(fig. 2b; online suppl. fig. 2a). In addition, the costimulatory molecules CD80 and CD86 on these cells were upregulated in response to poly I:C (fig. 2c). To further investigate the effect of poly I:C treatment on the function of $CD11b^+Gr1^+$ cells, we analyzed the gene expression of $CD11b^+Gr1^+$ cells isolated from B16 tumor-bearing mice, 4 h after injection with poly I:C or PBS. We found an increase in mRNA for IFN-α4, IL-15, IL-18 and INAM (fig. 2d) [21, 32]. Furthermore, in vivo poly I:C treatment for 8 h resulted in upregulation of RAE-1, PVR (CD155), CD70, IL-15RA, SLAM (CD150) and CD40 on $CD11b^+Gr1^+$ cell surface (fig. 2c). These molecules are involved in DC-mediated NK cell activation [18, 34, 35]. However, mRNA for arginase-1, which is involved in MDSC-mediated inhibition of T cell proliferation, was not increased in $CD11b^+Gr1^+$ cells (fig. 2d). These results suggest that in vivo pretreatment of mice with poly I:C effectively induces the maturation of $CD11b^+Gr1^+$ cells, resulting in enhanced expression of NK cell-activating molecules.

$CD11b^+Gr1^+$ Cells from Poly I:C-Treated Tumor-Bearing Mice Activate NK Cells

To investigate whether $CD11b^+Gr1^+$ cells from poly I:C-injected tumor-bearing mice are capable of activating NK cells, we isolated $CD11b^+Gr1^+$ cells from the spleens of tumor-bearing mice after poly I:C administration and cocultured the cells with NK cells from naïve mice. NK cells upregulated CD69 on their surface in response to the $CD11b^+Gr1^+$ cells from poly I:C-injected B16 tumor-bearing mice. However, the level of CD69 on NK cells was not changed when the cells were mixed with $CD11b^+Gr1^+$ cells from PBS-injected tumor-bearing mice (fig. 3a, left panel). $CD11b^+Gr1^+$ cells from poly I:C-injected tumor-bearing mice also induced NK cell IFN-γ production (fig. 3a, right panel). Similar results were obtained with NK cells cocultured with $CD11b^+Gr1^+$ cells from mice bearing 3LL- or EL4 cell tumors after poly I:C treatment (fig. 3b, c; online suppl. fig. 2b, c). Furthermore, $CD11b^+Gr1^+$ cells from tumors of 3LL-implant mice had a similar ability to induce IFN-γ production and CD69 expression in NK cells after poly I:C treatment (fig. 3c). IFN-γ inhibits proliferation of B16 cells in vitro without affecting the cell viability (online suppl. fig. 3; data not shown). In contrast, $CD11b^+Gr1^+$ cells did not drive a cytotoxic phenotype from NK cells (fig. 3d). In vitro stimulation of $CD11b^+Gr1^+$ cells with poly I:C did not induce NK cytotoxicity in coculture (data not shown). These results demonstrated that when poly I:C was injected into tumor-bearing mice, $CD11b^+Gr1^+$ cells acquired the ability to prime NK cells as measured by CD69 expression and IFN-γ production, but did not induce cytotoxic activity.

Type-I IFN Signaling Is Essential for NK Cell Priming by $CD11b^+Gr1^+$ Cells

Next, we investigated the mechanisms by which $CD11b^+Gr1^+$ cells primed NK cells through the in vivo administration of poly I:C. Soluble factors and membrane-associated molecules induced by poly I:C are reportedly involved in in vivo NK cell activation [15, 18]. As shown in figure 2a, $CD11b^+Gr1^+$ cells from poly I:C-treated tumor-bearing mice produced IFN-α. To examine whether type-I IFN signaling through IFNAR was involved in NK cell activation, we added anti-IFNAR1 antibodies to cultures to inhibit type-I IFN signaling by both $CD11b^+Gr1^+$ and NK cells that express IFNAR1. CD69 upregulation on NK cells and IFN-γ production induced by activated $CD11b^+Gr1^+$ cells were completely abrogated by anti-IFNAR1 antibodies (fig. 4a). These results suggest that type-I IFN signaling is essential for NK cell priming by $CD11b^+Gr1^+$ cells.

To investigate type-I IFN signaling in NK cell priming, we prepared NK cells from $IFNAR1^{-/-}$ mice. $IFNAR1^{-/-}$ NK cells were cocultured with $CD11b^+Gr1^+$ cells. $CD11b^+Gr1^+$ cells from poly I:C-stimulated WT mice stimulated CD69 expression and IFN-γ production by WT NK cells but not $IFNAR1^{-/-}$ NK cells (fig. 4b). Therefore, type-I IFN signaling in NK cells is essential for NK priming by $CD11b^+Gr1^+$ cells.

