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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.

Abbreviations: AP-1, activator protein-1; CTL, cytotoxic T-cell; DC, dendritic cell; ECD, ectodomain; exRNA, extracellular RNA; HEK, human embryonic kidney; IFN, interferon; IL, interleukin; iPSC, induced pluripotent stem cell; IRF-3, IFN regulatory factor-3; LRR, leucine-rich repeat; LRR-CT, LRR C-terminal; LRR-NT, LRR N-terminal; MDA5, melanoma differentiation-associated gene 5; NF-$\kappa$B, nuclear factor $\kappa$B; NK, natural killer; ODN, oligodeoxynucleotide; PAMP, pathogen-associated molecular pattern; poly(I:C), polyriboinosinic:polyribocytidylic acid; PRR, pattern-recognition receptor; PV, poliovirus; RIG-I, retinoic acid inducible gene-I; RLR, retinoic acid inducible gene-I-like receptor; TICAM-1, Toll–IL-1 receptor domain-containing adaptor molecule-1; TIR, Toll–IL-1 receptor; TLR, Toll-like receptor.

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**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

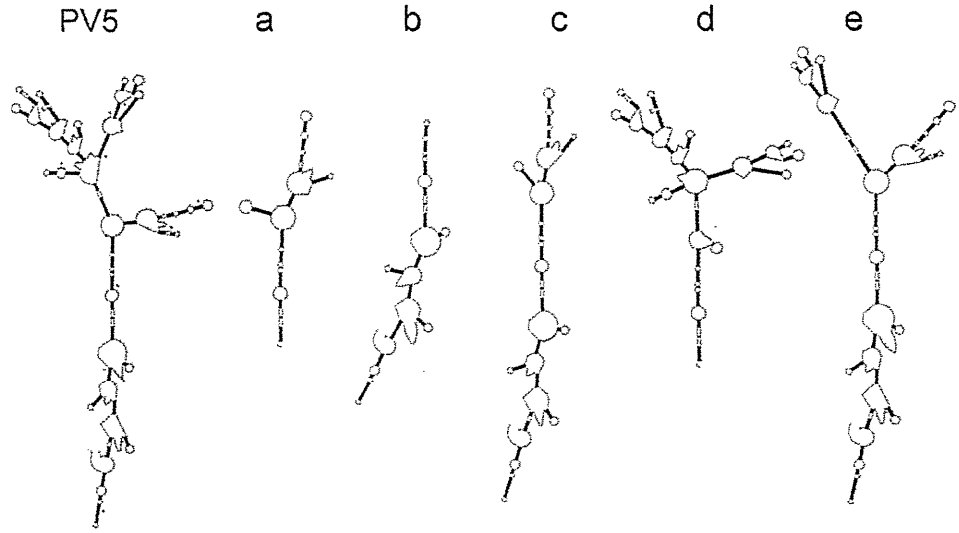
TLR3 recognizes viral or *in vitro* transcribed dsRNA in a sequence-independent manner and mediates downstream signalling via TICAM-1 (TIR domain-containing adaptor molecule-1; also known as TRIF) [27,28]. 5′-Triphosphorylation of dsRNA is dispensable for TLR3 recognition, differing from the dsRNA recognition 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

