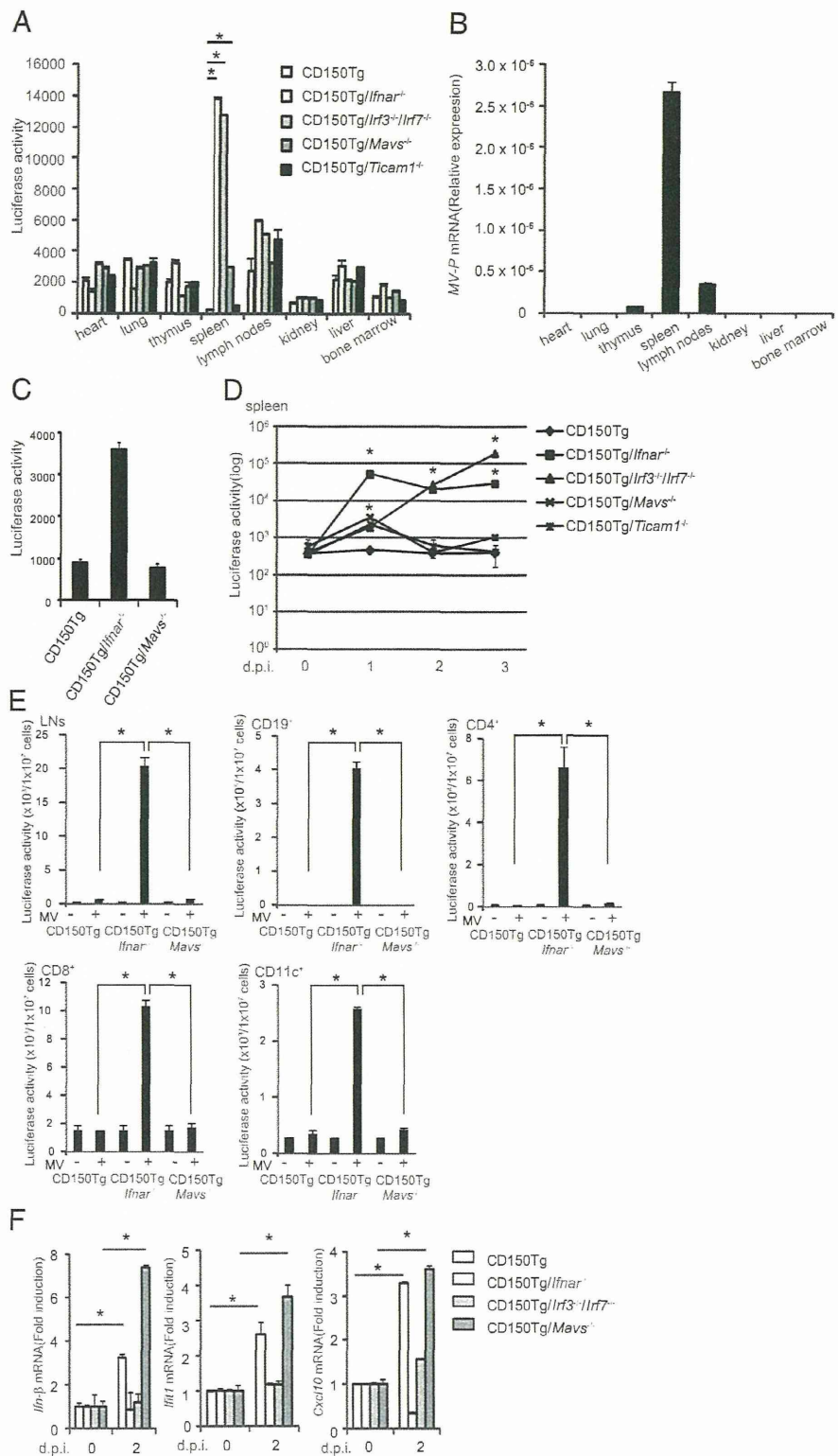


not differ among various spleen cells from CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, CD150Tg/*Irf3*<sup>-/-</sup>/*Irf7*<sup>-/-</sup>, CD150Tg/*Ticam1*<sup>-/-</sup> or CD150Tg/*Mavs*<sup>-/-</sup> mice (Supplemental Fig. 1). Each knockout mouse strain was i.p. injected with 1 × 10<sup>6</sup> PFU of MV-luciferase. In accord with previous data (16, 22), luciferase activity in various tissues derived from MV-infected CD150Tg mice was very low 2 d after inoculation (Fig. 1A). In contrast, luciferase activity was predominantly increased in spleen and lymph nodes (LNs) derived

from MV-infected CD150Tg/*Ifnar*<sup>-/-</sup> mice, compared with other tissues (Fig. 1A). *MV-P* mRNA expression was also increased in MV-infected CD150Tg/*Ifnar*<sup>-/-</sup> spleen and LNs, similarly to luciferase activity (Fig. 1B). These results indicate that spleen and LNs are the major target tissues of i.p.-injected MV in CD150Tg/*Ifnar*<sup>-/-</sup> mice, as shown in a previous report (24). Luciferase activity was increased in the lung of CD150Tg/*Ifnar*<sup>-/-</sup>, but not CD150Tg/*Mavs*<sup>-/-</sup>, mice after intratracheal injection of MV

**FIGURE 1.** CD150Tg/*Mavs*<sup>-/-</sup> mice were resistant to in vivo MV infection. **(A)** CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, CD150Tg/*Irf3*<sup>-/-</sup>/*Irf7*<sup>-/-</sup>, CD150Tg/*Mavs*<sup>-/-</sup>, and CD150Tg/*Ticam1*<sup>-/-</sup> mice were infected i.p. with 1 × 10<sup>6</sup> PFU MV-luciferase. After 2 d, cells were isolated from each organ and lysed with lysis buffer for luciferase assay. The amount of protein in each lysate was determined by bicinchoninic acid assay. Luciferase activity in each lysate was measured and normalized by the amount of protein. Data are shown as luciferase activity per 1 mg of protein and means ± SD of three independent samples. \**p* < 0.05. **(B)** CD150Tg/*Ifnar*<sup>-/-</sup> mice were infected with MV (1 × 10<sup>6</sup> PFU). At 2 d after inoculation, total RNA was collected from the indicated tissues, and the expression level of *MV-P* mRNA in each tissue was determined by real-time PCR. *MV-P* mRNA expression is shown as expression relative to *β-actin*. Data are means ± SD of three independent samples. **(C)** CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, and CD150Tg/*Mavs*<sup>-/-</sup> mice were intratracheally injected with MV-luciferase (8 × 10<sup>5</sup> PFU). At 3 d after inoculation, luciferase activity was measured in cells from lungs. Data are means ± SD of two independent samples. **(D)** At the indicated days post infection (d.p.i.), luciferase activity in 1 × 10<sup>7</sup> splenocytes was measured. Three mice were analyzed for each genotype. Data are representative of two independent experiments. \**p* < 0.05 versus CD150Tg. **(E)** CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, and CD150Tg/*Mavs*<sup>-/-</sup> mice were infected i.p. with 1 × 10<sup>6</sup> PFU MV-luciferase. At 2 d.p.i., CD19<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD11c<sup>+</sup> cells were isolated from splenocytes, using anti-CD19, anti-CD4, anti-CD8, and anti-CD11c MACS beads. Luciferase activity in CD19<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD11c<sup>+</sup> cells, as well as LNs, was measured and normalized by the total number of cells. Data are shown as the luciferase activity per 1 × 10<sup>7</sup> cells. Data are means ± SD of three independent samples. \**p* < 0.05. **(F)** CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, CD150Tg/*Irf3*<sup>-/-</sup>/*Irf7*<sup>-/-</sup>, and CD150Tg/*Mavs*<sup>-/-</sup> mice were infected i.p. with 1 × 10<sup>6</sup> PFU MV-luciferase. At 2 d.p.i., mRNA levels of *Ifn-β*, *Ifit1*, and *Cxcl10* in spleens were determined by real time-PCR. Data are means ± SD of three independent samples. \**p* < 0.05.



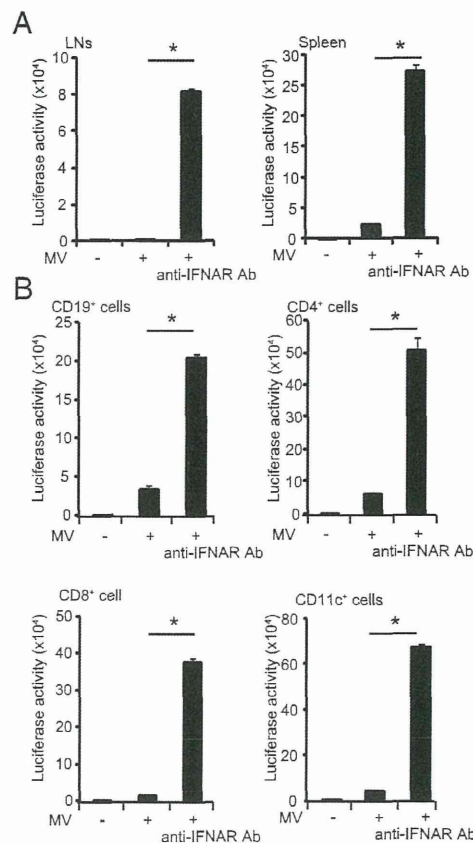
(Fig. 1C). However, the route of MV administration is an issue to be further investigated.

