# Chapter 17

# The Role of RNA Editing in the Immune Response

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## Abstract

The innate immune receptors in higher organisms have evolved to detect molecular signatures associated with pathogenic infection and trigger appropriate immune response. One common class of molecules utilized by the innate immune system for self vs. nonself discrimination is RNA, which is ironically present in all forms of life. To avoid self-RNA recognition, the innate immune sensors have evolved sophisticated discriminatory mechanisms that involve cellular RNA metabolic machineries. Posttranscriptional RNA modification and editing represent one such mechanism that allows cells to chemically tag the host RNAs as "self" and thus tolerate the abundant self-RNA molecules. In this chapter, we discuss recent advances in our understanding of the role of RNA editing/modification in the modulation of immune signaling pathways, and application of RNA editing/modification in RNA-based therapeutics and cancer immunotherapies.

**Key words** Innate immunity, RIG-I-like receptors, Toll-like receptors, PKR, ADAR, APOBEC, RNA modification, Editing

## 1 Introduction

Higher eukaryotes have evolved complex immune systems that prevent invasion by various infectious agents including bacteria, viruses, fungi, etc. One common immune strategy is to employ germline-encoded innate immune receptors known as pattern recognition receptors (PRRs) that detect infection at early stage and activate appropriate immune response. PRRs sense chemical signatures called pathogen-associated molecular patterns (PAMPs) that are broadly preserved in pathogens, but are generally absent in the host [1]. Classic examples of PAMP include lipopolysaccharides found in bacterial outer membranes or peptidoglycans present in bacterial cell walls. Upon engagement with their specific PAMPs, PRRs can initiate downstream signaling cascades that lead to production and secretion of proinflammatory cytokines and chemokines. Intriguingly, the last decade of research showed that many PRRs utilize nucleic acids for "self" vs. "nonself" discrimination. Considering that nucleic acids are universally present across all

Ernesto Picardi and Graziano Pesole (eds.), *RNA Editing: Methods and Protocols*, Methods in Molecular Biology, vol. 2181, https://doi.org/10.1007/978-1-0716-0787-9_17, 

forms of life, it raises questions of how these PRRs selectively recognize pathogen-derived nucleic acids against those from the host, and whether mis-recognition of self-nucleic acids occurs under some pathologic conditions. In this chapter, we discuss RNA-specific PRRs and cellular mechanisms, in particular RNA editing and modification, that modulate immune-stimulatory activity of RNAs.

### 1.1 RNA Sensors in Innate Immunity

There are three major RNA-sensing pathways in humans. Among these, RIG-I-like receptors (RLRs) and Toll-like receptors (TLRs) are the conventional innate immune sensors that can initiate pro-inflammatory responses upon RNA recognition (Fig. 1). Protein kinase R (PKR), on the other hand, is a stress-response gene that suppresses global protein synthesis upon recognition of viral RNAs (Fig. 1). In the following section, we briefly introduce these RNA sensors and downstream immune responses.

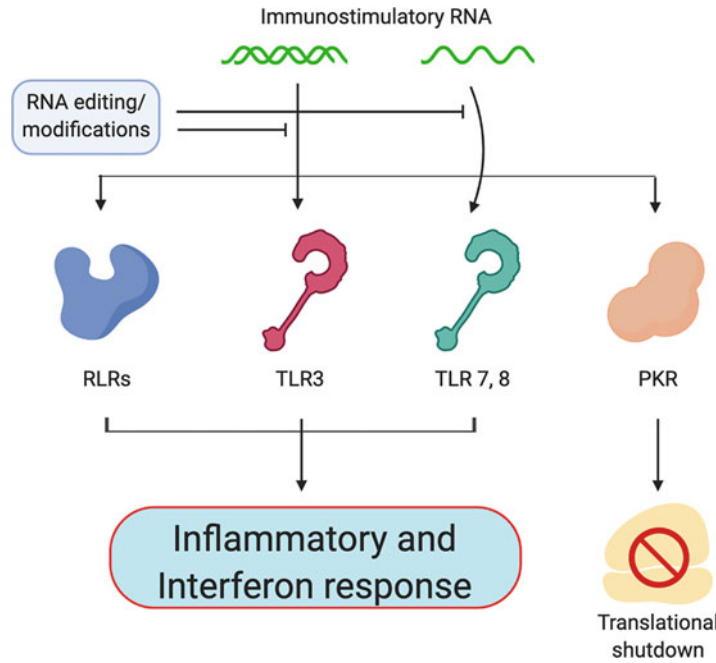


**Fig. 1** Foreign RNA sensors in human. RLRs, TLRs, and PKR are the three major types of foreign RNA sensors and are the focus of this review. Upon foreign RNA recognition, RLRs and TLRs initiate signaling cascades that lead to transcriptional upregulation of type I interferons and proinflammatory cytokines. PKR, on the other hand, induces global suppression of protein synthesis. RNA editing/modification were generally shown to suppress RNA recognition by these receptors. This observation led to the model that cellular RNAs utilize RNA editing/modification to maintain their immunological inert state