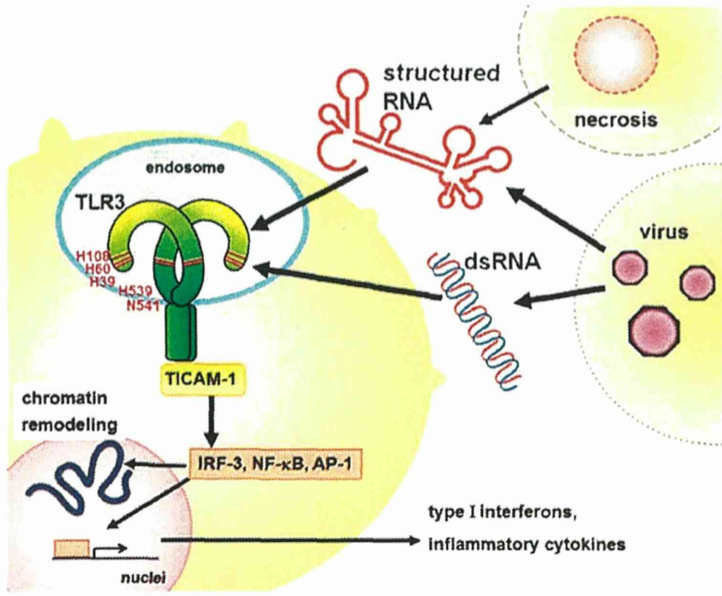



**Figure 2 Model for dsRNA/structured RNA-induced TLR3-mediated immunity**

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 pre-requisite 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-$\kappa$B (nuclear factor $\kappa$B) and AP-1, leading to the production of IFN-$\beta$ 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-$\beta$ 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 CD8$\alpha^+$ 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-$\beta$ 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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# MAVS-dependent IRF3/7 bypass of interferon β-induction restricts the response to measles infection in CD150Tg mouse bone marrow-derived dendritic cells

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ABSTRACT

Measles virus (MV) infects CD150Tg/*Ifnar* (IFN alpha receptor)$^{-/-}$ mice but not CD150 (a human MV receptor)-transgenic (Tg) mice. We have shown that bone marrow-derived dendritic cells (BMDCs) from CD150Tg/*Ifnar*$^{-/-}$ mice are permissive to MV in contrast to those from simple CD150Tg mice, which reveals a crucial role of type I interferon (IFN) in natural tropism against MV. Yet, the mechanism whereby BMDCs produce initial type I IFN has not been elucidated in MV infection. RNA virus infection usually allows cells to generate double-stranded RNA and induce activation of IFN regulatory factor (IRF) 3/7 transcription factors, leading to the production of type I IFN through the retinoic acid-inducible gene I (RIG-I)/melanoma differentiation-associated gene 5 (MDA5)-mitochondrial antiviral signaling protein (MAVS) pathway. In mouse experimental BMDCs models, we found CD150Tg/*Mavs*$^{-/-}$BMDCs, but not CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$BMDCs, permissive to MV. IFN-α/β were not induced in MV-infected CD150Tg/*Mavs*$^{-/-}$BMDCs, while IFN-β was subtly induced in CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$BMDCs. *In vivo systemic infection was therefore established by transfer of MV-infected CD150Tg/Mavs*$^{-/-}$ BMDCs to CD150Tg/*Ifnar*$^{-/-}$ mice. These data indicate that MAVS-dependent, IRF3/7-independent IFN-β induction triggers the activation of the IFNAR pathway so as to restrict the spread of MV by infected BMDCs. Hence, MAVS participates in the initial induction of type I IFN in BMDCs and IFNAR protects against MV spreading. We also showed the importance of IL-10-producing CD4$^+$ T cells induced by MV-infected BMDCs *in vitro*, which may account for immune modulation due to the functional aberration of DCs.



## 1. Introduction

Recognition of viral RNA in infected cells results in activation of IRF and induction of type I IFN, which initiates potent antiviral responses (Honda et al., 2006; Rathinam and Fitzgerald, 2011). RIG-I and MDA5 sense cytoplasmic viral RNA to activate IRF3/7 through the adaptor MAVS, while TLR3 recognizes extracellular RNA to signal IRF3/7 through the adaptor TICAM-1 (Kawai and Akira, 2006; Matsumoto et al., 2011). Each virus species has its own strategy to circumvent IFN induction, thereby successfully replicating in host cells.