In MV-infected human epithelial cells, signals via RIG-I and MDA5 are essential for production of type I IFN (9, 10). Cells lacking the MAVS protein, a common adaptor molecule for RIG-I and MDA5, are unable to produce type I IFN in response to members of the Paramyxoviridae family (8). To investigate the *in vivo* role of the MAVS pathway during MV infection, we first examined whether CD150Tg/*Mavs*<sup>-/-</sup> mice were permissive to MV infection. Although luciferase activity in splenocytes was slightly increased in CD150Tg/*Mavs*<sup>-/-</sup> mice compared with CD150Tg mice 1 d after i.p. inoculation, luciferase activity in splenocytes of CD150Tg/*Mavs*<sup>-/-</sup> mice was decreased to the same degree as in those of CD150Tg mice a few days after inoculation (Fig. 1A, 1D, 1E). In contrast, luciferase activity in splenocytes was significantly increased in CD150Tg/*Ifnar*<sup>-/-</sup> and CD150Tg/*Irf3*<sup>-/-</sup>/*Irf7*<sup>-/-</sup> mice compared with CD150Tg, CD150Tg/*Mavs*<sup>-/-</sup>, or CD150Tg/*Ticam1*<sup>-/-</sup> mice 2 d after inoculation (Fig. 1D). We also examined luciferase activity in LNs and CD4<sup>+</sup>, CD8<sup>+</sup>, CD19<sup>+</sup>, and CD11c<sup>+</sup> cells isolated from splenocytes of the mice. As shown in Fig. 1E, luciferase activity in these cells from MV-infected CD150Tg/*Mavs*<sup>-/-</sup> mice was much lower than in those from CD150Tg/*Ifnar*<sup>-/-</sup> mice. To investigate the reasons why CD150Tg/*Mavs*<sup>-/-</sup> mice were resistant to MV infection, we examined the expression level of mRNA coding antiviral protein in splenocytes (Fig. 1F). The expression of *Ifn*- $\beta$  and two IFN-inducible genes, *Ifit1* and *Cxcl10*, was upregulated by MV infection in splenocytes from CD150Tg/*Mavs*<sup>-/-</sup> mice, but not in those from CD150Tg/*Ifnar*<sup>-/-</sup> and CD150Tg/*Irf3*<sup>-/-</sup>/*Irf7*<sup>-/-</sup> mice (Fig. 1F). Type I IFN levels in sera were below the detection limit throughout the time course (data not shown). These data suggest that *in vivo* MV infection induces the expression of type I IFN and IFN-inducible genes to elicit resistance to MV infection in CD150Tg/*Mavs*<sup>-/-</sup>. Moreover, type I IFN induction by *in vivo* MV infection occurs via the IRF3/IRF7-dependent and MAVS-independent pathway.

Next, we performed *in vivo* infection experiments using IFNAR Ab. Type I IFNAR in CD150Tg/*Mavs*<sup>-/-</sup> mice was blocked by injecting 2.5 mg of the MAR1-5A3 mAb against IFNAR-1, 1 d before infection. This dose of Ab is reported to block antiviral effects of type I IFN *in vivo* (31). In MV-infected CD150Tg/*Mavs*<sup>-/-</sup> mice, blocking of IFNAR increased luciferase activity in LNs up to 180-fold, and in splenocytes up to 10-fold (Fig. 2A). Similarly, luciferase activity was increased up to 6-fold in CD19<sup>+</sup>, 8-fold in CD4<sup>+</sup>, 29-fold in CD8<sup>+</sup>, and 17-fold in CD11c<sup>+</sup> cells by IFNAR blocking (Fig. 2B). These results indicate that CD150Tg/*Mavs*<sup>-/-</sup> mice are permissive to MV infection once IFNAR is functionally neutralized.

#### CD11c<sup>+</sup> DCs produce type I IFN via the MAVS-independent pathway during MV infection

To determine which cell types were responsible for type I IFN induction in MV-infected CD150Tg/*Mavs*<sup>-/-</sup> mice, we performed an *in vitro* infection assay using splenocytes. When infected with MV *in vitro*, the luciferase activity in B cells and T cells isolated from CD150Tg/*Mavs*<sup>-/-</sup> mice was comparable to activity in those from CD150Tg/*Ifnar*<sup>-/-</sup> mice (Fig. 3A). Treatment with anti-IFNAR Ab did not affect the infection efficiency in CD150Tg/*Mavs*<sup>-/-</sup> T and B cells (Fig. 3A). Unlike T and B cells, CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs were not permissive to MV (Fig. 3A). These data suggest that CD11c<sup>+</sup> DCs, but not lymphocytes, are responsible for resistance to MV infection in CD150Tg/*Mavs*<sup>-/-</sup> mice. Because CD150Tg/*Mavs*<sup>-/-</sup> mice were permissive to MV *in vivo* when type I IFN signaling was blocked by anti-IFNAR Ab

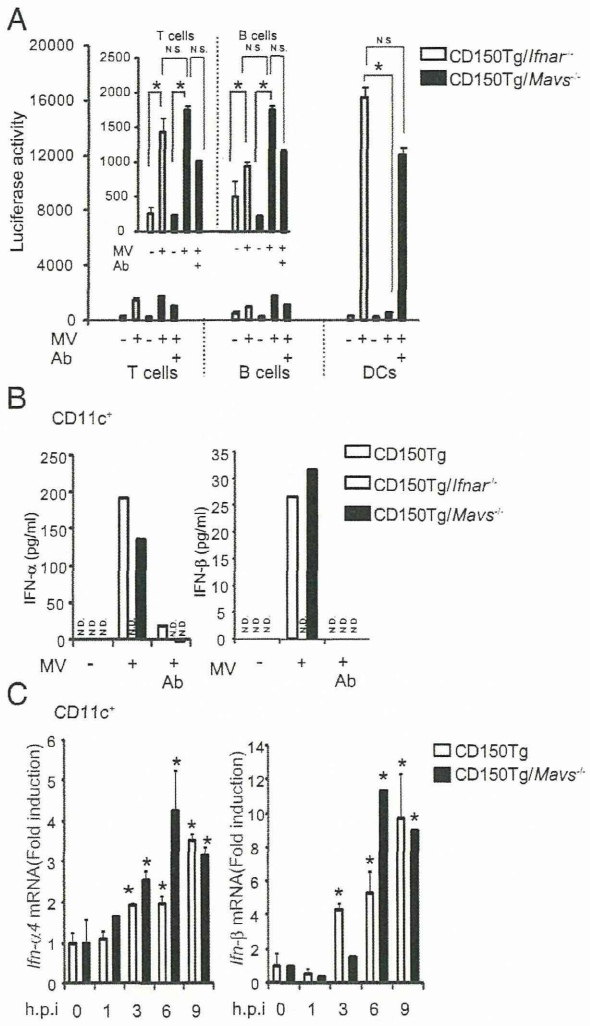


**FIGURE 2.** CD150Tg/*Mavs*<sup>-/-</sup> mice were permissive to MV after treatment with anti-IFNAR Ab *in vivo*. CD150Tg/*Mavs*<sup>-/-</sup> mice were injected i.p. with 2.5 mg of anti-IFNAR Ab at 1 d before MV-luciferase infection ( $1 \times 10^7$  PFU per mouse). At 2 d post infection, luciferase activity in LNs, splenocytes (A), and (B) CD19<sup>+</sup>, CD4<sup>+</sup>, CD8<sup>+</sup>, and CD11c<sup>+</sup> cells isolated from splenocytes was measured and normalized by the total number of cells. Data are shown as the luciferase activity per  $1 \times 10^7$  cells. Data are means  $\pm$  SD of three independent samples. \* $p$  < 0.05.

(Fig. 2A, 2B), we examined whether treatment with anti-IFNAR Ab increased the efficiency of MV infection *in vitro*. CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs were permissive to MV in the presence of anti-IFNAR Ab (Fig. 3A). Consistent with results in Fig. 3A, type I IFN was detectable in the culture supernatant of MV-infected CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs, but not of T or B cells (Fig. 3B and data not shown). Expression of *Ifn*- $\beta$  and *Ifn*- $\alpha$ 4 mRNAs in CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs was also upregulated within 6 h after MV infection (Fig. 3C). These data suggest that CD11c<sup>+</sup> DCs, but neither T nor B cells, mainly produce type I IFN through the MAVS-independent pathway in response to MV infection *in vitro*.