To examine whether IFNAR signaling is the only route for the induction of $CD11b^+Gr1^+$ cell-mediated NK priming, we added recombinant mouse IFN-α to cultures of NK cells or to cocultures of untreated $CD11b^+Gr1^+$ cells and WT NK cells. Recombinant mouse IFN-α in NK cell cultures resulted in induction of CD69 expression on the NK cells (fig. 4c, left panels). However, CD69 expression was minimally augmented in the NK cells cocultured with $CD11b^+Gr1^+$ cells. In contrast, NK cell IFN-γ production was clearly induced at high concentrations of IFN-α (2,000 IU/ml) and augmented by $CD11b^+Gr1^+$ cells (fig. 4c, right panel).

We investigated if cell-cell contact is involved in NK cell activation in cocultures of $CD11b^+Gr1^+$ cells and naïve NK cells using the Transwell system. Sufficient NK cell priming was detected when NK cells were cocultured with in vivo poly I:C-activated $CD11b^+Gr1^+$ cells. However, expression of CD69 and production of IFN-γ by NK cells was abrogated by separation of the cells by the Transwell membrane (online suppl. fig. 4a, b). These results,

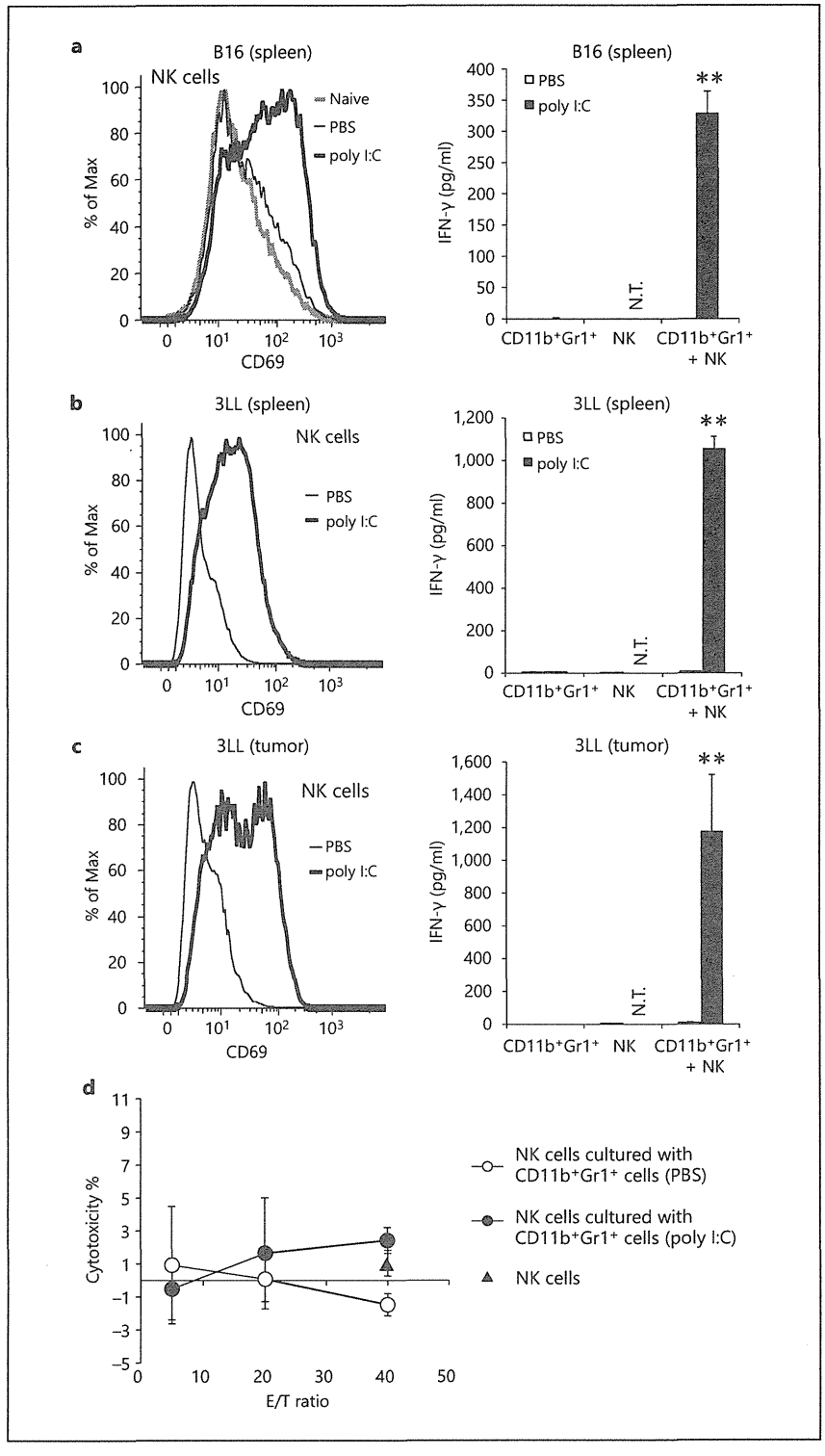

Fig. 3. NK cells are primed by $CD11b^+Gr1^+$ cells isolated from poly I:C-injected tumor-bearing mice. **a–c** $CD11b^+Gr1^+$ cells were isolated from spleens or tumors of B16 (**a**), 3LL (**b**, **c**) tumor-bearing mice pretreated with 200 μg poly I:C or PBS for 4 h and cultured with NK cells from naïve WT mice. After 24 h, CD69 expression on NK cells (**a–c**, left panels) and IFN-γ concentration in conditioned medium (**a–c**, right panels) were determined. CD69 expression of $NK1.1^+CD3\varepsilon^-$ cells is indicated (**a–c**). N.T. = Not tested. **d** Cytotoxic activity of NK cells cocultured with or without $CD11b^+Gr1^+$ cells isolated from poly I:C- or PBS-treated tumor-bearing mice was determined by standard ^{51}Cr release assay (n = 3). Triangle: NK cells not cultured with $CD11b^+Gr1^+$ cells. Data shown are representative of at least 3 independent experiments. ** $p < 0.01$.