MV is a negative-strand RNA virus, that infects human cells and rapidly induces a Th1 response in children which is characterized by high levels of IFN-γ and IL-2 in the early phase (Griffin et al., 1990). Paradoxically, MV infection is also accompanied by a severe suppression of the immune response that may last for months and this increases the vulnerability to secondary life-threatening infections (Schneider-Schaulies et al., 1995; Moss et al., 2004). Although consensus conclusions are limited in this issue, host dendritic cells (DCs) and acute type I IFN/IL-10 responses are critically implicated in a MV-mediated immune modulation.

It has been reported that V protein of MV wild-type strains blocks IFN-inducing signaling, thereby most wild-type strains can replicate in human cells without interfering with type I IFN

*Abbreviations:* BM, bone marrow; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation associated gene 5; MV, measles virus; RIG-I, retinoic acid inducible gene-I; TICAM1, Toll/IL-1 receptor homology domain-containing adaptor molecule 1; WT, wild-type.

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(Takeuchi et al., 2003; Shingai et al., 2007; Ikegame et al., 2010). Several laboratory-adapted strains of MV which produce defective interfering (DI) RNA (Shingai et al., 2007), and a rescued strain called Edmonston tag (Radecke et al., 1995) that harbors C272R-mutated V protein (Ohno et al., 2004), induces type I IFN and explains the mechanism of IFN induction by this MV clone (Takaki et al., 2011). Cytoplasmic RNA sensors, RIG-I and MDA5, are involved in MV RNA recognition and following type I IFN induction (Ikegame et al., 2010), that causes IFNAR-mediated amplification (Takeuchi et al., 2003). RIG-I and MDA5 deliver signals through mitochondrial antiviral signaling protein (MAVS, also called IPS-1/Cardif/VISA) (Yoneyama et al., 2008). Minimal participation of TLRs in MV replication has been reported in human cells including macrophages and dendritic cells (Murabayashi et al., 2002; Tanabe et al., 2003).

The dsRNA-sensing system is believed to be essentially the same in the human and mouse, except that the type I IFN basal level is relatively high in the intact mouse (Shingai et al., 2005). We have made mouse models for analysis of immune aberration induced by various virus infections (Matsumoto et al., 2011). Human CD150 is a main entry receptor for MV, and expressed on DCs, macrophages, T and B cells, (Tatsuo et al., 2000). *Ifnar*$^{-/-}$ mice with transgenic human CD150 (CD150Tg/*Ifnar*$^{-/-}$) have been used as a MV infection model mouse (Welstead et al., 2005; Shingai et al., 2005; Sellin et al., 2009; Koga et al., 2010) and shown that bone marrow-derived (BM)DCs are highly susceptible to MV (Shingai et al., 2005) as in human monocyte-derived or CD34$^+$ progenitor-derived DCs (Fugier-Vivier et al., 1997; Grosjean et al., 1997). Actually, transfer of MV-infected BMDCs to CD150Tg/*Ifnar*$^{-/-}$ mice facilitates establishing systemic MV infection in mice (Shingai et al., 2005).

Here, we generated CD150Tg/*Mavs*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, and CD150Tg/*Ticam1*$^{-/-}$ mouse sublines and compared the MV-permissiveness of their BMDCs to those of BMDCs from CD150Tg/*Ifnar*$^{-/-}$ mice by *in vitro* MV infection and *in vivo* BMDC-transfer analyses. We found that the IFN response initially elicited by MV was abolished in CD150Tg/*Mavs*$^{-/-}$ BMDCs, but not CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs, and therefore CD150Tg/*Mavs*$^{-/-}$ BMDCs are permissive to MV infection, similar to CD150Tg/*Ifnar*$^{-/-}$ BMDCs. We report here the results of an analysis of CD150Tg/*Mavs*$^{-/-}$ BMDCs in MV infection. Moreover, we show that MV-infected BMDCs induce the differentiation of naïve CD4$^+$ T cells into high levels of IL-10- and IFN-γ-producing T cells.

