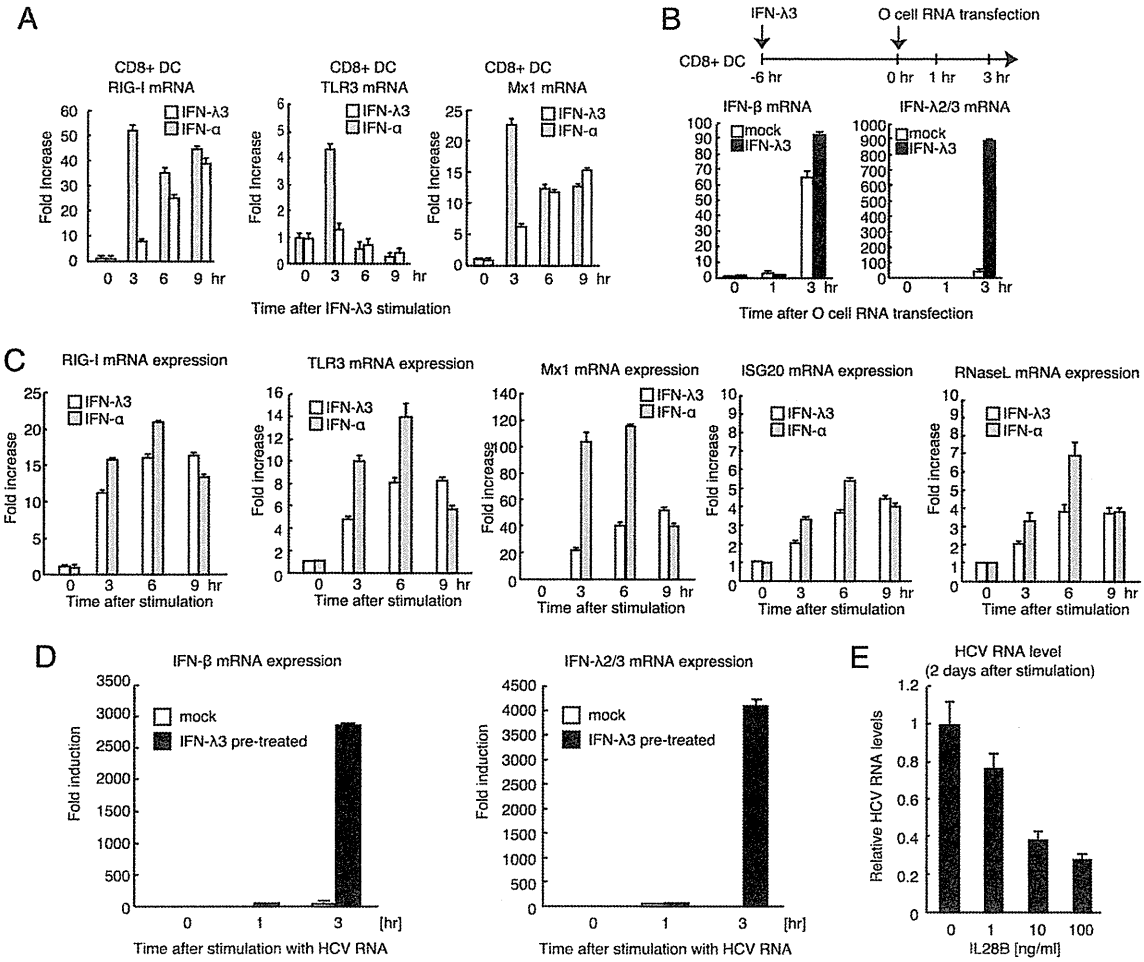

FIGURE 6. Antiviral responses induced by IFN-λ. (**A**) Mouse spleen CD8$^+$ DCs were stimulated with 100 ng/ml IFN-λ3 or 1000 IU/ml IFN-α, after which RIG-I, TLR3, and Mx1 mRNA levels were determined by quantitative RT-PCR. (**B**) Mouse spleen CD8$^+$ DCs were treated with 100 ng/ml IFN-λ3 for 6 h. 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 cross-priming, 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

IFN-λ1 and 2/3 reporter plasmids and O cells with HCV replicons were gifted from T. Imamichi (National Institutes of Health) and N. Kato (Okayama University), respectively.

Disclosures

The authors have no financial conflicts of interest.

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BJ Disease

Biochem. J. (2014) **458**, 195–201 (Printed in Great Britain) doi:10.1042/BJ20131492

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-κB, nuclear factor κB; 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

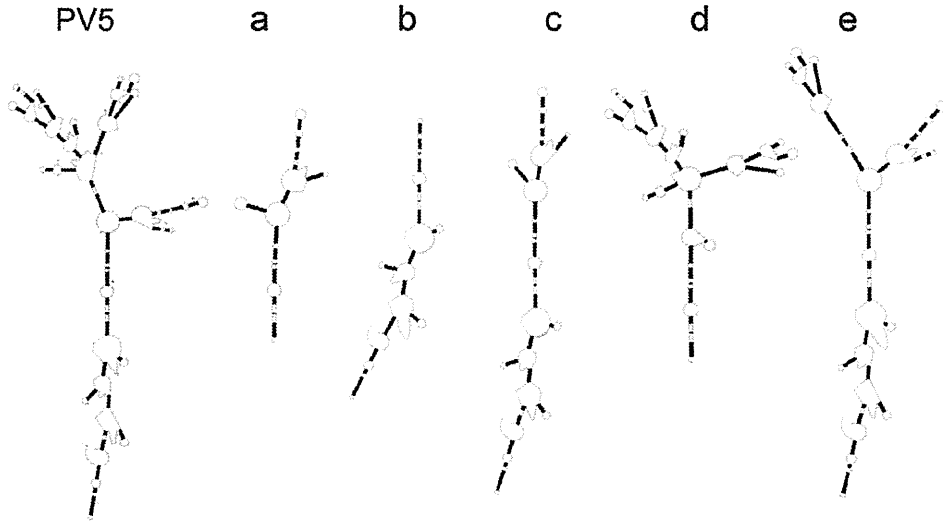
TLR3 recognizes viral or *in vitro* transcribed dsRNA in a sequence-independent manner and mediates downstream signalling via TICAM-1 (TIR domain-containing adaptor molecule-1; also known as TRIF) [27,28]. 5′-Triphosphorylation of dsRNA is dispensable for TLR3 recognition, differing from the dsRNA recognition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

