mRNAs in response to O cell RNA and polyI:C (Fig. 2C, 2D). IPS-1 KO severely reduced IFN-λ2/3 mRNA expression in BM-DCs and BM-Mfs in response to O cell RNA (Fig. 2C). These results indicated that IPS-1 in BM-DCs and BM-Mfs plays a crucial role in IFN-λ2/3 mRNA expression in response to cytoplasmic HCV RNA.

Mice have CD4⁺, CD8⁺, and DN DCs. Thus, we next examined the IFN-β and -λ2/3 mRNA expression in these mouse DC subsets. As seen with BM-DCs, the mouse DCs expressed IFN-β and -λ2/3 mRNA in response to polyI:C but not O cell RNA in the culture medium, whereas stimulation with polyI:C or O cell RNA by transfection strongly induced their expression (Fig. 3A, 3B). Interestingly, CD8⁺ DCs highly expressed IFN-λ2/3 mRNA in response to stimulation with polyI:C or O cell RNA by transfection compared with CD4⁺ and DN DCs (Fig. 3A, 3B), and IPS-1 KO but not TICAM-1 KO severely reduced IFN-λ2/3 expression in CD8⁺ DCs in response to O cell RNA transfection (Fig. 3C, 3D). This indicated that IPS-1 was essential for IFN-λ2/3 mRNA expression in CD8⁺ DCs in response to cytoplasmic HCV RNA.

It was recently reported that exosomes mediate cell-to-cell transfer of HCV RNA from infected cells to cocultured DCs (27). We examined the production of IFN-β and -λ2/3 by CD8⁺ DCs that were cocultured with O cells and Oc cells. Coculture with O cells but not Oc cells induced IFN-β and -λ2/3 production by CD8⁺ DCs (Fig. 4A, 4B). Interestingly, TICAM-1 KO abolished IFN-λ2/3 mRNA expression and protein production, whereas IPS-1 KO failed to reduce IFN-λ2/3 mRNA expression and protein production in CD8⁺ DCs (Fig. 4C, 4D). This suggested that TICAM-1 but not IPS-1 was essential for IFN-λ2/3 production by CD8⁺ DCs when cocultured with hepatocytes with HCV replicons.

Type III IFN increases RIG-I expression in CD8⁺ DC

The receptor for type III IFN consists of IL-10RB and IL-28Rα subunits (8). DN and CD4⁺ DCs and NK cells did not express IL-28Rα mRNA, whereas CD8⁺ DCs expressed both IL-10RB and IL-28Rα mRNAs (Fig. 5A). Thus, we investigated the effects of IFN-λ on DC function.

First, we examined DC cell surface markers. Unlike IFN-α, IFN-λ3 hardly increased CD40, 80, and 86 surface marker expressions on CD8⁺ DCs (Fig. 5B). Second, we examined the effects of IFN-λ3 on cross-priming because CD8⁺ DCs have high cross-priming capability. OVA, IFN-α, and/or IFN-λ3 were i.p. injected into mice according to the indicated schedules (Fig. 5C). Seven days after injection, OVA (SL8)-specific CD8⁺ T cells in spleens were quantified by tetramer staining. For a positive control, OVA and polyI:C were i.p. injected into mice. The results showed that IFN-

λ3 failed to increase OVA-specific CD8⁺ T cells in the spleens and suggested that IFN-λ3 failed to promote cross-priming at least in our experimental condition (Fig. 5C).

Third, we examined NK cell activation by DCs. NK cells and DCs were isolated from mouse spleens and were cocultured for 24 h in the presence of IFN-α, λ3, or polyI:C. Although IFN-γ production was increased by IFN-α stimulation, IFN-λ3 failed to increase IFN-γ production (Fig. 5D). Next, we investigated a cell surface marker for NK cells when cocultured with DCs. The expression of CD69, a NK cell activation marker, was not increased by IFN-λ3 stimulation (Fig. 5E). These results indicated that, unlike IFN-α, IFN-λ3 failed to enhance the activation of NK cells by DCs.

Fourth, we investigated the expression of antiviral genes in CD8⁺ DCs in response to IFN-λ3 stimulation. Interestingly, IFN-λ3 stimulation increased RIG-I and Mx1 but not TLR3 mRNA expression in CD8⁺ DCs (Fig. 6A). In addition, pretreatment with IFN-λ3 augmented IFN-λ2/3 mRNA expression in CD8⁺ DCs in response to HCV RNA (Fig. 6B). Taken together, type III IFN induced RIG-I and antiviral protein expression but failed to promote DC-mediated NK cell activation and cross-priming.

Hepatocytes express type III IFN receptors. Thus, we examined the effects of IFN-λ on mouse hepatocytes. As with IFN-α, IFN-λ3 stimulation induced both TLR3 and RIG-I mRNA expression in mouse hepatocyte (Fig. 6C). Antiviral nucleases, ISG20 and RNaseL, and an IFN-inducible gene, Mx1, were induced by IFN-λ3 or IFN-α treatment (Fig. 6C). Pretreating mouse hepatocytes with IFN-λ3 enhanced IFN-β and -λ2/3 mRNA expression in response to stimulation with HCV RNA by transfection (Fig. 6D). These results indicated that IFN-λ3 induced cytoplasmic antivirus protein expression in mouse hepatocytes. We confirmed that IFN-λ3 treatment significantly reduced HCV RNA levels in O cells with HCV replicons (Fig. 6E). A previous study also reported that IFN-λ inhibits HCV replication (13).

Discussion

Previous studies have established the importance of the TLR3 pathway for type III IFN production in response to polyI:C (15) or HCV (17). In this study, we established the importance of IPS-1–dependent pathway for type III IFN production in response to cytoplasmic HCV RNA in vivo and in vitro using a mouse model. These data indicated that there are at least two main pathways for type III IFN production in vivo, as follows: one is TICAM-1 dependent, and the other is IPS-1 dependent.