## 2. Materials and methods

### 2.1. Mice

All knockout mice were backcrossed with C57BL/6 mice more than eight times before use. CD150Tg (Shinagi et al., 2005), *Ticam1*$^{-/-}$ (Akazawa et al., 2007) and *Mavs*$^{-/-}$ (Oshiumi et al., 2011) mice were generated in our laboratory. *Irf3*$^{-/-}$/*Irf7*$^{-/-}$ double knockout (DKO) mice (Sato et al., 2000) and IL-10 Venus mice (Atarashi et al., 2011) were provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan) and Dr. K. Honda (RIKEN Research Center for Allergy and Immunology), respectively. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, who approved this study as no.08-0244. All inoculation and experimental manipulation was performed under anesthesia that was induced and maintained with pentobarbital sodium, and all efforts were made to minimize suffering. All mice were maintained under specific pathogen-free conditions in the Animal Facility at Hokkaido University Graduate School of Medicine (Sapporo, Japan) and used when they were between 6 and 12 weeks of age.

### 2.2. Cell culture

Vero/CD150 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. BMDCs were generated from bone marrow according to the method described by Inaba et al. (1992), with slight modifications. Briefly, bone marrow samples from the femurs and tibiae of mice were cultured in RPMI 1640 (GIBCO) with 10% heat-inactivated FBS containing GM-CSF (J558 supernatant) for 6 days with replenishment of the medium every other day. Splenic naïve CD4$^+$ CD25$^-$ T cells were isolated by negative selection using the biotin-CD8a, CD11b, B220, Dx5, Gr1, CD25 anitbody and streptavidin beads (Miltenyi Biotec) (typically >90% purity) (Akazawa et al., 2007). For coculture experiment, $2 \times 10^5$ CD4$^+$ T cells and $1 \times 10^4$ mock or with MV-infected BMDCs were cocultured with or without anti-CD3 antibody (0.1 μg/ml) for 4 or 6 days. For restimulation, $4 \times 10^5$ CD4$^+$ T cells were cultured with the plate bound anti-CD3 antibody (0–1 μg/ml) for 48 h.

### 2.3. Virus

IC323, corresponding to the IC-B strain of MV was recovered from the plasmid p(+)MV323 encoding the antigenomic IC-B sequence (Takeda et al., 2000). IC323-Luci (MV-luciferase) was kindly gifted from Dr. M. Takeda (Department of Virology III, National Institute of Infectious Disease, Tokyo, Japan) (Takeda et al., 2007). MV-luciferase and MV-GFP (Shingai et al., 2005) were maintained in Vero/CD150 cells (Shingai et al., 2007). Virus titer was determined as plaque forming units (PFUs) on Vero/CD150 cells and the MOI of each experiment was calculated based on this titer (Kobune et al., 1990). To measure the efficiency of *in vitro* infection, cells ($5 \times 10^4$ to $2 \times 10^5$) were harvested in 25 μl of lysis buffer for luciferase assays. Luciferase assays were performed using a Dual-Luciferase reporter assay system (Promega), and luciferase activity was read using Lumat LB 9507 (Berthold Technologies). Luciferase activity is shown as means ± S.D. of three samples.

### 2.4. In vivo infection and BMDCs transfer

Six- to 12-week-old mice were used throughout this study. Mice were infected i.p. with MV-GFP at dose of $1 \times 10^6$ pfu. At 3 and 6 days after inoculation, sera were collected from MV- or mock-infected mice. At 4 days after inoculation, CD4$^+$ cells, CD8$^+$ cells, CD11c$^+$ cells and CD19$^+$ cells were isolated from splenocytes of MV or mock infected mice using anti-CD4, anti-CD8, anti-CD11c and anti-CD19 MACS beads (Miltenyi Biotec). The purity of isolated cells was >90%. For BMDCs transfer, CD150Tg/*Mavs*$^{-/-}$ BMDCs were infected with MV (MOI = 0.25) or mock for 24 h. BMDCs were washed 4 times and resuspended with PBS. Cells ($1 \times 10^6$ cells) were intravenously transferred to CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice. After 4 days, splenocytes ($1 \times 10^7$ cells) and LNs ($1 \times 10^7$ cells) were collected and CD4$^+$ cells, CD8$^+$ cells, CD11c$^+$ cells and CD19$^+$ cells were isolated from the splenocytes. MV titers in these cells were determined by measuring luciferase activity.