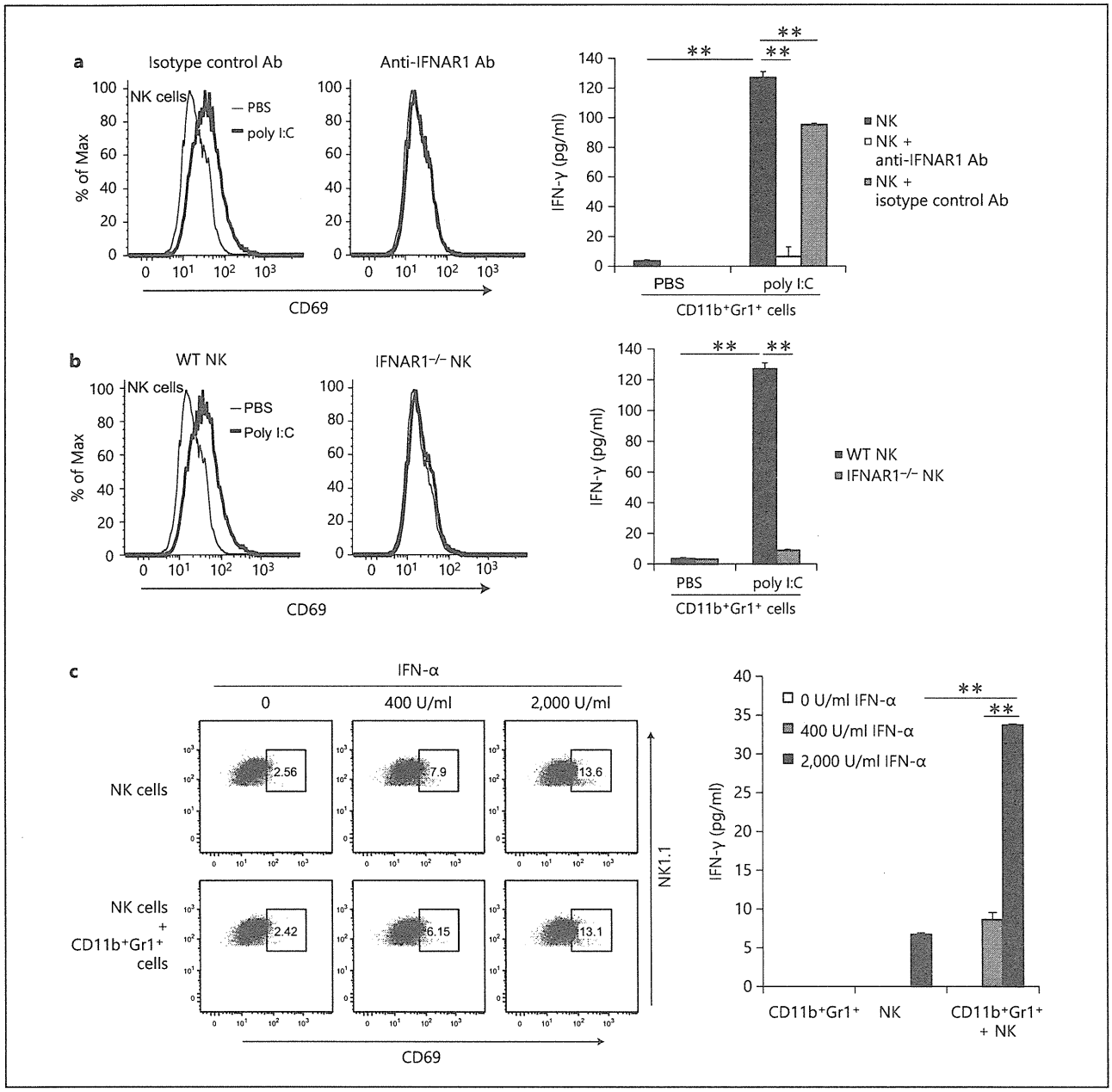

Fig. 4. Type-I IFNs from CD11b$^+$Gr1$^+$ cells and IFNAR in NK cells are indispensable for NK cell activation. **a** CD11b$^+$Gr1$^+$ cells were isolated from spleens of B16 tumor-bearing mice treated with 200 μg poly I:C or PBS for 4 h and cultured with NK cells from naïve WT mice in the presence or absence of 10 μg/ml anti-IFNAR1 antibody (Ab). After 24 h, CD69 expression on NK cells (left panels) and IFN-γ concentration in conditioned medium (right panel) was determined (n = 3). **b** CD11b$^+$Gr1$^+$ cells isolated as described in **a** were cultured for 24 h with NK cells from naïve WT mice or IFNAR1$^{-/-}$ mice, and CD69 expression (left panels) and IFN-γ production were measured (n = 3) (right panel). **c** Recombinant IFN-α was added to cultures of naïve NK cells with or without CD11b$^+$Gr1$^+$ cells from nontreated tumor-bearing mice. After incubation for 24 h, CD69 expression on NK cells (left panels) and IFN-γ concentration in conditioned medium (right panel) were determined (n = 3). CD69 expression of NK1.1$^+$CD3ε$^-$ cells is indicated (**a**–**c**). Data shown are representative of two independent experiments. ** $p < 0.01$.