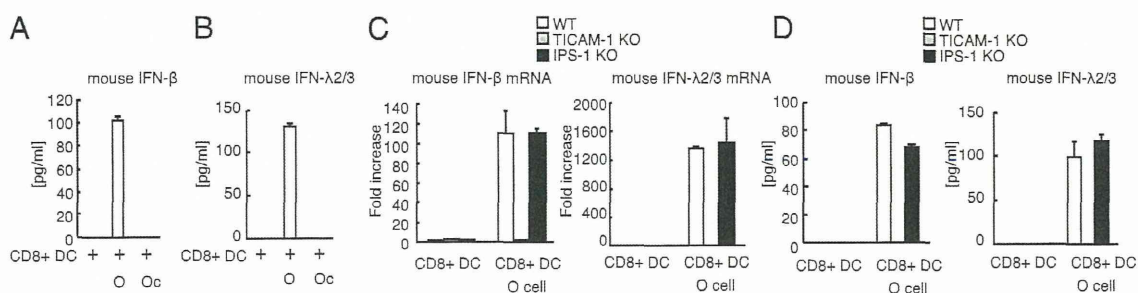We revealed that IFN-λ was efficiently produced by CD8⁺ DCs, the mouse counterpart of human BDCA3⁺ DCs, in response to

FIGURE 4. IFN-β and –λ production by CD8⁺ DCs cocultured with hepatocytes with HCV replicons. (**A** and **B**) CD8⁺ DCs isolated from wild-type spleens were cocultured with O cells (with HCV replicons) or Oc cells (without HCV replicons). After 24 h of coculture, IFN-β (A) and –λ2/3 (B) concentrations in culture medium were determined by ELISA. (**C**) CD8⁺ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons for six hours, and then IFN-β and –λ2/3 mRNA expression was determined by RT-qPCR. (**D**) CD8⁺ DCs isolated from wild-type, TICAM-1 KO, or IPS-1 KO spleens were cocultured with O cells with HCV replicons. IFN-β and –λ2/3 concentrations in culture medium were determined by ELISA.
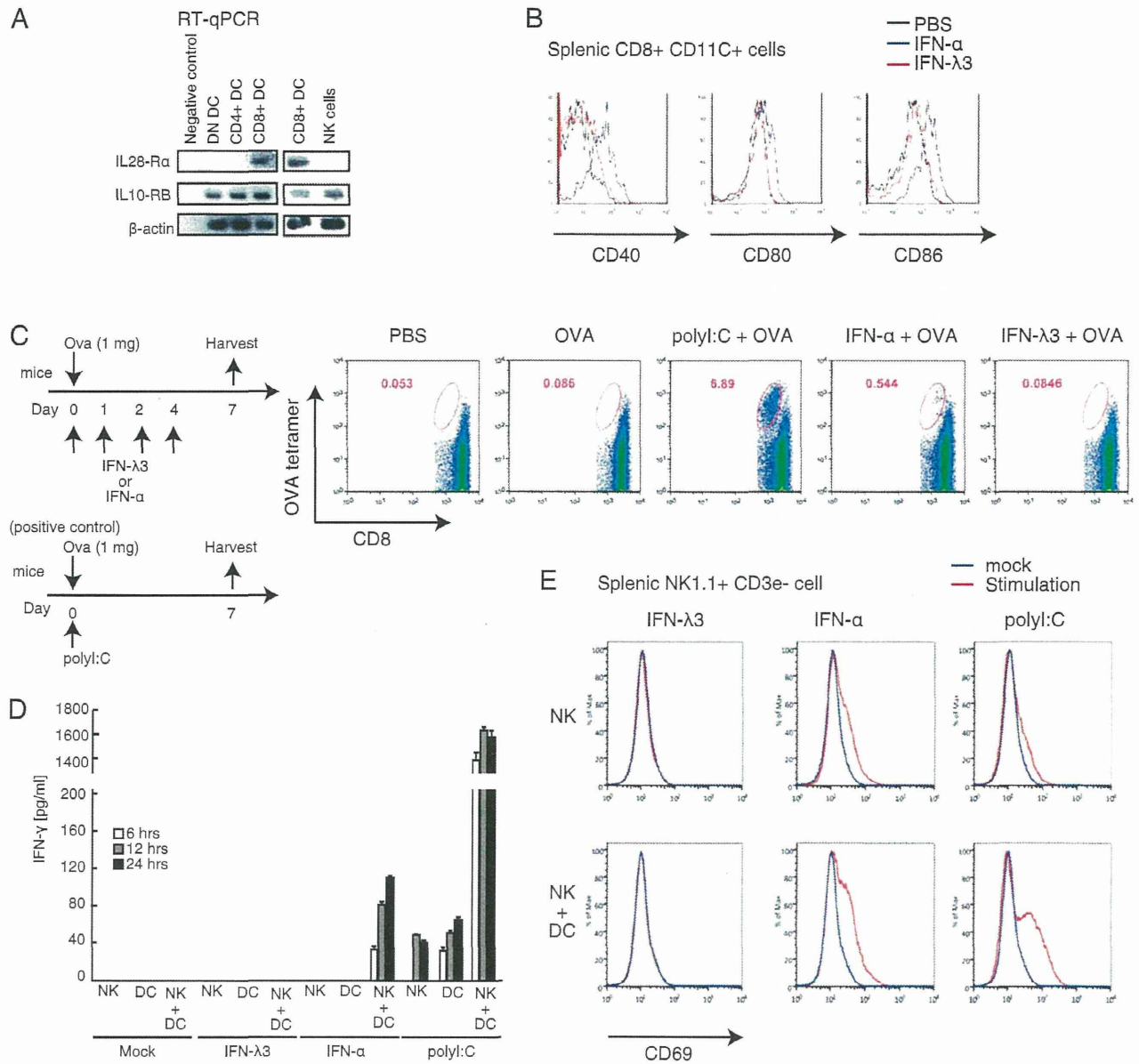
FIGURE 5. IFN-λ effects on DC functions. (**A**) DN, CD4^{+}, CD8^{+} DCs, and NK cells were isolated from wild-type mouse spleens. IL-28Rα and IL-10RB mRNA were determined by RT-PCR. (**B**) A total of 0.5 μg IFN-λ3 or 1 × 10^{5} IU IFN-α was i.p. injected into mice. Six hours after injection, spleen CD8^{+} DCs were isolated, and cell surface expressions of CD40, 80, and 86 were determined by FACS analysis. (**C**) OVA and IFN-λ or IFN-α were i.p. injected into mice on day 0, and then IFN-λ or IFN-α was injected into mice on days 1, 2, and 4. Spleens were excised on day 7, and OVA (SL8)-specific CD8^{+} T cells were determined by a tetramer assay. For a negative control, PBS in place of IFN was injected on days 0, 1, 2, and 4. For a positive control, polyI:C and OVA were injected into mice on day 0. (**D**) NK cells and CD11c^{+} DCs were isolated from mouse spleens and then stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 100 μg/ml polyI:C. IFN-γ concentrations in the culture medium at the indicated times were determined by ELISA. (**E**) NK cells were isolated from mouse spleens and then cultured with or without spleen CD11c^{+} DCs. Cells were stimulated with 1000 U/ml IFN-α, 100 ng/ml IFN-λ3, or 20 μg polyI:C. CD69 expression on NK cells was determined by FACS analysis.