### 1.1.1 RIG-I-Like Receptors (RLRs)

RLRs comprise a family of DExD/H box helicases that function as innate immune sensors of cytosolic viral RNAs [2, 3]. These include *R*etinoic acid-*I*nducible *G*ene *I* (RIG-I), *M*elanoma *D*ifferentiation-*A*ssociated factor *5* (MDA5), and *L*aboratory of *G*enetics and *P*hysiology *2* (LGP2). RIG-I and MDA5 show RNA-mediated antiviral signaling activity, but the exact function of LGP2 is yet unclear and seems to play only a regulatory role [4]. RIG-I and MDA5 utilize distinct RNA features to discriminate viral RNAs from endogenous RNAs. RIG-I can ideally recognize viral double-stranded RNAs (dsRNAs) that have triphosphate or diphosphate at 5′-ends [5–9]. This RNA specificity prevents RIG-I's aberrant activation by cellular RNAs, which are generally depleted of 5′-triphosphate. MDA5, on the other hand, recognizes very long RNA duplexes ($\geq$1 kb) that are characteristically absent in the host cell, but are present in cells infected with several positive-strand RNA viruses [10, 11].

RIG-I and MDA5 share the downstream signaling pathway. Upon activation by RNA ligands, both receptors can induce filament formation of the downstream adapter molecule *M*itochondrial *A*nti-*V*iral *S*ignaling protein (MAVS; also known as IPS-1, VISA, or Cardiff) [12–15]. This MAVS filament induction is mediated by homotypic interactions between *C*aspase *A*ctivation and *R*ecruitment *D*omains (CARD) of RIG-I (or MDA5) and that of MAVS [16–18]. Oligomerized MAVS then recruits TNF-receptor-associated factors (TRAFs) 2, 5, and 6 to activate the cytosolic protein kinases TANK-binding kinase 1 (TBK1) and IκB kinase (IKK) [19–22]. Active TBK1 complex phosphorylates transcription factors IRF3 and IRF7, which then homo-dimerize and translocate to the nucleus to induce the expression of type I interferon (IFN) genes. The activated IKK complex, on the other hand, induces the NF-κB signaling pathway resulting in the expression of pro-inflammatory cytokines.

### 1.1.2 Toll-Like Receptors (TLRs)

Among the nearly a dozen TLRs identified in humans, TLR3, 7, and 8 are sensors for foreign RNAs. The self vs. nonself RNA discrimination by these TLRs is primarily based on the mechanism of spatial segregation since these TLRs are located in the endosomes, to which cellular RNAs have limited or no access. In addition to endosomal localization, dsRNA structure is required for TLR3 activation [23], while GU/U-rich sequence is important for TLR7 and 8 activation [24, 25].

Once activated, TLRs initiate a downstream inflammatory signaling cascade via an adaptor protein, TRIF [26] or MyD88 [27]. Recruitment of TRIF leads to the formation of the signaling complex that includes TRAF3, TBK1, and IKK. The TRIF complex can then activate both IRF3/IRF7-mediated type I IFN response and NF-κB-mediated expression of pro-inflammatory cytokines. MyD88-mediated pathway, on the other hand, involves the

formation of MyD88 signaling complex known as Myddosome [28]. This complex also includes multiple copies of MyD88, IRAK1/2, and IRAK4, which recruit TRAF6 and TRAF3 for the activation of IFN and inflammatory responses through IRF7 and NF-κB, respectively. TLR3 has been shown to trigger TRIF-dependent pathway while TLR7 and 8 activate MyD88-dependent pathway.

#### 1.1.3 Protein Kinase R (PKR)

Unlike RLRs and TLRs, PKR is not a canonical pattern recognition receptor in a sense that it does not directly induce antiviral or pro-inflammatory cytokine responses. However, it has an important antiviral activity by inhibiting global protein synthesis, for both viral and host mRNAs, and suppressing cellular proliferation [29]. PKR is a cytoplasmic Ser/Thr kinase and has two tandem dsRNA-binding domains (dRBDs) along with the catalytic kinase domain [30]. PKR exists in the latent form in the absence of viral dsRNA. Only upon dsRNA binding to the dRBDs PKR forms a dimer and concomitantly autophosphorylates itself to switch to the active state [31, 32]. Once activated, PKR phosphorylates multiple target molecules, including the eukaryotic translation initiation factor 2α (eIF2α), which results in the inhibition of eIF2-dependent translational initiation [33].

The antiviral pathways discussed above need to be efficiently regulated in order to prevent their aberrant activation by cellular RNAs and constitutive innate immune responses. In order to limit self-recognition by these receptors, cells generally employ a multi-pronged approach, including those mentioned above that utilize differences in spatial, temporal, or chemical properties of RNAs between self and nonself RNAs. In this review, we examine one such strategy, namely RNA editing and modification, that is commonly used for self vs. nonself RNA discrimination by RLRs, TLRs, and PKR. We will not discuss RNA metabolic processes, such as splicing, capping, and polyadenylation. Although RNA editing/modifications play an important role in a number of biological processes including development, stress response, etc., we will here focus on their roles in antiviral immunity and immune regulation.