#### CD4<sup>+</sup> DCs and pDCs are responsible for MV-induced type I IFN production in CD150Tg/*Mavs*<sup>-/-</sup> mice

Our data indicate that type I IFN induction following the MV recognition in mouse DCs is independent of the MAVS pathway. Various types of DCs have been identified in mouse secondary lymphoid tissues, including three CD11c<sup>high</sup> subsets: CD8 $\alpha$ <sup>+</sup>, CD4<sup>+</sup>, and DN CD4<sup>-</sup>CD8 $\alpha$ <sup>-</sup> DCs (32), and one subset of CD11c<sup>low</sup> pDCs (33). To identify the type I IFN-producing subsets, we used a cell sorter to isolate CD11c<sup>high</sup> (cDCs) and CD11c<sup>low</sup> pDCs from splenocytes, with purity > 98% (data not shown). CD150Tg/*Ifnar*<sup>-/-</sup> cDCs and pDCs were permissive to MV (Fig. 4A and 4C). In contrast, both CD150Tg/*Mavs*<sup>-/-</sup> cDCs and pDCs



**FIGURE 3.** CD11c<sup>+</sup> DCs mainly produced type I IFN during MV infection in vitro. (A) T cells, B cells, and DCs were isolated from CD150Tg/*Ifnar*<sup>-/-</sup> and CD150Tg/*Mavs*<sup>-/-</sup> splenocytes, using MACS beads. Cells were treated with or without 10 μg/ml of anti-IFNAR Ab, then infected with mock or MV-luciferase at an MOI of 0.25. At 24 h post infection, luciferase activity was measured. Data are means ± SD of three independent samples. \**p* < 0.05. (B) CD11c<sup>+</sup> cells were prepared from splenocytes of CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, and CD150Tg/*Mavs*<sup>-/-</sup> mice, using MACS beads. IFN-α and IFN-β protein production in culture supernatant of MV-infected cells (MOI = 0.25) was measured by ELISA. One of two experiments is shown. (C) CD11c<sup>+</sup> DCs were infected with MV (MOI of 0.25) for the indicated times, and type I IFN expression was determined by real-time PCR. Data are means ± SD of three independent samples. \*A *t* test between 0 h.p.i. and the indicated time in respective mouse lines is *p* < 0.05. N.D., Not detected.

produced type I IFN (Fig. 4B, 4D) and were not permissive to MV (Fig. 4A, 4C). The resistance to MV infection was abolished by neutralizing type I IFNR with anti-IFNAR Ab (Fig. 4A, 4C). These data indicate that, like CD11c<sup>+</sup> DCs, CD150Tg/*Mavs*<sup>-/-</sup> pDCs and cDCs were barely permissive to MV because of the type I IFN production.

For more detailed study on the type I IFN-producing subsets, we further separated cDCs into CD8α<sup>+</sup>, CD4<sup>+</sup>, and DN DCs, using MACS beads. The purity of these subsets was > 90% (data not shown). *Ifn*-β mRNA was not induced in CD150Tg/*Mavs*<sup>-/-</sup> CD8α<sup>+</sup> DCs or DN DCs in response to MV infection (Fig. 5A, 5B). However, MV infection induced expression of *Ifn*-β mRNA

in CD4<sup>+</sup> DCs from CD150Tg/*Mavs*<sup>-/-</sup> mice (Fig. 5C). Different from CD150Tg/*Mavs*<sup>-/-</sup> DCs, CD150Tg CD8α<sup>+</sup> and DN DCs expressed *Ifn*-β mRNA in response to MV infection (Fig. 5A, 5B). These data indicate that the expression of *Ifn*-β mRNA induced by MV infection is MAVS dependent in CD8α<sup>+</sup> DCs and DNs, but MAVS independent in CD4<sup>+</sup> DCs.

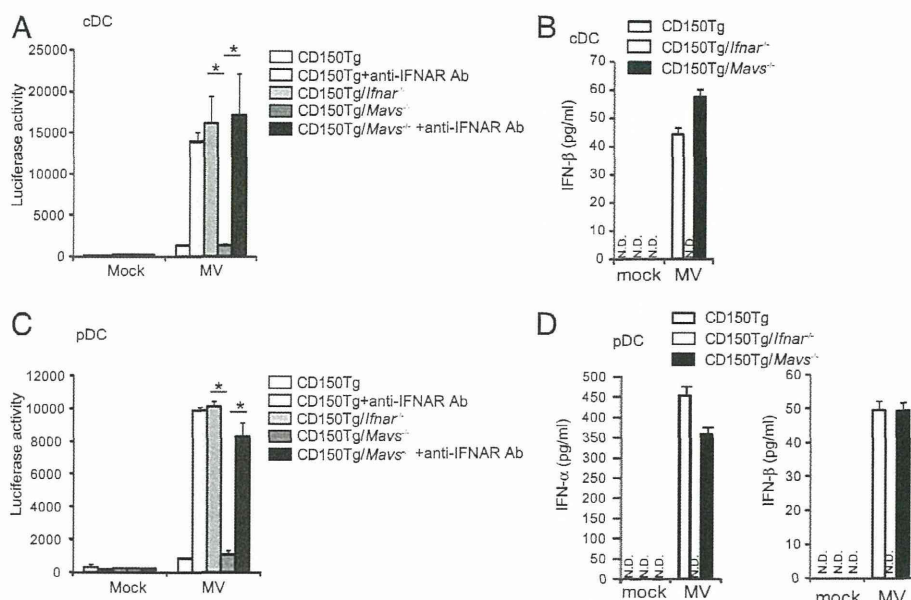
*The MyD88-dependent pathway is essential for MV-induced type I IFN production in pDCs and CD4<sup>+</sup> DCs under MAVS deficiency*

Next, we tried to identify the signaling pathway involved in type I IFN induction during MV infection. pDCs and CD4<sup>+</sup> DCs express TLR7 as a sensor for single-stranded RNA (34, 35). Recognition of single-stranded RNA through the TLR7-MyD88 pathway induces type I IFN expression in human and mouse pDCs (36–38). Therefore, we examined whether the TLR7-MyD88 pathway participated in the host defense during MV infection in mouse DCs. First, we analyzed the expression of TLR7 in pDCs and CD4<sup>+</sup> DCs. As reported in previous studies (34, 35), we were able to detect the expression of TLR7 at both mRNA and protein levels (Supplemental Fig. 2). RNA interference cannot be used in primary pDCs because the RNA molecules induce type I IFN expression (39); therefore, we used a peptide that is fused to an antennapedia-derived cell permeant motif and inhibits MyD88 homodimerization by binding to the MyD88 TIR domain (40). pDCs from wild-type mice were cultured for 6 h in the presence of the MyD88 inhibitory peptide or the control peptide and activated with R837, a TLR7 ligand, for 24 h. The control peptide marginally affected IFN-β secretion induced by R837, and the MyD88 inhibitory peptide at 50 μM significantly reduced IFN-β production induced by R837 (Supplemental Fig. 3A). To evaluate the effects of the inhibitory peptide on cell viability, we counted the number of live cells using trypan blue staining (Supplemental Fig. 3B). Although stimulation with R837 partially induced cell death (41.3% ± 5.4%), cell viability was not changed by the addition of the inhibitory peptide at 50 μM (Supplemental Fig. 3B). MyD88 inhibitory peptide has no effects on polyinosinic/polycytidylic acid-induced IFN-β production in CD11c<sup>+</sup> DCs (Supplemental Fig. 3C). Next, we tested the effect of MyD88 inhibitory peptide on permissiveness and type I IFN production in MV-infected pDCs from CD150Tg/*Mavs*<sup>-/-</sup> mice. Although CD150Tg/*Mavs*<sup>-/-</sup> pDCs were not permissive to MV in the presence of control peptide, infection efficiency was increased 4-fold by treatment with MyD88 inhibitory peptide (Fig. 6A). As with pDCs, the infection efficiency of CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs increased 3-fold by MyD88 inhibitory peptide treatment (Fig. 6B). When CD150Tg/*Mavs*<sup>-/-</sup> CD11c<sup>+</sup> DCs, CD4<sup>+</sup> DCs, and pDCs were pretreated with MyD88 inhibitory peptide, they did not induce type I IFN mRNA after MV infection (Fig. 6C–E). These data suggest that the MyD88 pathway is responsible for MV-induced type I IFN production in DCs under MAVS deficiency. To confirm that the MyD88 pathway is involved in type I IFN induction in MV-infected DCs, CD150Tg/*Myd88*<sup>-/-</sup> pDCs were infected with MV in vitro. *Ifn*-β mRNA expression was not induced in MV-infected CD150Tg/*Myd88*<sup>-/-</sup> pDCs (Fig. 6F).

**Discussion**

This study demonstrated that CD150Tg/*Mavs*<sup>-/-</sup> mice were not susceptible to MV infection in vivo (Fig. 1). This is an unexpected result because many reports have highlighted the important role of the RIG-I/MDA5-MAVS pathway in type I IFN production in MV-infected cells as a means to suppress MV replication. Ultimately, the MyD88 signal, rather than the MAVS signal, serves as a critical inducer of primary type I IFN for cell protection against

**FIGURE 4.** CD150Tg/*Mavs*<sup>-/-</sup> pDCs produced type I IFN in response to MV infection. The cDCs (**A, B**) or pDCs (**C, D**) isolated from CD150Tg, CD150Tg/*Ifnar*<sup>-/-</sup>, or CD150Tg/*Mavs*<sup>-/-</sup> splenocytes were infected by MV (MOI of 0.25). At 24 h post infection, luciferase activity was measured (**A, C**). Production of type I IFN in culture supernatant was measured by ELISA (**B, D**). Data are means  $\pm$  SD of three independent samples. N.D., Not detected.