### 2.5. ELISA

Culture supernatants of cells ($3–5 \times 10^5$) seeded on 24-well plates were collected and analyzed for cytokine levels with enzyme-linked immunosorbent assay (ELISA). ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories. ELISA kits for mouse IL-10, IL-13 and IFN-γ were purchased from

eBiosciences. ELISA was performed according to the manufacturer's instructions.

### 2.6. RT-PCR and real-time PCR

Total RNA was prepared using TRIzol Reagent (Invitrogen) following the manufacturer's instructions. RT-PCR was carried out using the High Capacity cDNA Reverse Transcripition kit (Applied Biosystems) according to the manufacturer's instructions. The nucleotide sequences of the primers for real-time PCR are shown in Supplemental Table I. Real-time PCR was performed using a Step One real-time PCR system (Applied Biosystems). Expression levels of target mRNA were normalized to *β-actin* and fold inductions of transcripts were calculated using the ddCT method relative to unstimulated cells.

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.08.007.

### 2.7. FACS analysis

BMDCs were stained with anti-CD11c-APC (eBiosciences) and anti-human CD150-FITC (eBiosciences) and fluorescence intensity was measured by FACS Calibur. For Foxp3 intracellular staining, cells were stained with anti-CD25-PE (eBiosciences), anti-CD4-FITC (eBiosciences) and anti-Foxp3-APC using Foxp3 staining kit (eBiosciences). For IFN-$\gamma$ intracellular staining, cells were stained with anti-IFN-$\gamma$-APC using BD Cytofix/Cytoperm kit (BD Biosciences). Stained cells were analyzed by flow cytometry.

### 2.8. Statistical analyses

Statistical significance of differences between groups was determined by the Student *t* test using Microsoft Excel software. Values of $p < 0.05$ were considered significant.

## 3. Results

### 3.1. CD150Tg/Mavs$^{-/-}$ BMDCs were permissive to MV infection

To identify the induction pathway for the type I IFN response to MV infection, we crossed CD150Tg mice with *Irf3*$^{-/-}$/*Irf7*$^{-/-}$, *Ticam1*$^{-/-}$ and *Mavs*$^{-/-}$ mice. First, we measured the expression levels of human CD150 in BMDCs derived from the CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice using FACS analysis (Fig. 1A). The expression levels of human CD150 were not changed in the BMDCs from any of the CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice (Fig. 1A). In all of the different BMDC genotypes used in this study, human CD150 expression was upregulated in response to LPS and PolyI:C and downregulated by infection with live MV and heated MV (Supplemental Fig. 1). BMDCs were infected with MV-GFP at MOI of 0.25 for 24 h and the percentage of GFP$^+$ cells was determined by FACS analysis. While CD150Tg BMDCs were barely permissive to MV compared to mock, ~5% of the CD11c$^+$ BMDCs derived from the CD150Tg/*Ifnar*$^{-/-}$ mice were infected (Fig. 1B). We expected that CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs would be permissive to MV infection, because IRF3 and IRF7 are essential molecules for type I IFN induction in response to viral infection (Sato et al., 2000). However, MV only marginally infected the BMDCs derived from the CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ mice (Fig. 1B). The CD150Tg/*Ticam1*$^{-/-}$ BMDCs were hardly as permissive to MV as CD150Tg BMDCs (Fig. 1B). Approximately 6% of CD150Tg/*Mavs*$^{-/-}$ BMDCs were infected with MV and the infection efficiency in CD150Tg/*Mavs*$^{-/-}$ BMDCs was comparable to that in CD150Tg/*Ifnar*$^{-/-}$ BMDCs (Fig. 1B). A previous report suggested that the IFN-inducing pathway in CD11c$^+$ BMDCs is critically implicated in establishment of MV infection (Shingai et al., 2005). Here, we show the molecular evidence that MAVS and IFNAR are crucial for protection against MV.