## 2 Role of RNA Editing/Modification in Immunomodulation

### 2.1 Adenosine-to-Inosine (A-to-I) Modification

In 1987, two simultaneous studies reported that exogenously added antisense RNAs inefficiently hybridize with the cognate sense RNA in *Xenopus* eggs and early embryos, compared to other *Xenopus* cells at different developmental stages [34, 35]. This observation was attributed to a RNA duplex "unwinding activity" present in early stages of *Xenopus* fertilized eggs [34, 35] and later shown in a number of mammalian cell lines

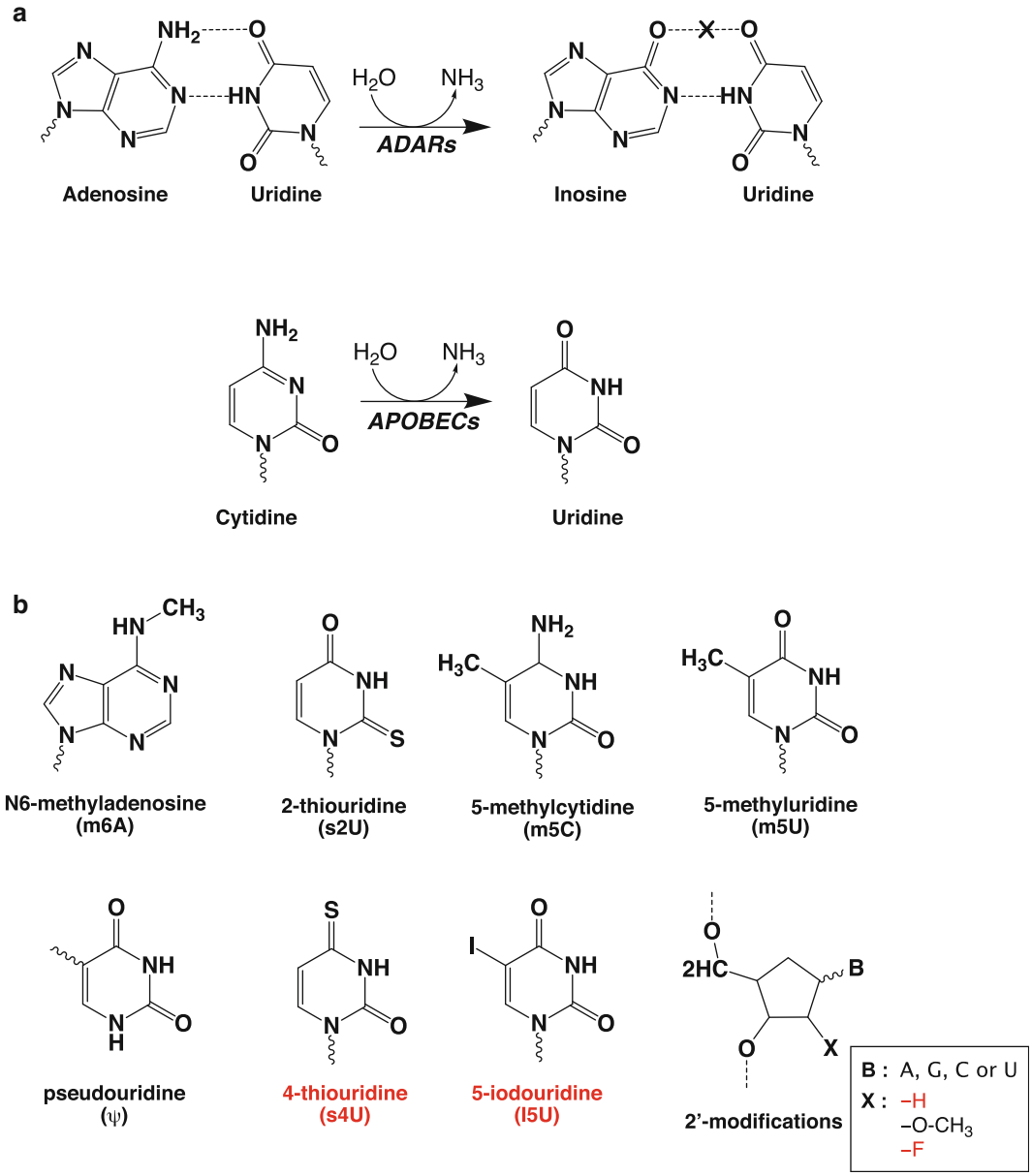


**Fig. 2** Chemical structures of RNA modifications in immunomodulation. (**a**) Editing reactions that give rise to A-to-I (upper panel) or C-to-U (lower panel) modifications. Note: A-to-I modification in dsRNA disrupts the base pair due to the loss of a hydrogen bond. (**b**) Other immune-modulatory modifications. Red indicates nonnatural modification

[36]. It turned out that this apparent duplex "unwinding activity" is caused by covalent modification of adenosines to inosines (A to I) that replaces the stable A:U base pairs by less stable I:U pairing (Fig. 2a) [37–39]. Although A-to-I modifying activity was already known at that time on tRNA substrates [40, 41], its discovery in the context of dsRNA was novel and its biological role was

unknown [42]. The enzyme responsible for A-to-I editing in dsRNAs, adenosine deaminase acting on RNA (ADAR), was first purified from *Xenopus laevis* eggs and later from multiple other sources [43–46].