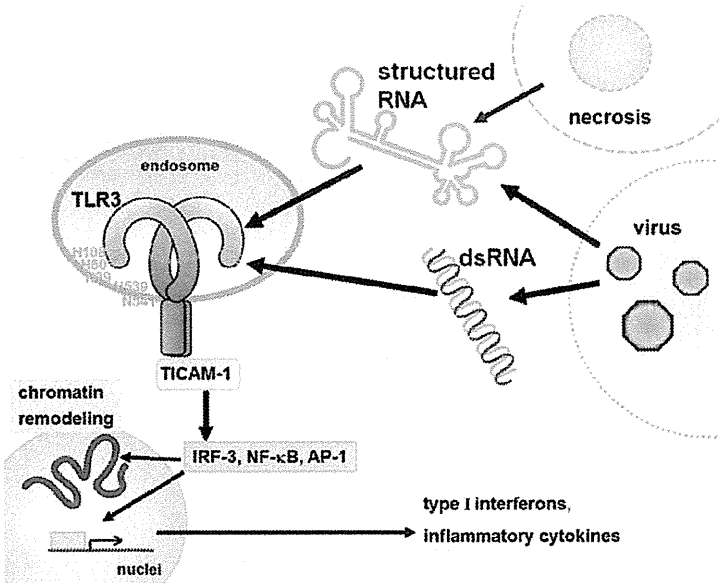


Figure 2 Model for dsRNA/structured RNA-induced TLR3-mediated immunity

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-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.

ACKNOWLEDGEMENTS

We thank Dr Fumiko Nishikawa and our laboratory members for their valuable discussions.

FUNDING

This work was supported, in part, by Grants-in-Aid from the Ministry of Education, Science, and Culture, the Ministry of Health, Labor and Welfare of Japan, and by the Akiyama Life Science Foundation.

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Received 13 November 2013; accepted 8 January 2014
Published on the Internet 14 February 2014, doi:10.1042/BJ20131492

Molecular Immunology 57 (2014) 100–110

Contents lists available at ScienceDirect

Molecular Immunology

journal homepage: www.elsevier.com/locate/molimm

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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ARTICLE INFO

Article history:
Received 7 June 2013
Received in revised form 7 August 2013
Accepted 15 August 2013
Available online 4 October 2013

Keywords:
Innate immunity
Dendritic cells
Type I interferon
Mitochondrial antiviral signaling protein (MAVS)
Measles virus

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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http://dx.doi.org/10.1016/j.molimm.2013.08.007

(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, 2×10^5 CD4$^+$ T cells and 1×10^4 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, 4×10^5 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 (5×10^4 to 2×10^5) 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 1×10^6 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 (1×10^6 cells) were intravenously transferred to CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice. After 4 days, splenocytes (1×10^7 cells) and LNs (1×10^7 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-γ intracellular staining, cells were stained with anti-IFN-γ-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.

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.

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*.

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.

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

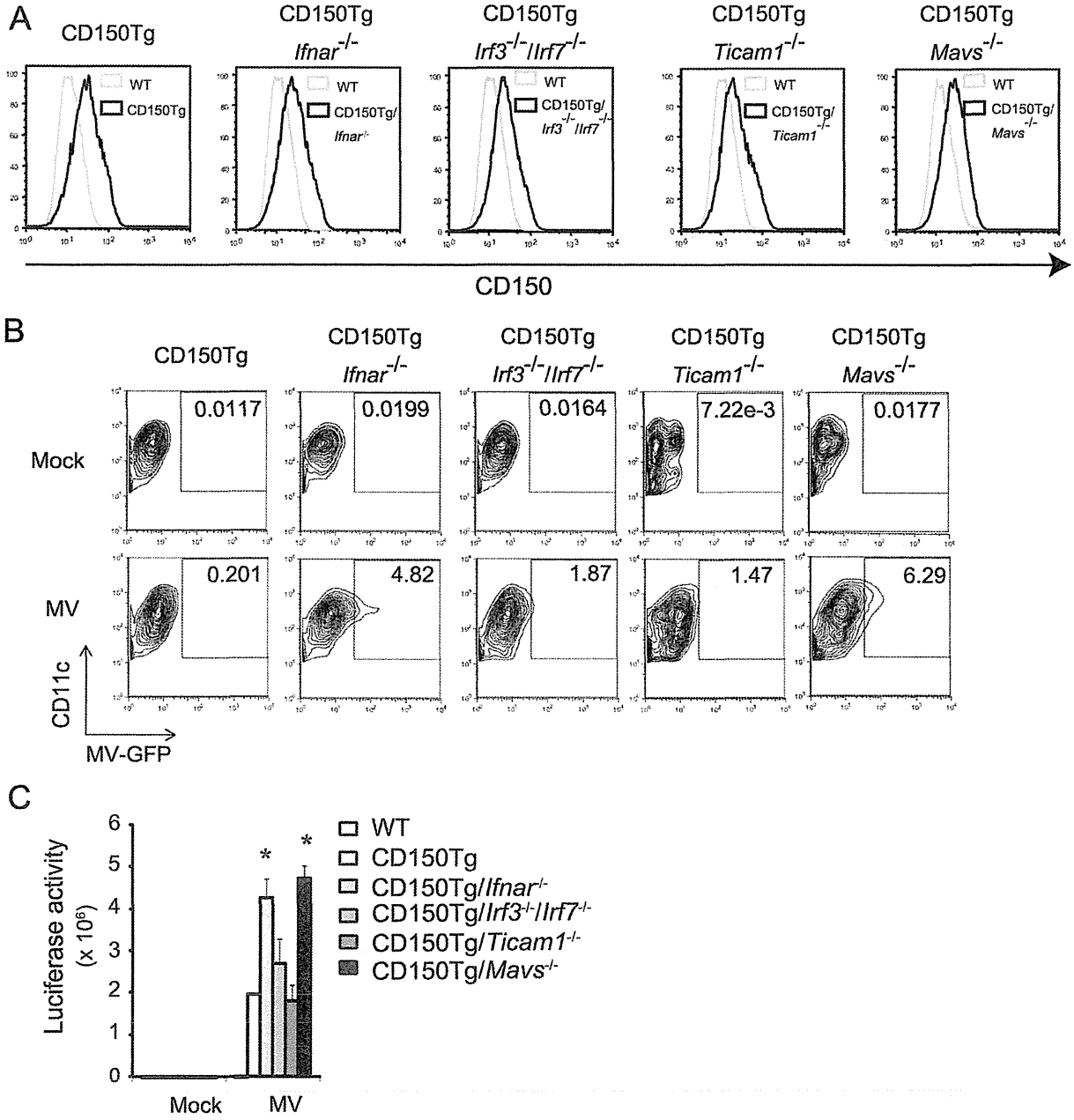

Fig. 1. CD150Tg/*Mavs*$^{-/-}$ BMDCs were permissive to MV infection. (A) Expression levels of human CD150 in BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were measured by FACS. The results are representative of three different experiments. (B) BMDCs generated from CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-GFP (MOI = 0.25). At 24 h after infection, the efficiency of virus infection was evaluated by GFP expression using FACS. The numbers indicate the percentages of cells expressing GFP. The results are representative of three different experiments. (C) BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-luciferase (MOI = 0.25). At 24 h after infection, the luciferase activity in BMDCs was measured. The data are the means ± SD of three independent samples. $^{*}p < 0.05$, MV-infected CD150Tg BMDCs vs. MV-infected knockout BMDCs.

was able to infect CD150Tg/*Mavs*$^{-/-}$ BMDCs. Moreover, type I IFN expression in response to MV infection depends on the MAVS pathway in BMDCs.