cytoplasmic HCV RNA. Moreover, our data showed that IFN-λ stimulation increased the mRNA expression of RIG-I but not that of TLR3 in CD8^{+} DCs, and CD8^{+} DCs required IPS-1 to produce IFN-λ in response to stimulation with cytoplasmic HCV RNA. Furthermore, IFN-λ enhanced the mRNA expression of IFN-λ itself in CD8^{+} DCs, which suggested a positive feedback loop for IFN-λ mRNA expression in CD8^{+} DCs. IFN-λ failed to promote DC-mediated NK activation or cross-priming at least in our experimental conditions, whereas antiviral proteins, such as ISG20 and RNaseL, were efficiently induced by IFN-λ stimulation in hepatocytes and CD8^{+} DCs. These results established a novel role of IPS-1 in innate immune response against HCV via IFN-λ

production. IFN-λ pretreatment markedly increased IFN-β mRNA expression in response to HCV RNAs in mouse hepatocyte but not in CD8^{+} DCs (Fig. 6B, 6D). Although the underlying mechanism is unclear, it is possible that there is a cell-type–specific role of IFN-λ.

It was recently reported that BDCA3^{+} DCs require TLR3 for type III IFN production in response to cell-cultured HCV (17). They used a HCV 2a JFH1 strain that cannot infect human DCs in vitro (5). We also showed that the TLR3 adaptor TICAM-1 was essential for type III IFN production by CD8^{+} DCs when cocultured with O cells with HCV replicons. Thus, TLR3 appears to be essential for type III IFN production by DCs that are not infected with HCV. It
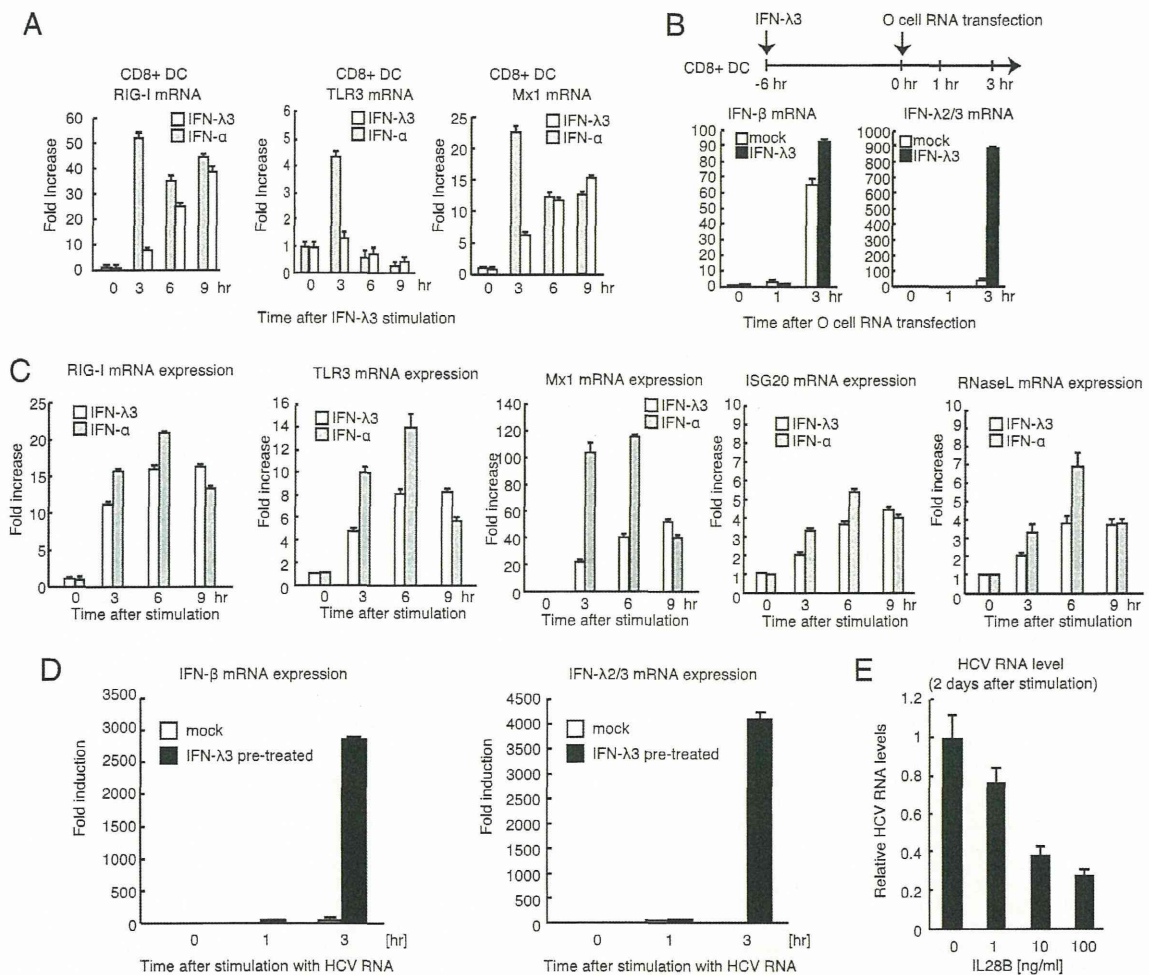
FIGURE 6. Antiviral responses induced by IFN-λ. (**A**) Mouse spleen CD8⁺ DCs were stimulated with 100 ng/ml IFN-λ3 or 1000 IU/ml IFN-α, after which RIG-I, TLR3, and Mx1 mRNA levels were determined by quantitative RT-PCR. (**B**) Mouse spleen CD8⁺ DCs were treated with 100 ng/ml IFN-λ3 for 6 h. O cell RNA was transfected into CD8⁺ DCs, and IFN-β and -λ2/3 mRNA levels were determined by quantitative RT-PCR at the indicated times. (**C**) Mouse hepatocyte cell line cells were stimulated with 1000 U/ml IFN-α or 100 ng/ml IFN-λ3. RIG-I, TLR3, Mx1, ISG20, and RNaseL mRNA levels were determined by quantitative RT-PCR. (**D**) Mouse hepatocyte cell line cells were treated with 100 ng/ml IFN-λ3 for 6 h, and then O cell RNA was transfected into these cells. IFN-β and -λ2/3 mRNA levels were measured by quantitative RT-PCR at the indicated times. (**E**) O cells that contain HCV 1b full-length replicons were treated with human IL-28B at indicated concentration for 2 d. HCV RNA levels were determined by quantitative RT-PCR. HCV RNA levels were normalized to GAPDH mRNA expression.