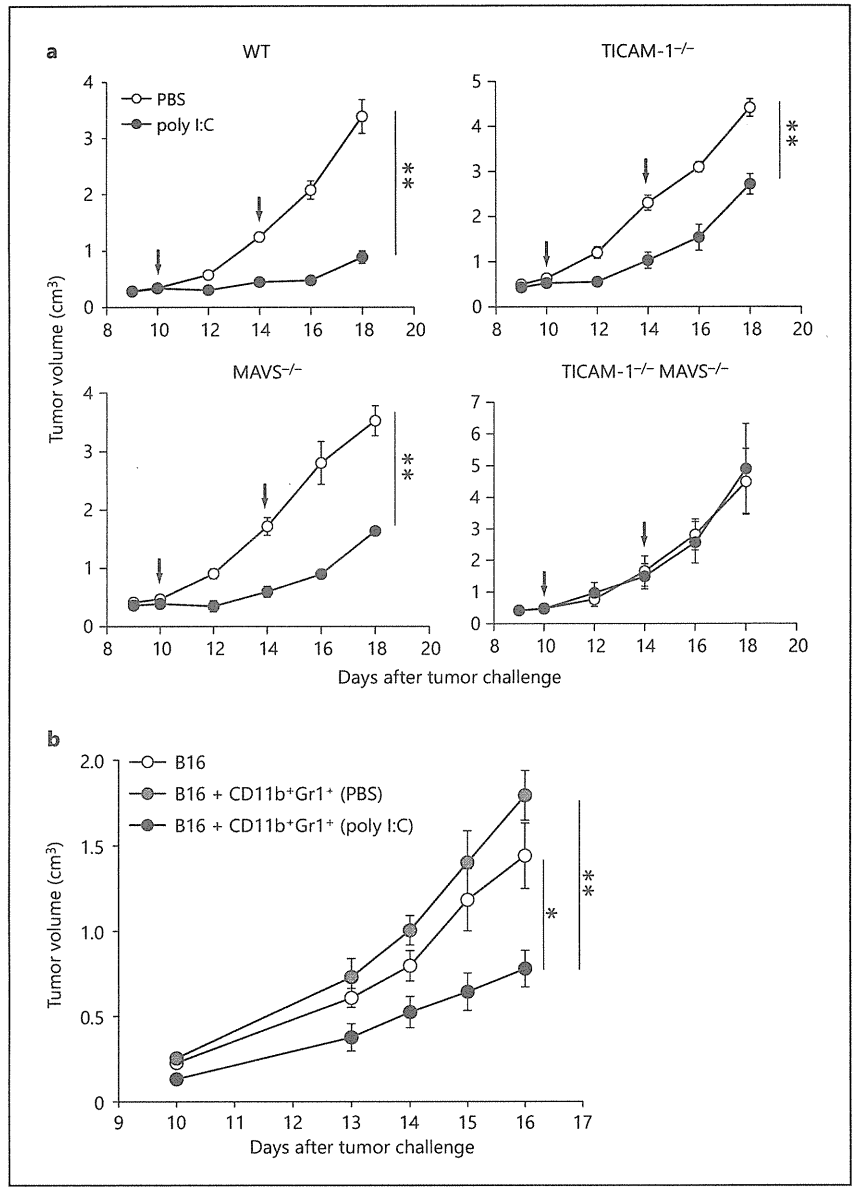

Fig. 5. Retardation of B16 tumor growth by poly I:C treatment in mouse models. **a** Both TICAM-1 and MAVS signals are involved in B16 tumor growth retardation after poly I:C therapy. B16 cells (6×10^5) were implanted s.c. into WT, TICAM-$1^{-/-}$, MAVS$^{-/-}$ and TICAM-1 and MAVS double-knockout mice. Tumor-bearing mice were treated with 200 μg poly I:C or PBS on days 10 and 14 (arrows) (n = 3–5 per group). Data are average ± SEM. **b** In vivo poly I:C-activated CD11b$^+$Gr1$^+$ cells inhibit B16 tumor growth. B16 cells (6×10^5) were mixed with or without CD11b$^+$Gr1$^+$ cells (1×10^6) from spleens of B16 tumor-bearing mice treated with 200 μg poly I:C or PBS for 4 h. Cell mixtures were implanted s.c. into WT mice on day 0 (n = 4 per group). Data are average ± SEM and are representative of 2 independent experiments. ** $p < 0.01$, * $p < 0.05$.

together with the results in figure 4, suggest that two modes of NK priming occur simultaneously in CD11b$^+$Gr1$^+$ cells: one mode is through type-I IFN production and the other is via cell-cell contact.

MAVS and IFNAR Are Required to Activate MDSCs with in vivo Poly I:C Treatment

Poly I:C induces growth retardation of B16 tumors implanted in WT mice [4–6, 36]. To determine the signaling pathway that is essential for the retardation of B16 tumor growth in vivo, we implanted B16 melanoma cells s.c. into TICAM-$1^{-/-}$ and MAVS$^{-/-}$ mice. B16 tumor growth was monitored after poly I:C injection. Marked tumor growth retardation was observed in poly I:C-treated mice (fig. 5a). The poly I:C antitumor effect was only partly abrogated in either TICAM-$1^{-/-}$ or MAVS$^{-/-}$ mice and was completely abolished in TICAM-$1^{-/-}$/MAVS$^{-/-}$ mice (fig. 5a). Therefore, both TICAM-1 and MAVS signals are involved in the antitumor activity of poly I:C, consistent with earlier reports [4, 5, 33].