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.

We next examined whether MV-infected CD150Tg/*Mavs*$^{-/-}$ BMDCs were able to transmit virus to lymphoid cells *in vivo*. CD150Tg/*Mavs*$^{-/-}$ BMDCs infected with MV-luciferase (MOI = 0.25) were intravenously transferred into CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice (Fig. 2D). After 4 days, the spleens and lymph nodes (LNs) were harvested and the MV luciferase activity was measured. Luciferase activity was not detected in CD150Tg splenocytes and LNs when mock-infected CD150Tg/*Mavs*$^{-/-}$ BMDCs were transferred. The luciferase activity in the spleen and LNs was increased when MV-infected CD150Tg/*Mavs*$^{-/-}$ BMDCs were transferred to CD150Tg mice (Fig. 2D). This result shows that MV-infected CD150Tg/*Mavs*$^{-/-}$ BMDCs transmit virus to spleen and LN cells in CD150Tg mice. The luciferase activity

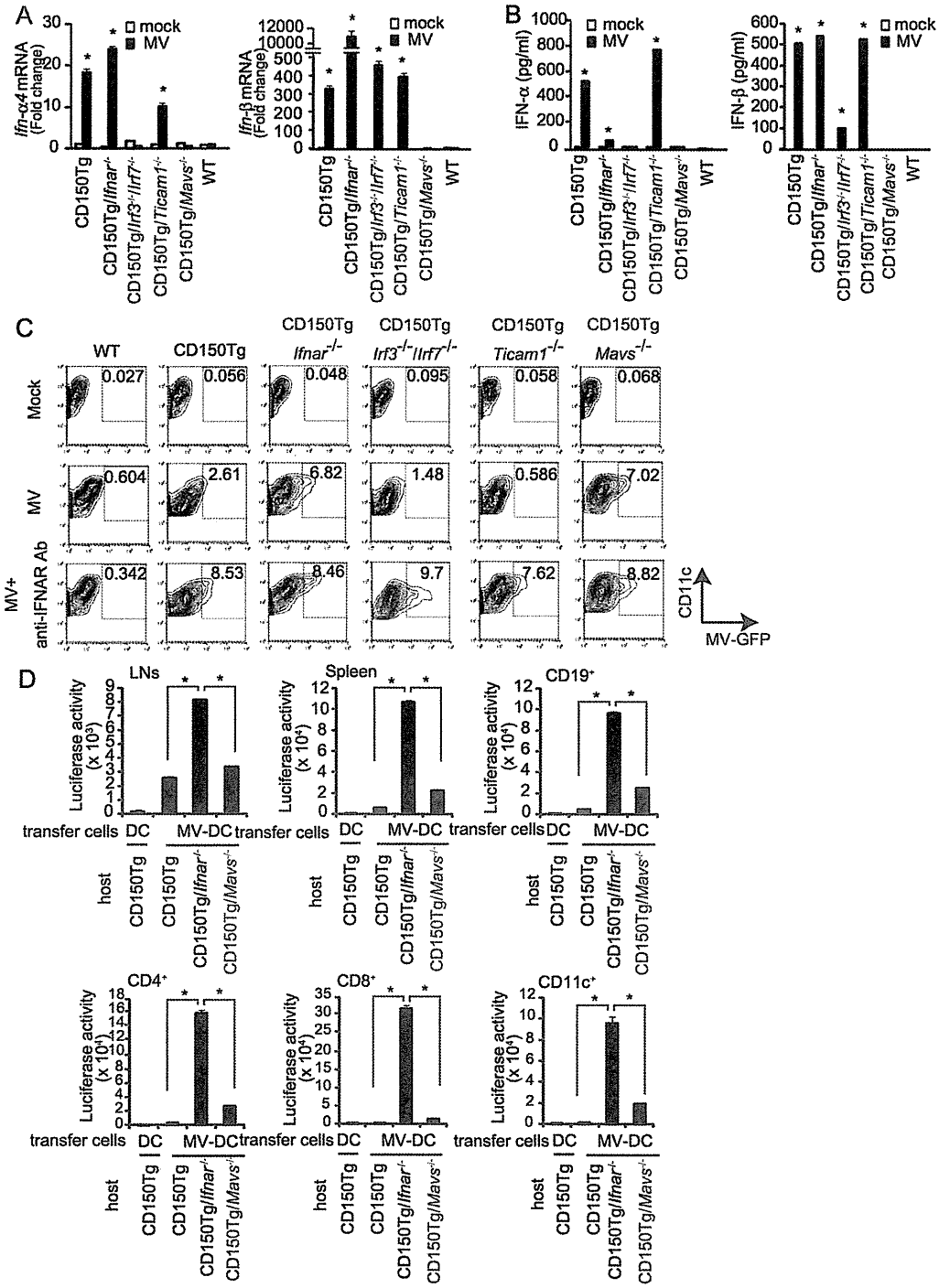

Fig. 2. MV infection did not induce type I IFN in CD150Tg/*Mavs*$^{-/-}$ BMDCs. BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-GFP (MOI = 0.25) or mock infected. (A) At 24 h after infection, *Ifn-α4* and *Ifn-β* mRNA expression was determined by real-time PCR. The data are the means ± SD of three independent samples. *$p < 0.05$, vs. mock-infected. (B) At 24 h after infection, IFN-α and IFN-β in the culture supernatants were measured by ELISA. The data are the means ± SD of three independent samples. * $p < 0.05$, vs. mock-infected. (C) BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-GFP (MOI = 0.25) or mock infected in the presence or absence of an anti-IFNAR antibody (10 μg/ml). At 24 h after infection, GFP expression was measured by FACS. The numbers shown are the percentages of cells expressing GFP. The results are representative of three different experiments. (D) BMDCs derived from CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-luciferase (MOI = 0.25) or mock infected for 24 h. BMDCs (1×10^6 cells) were washed 4 times and intravenously transferred to CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice. At 4 days after the transfer, splenocytes and LNs were collected and measured lucifrease activity. Luciferase activity was normalized by the total number of cells. Data are shown as the luciferase activity per 1×10^7 cells. The data are the means ± SD of three independent samples. * $p < 0.05$.

obtained from spleens and LNs of CD150Tg/*Ifnar*$^{-/-}$ mice with MV-infected BMDCs was much higher than CD150Tg mice. On the other hand, the efficiency of infection in the spleen and LNs of CD150Tg/*Mavs*$^{-/-}$ mice with MV-infected BMDCs was less than that for CD150Tg/IFNAR$^{-/-}$ mice. These results were confirmed with CD19$^+$, CD4$^+$, CD8$^+$ and CD11c$^+$ cells isolated from splenocytes (Fig. 2D). These results infer that the spread of MV infection is dependent on IFNAR rather than MAVS in host cells.