has been shown that exosomes are internalized efficiently by DCs and sorted into early endosomes, where TLR3 is localized (28, 29). Unlike the transfected HCV RNA, exosome-enclosed HCV RNA might be efficiently sorted and released within early endosomes of CD8⁺ DC, where TLR3 is localized, leading to TLR3-dependent IFN-λ2/3 production. Although HCV JFH1 infection particles fail to infect DCs in vitro, previous studies indicated that HCV infects DCs in chronically infected patients (23, 24, 30). In human patient DCs and hepatocytes infected with HCV, the IPS-1 pathway could play a pivotal role in type III IFN production.

Knockout of TICAM-1 failed to reduce IFN-λ2/3 mRNA expression in mouse liver after HCV RNA hydrodynamic injection, whereas knockout of TICAM-1 abolished IFN-λ2/3 levels in sera after HCV RNA hydrodynamic injection (Fig. 1B, 1D). Considering that there is a positive feedback loop for IFN-λ production, it is possible that TICAM-1 and IPS-1 pathways augment IFN-λ production each other in vivo; however, we do not exclude a possibility that TICAM-1 is involved in posttranscriptional step of IFN-λ production.

HCV NS3-4A protease cleaves IPS-1 to suppress host innate immune responses (31, 32). However, it is notable that a mutation

within the *RIG-I* gene in HuH7.5 cells increases cellular permissiveness to HCV infection (33). This indicates that the RIG-I pathway is functional at least during the early phase of HCV infection before NS3-4A cleaves IPS-1. Thus, we propose that IPS-1 is important for type III IFN production during the early phase of HCV infection.

IFN-α augmented DC-mediated NK cell activation and crosspriming, whereas IFN-λ failed to augment DC-mediated NK cell activation and cross-priming in our experimental conditions. However, as seen with IFN-α, IFN-λ could induce RNaseL and ISG20 mRNA expression. These data indicated that IFN-λ induces cytoplasmic antiviral proteins to eliminate infected virus. A previous study showed that IPS-1 is required for initial antiviral response but dispensable for the protective adaptive immune response to influenza A virus (34). Thus, it is expected that IPS-1–mediated IFN-λ production would be required for initial antiviral response to HCV infection.

In summary, our results provide insights into type III IFN production mechanism in response to HCV RNA in vivo and identify IPS-1 as a molecule crucial for producing type III IFN from hepatocyte and CD8⁺ DCs in response to cytoplasmic HCV RNA.

Acknowledgments

Disclosures

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REVIEW ARTICLE

Beyond dsRNA: Toll-like receptor 3 signalling in RNA-induced immune responses

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The innate immune system recognizes pathogen- and damage-associated molecular patterns using pattern-recognition receptors that activate a wide range of signalling cascades to maintain host homoeostasis against infection and inflammation. Endosomal TLR3 (Toll-like receptor 3), a type I transmembrane protein, senses RNAs derived from cells with viral infection or sterile tissue damage, leading to the induction of type I interferon and cytokine production, as well as dendritic cell maturation. It has been accepted that TLR3 recognizes perfect dsRNA, but little has been addressed experimentally with regard to the structural features of virus- or host-derived RNAs that activate TLR3. Recently, a TLR3 agonist was identified, which was a virus-derived 'structured' RNA with incomplete stem structures. Both dsRNA and structured RNA are similarly internalized

through clathrin- and raftlin-dependent endocytosis and delivered to endosomal TLR3. The dsRNA uptake machinery, in addition to TLR3, is critical for extracellular viral RNA-induced immune responses. A wide spectrum of TLR3 ligand structures beyond dsRNA and their delivery systems provide new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present paper, we focus on the system for extracellular recognition of RNA and its delivery to TLR3.

Key words: dendritic cell, dsRNA, endocytosis, innate immunity, structured RNA, Toll-like receptor (TLR), type I interferon, uptake receptor, viral infection.

INTRODUCTION

The immune system has developed a strategy for maintaining host homoeostasis through its interaction with environmental microbes. An array of PRRs (pattern-recognition receptors) in the innate immune system recognizes PAMPs (pathogen-associated molecular patterns) and induces anti-microbial immune responses [1]. Endosomal TLRs (Toll-like receptors) 3, 7, 8 and 9 serve as sensors of exogenous nucleic acids, whereas cytoplasmic RLRs (RIG-I-like receptors), AIM2-like receptors and DDX family members recognize intracellular viral nucleic acids [2,3]. The compartmentalization of PRRs is important for sensing both extra- and intra-cellular PAMPs and transmitting signals via distinct adaptor molecules.

Among the nucleic acid-sensing TLRs, TLR3 that recognizes dsRNA has a unique expression profile and subcellular localization [4,5]. It is expressed in immune cells, including myeloid DCs (dendritic cells) and macrophages, and in non-immune cells such as fibroblasts, epithelial cells and neurons [5–7]. TLR3 localizes to the early endosome in myeloid DCs [8], whereas macrophages, fibroblasts and some epithelial cell lines express TLR3 both on the cell surface and in the early endosome [5,9]. Although TLR3s on the cell surface participate in dsRNA recognition [5], TLR3-mediated signalling is initiated from endosomal compartments in either cell type [8].