Three different isotypes of ADAR (ADAR1, ADAR2, and ADAR3) have been reported in mammals. ADAR1 and ADAR2 are ubiquitously expressed and are catalytically active, while ADAR3 lacks the deaminase activity and its expression is largely restricted to the brain [47]. Two other distantly related testis-specific members of ADAR family, ADAD1 and 2, also lack deaminase activity but play a role in spermatogenesis [48]. Therefore, A-to-I editing in the mammalian cells is carried out by ADAR1 and ADAR2, while ADAR3 and ADADs have been suggested to play a regulatory role via their RNA-binding domains [49, 50].

There are two alternatively spliced isoforms of ADAR1, a constitutive p110 and an IFN-inducible p150 [51]. Both isoforms have a C-terminal catalytic deaminase domain and three tandem repeats of dRBDs at the center, which harbor a nuclear localization signal (NLS). At the N-terminus, p110 has a single Z-DNA-binding domain (Zβ), while p150 has two (Zα and Zβ) and a nuclear export signal. As such, p110 is localized in the nucleus while p150 shuttles between nuclear and cytosolic compartments. Among the Z-DNA-binding domains, Zα has been shown to preferentially bind dsRNA with high propensity to form Z-form duplex conformation (e.g., alternating CG sequence) over the typical A-form duplex conformation [52]. Zβ, on the other hand, lacks nucleic acid binding on its own [53, 54] but it can alter the sequence specificity of Zα when present in the same peptide [53, 55]. It is expected (but not formally proven) that Zα and Zβ contribute to RNA specificities of ADARs [56, 57]. The Zα domain of p150 was also shown to help its localization to stress granules in response to IFN-induced or oxidative stress, although the mechanism remains unclear [58, 59]. The domain architectures of ADAR2 and ADAR3 are very similar to each other, wherein the C-terminal deaminase domain is preceded by two tandem dRBDs. Both ADAR2 and ADAR3 have an NLS upstream of the dRBDs and no Z-DNA-binding domain. ADAR3 has an additional arginine-rich region (R domain) not found in any other ADAR, which seems to confer an additional RNA-binding activity [47]. On the other hand, both ADADs 1 and 2 have a single dRBD followed by a deaminase domain that lacks the key catalytic residues.

Much of the current mechanistic understanding of how ADARs function has come from the studies on ADAR2. This is because of its simpler domain architecture and ease of protein purification from heterologous systems. The structure of ADAR2 revealed that inositol hexakisphosphate (IP6) is buried deep inside the protein core, likely playing an important role in protein folding [60]. A $Zn^{2+}$ ion was also identified in the catalytic site that

positions the nucleophilic water for the deamination reaction [60]. The crystal structure of ADAR2 in complex with dsRNA further showed that the deaminase domain alone has an intrinsic affinity to bind dsRNA, and utilizes slight backbone distortion and base-flipping mechanism to access the deamination target base [61]. It is possible that the base-flipping mechanism by the catalytic domain may account for the moderate sequence preference found near the deamination site [61].

### 2.2 A-to-I Modification in Innate Immunity

The role of A-to-I editing in antiviral immunity was initially studied in the context of its mutagenic effect on the viral genome. This is because inosine is recognized by reverse transcriptase and ribosome as guanosine instead of adenosine. This recoding effect of A-to-I editing also occurs in host RNAs and plays an important role especially in the central nervous system (CNS) [62–65] (also reviewed in [66]). It has been shown that these essential site-specific recoding events are carried out mainly by ADAR2, not ADAR1 [67, 68]. ADAR1, on the other hand, is involved in promiscuous hyperediting of long dsRNAs, either from cellular or viral origins. This activity of ADAR1 has now been shown to prevent self-triggered immune response while risking suppression of virally triggered antiviral immunity (*see* below).

Initial studies established an essential role of ADAR1 in development. Knocking out *Adar1* gene in mice is embryonically lethal due to defects in erythropoiesis and liver disintegration [69, 70] and widespread apoptosis in multiple tissue types [71]. Although involvement of A-to-I editing in immune regulation was initially suspected based on the existence of IFN-inducible isoforms of ADAR1 [51], its first direct role as an immune suppressor was reported only in 2009 [72]. In this study, disruption of *Adar1* gene in mice increased global IFN response characteristic of virus-infected cells. More importantly, in 2012, loss-of-function mutations in ADAR1 were found to cause a rare auto-inflammatory disease, Aicardi-Goutières syndrome (AGS) [73]. AGS is a severe neurodegenerative disease characterized by heightened type I IFN in cerebrospinal fluid (CSF) and peripheral blood, and has phenotypic overlap with a more common autoimmune disease, such as systemic lupus erythematosus (SLE) [74]. Aside from ADAR1, AGS mutations have also been mapped to genes encoding for TREX1 [75], RNASEH2A, RNASEH2B, RNASEH2C [76], SAMHD1 [77], and MDA5 [78], highlighting the link between AGS and nucleic acid metabolism/sensing.