3.3. CD4$^+$ T cells produced IL-10 when CD4$^+$ T cells were cocultured with MV-infected BMDCs

Next, we focused on CD150Tg/*Ifnar*$^{-/-}$ cells because type I IFN induction in response to MV infection is known to be an important determinant of permissiveness to MV. MV infection reportedly induces immunosuppression in humans, non-human primates and mice (Schneider-Schaulies et al., 1995; Moss et al., 2004). DCs are thought to play a pivotal role in the pathogenesis of MV infection and elicit immunosuppressive effects during and after acute MV infection (Schneider-Schaulies et al., 2003; Servet-Delprat et al., 2003). Inducible regulatory T cells (iTreg) have also been reported to participate in immunosuppression during MV infection (Welstead et al., 2005). CD4$^+$ T cells prepared from MV-infected CD150Tg/*Ifnar*$^{-/-}$ mice produced the Th2 cytokines, IL-10 and IL-4, and the blocking of IL-10 ameliorated immunosuppression in the MV infected mice (Koga et al., 2010). Therefore, we examined whether MV-infected BMDCs affected Treg induction and the production of cytokines from CD4$^+$ T cells. MV-infected CD150Tg/*Ifnar*$^{-/-}$ BMDCs were cocultured with naïve CD4$^+$ T cells prepared from wild type (WT) mice for 6 days and then cells were subjected to intracellular staining with an anti-Foxp3 antibody, which is known to be a marker of Treg. Approximately 3% of the CD4$^+$ T cells expressed Foxp3, which was comparable to the percentage in naïve CD4$^+$ T cells cocultured with uninfected BMDCs (Fig. 3A). Population of CD25$^+$ T cells was increased when naïve T cells were cocultured with MV-infected BMDCs (Fig. 3A). A large amount of IL-10 was produced in the supernatant of naïve CD4$^+$ T cells cocultured with MV-infected BMDCs and the amount was markedly high compared to that in naïve CD4$^+$ T cells cocultured with uninfected BMDCs (Fig. 3B). Moreover, IL-10 production was dependent on anti-CD3 stimulation (Fig. 3B). IFN-γ, a Th1 cytokine, was also detected in the supernatant from naïve CD4$^+$ T cells cocultured with MV-infected BMDCs at a level that was comparable to that from naïve CD4$^+$ T cells cocultured with uninfected BMDCs (Fig. 3B). To confirm these data, we performed intracellular staining for IL-10 and IFN-γ using IL-10 reporter mice, in which a cassette containing an internal ribosomal entry site and Venus was inserted immediately before the polyadenylation signal of the *Il10* gene (referred to IL-10 Venus mice) (Atarashi et al., 2011). IL-10 Venus$^+$ CD4$^+$ T cells and IFN-γ$^+$ CD4$^+$ T cells were significantly increased when T cells were cocultured with MV-infected BMDCs (Fig. 3C). On the other hand, T cells cocultured with uninfected BMDCs expressed IFN-γ but not IL-10 Venus (Fig. 3C).

We further examined whether these CD4$^+$ T cells produced IL-10. BMDCs, either MV-infected or non-infected, were mixed with T cells in anti-CD3-coated wells (Kemper et al., 2003). After 4 days, BMDC/CD4$^+$ T-coculture cells were restimulated with plate-bound anti-CD3 antibody for 3 days and the amount of IL-10 and IFN-γ production from CD4$^+$ T cells was determined (Fig. 3D). CD4$^+$ T cells cocultured with MV-infected BMDCs produced high levels of IL-10 and IFN-γ in a manner that was dependent upon anti-CD3 stimulation (Fig. 3D). Without CD4$^+$ T cells, the IL-10 level in the MV-infected BMDCs was not increased compared to the mock-infected BMDCs (Supplemental Fig. 5). This result indicates that MV-infected BMDCs induce the differentiation of naïve CD4$^+$ T cells into IL-10- and IFN-γ-producing T cells. The expression level of *Gata3* mRNA, a master regulator of Th2, was increased when naïve CD4$^+$ T cells were cocultured with MV-infected BMDCs (Fig. 3E). *c-Maf* mRNA, a master regulator of Tr1, and *Rorgt* mRNA, a master regulator of Th17, and *Foxp3* mRNAs were decreased in CD4$^+$ T cells cocultured with BMDCs (Fig. 3E). The expression level of *T-bet* mRNA, a master regulator of Th1, was increased when naïve CD4$^+$ T cells were cocultured with BMDCs (Fig. 3E). Taken together, the results indicate that MV-infected BMDCs affect naïve CD4$^+$ T cells in such a manner as to induce IL-10- and IFN-γ-producing T cells without any induction of Treg. Although recent reports have demonstrated that IL-27 promotes IL-10 production by CD4$^+$ T cells (Stumhofer et al., 2007; Fitzgerald et al., 2007; Awasthi et al., 2007), in this setting, IL-27 only partially contributed to MV-induced IL-10 production (Supplemental Fig. 6).

Supplementary material related to this article found, in the online version, at http://dx.doi.org/10.1016/j.molimm.2013.08.007.

3.4. CD4$^+$ T cells produced IL-10 in response to MV infection

The IL-10 level in the serum prepared from MV-infected CD150Tg/*Ifnar*$^{-/-}$ mice was not different from the level in mock-infected mice (Fig. 4A). To identify cell types that produce IL-10, we isolated subsets of the splenocytes from MV- or mock-infected CD150Tg/*Ifnar*$^{-/-}$ mice and restimulated. When CD4$^+$ T cells were isolated from MV-infected CD150Tg/*Ifnar*$^{-/-}$ mice, CD4$^+$ T cells produced a large amount of IL-10 in response to an anti-CD3 antibody (Fig. 4B) (Kemper et al., 2003). CD8$^+$ T cells, CD11c$^+$ DCs and CD19$^+$ B cells did not produce any evident IL-10 even in the presence of the anti-CD3 antibody, LPS or PMA plus ionomycin, respectively. CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/IL-10 Venus/*Ifnar*$^{-/-}$ mice were infected with MV. Four days after inoculation, splenocytes were restimulated with PMA, ionomycin and brefeldin A for 6 h and subjected to FACS analysis. IL-10 Venus expression significantly induced in CD4$^+$ T cells but not CD8$^+$ T cells, CD11c$^+$ DCs nor CD19$^+$ B cells derived from MV-infected CD150Tg/IL-10 Venus/*Ifnar*$^{-/-}$ mice (Fig. 4C). Moreover, IL-10 producing CD4$^+$ T cells were different subsets from IFN-γ producing T cells (Fig. 4D).