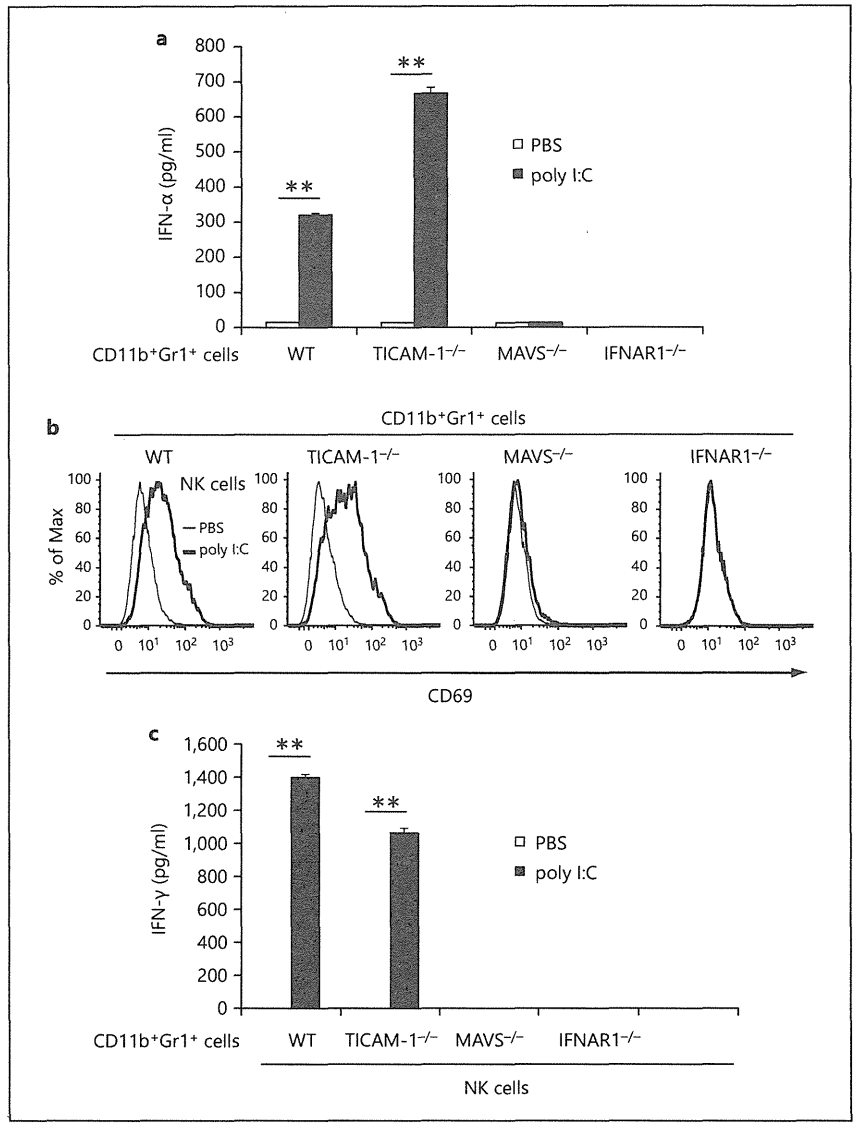

Fig. 6. MAVS and type-I IFN signaling pathways are critical for CD11b$^+$Gr1$^+$ cell activation in vivo. **a** B16 cells (6×10^5) were implanted into WT, TICAM-1$^{-/-}$, MAVS$^{-/-}$ or IFNAR1$^{-/-}$ mice. Tumor-bearing mice were treated with 200 μg poly I:C or PBS for 4 h and CD11b$^+$Gr1$^+$ cells were isolated from spleens, and allowed to stand for 24 h. IFN-α concentration in conditioned medium was determined (n = 3). **b**, **c** CD11b$^+$Gr1$^+$ cells were isolated from KO mouse lines as described in **a**, and were cultured with naïve WT NK cells for 24 h. CD69 expression on NK cells (**b**) and IFN-γ concentration in conditioned medium (**c**) were determined (n = 3). Data shown are representative of 3 independent experiments. ** $p < 0.01$.

Next, we determined the mechanisms involved in the in vivo activation of CD11b$^+$Gr1$^+$ cells by poly I:C. To investigate the signaling pathway that was important for poly I:C-induced activation of CD11b$^+$Gr1$^+$ cells in vivo, we challenged TICAM-1$^{-/-}$ and MAVS$^{-/-}$ mice with B16 melanoma cells. After tumor formation, poly I:C was injected i.p. into the mice and CD11b$^+$Gr1$^+$ cells were isolated from the spleen and were cocultured with naïve WT NK cells. CD11b$^+$Gr1$^+$ cells from tumor-bearing TICAM-1$^{-/-}$ mice produced IFN-α at levels comparable to cells from WT mice (fig. 6a). In parallel, CD69 expression on NK cells and IFN-γ production was observed in conditioned medium from mixed cultures of TICAM-1$^{-/-}$ CD11b$^+$Gr1$^+$ cells and naïve WT NK cells. The results suggest that in vivo TICAM-1 signaling is not mandatory for CD11b$^+$Gr1$^+$ cell activation to induce NK cell priming (fig. 6a–c). CD11b$^+$Gr1$^+$ cells from tumor-bearing MAVS$^{-/-}$ mice treated with poly I:C did not produce IFN-α or induce CD69 expression and IFN-γ production in NK cells (fig. 6a–c). Similar results were obtained with CD11b$^+$Gr1$^+$ cells from B16 tumor-bearing IFNAR1$^{-/-}$ mice (fig. 6a–c). These results suggest that MAVS as well as type-I IFN signaling is crucial for poly I:C-dependent NK cell priming in CD11b$^+$Gr1$^+$ cells of tumor-bearing mice.