The mechanism by which the ADAR1 deficiency causes auto-inflammation became obvious when subsequent mouse model studies showed that the embryonic lethality and inflammatory phenotype of *Adar1* deficiency could be at least partially rescued by additional knockout of MDA5 and MAVS, but not RIG-I or STING [79–81]. The MDA5 regulatory activity was attributed to

the IFN-inducible isoform of ADAR1, p150. The constitutive nuclear isoform p110 was shown to be involved in organ development along with the p150 isoform [81]. A more recent study suggested that the dsRNA-binding activity of ADAR1 also plays an immunoregulatory role, independent of the deamination activity [82]. However, studies with knock-in mouse that expresses catalytically deficient, but RNA-binding-competent ADAR1 showed that deficiency of A-to-I modification is sufficient to constitutively activate MDA5 and that the phenotype of the knock-in mouse largely recapitulates the embryonic lethality of *Adar*$^{-/-}$ [80].

How does the lack of A-to-I modification lead to constitutive activation of MDA5? Studies showed that ~300 bp long cellular RNA duplexes formed by invert repeat Alu (IR-Alus) transcripts frequently present in 3′ UTR of mRNAs are the primary targets of ADAR1 and are at the heart of this interplay [83–86]. MDA5 is normally not activated by A-to-I-modified IR-Alus, because the structural irregularities in modified duplex structure prevent stable filament formation of MDA5 [87]. In the absence of A-to-I modification, however, IR-Alus form a more regular duplex structure, supporting MDA5 to form filaments and constitutively activate antiviral signaling pathways [87]. Later studies found that IR-Alus can also activate MDA5 even in the absence of the ADAR1 deficiency. In this case, gain-of-function (GOF) mutations in *IFIH1* (the gene encoding MDA5) found to cause AGS and related diseases [78, 88–90] over-stabilize MDA5 filaments, thereby allowing robust signal activation on otherwise inert IR-Alus [87].

Besides MDA5, A-to-I editing can also regulate PKR. A-to-I editing and consequent partial melting of dsRNA structure suppress PKR activity both in the presence [91–93] and absence of viral infection [86, 94–96]. The proviral role of ADAR1 editing through PKR suppression has been reported in the case of measles virus [91–93], human immunodeficiency virus (HIV) [97], vesicular stomatitis virus (VSV) [98, 99], human T-cell leukemia virus (HTLV) [100], and Orf virus [101]. Some reports have also proposed editing-independent mechanisms for PKR suppression by ADAR1 possibly through RNA sequestration [98] or through direct interaction with PKR [97].

A-to-I editing was also proposed to regulate the activities of TLR3, 7, and 8, although in this case the role of A-to-I editing was observed for single-stranded RNA (ssRNA). A study revealed that single-stranded poly(I), when presented extracellularly, could mount a potent immune response in B cells, dendritic cells (DCs), and macrophages through TLR3 [102]. Later, it was shown that around 10% inosine incorporation in ssRNA was sufficient to trigger a TLR3-mediated immune response [103]. A subsequent study showed that A-to-I editing of viral RNAs could potentiate signal activation by TLR7/8 [104]. However, the biological significance and molecular mechanism for these observations remain to be further investigated.

### 2.3 A-to-I Editing in Adaptive Immunity

A recent study showed that A-to-I editing also plays an important role in the maturation and development of T and B cells [105, 106]. Nakahama et al. showed that the p150 isoform of ADAR1 expresses abundantly in thymus and spleen [105]. Conditional knockout of *Adar1* in T cells in mice resulted in abnormal thymic maturation of T cells, decreased self-tolerance, and autoimmune symptoms including spontaneous colitis accompanied by diarrhea, bloody stools, and rectal prolapse. The phenotype could be rescued by deleting MDA5, suggesting that aberrant activation of MDA5 by unedited self-RNAs underlies T-cell developmental abnormality. Indeed, the levels of ISGs were found to be elevated in these cells limiting the intensity of T-cell receptor (TCR) signal transduction required for appropriate T-cell selection. Similarly, Marcu-Malina et al. examined B-cell conditional knockout of *Adar1* and found the importance of ADAR1 in B lymphopoiesis in mice [106]. These studies suggest that A-to-I editing indirectly contributes to the adaptive immune system development through the loss of innate immune tolerance to self-RNAs.

### 2.4 A-to-I Editing in Cancer Biology

Numerous studies have reported elevated levels of ADAR1 in cancers [107–111] leading to the speculation that RNA editing possibly assists tumors in escaping immune surveillance [112]. Three recent studies have established a more definitive role of ADAR1 in tumor proliferation and immune evasion [113–115]. These studies found that tumors often display a high level of basal type I IFN signaling, possibly initiated by certain genomic instability and its sensing by cGAS-STING pathways. This basal IFN signaling upregulates ADAR1 (p150), MDA5, and PKR, as all of them are IFN-inducible genes. Under these conditions, tumors become highly dependent on ADAR1 [114, 115]. Depletion of ADAR1 leads to MDA5-mediated inflammatory response and PKR-mediated cell death and growth arrest [113–115]. MDA5-mediated production of type I IFNs then reenforces a feedforward cycle within the tumor to perpetually produce type I IFN and maintain the level of MDA5 and PKR. IFNs also recruit CD8+ T cells to boost antitumor adaptive immune response and synergize with checkpoint blockade immunotherapy [113].