4. Discussion

We have demonstrated that CD150Tg/*Mavs*$^{-/-}$ BMDCs were permissive to MV *in vitro*. MV infection did not induce the expression of type I IFN mRNA or protein in CD150Tg/*Mavs*$^{-/-}$ BMDCs. These data suggest that MV-derived primary type I IFN depends on the MAVS pathway in BMDCs, the result being consistent with the fact that CD11c$^+$ DCs are a primary target for replication of MV (Shingai et al., 2005).

Unexpectedly, MV infection minimally occurred in BMDCs prepared from CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ mice, because of their capacity to produce IFN-β. When anti-IFNAR antibody was present, MV was able to infect CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs. Therefore, MV-induced type I IFN production depends on not only the primary MAVS-IRF3/7 pathway but also the amplifiable IFNAR pathway in BMDCs, and that unidentified transcription factors, rather than IRF3/IRF7, participate in the primary induction of IFN-β. TLR3 signals the presence of exogenous RNA *via* the TICAM-1 adaptor (Oshiumi et al., 2003). Although TLR3/TICAM-1 participate in BMDC maturation in response to cell-derived virus RNA in RNA virus infections (Ebihara et al., 2008; Oshiumi et al., 2011), this is not the case in MV infection.

Ifn-β is reportedly induced in conjunction with the activation of transcription factors, IRF3, IRF7, ATF-2/c-Jun and NF-κB