The MAVS pathway is conserved in most cell types in mice. We examined whether NK cell priming induced by poly I:C (i.e. MAVS signal)-activated $CD11b^+Gr1^+$ cells was involved in retardation of B16 tumor growth. NK-sensitive B16 tumor cells were mixed with $CD11b^+Gr1^+$ cells isolated from poly I:C (or control PBS)-injected tumor-bearing mice, and inoculated s.c. into WT mice (fig. 5b). Significant B16 growth retardation was detected only in those tumors containing poly I:C-treated $CD11b^+Gr1^+$ cells (fig. 5b). The B16 tumors with intact $CD11b^+Gr1^+$ cells showed higher growth rates than B16 tumor cells only, which might reflect the previously-reported tumor-supporting activity of MDSCs [37]. Thus, MDSC-like $CD11b^+Gr1^+$ cells can be converted to cells with an NK-priming activity that induces growth retardation of NK-sensitive tumors in mice. NK-priming would be a condition prior to full activation of antitumor NK cells.

Discussion

We demonstrated that in vivo poly I:C treatment led to $CD11b^+Gr1^+$ MDSC maturation and cytokine production in tumor-bearing mice. Poly I:C treatment rendered tumor and spleen MDSCs competent for $DX5^+$ NK cell priming as measured by CD69 expression and IFN-γ production. Poly I:C-dependent NK priming raises through the MAVS pathway (fig. 6). Among a number of proteins that were upregulated after poly I:C treatment, type-I IFN produced by activated MDSCs was critical for NK cell priming since it activated the IFNAR pathway in NK cells. However, NK cells barely exerted direct cytotoxic activity to B16 cells in response to poly I:C-matured MDSCs. This NK activation profile resembles that of IFN-γ-producing innate lymphoid cells. Some populations of these innate lymphocytes produce IFN-γ but exhibit little cytotoxic activity [38], similar to the NK cells affected by MDSC. These findings would allow us to speculate that the production of IFN-γ without cytotoxic activity is an activation state of NK cells or innate lymphocytes where MDSCs contribute.

In tumor-bearing hosts, poly I:C treatment resulted in tumor regression. Poly I:C induces direct killing of 3LL tumor cells by M2-M1 conversion of tumor-associated macrophages [7]. The TICAM-1 signal facilitates tumor-associated macrophage conversion as well as cross-presentation by DCs leading to antigen-specific CTL induction, which is also evoked by poly I:C [8]. The action of $CD11b^+Gr1^+$ MDSCs on implant B16 tumor was tumor-supporting when MDSCs were embedded into the tumor (fig. 5b). However, once MDSCs were pretreated with poly I:C and mixed with B16 cells, tumor growth was prohibited (fig. 5b). The result suggests that MDSC has plasticity to change the function from tumor-supporting to tumor-suppressing even in vivo. Here, we highlight the first evidence of MDSCs to evoke NK cell priming, which ultimately associates with retardation of tumor growth.

Although the exact mechanism of tumor regression by MDSC-NK activation remains to be elucidated, we speculate that IFN-γ produced by the primed NK cells could evoke antitumor activity. One possibility is that IFN-γ directly inhibits the growth of a certain tumor line including B16 melanoma by inducing cell cycle arrest. IFN-γ has a synergistic effect on type-I IFNs, arresting cell cycle to cell death in some tumor cell lines independent of p53 [39]. IFN-γ also induces angiostasis, which prevents rapid tumor progression [19], and inhibits metastasis and proliferation of B16 melanoma [17, 20]. In fact, we observed that IFN-γ directly inhibits proliferation of B16 cells in vitro, suggesting that NK cell-derived IFN-γ might inhibit B16 tumor growth during poly I:C treatment (online suppl. fig. 3).

Retardation of B16 growth was partially abrogated in TICAM-$1^{-/-}$ or $MAVS^{-/-}$ mice and completely abrogated in TICAM-$1^{-/-}$, $MAVS^{-/-}$ double KO mice (fig. 5a), the two pathways contributing to in vivo poly I:C-derived tumor suppression. In addition, MDSC activation is completely abrogated in $IFNAR1^{-/-}$ mice and IFNAR in NK cells is involved in efficient IFN-γ production induced by activated MDSCs. Type-I IFN receptor signaling is crucial for growth retardation of B16 tumor in poly I:C therapy. Therefore, a variety of situations result in NK activation/priming in the therapeutic use of poly I:C in tumor-bearing mice, although IFNAR is the common factor.