### 2.5 Cytidine-to-Uridine (C-to-U) Modification

Deamination at C4 position of cytosine results in its conversion to uracil (Fig. 2a). This editing reaction is carried out by a family of enzymes known as *APO*lipoprotein *B* mRNA *E*diting enzyme, *C*atalytic polypeptide-like (APOBEC), named after the first reported substrate ApoB mRNA [116]. In vertebrates, up to 11 members of this family have been identified to date [117]. While most of the APOBEC enzymes make dC-to-dU modification in DNA, APOBEC1 and APOBEC3A have been shown to edit RNA as well as DNA. APOBEC1-mediated RNA

editing was reported in intestine and liver tissues [118], while APOBEC3A-mediated RNA editing was found in macrophages and other monocytes [119].

Multiple studies have implicated APOBEC enzymes in antiviral defense. The antiretroviral role of APOBEC enzymes has been especially well documented for HIV [120–123]. While most studies point to the importance of DNA editing, some studies found the role of RNA editing enzymes in antiviral immunity. For example, APOBEC1 was found in model organisms to suppress propagation of HIV-1, simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), and murine leukemia virus (MLV) by causing accumulation of mutations in viral genomic RNA [124, 125]. So far, evidence in support of antiviral function of C-to-U RNA editing in humans is lacking.

Activation-induced deaminase (AID) is another member of APOBEC family that is expressed in B cells [126] and liver cells [127, 128], and plays a key role in antibody diversification through somatic hypermutation (SHM) and class switch recombination (CSR). Although these functions of AID are broadly accepted to be mediated through DNA cytidine deaminase activity [129], some speculate that AID can edit RNA and this RNA editing activity may also play a role [130–132]. Some reports suggest that AID can also function as an antiviral protein [133], although whether this activity is mediated by DNA or RNA editing remains to be further examined.

### 2.6 Other Modifications

Aside from A-to-I and C-to-U editing, about 150 different post-transcriptional RNA modifications have been identified to date [134]. The extent and diversity of modifications in higher eukaryotes are significantly greater than in prokaryote or lower eukaryotes [135]. As such, these differences appear to be favorably exploited by the vertebrate innate immune system for selective recognition of RNAs derived from microorganism against the background of cellular RNAs. In a study conducted in 2005, Karikó et al. showed that multiple types of naturally occurring RNA modifications impair TLR response, albeit to varying degrees [136]. Among the modifications tested, N6-methyladenosine (m6A) and 2-thiouridine (s2U) almost completely abrogated the TLR3 stimulatory activity. TLR7 and TLR8, on the other hand, were sensitive to 5-methylcytidine (m5C), 5-methyluridine (m5U), and pseudouridine (Ψ) modifications in addition to m6A and s2U (*see* Fig. 2b for structures; Table 1). The observed effect of chemical modification on RNA immune evasion has also provided key strategies to lower undesirable immunogenicity of therapeutic RNAs. For example, 2′ modification of uridines (2′-fluoro, 2′-deoxy, or 2′-*O*-methyl uridines) was found to significantly reduce TLR 7/8 stimulation [137, 138], while 2′-*O*-methylation not only reduced the stimulatory potential of the RNA bearing the modification but

**Table 1**
**List of RNA editing/modifications and their effects on the innate immune response**

| Modification | Catalyzing enzyme in humans | Effect on RNA sensors [reference] | | | |
|---|---|---|---|---|---|
| | | RIG-I | MDA5 | TLRs | PKR |
| A-to-I editing | ADAR 1,2; ADAT 1,2,3[a] | None [81] | Suppression [79–81, 87] | Activation [102–104] | Suppression [86, 91–96, 99, 100] |
| C-to-U editing | APOBEC1, 3A; AID | None | None | None | None |
| Other modifications: | | | | | |
| N6-methyladenosine (m6A) | METTL 3,14 | Suppression [146, 160] | None [148] | Suppression [136] | Suppression in ssRNA<br>No effect in dsRNA [151] |
| 2-Thiouridine (s2U) | MTU1 | Suppression [5] | Unknown | Suppression [136] | Suppression [151] |
| 5-Methylcytidine (m5C) | NSUN family, DNMT2 | Suppression [160] | None [148] | Suppression of TLR7, TLR8 [136, 143] | Suppression [161] |
| 5-Methyluridine (m5U) | TRM2A | Unknown | Unknown | Suppression of TLR7, TLR8 [136] | Suppression in ssRNA<br>No effect in dsRNA [151] |
| Pseudouridine (Ψ) | Pseudouridine synthase (PUS) family | Suppression [5, 160] | None [148] | Suppression of TLR7, TLR8 [136, 143] | Suppression in ssRNA, moderate suppression in dsRNA [151] |
| 2′-*O*-methylation | FTSJ 1,2,3 | Suppression [5, 144, 145] | Suppression [149] | Suppression [137–142] | Suppression [152] |

[a]ADAT enzymes are involved in tRNA processing and not linked to immune regulation

also actively antagonized TLR 7/8 activation by other unmodified stimulatory RNAs [139–142]. Substitution of all C residues with 5mC or all Us with Ψ in a synthetically designed mRNA significantly reduced the IFN response and increased mRNA translation, suggesting that immunomodulatory modifications could have a beneficial impact on the therapeutic efficacy of RNA [143].