MV replication in a mouse model. This finding would be a novel feature of MV, a negative single-stranded RNA virus, and has reminded us that cell-level studies on host innate immunity in coping with infection cannot always predict critical virus-sensing factors in whole-animal studies.

Because blockage of type I IFN $\alpha$  function resulted in permissiveness of CD150Tg/*Mavs*<sup>-/-</sup> mice to MV, MAVS-independent type I IFN induction protects neighboring cells (that express IFNAR) from MV infection (Fig. 2). Among the cell subsets tested, only pDCs and CD4<sup>+</sup> DCs from CD150Tg/*Mavs*<sup>-/-</sup> produced type I IFN after MV infection (Figs. 3–5). Treatment with inhibitory peptide for MyD88 drastically decreased type I IFN expression in CD150Tg/*Mavs*<sup>-/-</sup> DCs and rendered these cells MV permissive to MV (Fig. 6). These data indicate that the MyD88 pathway is involved in type I IFN induction in MV-infected pDCs and CD4<sup>+</sup> DCs under a MAVS-deficient state. The results were confirmed with CD150/*Myd88*<sup>-/-</sup> mice (Fig. 6F). In this context, the MyD88 pathway plays a primary role in the initial phase of MV protection in vivo. However, CD150Tg/*Myd88*<sup>-/-</sup> mice were not permissive to MV in vivo (data not shown), suggesting that not only MyD88 in pDCs/CD4<sup>+</sup> DCs but also MAVS in other cells contributes to the protection against systemic infection by MV. Because the MyD88 pathway participates in the initial type I IFN induction in pDCs, only a very weak but significant luciferase activity was detected in the spleen of CD150Tg/*Mavs*<sup>-/-</sup> mice at day 1 after inoculation (Fig. 1C). Moreover, CD8 $\alpha$ <sup>+</sup> DCs and DN DCs from CD150Tg/*Mavs*<sup>-/-</sup> mice could not induce *Ifn*- $\beta$  mRNA expression in response to MV infection in vitro (Fig. 5). These results suggest that the MAVS pathway also participates in host defense against MV infection as an alternative pathway in splenic DCs, at least in these experimental conditions.

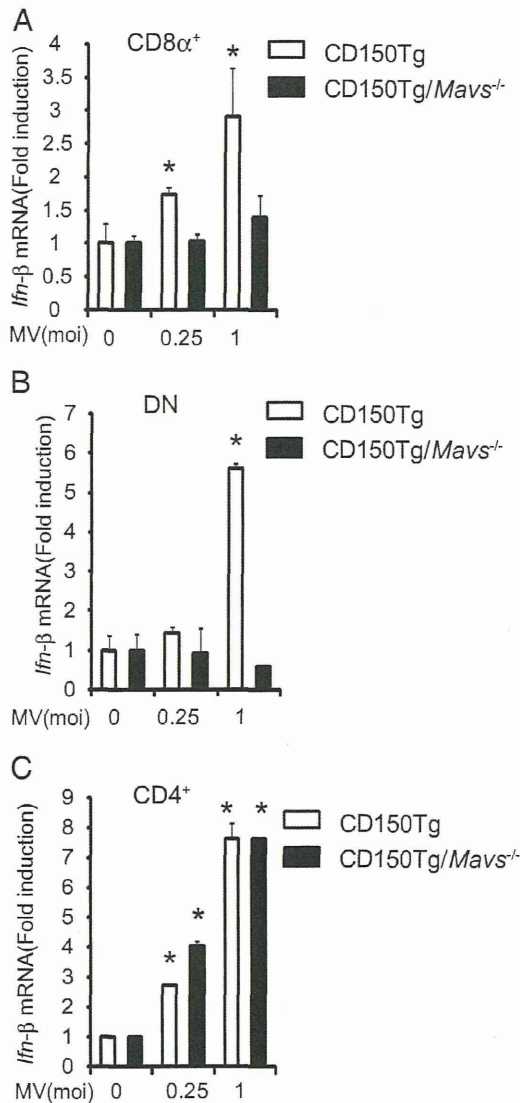
Another target cell of MV infection in nonhuman primates is the AM (18). AMs in the CD150Tg/*Ifnar*<sup>-/-</sup> mouse model are also permissive to MV infection (17). AMs derived from CD150Tg/*Mavs*<sup>-/-</sup> mice were permissive to MV infection in vitro (data not shown). This result is consistent with a previous report indicating that the RLR-MAVS pathway is predominantly used for type I IFN induction in virus-infected AMs (41). However, intratracheal inoculation with MV in CD150Tg/*Mavs*<sup>-/-</sup> mice resulted in a lower luciferase activity in AMs from CD150Tg/*Mavs*<sup>-/-</sup> mice

than in those from CD150Tg/*Ifnar*<sup>-/-</sup> mice (data not shown), similar to the results from i.p. inoculation. These data indicate that a cell population other than AMs produces type I IFN in MV-infected lungs. In this case, pDCs in lung might act as IFN-producing cells, because pDCs in lung are reported to produce type I IFN and act as immune system defenders against infection (41). Taken together, the recognition pathways for MV to induce type I IFN differ among cell types. Existence of the several alternative pathways might engage the protection from systemic MV infection (Supplemental Fig. 4).

In CD8 $\alpha$ <sup>+</sup> DCs and pDCs, the TLR-MyD88 pathway is preferentially used to induce type I IFN in response to infections. TLR7, a sensor for viral RNA, is known to be expressed specifically in these DC subsets (34, 35) (Supplemental Fig. 2). The restricted expression pattern of TLR7 seems to reflect the ability of CD4<sup>+</sup> DCs and pDCs to produce type I IFN in MV-infected CD150Tg/*Mavs*<sup>-/-</sup>. Therefore, TLR7 may be one of the candidate receptors for recognition of MV RNA. Because viral RNA is recognized mainly through the RLR-MAVS pathway in CD4<sup>+</sup> DCs at steady state (35), it would be difficult to examine the role of the TLR7-MyD88 pathway in CD4<sup>+</sup> DCs during viral infection in WT mice. Thus, our assay system using CD150Tg mice crossed with given knockout mice is a powerful tool to investigate the signaling pathway for host defense against MV infection.

Previous studies were mainly done using bone marrow-derived DCs (BMDCs) to analyze the immunosuppressive effects of MV in a mouse model (16, 42, 43). However, BMDCs from CD150Tg/*Mavs*<sup>-/-</sup> mice completely lacked the ability to produce type I IFN in response to MV infection. In contrast to the results in splenic DCs (44). Because the expression profile of pattern-recognition receptors in BMDCs was different from the profile in splenic DCs (34, 35, 45), it is reasonable to think that each cell type has its own unique type I IFN induction pathway in response to viral infection. Therefore, further investigations on immunosuppression and immunopromotion of MV in infected DCs should be performed using splenic DCs, taking into consideration the properties of resident DCs.

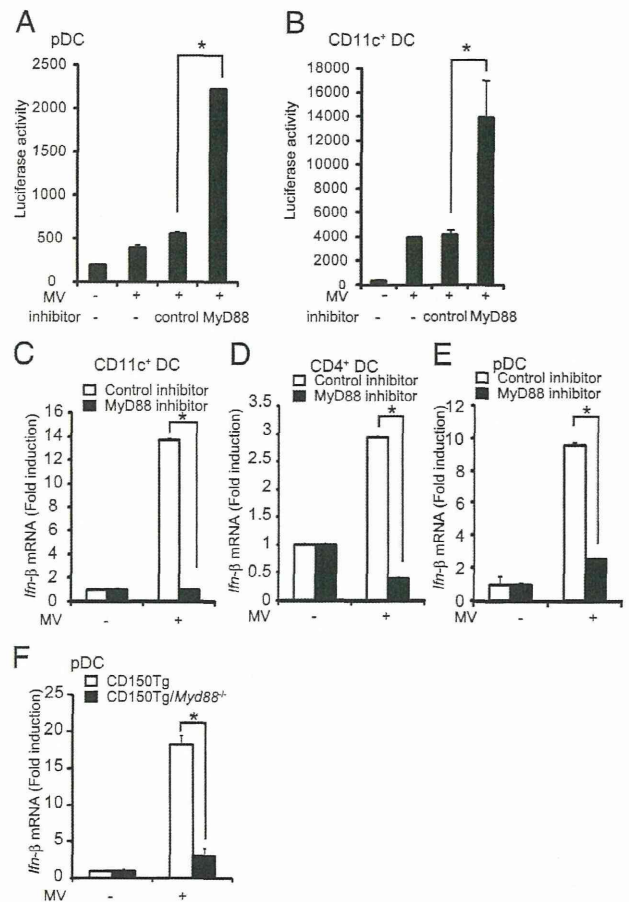
In this study, we used recombinant MV-luciferase that expresses the reporter *Renilla* luciferase from the first gene position of MV genome (29). The MV-luciferase is suitable to evaluate the efficiency of infection in vivo and in vitro correctly because the assay



**FIGURE 5.** MV-infected CD150Tg/Mavs<sup>-/-</sup> CD4<sup>+</sup> DCs expressed type I IFN mRNA. CD8α<sup>+</sup> DCs (A), DN DCs (B), and CD4<sup>+</sup> DCs (C) were isolated by MACS beads. Cells were infected with MV (MOI of 0.25 or 1.0) for 9 h, and levels of *Ifn-β* mRNA were measured by real-time PCR. Data are means ± SD of three independent samples. \*A *t* test between 0 h. p.i. and the indicate time in respective mouse line is *p* < 0.05.

system worked well using the CD150Tg/*Ifnar*<sup>-/-</sup> mouse model. Luciferase activity and MV-P mRNA in CD150Tg/*Ifnar*<sup>-/-</sup> was significantly increased compared with CD150Tg mice during infection (Fig. 1A, 1B and data not shown). Luciferase activity obtained from MV-infected Vero/CD150 cells was correlated with viral titer in culture supernatant from MV-infected cells (data not shown). Recombinant MV strains expressing a foreign gene from a first gene position of the MV genome were used in several groups for in vivo infection assay without any problem (17, 18, 24). However, the infection efficiency of recombinant *Morbillivirus* expressing a foreign gene from position one is reported to be attenuated in vivo (46), which is well reflected in the relatively low virus titer observed in the spleen from CD150Tg mice infected with MV-luciferase.