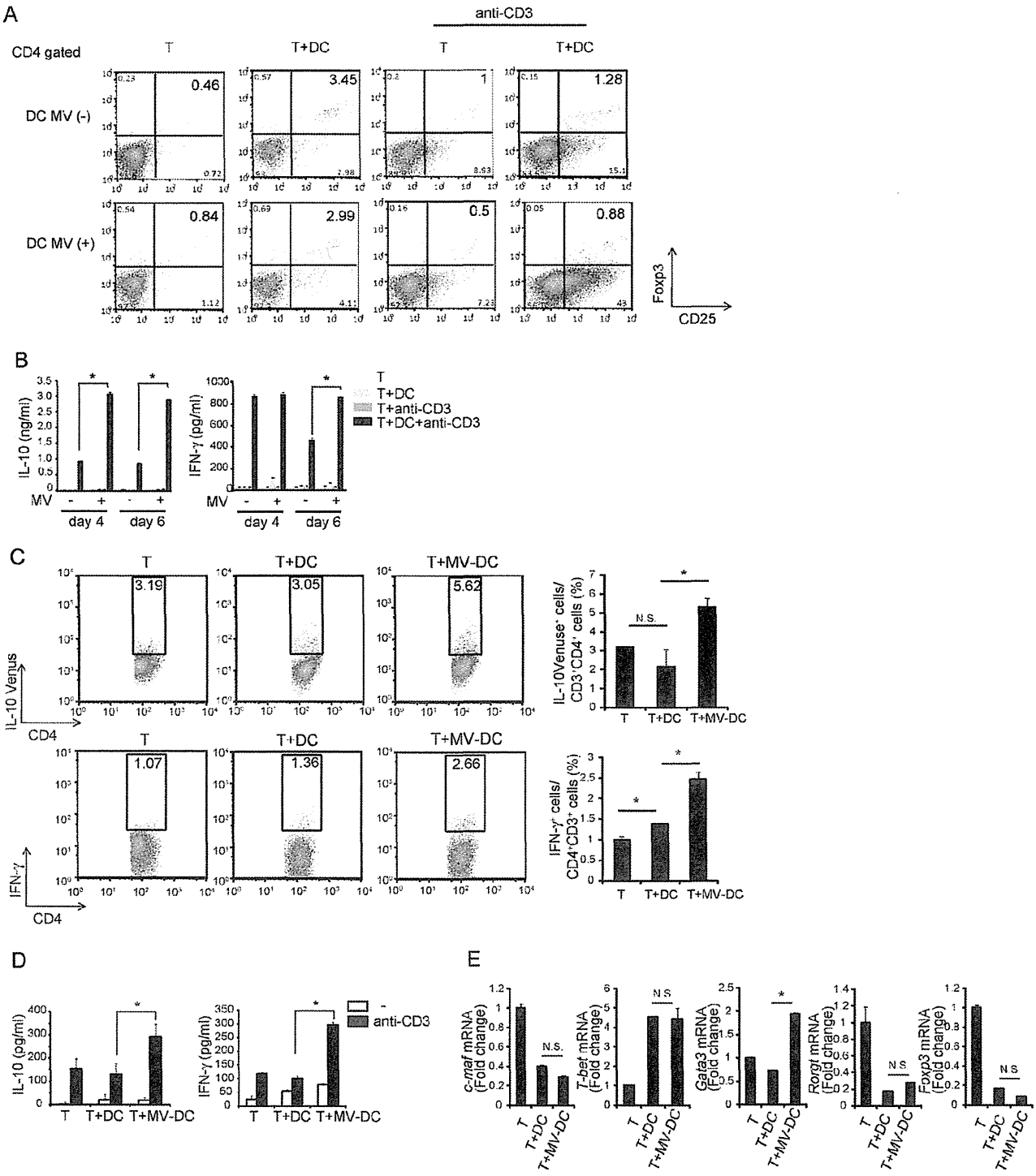

Fig. 3. MV-infected BMDCs induced IL-10 and IFN-γ producing CD4+ T cells. CD150Tg/*Ifnar*$^{-/-}$ BMDCs were infected with MV (MOI = 0.25) or mock for 24 h. Naïve CD4+CD25− T cells (2×10^5) isolated from WT mice were cocultured with 1×10^4 BMDCs in the presence or absence of 0.1 μg/ml of the anti-CD3 antibody. (A) At 6 days after coculture, cells were stained with anti-CD4, anti-CD25 and anti-Foxp3 antibodies and subjected to FACS analysis. The numbers shown are the percentage of CD25+Foxp3+ cells. The results are representative of three different experiments. (B) At 4 or 6 days after coculture, IL-10 and IFN-γ in the coculture supernatant were measured by ELISA. The data are the means ± SD of three independent samples. *$p < 0.05$. (C) CD4+ T cells isolated from IL-10 Venus mice were cocultured with uninfected or MV-infected CD150Tg/*Ifnar*$^{-/-}$ BMDCs for 4 days. Cells were stained with anti-CD3, anti-CD4 and anti-IFN-γ antibodies and analyzed by flow cytometry. The numbers shown are the percentage of CD4+IL-10 Venus+ cells and CD4+IFN-γ+ cells. The right graphs represent the fraction of the IL-10 Venus+ cell and IFN-γ+ cell populations. The data are the means ± SD of three independent samples. *$p < 0.05$. (D) At 4 days after the coculture, cells were collected and washed twice. Two x 10^5 cells were restimulated with the anti-CD3 plate-bound antibody for 3 days. At 3 days after restimulation, IL-10 and IFN-γ in the culture supernatants were measured by ELISA. The data are the means ± SD of three independent samples. *$p < 0.05$. (E). At 4 days after the cocultured, the expression level of *c-Maf*, *T-bet*, *Gata-3*, *Rorgt* and *Foxp3* mRNA in the CD4+ T cells cocultured with MV- or mock-infected BMDCs were determined by real-time PCR. The data are the means ± SD of three independent samples. N.S.; not significant, *$p < 0.05$.

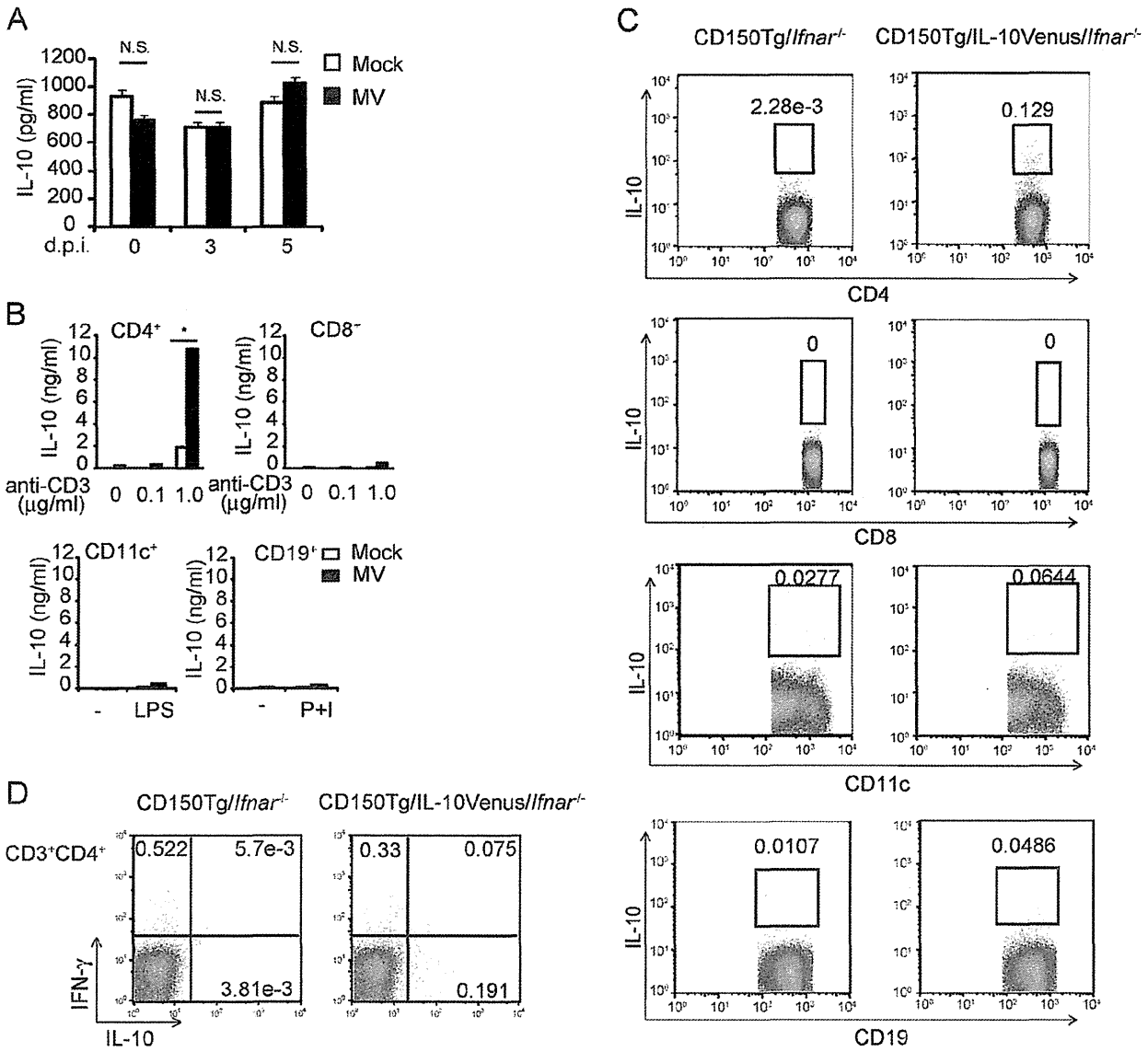

Fig. 4. CD4+ T cells produced IL-10 *ex vivo*. (A) CD150Tg/*Ifnar*$^{-/-}$ mice were infected i.p. with 1×10^6 pfu MV-GFP or mock. At the indicated days after infection, IL-10 production in sera was measured by ELISA. The data are the means ± SD of three independent samples. N.S.; not significant. (B) CD150Tg/*Ifnar*$^{-/-}$ mice were infected i.p. with 1×10^6 pfu MV-GFP or mock infected. At 4 days after infection, CD4+, CD8+, CD11c+ and CD19+ cells were isolated from splenocytes and restimulated with plate-bound anti-CD3 (0–1.0 μg/ml), LPS (100 ng/ml) or PMA (1 μg/ml) plus ionomycin (1 μg/ml) (P+I), respectively. At 3 days after restimulation, IL-10 production in the culture supernatant was measured by ELISA. The data are the means ± SD of three independent samples. $^*p < 0.05$. (C, D) CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/IL-10 Venus/*Ifnar*$^{-/-}$ mice were infected with MV (1×10^6 pfu). At 2 days after inoculation, splenocytes were stained with anti-CD3, anti-CD4, anti-CD8, anti-CD11c, anti-CD19, and anti-IFN-γ antibodies and subjected to FACS analysis. (C) The numbers shown are the percentage of IL-10+ cells. (D) The numbers shown are the percentage of the gated populations. The results are representative of three different experiments.