In the case of TLR3, virus-derived dsRNA and poly(I:C) (polyriboinosinic:polyribocytidylic acid), a synthetic dsRNA,

were first identified as TLR3 ligands [4,5]. dsRNA exists as a viral genome or is generated in the cytosol during replication of positive-strand RNA viruses and DNA viruses [10]. Thus TLR3 appears to sense extracellular viral dsRNA released from infected cells and activates antiviral immunity [11]. Indeed, TLR3 mediates a protective response against positive-strand RNA virus infection, including PV (poliovirus), coxsackievirus group B serotype 3 and encephalomyocarditis virus, and DNA virus infection such as herpes simplex virus 1 and murine cytomegalovirus (Table 1) [12–19]. On the other hand, detrimental effects of TLR3 in host immunity to some RNA and DNA viruses also have been demonstrated [20–23]. Notably, TLR3-mediated signalling exacerbates negative-strand RNA virus infection, in which dsRNA is barely detectable [22,23]. In addition, RNA released from damaged cells or mRNA is also recognized by TLR3 [24,25]. However, little is known about which RNA molecules or structures activate TLR3 during infection or inflammation. We identified recently a structural unit that can activate TLR3; surprisingly, this 'structured' RNA recognized by TLR3 contains an incomplete stem with bulge and internal loops, but sufficiently induces type I IFNs (interferons) and pro-inflammatory cytokines in both human and mouse cells [26]. Hence the spectrum of TLR3 ligand structures appeared to be beyond the canonical dsRNA. The results offer new insights into the physiological role of TLR3 in virus- or host-derived RNA-induced immune responses. In the present review, we focus on exRNA (extracellular RNA) recognition and signalling by TLR3.

Table 1   Role of TLR3 in viral infections

Viral genome	Protection	Deterioration
(+) ssRNA	Poliovirus [12,13], coxsackievirus group B serotype 3 [14] and encephalomyocarditis virus [15]	West Nile virus [20]
dsRNA	Rotavirus [16]	
dsDNA	Herpes simplex virus 1 [17,18] and murine cytomegalovirus [19]	Vaccinia virus [21]
(−) ssRNA		Influenza A virus [22] and phlebovirus [23]

Table 2   Representative TLR3 ligands identified by *in vivo* or *in vitro* experiments using reporter assay and TLR3-deficient mouse DC/macrophage stimulation

Ab, antibody; PBMC, peripheral blood mononuclear cell.

RNA ligands for TLR3	Details	*In vitro* assay	Reference(s)
Exogenous RNA			
Viral dsRNA	Reovirus genome dsRNA	Mouse DC	[4]
Viral mRNA	*In vitro* transcribed HIV gag mRNA	HEK-293/TLR3	[24]
Viral structured RNA	*In vitro* transcribed PV RNA	Mouse DC and HEK-293/TLR3	[26]
Bacterial RNA	*Escherichia coli* total RNA	HEK-293/TLR3	[73]
	dsRNA of lactic acid bacteria	Mouse DC	[74]
Endogenous RNA			
RNA from necrotic cells	RNA from necrotic neutrophils	Macrophage	[25]
	UVB-irradiated U1 RNA (small nuclear RNA)	Human PBMC	[42]
Synthetic dsRNA	Poly(I:C)	HEK-293/TLR3 and Ab inhibition	[5]
	Poly(I:C$_{12}$U)	Mouse DC	[75]
In vitro transcribed dsRNA	Measles virus cDNA	HEK-293/TLR3	[31]
	pFastBac-CPrME plasmid	Mouse DC and HEK-293/TLR3	[37,41]

RECOGNITION OF dsRNA BY TLR3

TLR3 recognizes viral or *in vitro* transcribed dsRNA in a sequence-independent manner and mediates downstream signalling via TICAM-1 (TIR domain-containing adaptor molecule-1; also known as TRIF) [27,28]. 5′-Triphosphorylation of dsRNA is dispensable for TLR3 recognition, differing from the dsRNA recognition mode of RIG-I (retinoic acid inducible gene-I) [29,30]. Furthermore, 2′-hydroxy groups are essential for TLR3 activation by poly(I:C), because 2′-O-methyl or 2′-fluoro modification of cytidylic acid abolishes the TLR3 activating ability of the I/C duplex [31].

TLR3 consists of an ECD (ectodomain) containing 23 LRRs (leucine-rich repeats) and the LRR-NT (LRR N-terminal) and LRR-CT (LRR C-terminal) regions, the transmembrane domain, the cytoplasmic linker region and the TIR (Toll–IL-1 receptor) domain [32]. Crystallized human TLR3 ECD is a horseshoe-shaped solenoid assembled from 23 LRRs, of which one face is largely masked by carbohydrate, whereas the other is unglycosylated [33,34]. The N-terminal histidine residues (His[39] in LRR-NT, His[60] in LRR1 and His[108] in LRR3) and the C-terminal His[539] and Asn[541] in LRR20 of TLR3 ECD are indispensable for dsRNA binding [33–36]. The histidine residues are protonated at endosomal pH (~pH 6.0), generating an ionic attraction with the negatively charged phosphate backbone of dsRNA. Leonard et al. [37] showed that the TLR3 ECD binds as a dimer to a 40–50 bp length of dsRNA and that multiple TLR3 ECD dimers bind to long dsRNA strands. Binding affinities increase with both buffer acidity and dsRNA length. Structural analysis of the mouse TLR3 ECD–46-bp dsRNA complex revealed that dsRNA interacts with both an N- and a C-terminal-binding site on the glycan-free surface of each TLR3 ECD, which are on opposite sides of the dsRNA [38]. The ribose-phosphate backbone is the major determinant of binding, accounting for sequence-

independent dsRNA binding to TLR3. In addition, the two LRR-CT regions come together, which is essential for stable receptor–ligand complex formation and facilitates the dimerization of the cytoplasmic TIR domain [39]. Indeed, a TLR3 mutant lacking LRR21 is constitutively active, probably because of ligand-independent dimer formation due to the altered configuration of the C-terminal TLR3 ECD structure [40].