In mouse models, the route of MV inoculation does not always reflect the natural infection route in human MV infection. Our results obtained from experiments using the i.p. infection model also may not faithfully reflect the process of natural MV infection



**FIGURE 6.** The MyD88 pathway is responsible for MV-induced type I IFN induction in CD150Tg/Mavs<sup>-/-</sup> DCs. (A) pDCs were isolated by FACS or (B) CD11c<sup>+</sup> DCs were isolated by MACS beads from CD150Tg/Mavs<sup>-/-</sup> splenocytes. Cells were treated with 50 μM of the control peptide or 50 μM of the MyD88 inhibitory peptide for 6 h and then infected with MV (MOI of 0.25). After 18 h, luciferase activity was measured. Data are means ± SD of three independent samples. CD150Tg/Mavs<sup>-/-</sup> CD11c<sup>+</sup> DCs (C), CD4<sup>+</sup> DCs (D), and pDCs (E) were pretreated with 50 μM control peptide or 50 μM MyD88 inhibitory peptide for 6 h following infection with MV (MOI of 0.25) for 9 h. Expression levels of *Ifn-β* mRNA were measured by real-time PCR. Expression levels of data are means ± SD of three independent samples. (F) *Ifn-β* mRNA expression in MV-infected CD150Tg and CD150Tg/Myd88<sup>-/-</sup> pDCs. pDCs were infected with MV (MOI = 0.25) for 9 h. Expression levels of *Ifn-β* mRNA were measured by real-time PCR. Expression levels of data are means ± SD of three independent samples. \**p* < 0.05.

involving epithelial cells in humans. However, as described above, intratracheal and i.p. administration gave rise to similar results in MV infection. This issue may reflect the viral strategy of targeting myeloid cells for initial propagation. Injected MV reaches DCs or macrophages without affecting other bystander cells in any route of MV administration. However, this is not the case in other virus species with different target cells for initial infection.

We conclude that MyD88-dependent type I IFN production in CD4<sup>+</sup> DCs and pDCs results in initial protection against MV; thereafter, the produced type I IFN induces an antiviral state in the neighboring cells, expressing IFNAR in the mouse model.

### Acknowledgments

We thank Drs. T. Taniguchi (University of Tokyo, Tokyo, Japan) and S. Akira (Osaka University, Osaka, Japan) for providing *Ir33*<sup>-/-</sup>/*Ir7*<sup>-/-</sup> and *Myd88*<sup>-/-</sup> mice, respectively, for this study, and Dr. Y. Yanagi (Kyushu University, Fukuoka, Japan) for providing MV-luciferase.

## Disclosures

The authors have no financial conflicts of interest.