RNA modifications also affect recognition by RLRs. In 2006, Hornung et al. showed that several naturally occurring

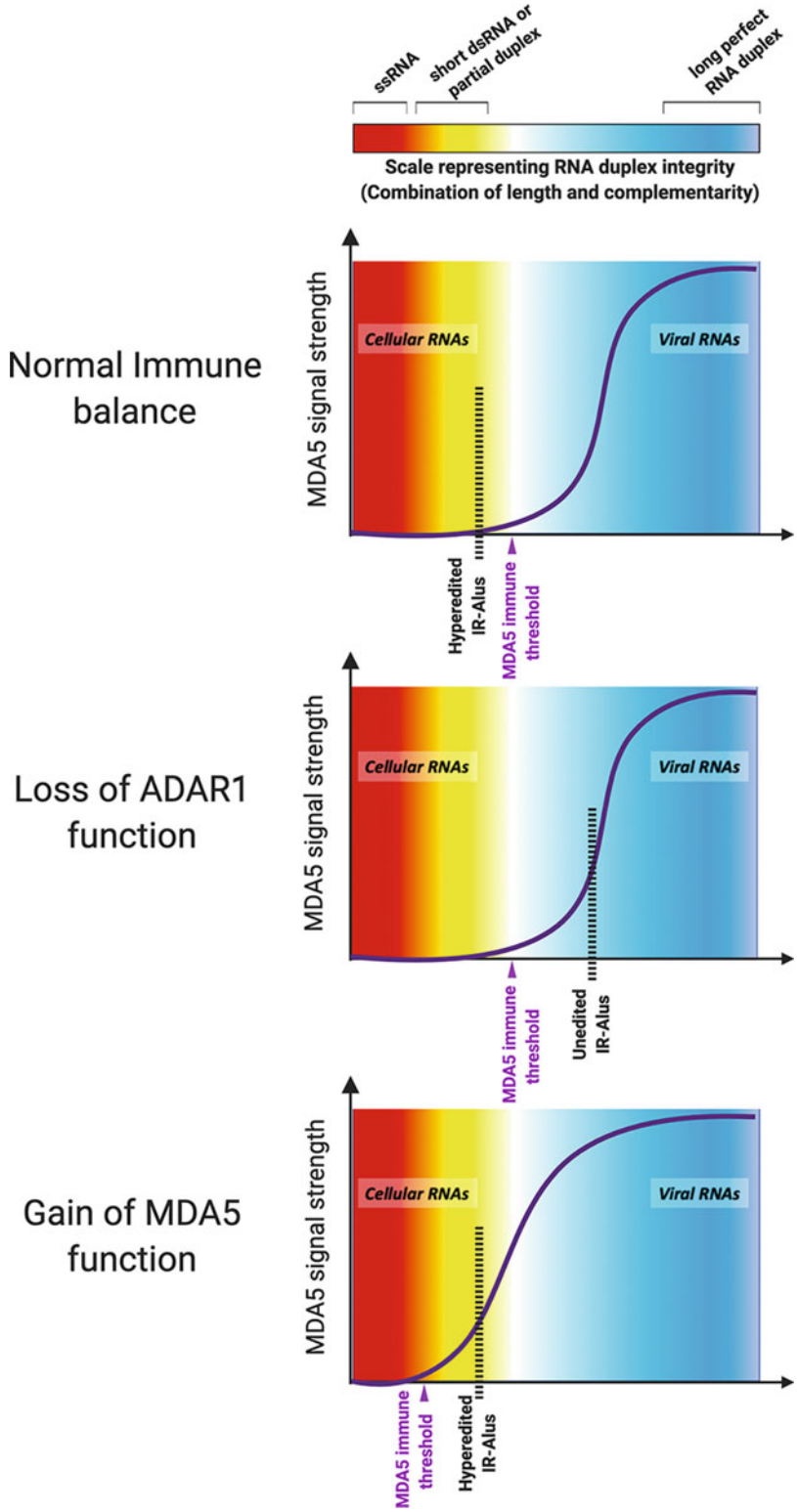


**Fig. 3** Model for ADAR1-MDA5 interplay in preventing autoinflammation. MDA5 signaling activity is dependent on dsRNA length and structural integrity. dsRNAs with longer and more regular duplex structure (as those

modifications, such as s2U, Ψ, and 2′-*O*-methylated uridine (2′-*O*-Me-U), strongly impair RIG-I stimulation [5] (Table 1). While 7-methylguanosine (m7G, Cap-0) capping of mRNA has long been thought as a primary mechanism by which RIG-I evades cellular mRNA recognition, recent studies showed that 2′-*O*-methylation at the 5′ end (Cap-1) is also required for suppressing RIG-I activity against cellular mRNAs [144, 145]. More recently, RIG-I was also found to be stimulated by circular RNAs despite the lack of an RNA end (through an unknown mechanism), and it was found that this recognition is blocked by m6A modification, a common modification in cellular circular RNAs [146, 147]. Unlike RIG-I, MDA5 is not affected by most of the RNA base modifications [148], with the exception of A-to-I editing. While viral 2′-*O*-methyltransferase that adds Cap-1 to viral mRNAs was reported to block MDA5 recognition [149], given that MDA5 is insensitive to RNA ends [150], the observed immune-suppressive role of the viral 2′-*O*-methyltransferase remains to be further examined.