Although a biochemical study showed that a dsRNA of 40–50 bp in length forms a stable complex with dimeric TLR3 ECD under acidic conditions (pH 5.5) [36], a dsRNA of >90 bp in length is required for TLR3-mediated cytokine production and DC maturation when added to mouse DCs [41]. Given that a dsRNA of >90 bp in length is required for stable complex formation with TLR3 at the pH within the early endosome (~pH 6.0–6.5), and that TLR3 localizes to the early endosome, TLR3 oligomerization in the early endosome is essential for downstream signalling.

RECOGNITION OF VIRUS- OR HOST-DERIVED RNA BY TLR3

Several reports suggest that TLR3 recognizes RNA molecules other than dsRNA (Table 2). In negative-sense RNA virus infections, such as influenza A virus and phlebovirus, which generate little dsRNA as intermediate replication products, TLR3-mediated inflammatory cytokine and chemokine production affects virus-induced pathology and host survival [22,23]. In addition, Karikó et al. [24] reported that *in vitro* transcribed HIV gag mRNA complexed with lipofectin activates TLR3. Cavassani et al. [25] also demonstrated that mouse macrophages responded to RNA from sterile necrotic neutrophils in a TLR3-dependent manner. However, which RNA molecules or structures of the virus- or host-derived RNAs activate TLR3 is unknown. A recent study showed that RNA from UVB-irradiated keratinocytes induces cytokine production in normal
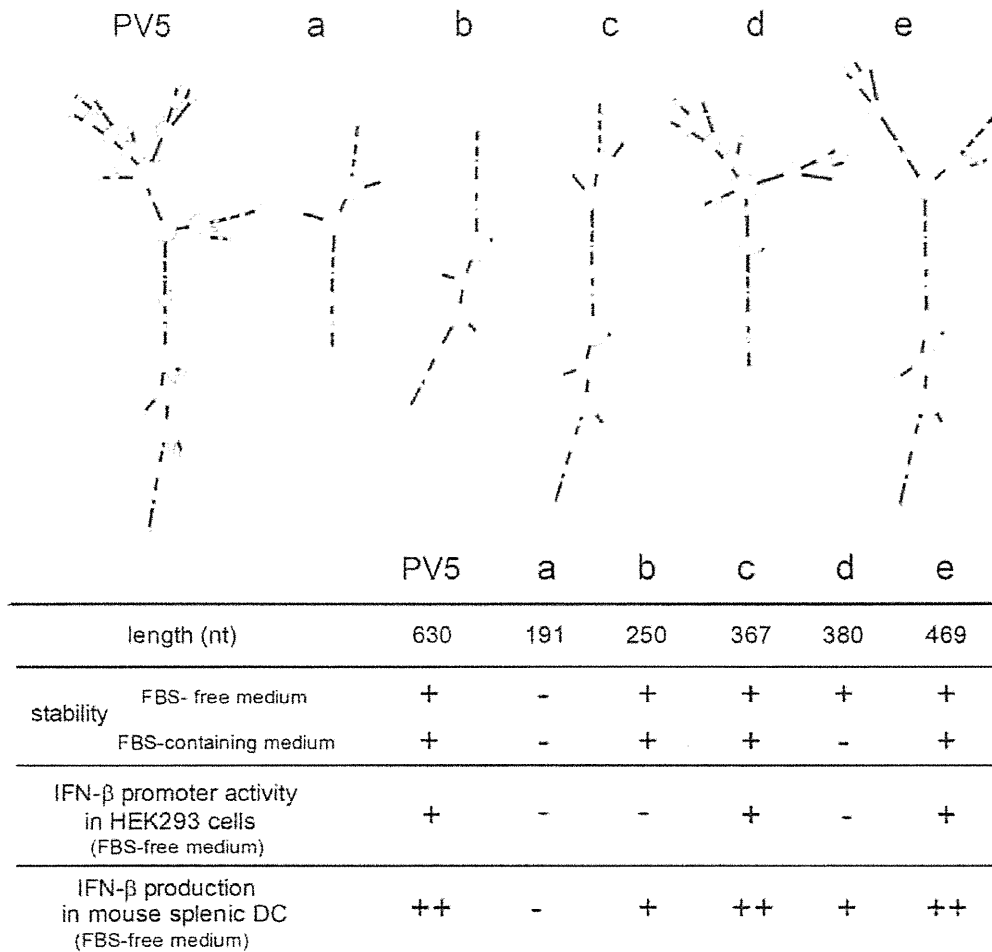
	PV5	a	b	c	d	e
length (nt)	630	191	250	367	380	469
stability — FBS- free medium	+	-	+	+	+	+
stability — FBS-containing medium	+	-	+	+	-	+
IFN-β promoter activity in HEK293 cells (FBS-free medium)	+	-	-	+	-	+
IFN-β production in mouse splenic DC (FBS-free medium)	++	-	+	++	+	++

Figure 1   RNA structures recognized by TLR3

PV5 and its derivatives (RNA a–e) were transcribed *in vitro* using PV cDNA as a template. Upper panel, secondary structure of PV5 and its derivatives (RNA a–e) predicted by the Mfold software. Thick lines indicate dsRNA regions (1–11 bp). RNAs were incubated in FBS-free or -containing medium at 37°C for 30 min. The degradability of RNAs was assessed by electrophoresis on agarose gel. The TLR3-activating ability of RNAs was assessed by IFN-β promoter reporter assay with HEK-293 cells transiently expressing human TLR3 and IFN-β production from splenic DCs isolated from wild-type and TLR3-deficient mice in FBS-free medium [26]. All RNAs failed to induce IFN-β production in splenic DCs isolated from TLR3-deficient mice. IFN-β production in mouse splenic DCs. +, <150 pg/ml; + +, >150 pg/ml.

human epidermal keratinocytes via TLR3 [42]. UVB-damaged small nuclear RNAs, including U1 RNA (165 nt in length) were the determinants of TLR3 activation, but the precise mechanism underlying how UVB-damaged U1 RNA activates TLR3 is unknown.