## References

- Honda, K., A. Takaoka, and T. Taniguchi. 2006. Type I interferon [corrected] gene induction by the interferon regulatory factor family of transcription factors. *Immunity* 25: 349–360.
- Yoneyama, M., K. Onomoto, and T. Fujita. 2008. Cytoplasmic recognition of RNA. *Adv. Drug Deliv. Rev.* 60: 841–846.
- Takeuchi, O., and S. Akira. 2009. Innate immunity to virus infection. *Immunol. Rev.* 227: 75–86.
- Yamamoto, M., S. Sato, K. Mori, K. Hoshino, O. Takeuchi, K. Takeda, and S. Akira. 2002. Cutting edge: a novel Toll/IL-1 receptor domain-containing adaptor that preferentially activates the IFN-beta promoter in the Toll-like receptor signaling. *J. Immunol.* 169: 6668–6672.
- Oshiumi, H., M. Matsumoto, K. Funami, T. Akazawa, and T. Seya. 2003. TICAM-1, an adaptor molecule that participates in Toll-like receptor 3-mediated interferon-beta induction. *Nat. Immunol.* 4: 161–167.
- Kawai, T., K. Takahashi, S. Sato, C. Coban, H. Kumar, H. Kato, K. J. Ishii, O. Takeuchi, and S. Akira. 2005. IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat. Immunol.* 6: 981–988.
- Yamamoto, M., S. Sato, H. Hemmi, K. Hoshino, T. Kaisho, H. Sanjo, O. Takeuchi, M. Sugiyama, M. Okabe, K. Takeda, and S. Akira. 2003. Role of adaptor TRIF in the MyD88-independent toll-like receptor signaling pathway. *Science* 301: 640–643.
- Kumar, H., T. Kawai, H. Kato, S. Sato, K. Takahashi, C. Coban, M. Yamamoto, S. Uematsu, K. J. Ishii, O. Takeuchi, and S. Akira. 2006. Essential role of IPS-1 in innate immune responses against RNA viruses. *J. Exp. Med.* 203: 1795–1803.
- Ikegame, S., M. Takeda, S. Ohno, Y. Nakatsu, Y. Nakanishi, and Y. Yanagi. 2010. Both RIG-I and MDA5 RNA helicases contribute to the induction of alpha/beta interferon in measles virus-infected human cells. *J. Virol.* 84: 372–379.
- Berghäll, H., J. Sirén, D. Sarkar, I. Julkunen, P. B. Fisher, R. Vainionpää, and S. Matikainen. 2006. The interferon-inducible RNA helicase, mda-5, is involved in measles virus-induced expression of antiviral cytokines. *Microbes Infect.* 8: 2138–2144.
- Griffin, D. E. 1995. Immune responses during measles virus infection. *Curr. Top. Microbiol. Immunol.* 191: 117–134.
- Tatsuo, H., N. Ono, K. Tanaka, and Y. Yanagi. 2000. SLAM (CDw150) is a cellular receptor for measles virus. *Nature* 406: 893–897.
- Mühlebach, M. D., M. Mateo, P. L. Sinn, S. Prüfer, K. M. Uhlig, V. H. Leonard, C. K. Navaratnarajah, M. Frenzke, X. X. Wong, B. Sawatsky, et al. 2011. Adherens junction protein nectin-4 is the epithelial receptor for measles virus. *Nature* 480: 530–533.
- Noyce, R. S., D. G. Bondre, M. N. Ha, L. T. Lin, G. Sisson, M. S. Tsao, and C. D. Richardson. 2011. Tumor cell marker PVRL4 (nectin 4) is an epithelial cell receptor for measles virus. *PLoS Pathog.* 7: e1002240.
- Delpout, S., R. S. Noyce, R. W. Siu, and C. D. Richardson. 2012. Host factors and measles virus replication. *Curr. Opin. Virol.* 2: 773–783.
- Shingai, M., N. Inoue, T. Okuno, M. Okabe, T. Akazawa, Y. Miyamoto, M. Ayata, K. Honda, M. Kurita-Taniguchi, M. Matsumoto, et al. 2005. Wild-type measles virus infection in human CD46/CD150-transgenic mice: CD11c-positive dendritic cells establish systemic viral infection. *J. Immunol.* 175: 3252–3261.
- Ferreira, C. S., M. Frenzke, V. H. Leonard, G. G. Welstead, C. D. Richardson, and R. Cattaneo. 2010. Measles virus infection of alveolar macrophages and dendritic cells precedes spread to lymphatic organs in transgenic mice expressing human signaling lymphocytic activation molecule (SLAM, CD150). *J. Virol.* 84: 3033–3042.
- Lemon, K., R. D. de Vries, A. W. Mesman, S. McQuaid, G. van Amerongen, S. Yüksel, M. Ludlow, L. J. Rennick, T. Kuiken, B. K. Rima, et al. 2011. Early target cells of measles virus after aerosol infection of non-human primates. *PLoS Pathog.* 7: e1001263.
- Mesman, A. W., R. D. de Vries, S. McQuaid, W. P. Duprex, R. L. de Swart, and T. B. Geijtenbeek. 2012. A prominent role for DC-SIGN+ dendritic cells in initiation and dissemination of measles virus infection in non-human primates. *PLoS ONE* 7: e49573.
- Schneider-Schaulies, S., I. M. Klagge, and V. ter Meulen. 2003. Dendritic cells and measles virus infection. *Curr. Top. Microbiol. Immunol.* 276: 77–101.
- Servet-Delprat, C., P. O. Vidalain, H. Valentin, and C. Rabourdin-Combe. 2003. Measles virus and dendritic cell functions: how specific response cohabits with immunosuppression. *Curr. Top. Microbiol. Immunol.* 276: 103–123.
- Welstead, G. G., C. Iorio, R. Draker, J. Bayani, J. Squire, S. Vongpunswad, R. Cattaneo, and C. D. Richardson. 2005. Measles virus replication in lymphatic cells and organs of CD150 (SLAM) transgenic mice. *Proc. Natl. Acad. Sci. USA* 102: 16415–16420.
- Sellin, C. I., N. Davoust, V. Guillaume, D. Baas, M. F. Belin, R. Buckland, T. F. Wild, and B. Horvat. 2006. High pathogenicity of wild-type measles virus infection in CD150 (SLAM) transgenic mice. *J. Virol.* 80: 6420–6429.
- Ohno, S., N. Ono, F. Seki, M. Takeda, S. Kura, T. Tsuzuki, and Y. Yanagi. 2007. Measles virus infection of SLAM (CD150) knockin mice reproduces tropism and immunosuppression in human infection. *J. Virol.* 81: 1650–1659.
- Akazawa, T., T. Ebihara, M. Okuno, Y. Okuda, M. Shingai, K. Tsujimura, T. Takahashi, M. Ikawa, M. Okabe, N. Inoue, et al. 2007. Antitumor NK activation induced by the Toll-like receptor 3-TICAM-1 (TRIF) pathway in myeloid dendritic cells. *Proc. Natl. Acad. Sci. USA* 104: 252–257.
- Oshiumi, H., M. Okamoto, K. Fujii, T. Kawanishi, M. Matsumoto, S. Koike, and T. Seya. 2011. The TLR3/TICAM-1 pathway is mandatory for innate immune responses to poliovirus infection. *J. Immunol.* 187: 5320–5327.
- Kobune, F., H. Sakata, and A. Sugiura. 1990. Marmoset lymphoblastoid cells as a sensitive host for isolation of measles virus. *J. Virol.* 64: 700–705.
- Takeda, M., K. Takeuchi, N. Miyajima, F. Kobune, Y. Ami, N. Nagata, Y. Suzuki, Y. Nagai, and M. Tashiro. 2000. Recovery of pathogenic measles virus from cloned cDNA. *J. Virol.* 74: 6643–6647.
- Takeda, M., M. Tahara, T. Hashiguchi, T. A. Sato, F. Jinouchi, S. Ueki, S. Ohno, and Y. Yanagi. 2007. A human lung carcinoma cell line supports efficient measles virus growth and syncytium formation via a SLAM- and CD46-independent mechanism. *J. Virol.* 81: 12091–12096.
- Shingai, M., T. Ebihara, N. A. Begum, A. Kato, T. Honma, K. Matsumoto, H. Saito, H. Ogura, M. Matsumoto, and T. Seya. 2007. Differential type I IFN-inducing abilities of wild-type versus vaccine strains of measles virus. *J. Immunol.* 179: 6123–6132.
- Sheehan, K. C., K. S. Lai, G. P. Dunn, A. T. Bruce, M. S. Diamond, J. D. Heutel, C. Duno-Arthur, J. A. Carrero, J. M. White, P. J. Hertzog, and R. D. Schreiber. 2006. Blocking monoclonal antibodies specific for mouse IFN-alpha/beta receptor subunit 1 (IFNAR-1) from mice immunized by in vivo hydrodynamic transfection. *J. Interferon Cytokine Res.* 26: 804–819.
- Vremec, D., J. Pooley, H. Hochrein, L. Wu, and K. Shortman. 2000. CD4 and CD8 expression by dendritic cell subtypes in mouse thymus and spleen. *J. Immunol.* 164: 2978–2986.
- Asselin-Paturel, C., A. Boonstra, M. Dalod, I. Durand, N. Yessaad, C. Dezutter-Dambuyant, A. Vicari, A. O'Garra, C. Biron, F. Brière, and G. Trinchieri. 2001. Mouse type I IFN-producing cells are immature APCs with plasmacytoid morphology. *Nat. Immunol.* 2: 1144–1150.
- Edwards, A. D., S. S. Diebold, E. M. Slack, H. Tomizawa, H. Hemmi, T. Kaisho, S. Akira, and C. Reis e Sousa. 2003. Toll-like receptor expression in murine DC subsets: lack of TLR7 expression by CD8 alpha+ DC correlates with unresponsiveness to imidazoquinolines. *Eur. J. Immunol.* 33: 827–833.
- Luber, C. A., J. Cox, H. Lauterbach, B. Fancke, M. Selbach, J. Tschopp, S. Akira, M. Wiegand, H. Hochrein, M. O'Keeffe, and M. Mann. 2010. Quantitative proteomics reveals subset-specific viral recognition in dendritic cells. *Immunity* 32: 279–289.
- Hemmi, H., T. Kaisho, O. Takeuchi, S. Sato, H. Sanjo, K. Hoshino, T. Horiuchi, H. Tomizawa, K. Takeda, and S. Akira. 2002. Small anti-viral compounds activate immune cells via the TLR7 MyD88-dependent signaling pathway. *Nat. Immunol.* 3: 196–200.
- Heil, F., H. Hemmi, H. Hochrein, F. Ampenberger, C. Kirschning, S. Akira, G. Lipford, H. Wagner, and S. Bauer. 2004. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. *Science* 303: 1526–1529.
- Diebold, S. S., T. Kaisho, H. Hemmi, S. Akira, and C. Reis e Sousa. 2004. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303: 1529–1531.
- Hornung, V., M. Guenther-Biller, C. Bourquin, A. Ablasser, M. Schlegel, S. Uematsu, A. Noronha, M. Manoharan, S. Akira, A. de Fougerolles, et al. 2005. Sequence-specific potent induction of IFN-alpha by short interfering RNA in plasmacytoid dendritic cells through TLR7. *Nat. Med.* 11: 263–270.
- Loiarro, M., C. Sette, G. Gallo, A. Ciacci, N. Fantò, D. Mastroianni, P. Carminati, and V. Ruggiero. 2005. Peptide-mediated interference of TIR domain dimerization in MyD88 inhibits interleukin-1-dependent activation of NF-kappaB. *J. Biol. Chem.* 280: 15809–15814.
- Kumagai, Y., O. Takeuchi, H. Kato, H. Kumar, K. Matsui, E. Morii, K. Aozasa, T. Kawai, and S. Akira. 2007. Alveolar macrophages are the primary interferon-alpha producer in pulmonary infection with RNA viruses. *Immunity* 27: 240–252.
- Hahn, B., N. Arbour, and M. B. Oldstone. 2004. Measles virus interacts with human SLAM receptor on dendritic cells to cause immunosuppression. *Virology* 323: 292–302.
- Koga, R., S. Ohno, S. Ikegame, and Y. Yanagi. 2010. Measles virus-induced immunosuppression in SLAM knock-in mice. *J. Virol.* 84: 5360–5367.
- Takaki, H., K. Honda, K. Atarashi, F. Kobayashi, T. Ebihara, H. Oshiumi, M. Matsumoto, M. Shingai, and T. Seya. 2013. MAVS-dependent IRF3/IRF7 bypass of interferon beta-induction restricts the response to measles infection in CD150tg mouse bone marrow-derived dendritic cells. *Molec. Immunol.* In press.
- Datta, S. K., V. Redecke, K. R. Prilliman, K. Takabayashi, M. Corr, T. Tallant, J. DiDonato, R. Dziarski, S. Akira, S. P. Schoenberger, and E. Raz. 2003. A subset of Toll-like receptor ligands induces cross-presentation by bone marrow-derived dendritic cells. *J. Immunol.* 170: 4102–4110.
- von Messling, V., D. Milosevic, and R. Cattaneo. 2004. Tropism illuminated: lymphocyte-based pathways blazed by lethal morbillivirus through the host immune system. *Proc. Natl. Acad. Sci. USA* 101: 14216–14221.

# A Distinct Role of Riplet-Mediated K63-Linked Polyubiquitination of the RIG-I Repressor Domain in Human Antiviral Innate Immune Responses

Hiroyuki Oshiumi\*, Moeko Miyashita<sup>‡</sup>, Misako Matsumoto, Tsukasa Seya

Department of Microbiology and Immunology, Graduate School of Medicine, Hokkaido University, Kita-ku, Sapporo, Japan

## Abstract

The innate immune system is essential for controlling viral infections, but several viruses have evolved strategies to escape innate immunity. RIG-I is a cytoplasmic viral RNA sensor that triggers the signal to induce type I interferon production in response to viral infection. RIG-I activation is regulated by the K63-linked polyubiquitin chain mediated by Riplet and TRIM25 ubiquitin ligases. TRIM25 is required for RIG-I oligomerization and interaction with the IPS-1 adaptor molecule. A knockout study revealed that Riplet was essential for RIG-I activation. However the molecular mechanism underlying RIG-I activation by Riplet remains unclear, and the functional differences between Riplet and TRIM25 are also unknown. A genetic study and a pull-down assay indicated that Riplet was dispensable for RIG-I RNA binding activity but required for TRIM25 to activate RIG-I. Mutational analysis demonstrated that Lys-788 within the RIG-I repressor domain was critical for Riplet-mediated K63-linked polyubiquitination and that Riplet was required for the release of RIG-I autorepression of its N-terminal CARDs, which leads to the association of RIG-I with TRIM25 ubiquitin ligase and TBK1 protein kinase. Our data indicate that Riplet is a prerequisite for TRIM25 to activate RIG-I signaling. We investigated the biological importance of this mechanism in human cells and found that hepatitis C virus (HCV) abrogated this mechanism. Interestingly, HCV NS3-4A proteases targeted the Riplet protein and abrogated endogenous RIG-I polyubiquitination and association with TRIM25 and TBK1, emphasizing the biological importance of this mechanism in human antiviral innate immunity. In conclusion, our results establish that Riplet-mediated K63-linked polyubiquitination released RIG-I RD autorepression, which allowed the access of positive factors to the RIG-I protein.