As with TLRs and RIG-I, PKR is also affected by a broad range of RNA modifications. s2U-, s4U-, and 2′-deoxy modifications of dsRNA showed strong inhibition, while m5U and m6A had little effect [151] (*see* Fig. 2b for structures; Table 1). Although PKR dRBDs bind only the minor groove of dsRNA [152, 153], there is no clear segregation of minor groove vs. major groove modifications in having a PKR-suppressive effect. Intriguingly, PKR-binding affinities were similar regardless of modification, suggesting that the mechanism could be more complex or indirect. PKR was proposed to be activated by certain ssRNA with hairpin structure, and it was found that a largely distinct set of modifications were shown to affect ssRNA- vs. dsRNA-mediated PKR activation [151]. More detailed studies are required to understand exactly how ssRNA can activate PKR, and how each RNA modification affects PKR activity (Fig. 3).

**Fig. 3** (continued) expected from virally infected cells) stimulate MDA5 more efficiently. On the other hand, shorter dsRNAs or dsRNAs with irregularities (bulges, mismatches, and A-to-I modification) that are commonly present in cellular environment do not stimulate MDA5 (top). ADAR1 deficiency, however, allows cellular dsRNAs to form a more stable and regular duplex structure and trigger MDA5 signaling (middle). Among all cellular dsRNAs in human, IR-Alu stands out as the most foreign-like RNA element because it forms ~300 bp long duplex and resembles regular dsRNA the most [87]. Even in the presence of A-to-I editing, IR-Alus can activate MDA5 when MDA5 activity is altered by gain-of-function (GOF) mutations. GOF mutations over-stabilize MDA5 filaments on dsRNA, overriding the destabilizing effect of A-to-I modification and allowing MDA5 filament formation and signaling on IR-Alus (bottom). Therefore, IR-Alu is the common culprit for causing sterile inflammation under the ADAR1 deficiency or the MDA5 GOF activity (adapted from [87])

### 2.7 Cautionary Tales

In examining the effect of RNA modification/editing on immune recognition, whether by TLRs, RLRs, or PKR, one needs to keep in mind that an RNA modification may alter the immune response through indirect mechanisms, rather than by directly changing the receptor-RNA interaction. There are several reasons to be concerned that this could in fact be an experimental artifact. First of all, most studies prepare modified RNA through T7 RNA polymerase-mediated in vitro transcription, and RNA modification is introduced co-transcriptionally by using modified nucleotides in the pool of NTP. Alarmingly, a recent study showed that T7 RNA polymerase generates highly immunogenic dsRNA by-products to varying degrees, and the level of dsRNA by-product is affected by the presence of modified nucleotides during transcription [148]. The exact mechanism for this observation is yet unclear, but one could speculate that modified nucleotides may affect kinetics of RNA polymerization, which could in turn affect the synthesis of dsRNA by-products. Thus, the apparent effect of modified nucleotides on the immune-stimulatory activity of the in vitro transcript may reflect a different level of immune-stimulatory by-products, rather than the bona fide immune-modulatory effect of RNA modification.

Secondly, RNA modification can also affect the in vitro or cellular stability of RNA, either of which could affect its apparent immune-stimulatory activity [154]. For example, a recent study revealed that m6A modification destabilizes *IFNB* mRNA and therefore acts as a negative regulator of IFN response, assisting viral propagation [155]. Alternatively, RNA modifications can increase the stability of the RNA, for example by blocking its recognition by RNases in the cell or tissue culture media. In fact, RNase resistance of modified RNA may have contributed to the observed negative effect of RNA modification on TLR7/8 activity. This is because TLR 7/8 recognize degradation products of endosomal RNA [156, 157]. Considering these undesirable possibilities, extreme caution is required for RNA preparation and data interpretation.

## 3 Conclusion and Future Perspective

While RNA editing and modifications have been known for a long time, their precise physiological functions, especially in the context of the immune system, have come to light only in the past decade or so. This progress was in part facilitated by rapidly evolving technology for detecting RNA modifications and recent explosion of our knowledge on foreign RNA sensors in the innate immune system. The new advent of RNA therapeutics also propelled our understanding of the immune-modulatory role of RNA modification from a clinical application perspective. Multiple modifications

have been tested to improve the efficacy of small RNA or mRNA therapeutics with minimal immune responses, whereas in some applications, RNAs are specifically designed to stimulate innate immune-signaling activities [158, 159]. More recently, RNA modification enzymes, such as ADAR1, are considered important drug targets, as the inhibition of ADAR1 would likely boost the immune response for immuno-oncology applications [112]. It remains to be seen whether enhancing RNA editing could also have a potential therapeutic application and whether RNA editing can be directed to a certain target in a site-specific manner (as with CRISPR-mediated DNA editing). We await the exciting era of new discoveries of RNA modifications and deeper understanding of their roles in the immune system and potential therapeutics.

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