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To confirm the efficiency of MV-GFP infection in BMDCs, we used a recombinant MV-luciferase which encodes the reporter *Renilla* luciferase (Takeda et al., 2007). Luciferase activity obtained from MV-infected Vero cells was correlated with the viral titer of MV-infected cells (Supplemental Fig. 2). BMDCs were infected with MV-luciferase at MOI of 0.25 for 24 h and luciferase activity was measured (Fig. 1C). As similar to the results from MV-GFP infection, CD150Tg, and CD150Tg/*Ticam1*$^{-/-}$ BMDCs were not permissive to MV infection compared with CD150Tg/*Ifnar*$^{-/-}$ BMDCs. On the other hand, a subtle increase of luciferase activity was observed in CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs. Furthermore, the luciferase activity levels obtained from MV-infected CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ BMDCs were approximately 2-fold higher than those in CD150Tg BMDCs (Fig. 1C). These data suggest that the loss of MAVS rather than IRF3/7 critically determines MV-permissiveness in CD150Tg BMDCs: *i.e.* an additional transcription factor other than IRF3/7 participates in the protection of CD150Tg BMDCs from MV infection *in vitro*.

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### 3.2. Type I IFN induction rendered CD150Tg/Irf3$^{-/-}$/Irf77$^{-/-}$ BMDCs MV-nonpermissive

Next, to clarify the reason why MV was barely able to infect CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$BMDCs, we evaluated type I IFN expression in MV-infected BMDCs (Fig. 2A). As expected, *Ifn-α4* mRNA was induced by MV infection in CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Ticam1*$^{-/-}$BMDCs, but not in CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ or CD150Tg/*Mavs*$^{-/-}$BMDCs (Fig. 2A). IFN-α protein was also induced in CD150Tg, CD150Tg/*Ticam1*$^{-/-}$BMDCs and to a lesser extent in CD150Tg/*Ifnar*$^{-/-}$ BMDCs (Fig. 2B). The message-protein discrepancy was observed with IFN-α4 in MV-infected CD150Tg/*Ifnar*$^{-/-}$ mice as reported (Marieí et al., 1998). In contrast, *Ifn-β* mRNA expression was observed in CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$BMDCs and CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$BMDCs (Fig. 2A). *Ifn-β* was barely detected in CD150Tg/*Mavs*$^{-/-}$BMDCs. We confirmed the production of the IFN-β protein from MV-infected CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs using ELISA, and found the protein level of IFN-β slightly but firmly detected in the MV-infected *Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs (Fig. 2B). This IRF3/IRF7-independent *Ifn-β* induction was almost completely abolished by an NF-κB inhibitor (BAY11-7082) but not ATF2 inhibitor (SB203580) (Supplemental Fig. 3). These data suggest that IFN-β, but not IFN-α, is induced in CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs in response to MV infection, and then CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs become relatively resistant to MV infection. To examine this possibility, BMDCs derived from mice of various genotypes were infected with MV in the presence of the anti-IFNAR antibody. As expected, MV infected CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs in the presence of the anti-IFNAR antibody (Fig. 2C). The effect of the anti-IFNAR antibody on MV infection in CD150Tg/*Mavs*$^{-/-}$ BMDCs was weak (Fig. 2C). These results were confirmed by using MV-luciferase (Supplemental Fig. 4). These data suggest that MV infection induces IFN-β production in BMDCs in part independent of IRF3/IRF7. In contrast, due to the absence of IFN-α/β induction in the MV-infected CD150Tg/*Mavs*$^{-/-}$ BMDCs (Fig. 2A and B), MV