these exoRNAs, the signal recognition particle-associated RNA RN7SL1 was the dominant stimulatory RNA and was specifically bound to RIG-I. RN7SL1 is normally shielded by the RBPs SRP9 and SRP14, but stromal exosomes contain unshielded RN7SL1[90]. RIG-I activation by exoRNA enhanced cancer cell growth and metastasis, emphasizing that IFN-I signaling does not exclusively have an anti-tumor effect[90].

Endogenous retroelements as self RNA ligands in cancer

Several studies correlate expression of certain classes of RNA with an RLR-dependent IFN-I response, although a direct interaction between RLRs and RNAs is not demonstrated, making it difficult to define the stimulatory RNA with much precision. Nonetheless, these studies unequivocally suggest that dsRNAs derived from ERVs and other REs augment intra-tumoral IFN-I production, mostly via MDA5, and enhance anti-tumor immunity. Transcription of a number of ERV families is indeed highly elevated in various cancers and this correlates with the induction of an IFN-I response and increased cytolytic activity[91–93]. In addition, treatment with DNA methylation inhibitors such as azacytidine leads to epigenetic derepression of many ERV families, and ERV-derived dsRNAs trigger IFN-I response via MDA5 and MAVS (and TLR3) in colorectal and ovarian cancer cells, reducing tumor growth *in vitro*[94,95]. Moreover, azacytidine synergizes with checkpoint inhibitors to increase tumor rejection in mice subcutaneously injected with the poorly immunogenic melanoma cell line B16[95]. Radiation therapy can also activate ERVs and mediate an MDA5- and MAVS-dependent IFN-I response[96]. This effect was enhanced upon loss of the chromatin regulator KAP1, which suppresses ERV transcription and was previously shown to inhibit an ERV-derived dsRNA-mediated IFN-I response[96,97].

Recent studies have identified key regulators that suppress RE expression in cancer cells. Their genetic loss enhances RE-derived dsRNA abundance, which yields a RIG-I- or MDA5-dependent IFN-I response and restricts tumor growth or sensitizes tumors to checkpoint blockade. Loss or inhibition of LSD1, a histone H3K4 demethylase, stimulates ERV expression, leading to increased appearance of ERV-derived dsRNA in the cytosol, which triggers an IFN-I response via MDA5 and TLR3, but not RIG-I and cGAS[98]. LSD1 ablation inhibits growth of B16 tumors *in vitro* and *in vivo*, and this phenotype can be reversed by concurrent deletion of MDA5 or IFN-β[98]. Consistently, intra-tumoral T cell infiltration was increased in LSD1-deficient melanomas in mice and LSD1 expression inversely correlated with T cell infiltration in a multitude of human tumors[98]. Finally, loss of LSD1 synergizes with checkpoint blockade *in*

vivo to enhance anti-tumor immunity[98]. Similarly, the histone H3K9 methyltransferase SETDB1 is highly expressed in many cancers and loss of SETDB1 in the acute myeloid leukemia human cell line THP1 increases the expression of many REs, in particular ERVs and LINEs, leading to a dsRNA-mediated IFN-I signature via MDA5 and RIG-I and cell death[99].

Besides these epigenetic regulators, loss of the RBP heterogeneous ribonucleoprotein C (HNRNPC) in breast cancer leads to unshielding of intronic Alu elements, which harbor HNRNPC binding sites. The resultant Alu-derived dsRNAs activate RIG-I and restrict proliferation[100]. These observations may explain why many cancers express high levels of HNRNPC. Finally, three independent studies have demonstrated that ADAR1 is not only a culprit in type I interferonopathies, but also an attractive target in cancer therapy[101–103]. Loss of ADAR1 unleashed an IFN-I signature and reduced viability in various tumor cell lines in a manner dependent on MDA5 and MAVS and the dsRNA-activated inhibitor of translation protein kinase R (PKR), respectively[101–103]. The loss of ADAR1 also increased the sensitivity of cancer cells to radiation and immunotherapy[103]. These effects were particularly evident in cancer cell lines with high pre-existing ISG expression levels (e.g. due to activation of DNA sensors)[101,102]. This is likely explained by elevated expression of dsRNA sensors in such cells combined with an IFN-I-mediated increase in transcription of SINEs that are normally (hyper) edited by ADAR1[102,103]. The precise RNA species that trigger an MDA5-mediated IFN-I response were not investigated in these studies.

These studies thus reveal novel checkpoints of RNA sensing and the IFN-I response, and their therapeutic targeting holds much promise for the design of complementary cancer therapies. This conclusion does require a critical note, as sustained IFN-I production in tumors may have pro-tumor effects[84]. Accordingly, a recent study identified an IFN-inducible subclass of ERVs in therapy-resistant small-cell lung cancer whose expression is associated with T cell and myeloid cell infiltration. This also correlates with markers of an immune-suppressed microenvironment, indicating that the overall outcome of ERV expression in cancer may depend on cell type or ERV family[104]. Insight into the precise ligands that trigger an IFN-I response in various cancers would allow us to better understand how to exploit and steer the RNA sensing machinery towards a beneficial anti-tumor response.

Conclusions

The notion that self RNAs interact with RLRs and modulate their activity is rapidly emerging, and additional self RNA ligands are likely to be uncovered in different disease contexts. Several reports correlate increased abundance of certain RNA species to RIG-I- or MDA5-dependent IFN-I responses, yet in absence of direct association studies it is difficult to understand which RNA ligands have most stimulatory potential. In addition, no self RNA ligands have been identified for LGP2, despite its high affinity for RNA[41]. Novel technologies, such as individual-nucleotide resolution ultraviolet crosslinking and immunoprecipitation (iCLIP), are likely to aid the identification of additional self-derived RLR ligands[105].

Do viruses modulate sensing of self RNAs by RLRs? Perhaps viruses have evolved protein- or RNA-based immune evasion strategies to prevent activation of RLRs by self RNAs. In addition, several DNA viruses encode small ncRNAs transcribed by Pol III and one could envision that such ncRNAs can bind and modulate RLRs[43].

It is also unclear how the mechanisms that underlie self RNA sensing during viral infection impact on autoinflammatory and autoimmune diseases or cancer. For example, does increased availability of self RNA ligands such as RNA5SP141 or loss of regulatory mechanisms

enhance inflammation in such disorders? And in the case of cancer, does this contribute to disease progression or immunosurveillance?

Finally, while existing literature has focused on self RNA sensing in disease, it is tempting to speculate that self RNAs may also occupy the RNA-binding pocket of RLRs, in particular those with an 'open conformation' such as MDA5, during homeostatic conditions to either keep them inactive (i.e. by setting a threshold for RLR activation) or to ensure tonic signaling. All in all, it is evident that many questions remain outstanding in this rapidly unraveling field.

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