**Citation:** Oshiumi H, Miyashita M, Matsumoto M, Seya T (2013) A Distinct Role of Riplet-Mediated K63-Linked Polyubiquitination of the RIG-I Repressor Domain in Human Antiviral Innate Immune Responses. *PLoS Pathog* 9(8): e1003533. doi:10.1371/journal.ppat.1003533

**Editor:** Michael Gale Jr, University of Washington, United States of America

**Received:** January 30, 2013; **Accepted:** June 17, 2013; **Published:** August 8, 2013

**Copyright:** © 2013 Oshiumi et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This work was supported in part by a grant-in-aid from the Ministry of Education, Science and Culture of Japan, and the Ministry of Health Labour, and Welfare of Japan, Kato Memorial Bioscience Foundation. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: oshiumi@med.hokudai.ac.jp

<sup>‡</sup> Current address: Hokkaido Pharmaceutical University School of Pharmacy, Katsuraoka-cho, Otaru, Hokkaido, Japan.

## Introduction

The innate immune system is essential for controlling virus infections, and several viruses have evolved strategies to evade host innate immune responses. Cytoplasmic viral RNA is recognized by RIG-I-like receptors, including RIG-I and MDA5 [1,2]. The RIG-I protein comprises N-terminal Caspase Activation and Recruitment Domains (CARDs), a central RNA helicase domain, and a C-terminal Repressor domain (RD) [3]. RD consists of C-terminal RNA binding domain (CTD) and a bridging domain between CTD and helicase [4]. RIG-I CARDs are essential for triggering the signal that induces type I interferon (IFN). In resting cells, RIG-I RD represses its CARDs signaling [3]. After viral infection, RIG-I RD recognizes 5'-triphosphate double-stranded RNA (dsRNA), which results in a conformational change in the RIG-I protein [3]. This conformational change leads to the release of RD autorepression of CARDs, after which CARDs associate with an IPS-1 adaptor molecule (also called MAVS, Cardif, and VISA) localized at the outer membrane of mitochondria [3,5,6,7,8]. IPS-1 activates downstream factors such as TBK1,

IKK-ε, and NEMO [9,10,11]. NEMO forms a complex with TBK1 and IKK-ε and has a polyubiquitin binding region [12]. These protein kinases are essential for activating transcription factors such as IRF-3 to induce type I IFN production [13].

Several ubiquitin ligases are involved in regulating the RIG-I-dependent pathway, and RIG-I itself is regulated by ubiquitin chains [14]. Gack MU and colleagues firstly reported that TRIM25 ubiquitin ligase mediates K63-linked polyubiquitination of RIG-I N-terminal CARDs, which results in RIG-I activation [15]. Other groups also detected a RIG-I-anchored polyubiquitin chain after ligand stimulation or viral infection [16,17]. It was recently demonstrated that an unanchored polyubiquitin chain but not ubiquitination is essential for RIG-I activation [18,19]. However, RIG-I anchored K63-linked polyubiquitin chains are detected after viral infection [15,20].

Another E3 ubiquitin ligase, Riplet, binds RIG-I RD and mediates the K63-linked polyubiquitination of RIG-I RD [21]. In contrast, Chen DY and colleagues reported that Riplet (also called Reul) ubiquitinated RIG-I CARDs [22]. A study that used Riplet knockout mice showed that mouse Riplet is essential for RIG-I-

## Author Summary

The cytoplasmic viral RNA sensor RIG-I recognizes various types of pathogenic viruses and evokes innate immune responses, whereas several viruses have evolved strategies to escape the host innate immune responses. RIG-I triggers a signal to induce type I interferon and inflammatory cytokines. RIG-I activation is regulated by K63-linked polyubiquitin chains mediated by the ubiquitin ligases TRIM25 and Riplet; however, the functional difference between the two ubiquitin ligases remains unclear, and the molecular mechanism underlying Riplet-mediated RIG-I activation is unknown. We revealed sequential roles of the two ubiquitin ligases during RIG-I activation and found that Riplet-mediated polyubiquitination of the RIG-I repressor domain released RIG-I autorepression of its N-terminal CARDs responsible for triggering the signal, which resulted in an association with TRIM25 ubiquitin ligase and TBK1 protein kinase. Interestingly, we found that this mechanism was targeted by hepatitis C virus, which is a major cause of hepatocellular carcinoma. This result emphasizes the vital role of Riplet-mediated release of RIG-I RD autorepression in antiviral responses. Our results establish that Riplet releases RIG-I RD autorepression and demonstrated the biological significance of this mechanism in human innate immune responses.

mediated type I IFN production in response to vesicular stomatitis virus (VSV), Flu, and Sendai virus (SeV) infections [23]. However, the functional difference between Riplet and TRIM25 remains unclear, and the molecular mechanism of how Riplet-mediated RIG-I ubiquitination activates RIG-I signaling remains unresolved.

Hepatitis C virus (HCV) is a major cause of hepatocellular carcinoma (HCC) worldwide. HCV RNA is primarily recognized by RIG-I *in vitro* and *in vivo* [24]. The HCV protease NS3-4A can suppress type I IFN production [25]. NS3-4A cleaves IPS-1 to suppress RIG-I-mediated innate immune responses [7,26]. Human monocyte-derived dendritic cells recognize HCV RNA through Toll-like receptor 3, and NS3-4A has the ability to cleave TICAM-1, which is a solo adaptor molecule of Toll-like receptor 3 [27,28]. In this study, we found that Riplet was another target of NS3-4A. Here, we demonstrated the molecular mechanisms of how Riplet-mediated RIG-I polyubiquitination triggered the type I IFN production signal and showed that this mechanism was targeted by HCV.

## Results

### Riplet and TRIM25 ubiquitin ligases play different roles in RIG-I activation

In mouse embryonic fibroblasts (MEFs), TRIM25 is essential for type I IFN production in response to SeV infection [15]. As with TRIM25 knockout, Riplet knockout abolished IFN- $\alpha$ 2 and IFN- $\beta$  mRNA expressions in response to SeV infection in MEF (Figure 1A and 1B), which suggested that both ubiquitin ligases were essential for type I IFN expression in MEFs in response to SeV infection. Thus, we examined whether these two ubiquitin ligases played different roles in RIG-I activation.

We performed reporter gene assays with p125luc (IFN- $\beta$  reporter) using either RIG-I CARDs fragment or full-length RIG-I expression vectors. It is known that ectopic expression of RIG-I CARDs fragment activates the signaling even in the absence of stimulation with RIG-I ligand [2,3] and that the auto-activation is observed when full-length RIG-I is ectopically