(Thanos and Maniatis, 1995; Panne et al., 2007). The coordinated binding of these regulatory factors synergistically augments transcription of the *Ifn-β* gene in several different cell types (Thanos and Maniatis, 1995). MAVS-dependent IRF3/IRF7-bypassed *Ifn-β* induction has also been reported to take place through the NF-κB signaling pathway in West Nile virus infection, the case being not only for DCs (Daffis et al., 2009). Recently, the MAVS/IRF5-dependent pathway was identified to participate in type I IFN induction in West Nile virus-infected myeloid cells BMDCs (Lazear et al., 2013). IRF1 is also involved in TLR9-mediated IFN-β production in BMDCs (Schmitz et al., 2007). In the case of MV infection, IRF5 and IRF1 might be candidate transcription factors for MAVS-dependent and IRF3/IRF7-independent type I IFN induction in BMDCs.

In this context, we looked for possible transcription factors other than typical IRFs. We found from a pharmacological test that the treatment of CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ BMDCs with an NF-κB inhibitor (BAY11-7082) resulted a significant reduction of MV-induced *Ifn-β* mRNA expression (Supplemental Fig. 3), suggesting that NF-κB is involved in MV-induced type I IFN expression in BMDCs. The result infers that MV mouse models harbor multiple IFN-inducing pathways and the MAVS-NF-κB axis predominantly functions in transferred BMDCs even with no IRF3/IRF7 for protection against MV infection in mice. Yet, the possible participation of IRF1 or IRF5 in NF-κB-mediated type I IFN induction has remained to be determined. This MAVS-NF-κB-mediated IFN-β induction and resultant protection against MV spread is unique to mouse BMDCs: other immune cells are protected from MV by IFNAR-STAT signaling

in the MV-infected BMDC transfer system (Shingai et al., 2005). The result reflects the essential protective role of IFNAR (that is activated by primary MAVS-derived IFN-β) from establishing systemic MV infection in mouse models (Welstead et al., 2005; Shingai et al., 2005; Sellin et al., 2009; Koga et al., 2010).

Each successful virus species has developed its own means of circumventing the host IFN system, and the RNA-sensing system was developed in the course of stepwise mutation of the viral genomes. In an earlier study, RIG-I and MDA5 were reported to be sensors for RNA structures characteristic of virus species (Kato et al., 2006). This concept was adapted to MV in human epithelial cells (Ikegame et al., 2010). However, these typical cases appear rather rare in *in vivo* virus infections, which are more complicated than the situation found in RIG-I/MDA5 knockout mice (Kato et al., 2006), depending upon the host tropism, phases and stages of virus infection. *In vivo*, RIG-I and MDA5 in epithelial cells are implicated in the formation of an infectious milieu and type I IFN production in laboratory-adapted or genetically-mutated MV strains (Takaki et al., 2011; Shingai et al., 2005), but there appears to be no *in vivo* data supporting this finding. In general, each cell type has its own dominant IFN-inducing systems by which viral infections are differentially sensed and rapidly prevented in a cell-specific manner. Here, we show that the MAVS-dependent but IRF-3/7-independent IFN-β production actually does function in CD150Tg BMDCs in response to MV infection, this pathway being unique to BMDCs for primary MV protection. Secondary protection against MV spreading to other cells is accomplished by IFNAR which prevents systemic MV infection due to BMDCs transfer. There are a number of subsets in mouse DCs, which differentially respond to MV with their IFN-inducing pathways (Takaki et al., 2013). It will be of interest to determine whether the results are reproducible in other DC subsets in the mouse MV-infection model.

In patients with measles, alteration of the cytokine profile has been reported earlier (Griffin et al., 1990). The early Th1 response is shifted to a Th2 response, which occurs during the late stages of measles, with an increase in the secretion of IL-4 and a decrease in the IL-12 levels (Naniche and Oldstone, 2000; Atabani et al., 2001). Consistent with these reports, we detected a high level of IL-13 production in the coculture supernatant of $CD4^+$ T cells and MV-infected BMDCs (data not shown). The plasma level of the anti-inflammatory cytokine IL-10 is increased in patients with measles (Atabani et al., 2001; Yu et al., 2008). This elevated level of plasma IL-10 probably contributes to the impaired cellular immunity and depressed hypersensitivity response following MV infection (Ryon et al., 2002). However, the primary DC response and source of IL-10 in MV-infected patients is at present not clear.

Recently a study reported that IL-10 is the cause of MV-induced immunosuppression in MV-infectious model mice (Koga et al., 2010). However, during MV infection, both the cells which produce IL-10 and the induction mechanism of IL-10 in these cells have yet to be elucidated. In this report, we showed that $CD4^+$ T cells are one of the cell types that produce IL-10 in response to MV infection both *ex vivo* and *in vitro*. MV-infected BMDCs induce IL-10- and IFN-γ-producing $CD4^+$ T cells, but not Treg cells. Previous reports showed that T regulatory (Tr1) cells became IL-10 and IFN-γ producing $CD4^+$ T cells (Vieira et al., 2004; Roncarolo et al., 2006), and that Tr1 cells in concert with IL-10-producing DCs were indispensable for a high level of IL-10 (Roncarolo et al., 2006). However, in Fig. 4A, IL-10 was neither produced in BMDCs nor up-regulated in mouse sera irrespective of MV-infection. It is $CD4^+$ T cells that produce IL-10 in response to MV and CD3 stimulation (Fig. 4B).

Recent reports have demonstrated that IL-27 promotes IL-10 production by $CD4^+$ T cells (Stumhofer et al., 2007; Fitzgerald et al., 2007; Awasthi et al., 2007), and the induction of c-Maf, IL-21 and ICOS has been proposed as a mechanism of IL-27-mediated Tr1 cell differentiation (Pot et al., 2009). We examined whether IL-27 was involved in MV-induced IL-10 and IFN-γ production in $CD4^+$ T cells with an anti-IL-27p28 neutralizing antibody. Blocking IL-27p28 partially suppressed IL-10 production in $CD4^+$ T cells which had been cocultured with MV-infected BMDCs (Supplemental Fig. 6), indicating that IL-27 might participate in the mechanisms of induction of MV-mediated Tr1-like cells *in vitro*.

CD150Tg/*Mavs*$^{-/-}$ BMDCs completely lack the ability to produce type I IFN, and thereby are permissive to MV infection (Fig. 2A and B). CD150Tg/*Ifnar*$^{-/-}$ mice have the full capacity to produce IFN-β in MV infection, but cannot compensate for the IFNAR-null state in BMDCs. The artificial unresponsiveness of the IFN amplification pathway to MV infection may have caused unusual immune aberrations (Welstead et al., 2005; Shingai et al., 2005; Sellin et al., 2009; Koga et al., 2010) due to the absence of any "idling" production of type I IFN in these gene-disrupted mice (Takaoka and Taniguchi, 2003). It would be likely that a lack of the amplification pathway of type I IFN also confers MV permissiveness on BMDCs in mice, even though the mice have intact MAVS pathway to produce sufficient IFN-β. The present analysis of CD150Tg/*Mavs*$^{-/-}$ BMDCs in MV infection allowed us to highlight the molecular mechanisms of initial type I IFN induction and IL-10 production by $CD4^+$ T cells in a mouse model. Further analyses using the model will contribute to elucidation of possible mechanisms by which MV induces immune modulation.