The point of our recent study was that TLR3 recognizes incomplete stem structures formed in viral ssRNA and induces innate immune signalling [26]. Analyses with *in vitro* transcribed PV-derived ssRNAs and dsRNAs revealed that some PV ssRNAs activate TLR3 extracellularly, but do not activate RLRs, in human and mouse cells. Stability and length of RNA are crucial factors for TLR3 activation in that case. Functional PV RNA, 630 nt in length (PV5), bound to TLR3 ECD with high affinity, and both the N- and C-terminal dsRNA-binding sites of TLR3 ECD are required for PV5-induced IFN-β promoter activation in HEK (human embryonic kidney)-293 cells that transiently express human TLR3 (Figure 1). Furthermore, PV5 was internalized into cells via clathrin- and raftlin-mediated endocytosis and co-localized with endosomal TLR3, as observed previously with poly(I:C) uptake [43,44]. The secondary structure of PV5 predicted by Mfold

software showed that PV5 possess double-strand regions (<11 bp in length) arranged in tandem, which are segmented with bulge or internal loops (Figure 1). The TLR3-activating ability of PV5 was abolished with RNaseIII treatment, indicating that the RNA duplex in PV5 is required for both the stability and functionality for the TLR3 activation. Analyses of PV5-derived RNAs partly having PV5 secondary structure (RNAs a–e in Figure 1) showed that longer stem structure with bulge and internal loops typically shown in RNA model c is the core RNA structure required for TLR3 activation in PV5 (Figure 1). Considering that dsRNA forms an A-type nucleotide duplex with 11 bp per turn [45], and that seven contiguous base pairs are needed for rapid duplex formation of DNA and RNA [46], incomplete stem structures containing contiguous base pairs may be required for stability that facilitates TLR3 binding. A fascinating model has been proposed for TLR3 dimer formation, in which shorter RNA duplexes (21–30 bp) can form less stable complexes with two TLR3 molecules [36]. Thus appropriate length or topology of multiple incomplete stems is required for TLR3 oligomerization, leading to the production of type I IFNs and pro-inflammatory
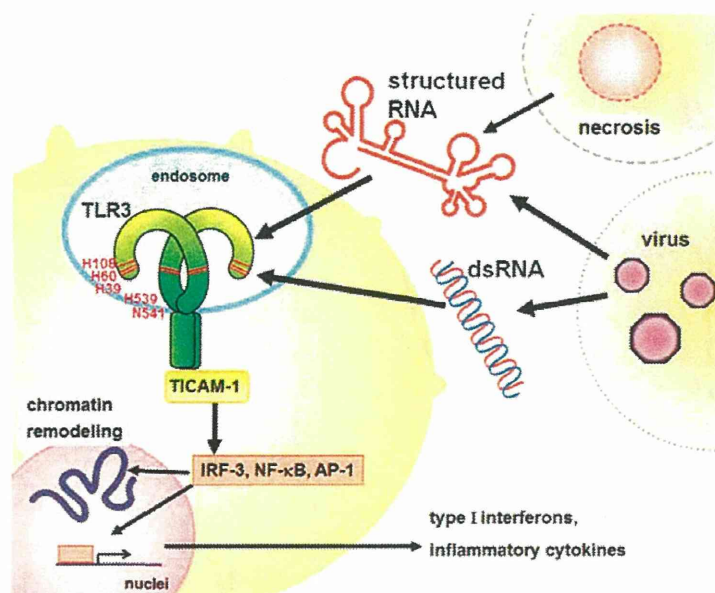
Figure 2   Model for dsRNA/structured RNA-induced TLR3-mediated immunity

Upon viral infection and sterile inflammation, virus- and host-derived RNAs are released from necrotic cells. In local environments, extracellular viral dsRNAs and virus/host-derived structured RNAs are rapidly taken up into cells via clathrin/raftlin-dependent endocytosis and delivered to endosomal TLR3. Once TLR3 is oligomerized by dsRNA/structured RNA, it recruits the adaptor protein TICAM-1 that activates the transcription factors, IRF-3, NF-κB and AP-1, leading to the production of type I IFNs and proinflammatory cytokines. The TLR3–TICAM-1 signal also induces chromatin modification in fibroblasts. In myeloid DCs, TLR3 activation triggers DC maturation capable of activating NK cells and CTLs. The key residues of TLR3, the N-terminal His[39] in LRR-NT, His[60] in LRR1, His[108] in LRR3 and the C-terminal His[539] and Asn[541] in LRR20, which are involved in RNA binding are shown.

cytokines. RNA tertiary structure is also important for both the stability and activity of PV RNA.

Notably, mouse splenic DCs responded to shorter RNAs with mismatched duplexes that failed to activate human TLR3 expressed in epithelial cells, suggesting cell type- or species-specific RNA recognition by TLR3 (Figure 1). The precise mechanisms underlying this are currently unknown, but the high density of TLR3 expression and the potent phagocytic activity of mouse splenic DCs are advantageous for RNA-induced oligomerization of TLR3 and effective RNA uptake. In a study by Ewald et al. [47], mouse TLR3 was reported to undergo cathepsin-mediated proteolytic processing in the macrophage cell line RAW in a manner similar to that of mouse TLR9. Subsequent studies also demonstrated that human TLR3 ECD is cleaved at the loop exposed in LRR12 by cathepsins in a cell-type dependent manner [48,49], and the N- and C-terminal halves of human TLR3 remain associated after cleavage [49]. Requirement of proteolytic cleavage in TLR3 signalling appears to depend on cell type [49,50]. Potentially shorter structured RNAs may be recognized by protease-processed TLR3 in mouse DCs. Further studies are required to clarify the cell type- or species-dependent RNA recognition by TLR3.