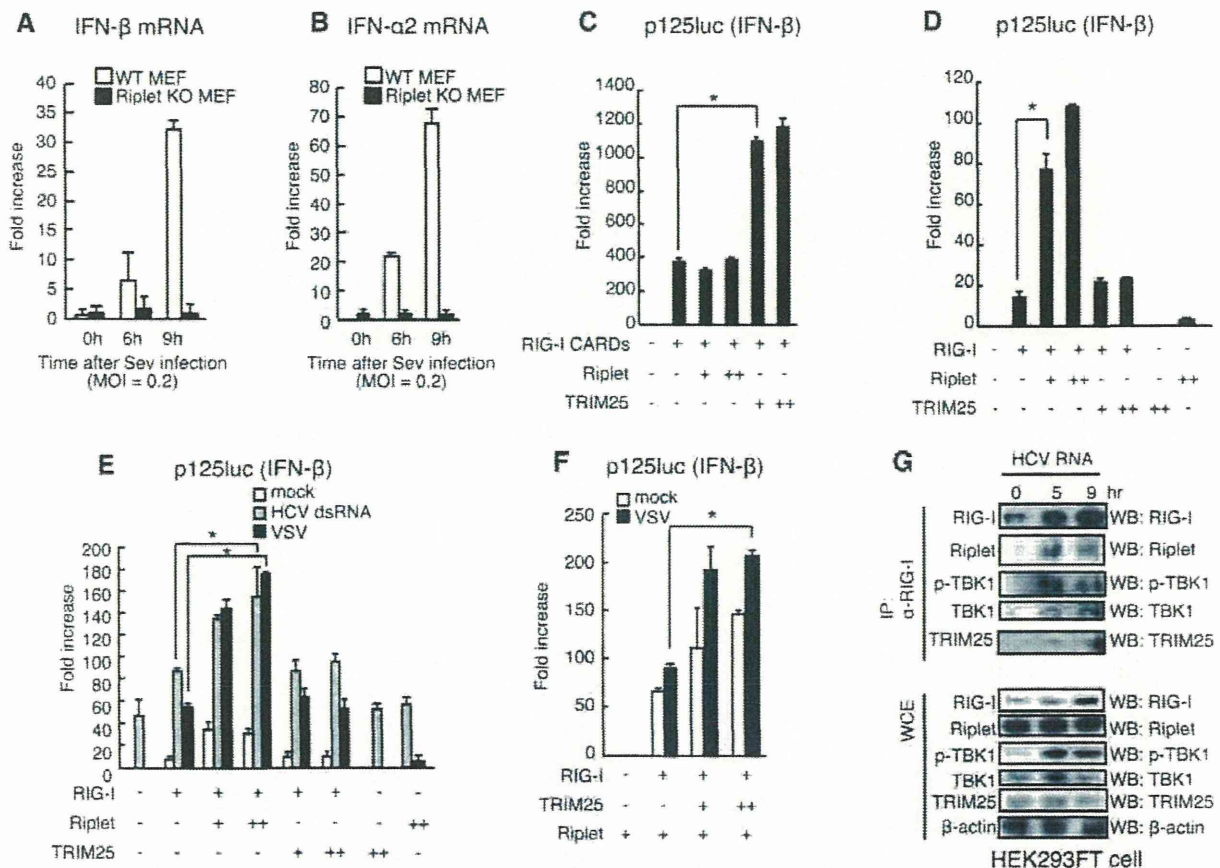
expressed in HEK293 cell [21,29]. As previously reported, TRIM25 ectopic expression efficiently increased RIG-I CARDs fragment-mediated signaling (Figure 1C). However, TRIM25 expression only mildly increased full-length RIG-I signaling (Figure 1D). In contrast, Riplet ectopic expression efficiently increased the full-length RIG-I-mediated signaling, although Riplet failed to increase the RIG-I CARDs-mediated signaling (Figure 1C and 1D). Interestingly, when Riplet was co-expressed with TRIM25, ectopically expressed TRIM25 could increase the full-length RIG-I-mediated signaling (Figure 1F). We observed the same effects of TRIM25 and Riplet expressions on the reporter activation in the presence of stimulation with HCV 3' UTR dsRNA (a RIG-I ligand) or VSV infection, which are recognized by RIG-I (Figure 1E and 1F). These different effects of Riplet and TRIM25 on the signaling suggested that Riplet and TRIM25 ubiquitin ligases played different roles in RIG-I activation.

We then investigated whether the two ubiquitin ligases associate with RIG-I after stimulation. First, we performed immunoprecipitation assay and found that endogenous Riplet and TRIM25 bound to endogenous RIG-I after stimulation with HCV dsRNA (Figure 1G). Second, we investigated subcellular localizations of the proteins by confocal immunofluorescence microscopy. In resting cells, RIG-I was barely detectable, whereas RIG-I exhibited punctate staining in cytoplasm after VSV infection (Figure 2A), and ectopically expressed Riplet was co-localized with RIG-I and TRIM25 in perinuclear region in VSV infected cells (Figure 2A–2C and 2E). This is consistent with a previous observation that RIG-I and TRIM25 co-localized extensively at cytoplasmic perinuclear bodies [15]. Pearson's correlation coefficient values also suggested the correlation of the colocalizations of those proteins (Figure 2D and 2F). Recently, it was reported that RIG-I recognized short polyI:C in stress granules [30]. G3BP is a marker of the stress granule [30]. In resting cells, G3BP dispersed in cytoplasm [30], and thus barely detectable (Figure 2G and 2H), whereas G3BP speckles were detected in cells stimulated with short polyI:C in the cytoplasm (Figure 2G and 2H). Riplet and TRIM25 localizations within G3BP speckles were detected in the stimulated cells (Figure 2G and 2H). Taken together, these data indicated that both Riplet and TRIM25 associated with RIG-I after stimulation.

Next, we assessed the RIG-I regions that bind to the two ubiquitin ligases using RIG-I fragments. As previously reported, TRIM25 bound to RIG-I CARDs fragment (Figure 2I). However, Riplet bound to RIG-I RD (735–925 aa), but not to CARDs fragment (Figure 2J). Deleting the Riplet binding region (RIG-I- $\Delta$ CTD) abrogated Riplet effect on RIG-I signaling (Figure 2K). This was contrast to TRIM25, which affects RIG-I CARDs. Taken together, our genetic and biochemical data indicated that the two ubiquitin ligases associated with RIG-I after stimulation but showed different effects on RIG-I activation. Thus, we next focused on Riplet specific role in RIG-I activation.

### Riplet-mediated RIG-I polyubiquitination is dispensable for RIG-I RNA binding activity

RIG-I CARDs harbor K63-linked polyubiquitination [15]. As RIG-I CARDs, RIG-I RD harbored K63-linked polyubiquitination (Supplemental Figure S1). Riplet expression increased the polyubiquitination of RIG-I RD but not that of CARDs (Figure 3A). RIG-I RD has two functions. One is RNA binding activity and the other is autorepression of its CARDs signaling. Firstly, we tested whether Riplet affects RIG-I RNA binding activity. In a pull-down assay using biotin-conjugated dsRNA and streptavidin beads, we found that both polyubiquitinated and non-ubiquitinated RIG-I were recovered (Figure 3B), which suggested that Riplet-mediated polyubiquitination was dispensable for RIG-I



**Figure 1. Riplet promotes TRIM25-mediated full-length RIG-I activation.** (A, B) Wild-type and Riplet KO MEFs were infected with SeV at MOI=0.2. The mRNA expressions of IFN- $\beta$  (A) and - $\alpha$ 2 (B) at the indicated times after viral infection were determined by RT-qPCR. Results are presented as mean  $\pm$  SD (n=3). (C, D) The activation of IFN- $\beta$  promoter was examined by reporter gene assay using a p125luc IFN- $\beta$  reporter. RIG-I CARDS (C) or full-length RIG-I (D) expression vectors were transfected into HEK293 cells together with a Riplet and/or TRIM25 expression vector as indicated. 24 hours after the transfection, the reporter activities were determined. (E, F) The activation of IFN- $\beta$  promoter was examined by reporter gene assay using a p125luc IFN- $\beta$  reporter. Full-length RIG-I expression vectors were transfected into HEK293 cells together with a Riplet and/or TRIM25 expression vector as indicated. 24 hours after the transfection, the cells were stimulated with 50 ng of HCV 3' UTR dsRNA by transfection or infected with VSV at MOI=1 for 24 hours, and then reporter activation was determined. Data are presented as mean  $\pm$  SD (n=3). \*p<0.05. (G) HEK293FT cells were stimulated with 0.8  $\mu$ g of HCV double-stranded RNA (HCV RNA) using lipofectamine 2000 in 6-well plate. Cell lysates were prepared at the indicated times, followed by immunoprecipitation with an anti-RIG-I mAb (Alme-1). doi:10.1371/journal.ppat.1003533.g001

RNA binding activity. As RIG-I is known to form homo-oligomers [3], it is possible that non-ubiquitinated RIG-I was recovered through ubiquitinated RIG-I by the pull-down assay. To exclude this possibility, we used RIG-I mutants, RIG-I 5KR and RIG-I K788R, which were barely ubiquitinated by Riplet (described below). We compared the binding abilities of the RIG-I mutants to that of wild-type RIG-I by pull-down assay using cell lysate isolated from cells without stimulation with RIG-I ligand to avoid any ubiquitination. The results showed that the RIG-I 5KR and RIG-I K788R mutant proteins were recovered by pull down assay as wild-type RIG-I (Figure 3C). This data also indicated that the polyubiquitination is dispensable for RIG-I RNA binding activity.

#### Riplet releases RIG-I RD autorepression of CARDS signaling

Secondly, we investigated whether Riplet expression affects RIG-I RD autorepression of CARDS signaling. To test this possibility, we constructed RIG-I mutant proteins (Figure 4A). We

previously showed that Lys to Ala amino acid substitutions at Lys-849, 851, 888, 907, and 909 of RIG-I (RIG-I 5KA) severely reduced RIG-I polyubiquitination and activation [21]. However, it is possible that the substitutions of Lys with Ala affect other function of RIG-I because the substitution abolishes positive charge of Lys residues. Thus, we constructed the RIG-I 5KR mutant protein, in which the five Lys residues were substituted with Arg, and examined the signal activation ability. The results showed that the 5KR mutation reduced RIG-I signaling, however residual activation of RIG-I 5KR was still detected (Figure 4B–4D). Thus, we assessed other Lys residues within RIG-I RD. Because Riplet is essential for RIG-I activation, it is expected that the Lys residues targeted by Riplet are conserved during evolution. Thus, we tested Lys residues within RIG-I RD conserved among vertebrate, and found that an RIG-I K788R mutation reduced RIG-I signaling at a level comparable to that by a K172R mutation, which abrogates TRIM25-mediated RIG-I activation [15,19] (Figure 4B–4D). Interestingly, the 5KR and K788R