Conflict of interest

There is no declared conflict of interest in this study.

Acknowledgements

We are grateful to Dr. Taniguchi (University of Tokyo) for providing *Irf3*$^{-/-}$ and *Irf7*$^{-/-}$ mice and Dr. Honda (RIKEN Research Center for Allergy and Immunology) for providing IL-10 Venus mice for this study. We also thank to Dr. Y. Yanagi (Kyushu University) for providing MV-luciferase. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research) and the Ministry of Health, Labor, and Welfare of Japan, and by the Ono Foundation and the Itoh Foundation. Financial supports by the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT, is gratefully acknowledged.

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Structures and interface mapping of the TIR domain-containing adaptor molecules involved in interferon signaling

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Edited by Shizuo Akira, Osaka University, Osaka, Japan, and approved October 29, 2013 (received for review January 3, 2013)

Homotypic and heterotypic interactions between Toll/interleukin-1 receptor (TIR) domains in Toll-like receptors (TLRs) and downstream adaptors are essential to evoke innate immune responses. However, such oligomerization properties present intrinsic difficulties in structural studies of TIR domains. Here, using BB-loop mutations that disrupt homotypic interactions, we determined the structures of the monomeric TIR domain-containing adaptor molecule (TICAM)-1 and TICAM-2 TIR domains. Docking of the monomeric structures, together with yeast two hybrid-based mutagenesis assays, reveals that the homotypic interaction between TICAM-2 TIR is indispensable to present a scaffold for recruiting the monomeric moiety of the TICAM-1 TIR dimer. This result proposes a unique idea that oligomerization of upstream TIR domains is crucial for binding of downstream TIR domains. Furthermore, the bivalent nature of each TIR domain dimer can generate a large signaling complex under the activated TLRs, which would recruit downstream signaling molecules efficiently. This model is consistent with previous reports that BB-loop mutants completely abrogate downstream signaling.

innate immunity | TLR signaling | MyD88-independent pathway | TRAM | TRIF

The extracellular domain of toll-like receptor 4 (TLR4) specifically binds lipopolysaccharides (LPSs) from Gram-negative bacteria, inducing dimerization and leading to the dimerization of cytosolic Toll/interleukin-1 receptor (TIR) domains. This activated conformation of TLR4 recruits the TIR domain of a downstream adaptor molecule, TIR domain-containing adaptor molecule-2 (TICAM-2) [also known as TRIF-related adaptor molecule (TRAM)], that subsequently recruits the TIR domain of another adaptor molecule, TIR domain-containing adaptor molecule-1 (TICAM-1) [also known as TIR domain-containing adaptor inducing IFN-β (TRIF)] (1–3) at endosomes. Eventually this process activates IFN response factors and generates type-I interferons (IFNs) (4–7). Elucidation of the homotypic and heterotypic interactions between TICAM-1 and TICAM-2 is essential for understanding of TLR4-mediated type-I IFN generation (8).

A large number of TIR domain structures, including receptors and adaptors, have been determined by X-ray crystallography and NMR. The receptors include TLR1 (9), TLR2 (10), and IL-1R accessory protein-like (IL-1RAPL) (11). Adaptors include myeloid differentiation factor 88 (MyD88) (12) and MyD88 adaptor-like (Mal) (13, 14). In addition, AtTIR (15, 16) derived from *Arabidopsis thaliana* and PdTIR (17) from bacteria have been solved. Each of these TIR domain structures has a ferredoxin fold with five β-strands (βA–βE), five α-helices (αA–αE), and loops connecting β-strands and α-helices (9). Although homotypic interactions of the TIR domains have been proposed based on the crystal structures, most proposed models have small interacting surfaces, possibly due to crystal contacts. Recently, however, a crystal structure of the TLR10 TIR domain was reported that forms a homotypic dimer mediated by the loop connecting βB and αB (designated "BB-loop") (18). Interestingly, BB-loop mutations in TLR4 were reported to be dominant-negative and abrogated downstream signaling (19). TICAM-1 and TICAM-2 harboring BB-loop mutations are also dominant-negative and unable to form homotypic interactions (1, 2), reinforcing the importance of BB-loop–mediated homotypic dimer formation in signal propagation.

Despite extensive structural studies, it is not known why homotypic interactions are essential for downstream signaling (20–27). To address this issue, it is necessary to discriminate residues required for homotypic and those required for heterotypic interactions. Here, we first determine the structures of the monomeric BB-loop mutants of the TICAM-1 and TICAM-2 TIR domains using NMR. Then, based on the solution structures of the BB-loop mutants, coupled mutagenesis/yeast two-hybrid experiments, and restrained docking calculations, we show that the homotypic interaction of TICAM-2 TIR is essential to form a scaffold for recruiting the TICAM-1 TIR domain.

Results

Monomerization of the TICAM-1 and TICAM-2 TIR Domains by BB-Loop Mutations. The TIR domains of TICAM-1 (387–545) and TICAM-2 (75–235) (Fig. 1*A*) oligomerized and precipitated in aqueous solution at ~200 μM concentration, so monomerization was

Significance

Toll/interleukin-1 receptor (TIR) homology domains mediate the downstream signaling of Toll-like receptors (TLRs), but the molecular mechanism of the signal transduction is elusive on the structural basis. Here, we determined the structures of TIR domain-containing adaptor molecule (TICAM) 1 and TICAM-2 TIR domains and demonstrated their homotypic and heterotypic interaction surfaces. Both TICAM-1 and TICAM-2 TIR domains form a BB-loop–mediated homodimer. The dimerization of TICAM-2 TIR presents an interaction surface for TICAM-1 TIR. The present result is consistent with the notion that the BB-loop mutant is dominant negative and that dimerization of upstream TIRs is crucial for recruiting downstream TIRs.

Author contributions: F.I. designed research; Y.E., K.F., M.H., M.M., and T.S. performed research; Y.E., H.K., J.S., K.Y., D.M.S., and F.I. analyzed data; and D.M.S., T.S., and F.I. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

Data deposition: The chemical shift assignments and NOE and dihedral restraint data have been deposited in the BioMagResBank, www.bmrb.wisc.edu [accession nos. 18883 (TICAM-1) and 18882 (TICAM-2)]. The atomic coordinates for the ensemble have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 2mlx (TICAM-1) and 2mlw (TICAM-2)].

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1222811110/-/DCSupplemental.