UPTAKE OF exRNA

The ability of exRNAs to induce cellular responses primarily depends on the stability of these RNAs in the extracellular milieu and whether they are taken up into cells. dsRNA is resistant to degradation compared with ssRNA and, thus, viral dsRNA released from infected cells can be a potent activator of neighbouring virus-uninfected cells, leading to the induction of anti-viral states. Poly(I:C) is the most common dsRNA in both *in vitro* and *in vivo* studies to induce cellular responses,

including type I IFN production and NK (natural killer) cell activation. Poly(I:C) is internalized into cells through clathrin-mediated endocytosis and delivered to endosomal TLR3 and to cytoplasmic MDA5 (melanoma differentiation-associated gene 5) [51]. Watanabe et al. [44] demonstrated that the cytoplasmic lipid raft protein raftlin is essential for poly(I:C) cellular uptake in human myeloid DCs and epithelial cells. In raftlin knockdown cells, surface-bound poly(I:C) neither enters the cells nor activates TLR3 and MDA5, indicating that cellular uptake is a prerequisite for dsRNA-induced cellular responses. Upon poly(I:C) stimulation, raftlin translocates from the cytoplasm to the cell surface, where it associates with the clathrin–AP-2 (clathrin-associated adaptor protein-2) complex and induces cargo delivery. Interestingly, structured PV RNA is also internalized into cells via raftlin-mediated endocytosis and is delivered to endosomal TLR3 [26]. B- and C-type ODNs (oligodeoxynucleotides) that share the uptake receptor with poly(I:C) in humans inhibit cellular uptake of PV RNA [26,43,44,52]. Hence extracellular dsRNA/structured RNA and ODNs are recognized by a common uptake receptor and their internalization is regulated by raftlin. Mouse DCs express raftlin-2 in addition to raftlin, and raftlin knockdown does not affect poly(I:C) cellular uptake, suggesting that raftlin-2 functionally compensates for raftlin [44].

The uptake receptors for poly(I:C) have been identified by several groups. Lee et al. [53] reported that CD14 enhances poly(I:C)-induced TLR3 activation by mediating poly(I:C) uptake in mouse macrophages. Furthermore, the scavenger receptor class-A was identified as a cell surface receptor for dsRNA in human bronchial epithelial cells and mouse cells [54,55]. However, knockout of these molecules does not result in complete abrogation of poly(I:C)-induced TLR3 activation, indicating the presence of another uptake receptor. Indeed, human myeloid DCs do not express CD14 on the cell surface and an inhibitor for the scavenger receptor does not affect poly(I:C) uptake in human

myeloid DCs and epithelial cells [44]. Additionally, DEC-205 was identified as a receptor for ODNs in mouse DCs [56], but this is not the case of human DCs (M. Tatematsu and M. Matsumoto, unpublished work). Hence there must be several uptake receptors that participate in the cell entry of RNAs/DNAs in a cell type- and/or species-specific manner.

exRNA-INDUCED TLR3–TICAM-1 SIGNALLING

Following TLR3 oligomerization, TICAM-1 is recruited to the TLR3–TIR domain that activates the transcription factors, IRF-3 (IRN regulatory factor-3), NF-κB (nuclear factor κB) and AP-1, leading to the production of IFN-β and proinflammatory cytokines, as well as DC maturation [57] (Figure 2). exRNA-induced TLR3–TICAM-1-mediated signalling is classified into two categories; one that induces innate responses and the other that induces adaptive immune responses. The fibroblasts and epithelial cells that express TLR3, but not TLR7, -8 and -9, produce IFN-β and proinflammatory cytokines in response to viral dsRNA and structured RNA, which induce anti-viral states by inducing IFN-stimulated genes [26]. Host RNAs released from damaged cells could be taken up through raftlin-mediated endocytosis and activate TLR3, if they form functional structures as observed in PV RNA. Bernard et al. [42] showed that small nuclear RNAs derived from UV-damaged cells induced inflammation through activation of TLR3, but how these RNAs are delivered to endosomes and interact with TLR3 remains unknown.

Another important TLR3 signal is the induction of adaptive immune responses in myeloid DCs. TLR3 is highly expressed in the professional antigen-presenting DCs, including mouse CD8α^+ DCs and human BDCA3$^+$ DCs [58,59]. Myeloid DCs mature as a result of TLR3 activation through the expression of co-stimulatory molecules, NK-activating molecules including INAM (IRF-3-dependent NK-activating molecule) [60], and unidentified molecules involved in cross-presentation pathways, leading to the activation of NK cells and CTLs (cytotoxic T-cells) [61]. The TICAM-1–TBK1–IRF3 axis downstream of TLR3 is critical for gene induction involved in mouse DC-mediated NK/CTL activation [62,63]. In addition, mouse DCs produce the Th1-type cytokines, IFN-β and IL-12 (interleukin-12), via the TLR3–TICAM-1 pathway. This facilitates NK/CTL induction. Mouse DCs efficiently phagocytose the cell debris of virus-infected cells and mature through virus RNA-induced TLR3 activation [64].

The most intriguing finding is a link between TLR3 signals and epigenetic modifications [65]. Knockdown of TLR3 or TICAM-1 blocks the induction of human iPSCs (induced pluripotent stem cells) by retroviral reprogramming in human fibroblasts [65]. Poly(I:C)-induced TLR3 activation accelerates the development of iPSCs induced by the non-viral methods in fibroblasts. TLR3 activation leads to chromatin modification in fibroblasts by promoting genome-wide epigenetic alterations. These findings enable us to offer a new concept that RNA is an extracellular mediator that accounts for a broad range of TLR3–TICAM-1-mediated gene expression compared with other RNA-sensing receptors.

CONCLUSIONS

In plants, insects and nematodes, dsRNA-induced Dicer-mediated RNA interference is a powerful strategy for protection against viral infection [66–68]. Extracellular dsRNA is taken up into cells and systematically induces gene silencing [69,70]. In *Caenorhabditis elegans*, the membrane proteins SID-1 and SID-2 act as transporters of extracellular dsRNA, whose ability is

dependent on the length of the dsRNA [71,72]. On the other hand, vertebrates have developed a wide range of anti-viral strategies, including an array of PRRs in the innate immune system, the IFN/cytokine system and the adaptive immune system. Extracellular dsRNAs are delivered to endosomal TLR3 that induce innate and adaptive anti-viral immunity. Additionally, structured RNAs with incomplete stem structures are recognized by both the dsRNA uptake receptor and TLR3, which may participate in the virus- or host-derived RNA-induced immune responses during infections or inflammation. The identification of the uptake receptor for dsRNAs and structured RNAs in human cells and also isolation of endogenous or exogenous TLR3-activating RNA molecules are important for improving our understanding of TLR3-mediated immunity.

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