Miyake et al. [5], reported that MAVS is responsible for NK cell-dependent tumor regression using $MAVS^{-/-}$ mice with poly I:C stimulation ; however, the cell types for the poly I:C response and the mechanism of induction of NK-sensitive tumor regression remain undefined. NK-activating ligands on the DC surface as well as soluble factors induced by IRF-3 and IFNAR stimulation are crucial for DC-mediated NK cell activation [15, 32]. In addition, MDSC is a cell type that specifically drives NK priming through the MAVS pathway (fig. 6). MAVS-dependent IRF-3 activation occurs through stromal cells other than DCs, and these cells including MDSCs participate in NK-sensitive tumor regression. The result of this MDSC function is in contrast to that of DCs where the TLR3/TICAM-1 pathway preferentially promotes NK cell acti-

vation [4]. Specific depletion of MDSCs in preformed tumors, if possible, would enable us to confirm the functional importance of MAVS in tumor regression reported by Miyake et al. [5], who postulated that stromal cells were a source of the cell type that induces MAVS-mediated NK priming. However, the function of poly I:C via the MDSC-NK cell pathway is only a part of the total antitumor activity of poly I:C, which would be difficult to detect by tumor-size reduction. MDSCs are expanded in a late phase of implant tumor, where poly I:C may act on MDSCs and exert antitumor activity via poly I:C-stimulated MDSCs.

A recent report suggested that type-I IFNs act on accessory cells such as DCs, leading to the production of IL-15, IL-12, IL-18 and NKG2D ligands such as RAE-1. IFN-α acted on NK cells and slightly induced IFN-γ production, which was augmented in the presence of MDSCs. Cell-cell interaction between MDSCs and NK cells appears to be required for robust IFN-γ production. Induction of NK-activating ligands in association with natural NK cytotoxicity is involved in cell-cell contact-mediated NK cell activation. We found mRNA for downstream genes of IRF-3, especially IL-15, IL-18, INAM and RAE-1 elevated in MDSCs after treatment with poly I:C. However, IL-15 and RAE-1 appear not to participate in IFN-γ production by NK cells with MDSCs because neutralizing antibodies to IL-15, RAE-1 or NKG2D did not inhibit IFN-γ production by NK cells. Therefore, other molecules should be involved in NK cell activation by MDSCs. In fact, INAM or other molecules expressed on the MDSC surface sustain NK cell activation following poly I:C treatment (fig. 2; data not shown) as in bone marrow-derived cells [32].

MDSCs that have expanded in tumor-bearing hosts strongly suppress antitumor immune responses [22, 23]. Reduction of MDSC population or function is achieved by treatment with reagents that are related to improvement in tumor-specific immunity [40]. Furthermore, maturation of MDSCs can be accomplished through IFN-α production by plasmacytoid DCs or direct TLR9 stimulation, which contributes to tumor regression [30, 31]. Direct administration of IFN-α, i.e. IFN therapy, however, has not been successful as a universal therapy in cancer patients. Serious side effects are associated with high therapeutic doses of type-I IFN as well as poly I:C. Development of less toxic reagents with sufficient IRF-3/7 activation would be important for anti-MDSC cancer therapy.

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MAVS-dependent IRF3/7 bypass of interferon β-induction restricts the response to measles infection in CD150Tg mouse bone marrow-derived dendritic cells

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ABSTRACT

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

1. Introduction

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

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

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

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

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

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

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

2. Materials and methods

2.1. Mice

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

2.2. Cell culture

Vero/CD150 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. BMDCs were generated from bone marrow according to the method described by Inaba et al. (1992), with slight modifications. Briefly, bone marrow samples from the femurs and tibiae of mice were cultured in RPMI 1640 (GIBCO) with 10% heat-inactivated FBS containing GM-CSF (J558 supernatant) for 6 days with replenishment of the medium every other day. Splenic naïve CD4$^+$ CD25$^-$ T cells were isolated by negative selection using the biotin-CD8a, CD11b, B220, Dx5, Gr1, CD25 anitbody and streptavidin beads (Miltenyi Biotec) (typically >90% purity) (Akazawa et al., 2007). For coculture experiment, 2×10^5 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.

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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.

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

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

Next, to clarify the reason why MV was barely able to infect CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$BMDCs, we evaluated type I IFN expression in MV-infected BMDCs (Fig. 2A). As expected, *Ifn-α4* mRNA was induced by MV infection in CD150Tg, CD150Tg/*Ifnar*$^{-/-}$ and CD150Tg/*Ticam1*$^{-/-}$BMDCs, but not in CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ or CD150Tg/*Mavs*$^{-/-}$BMDCs (Fig. 2A). IFN-α protein was also induced in CD150Tg, CD150Tg/*Ticam1*$^{-/-}$BMDCs and to a lesser extent in CD150Tg/*Ifnar*$^{-/-}$ BMDCs (Fig. 2B). The message-protein discrepancy was observed with IFN-α4 in MV-infected CD150Tg/*Ifnar*$^{-/-}$ mice as reported (Mariet 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

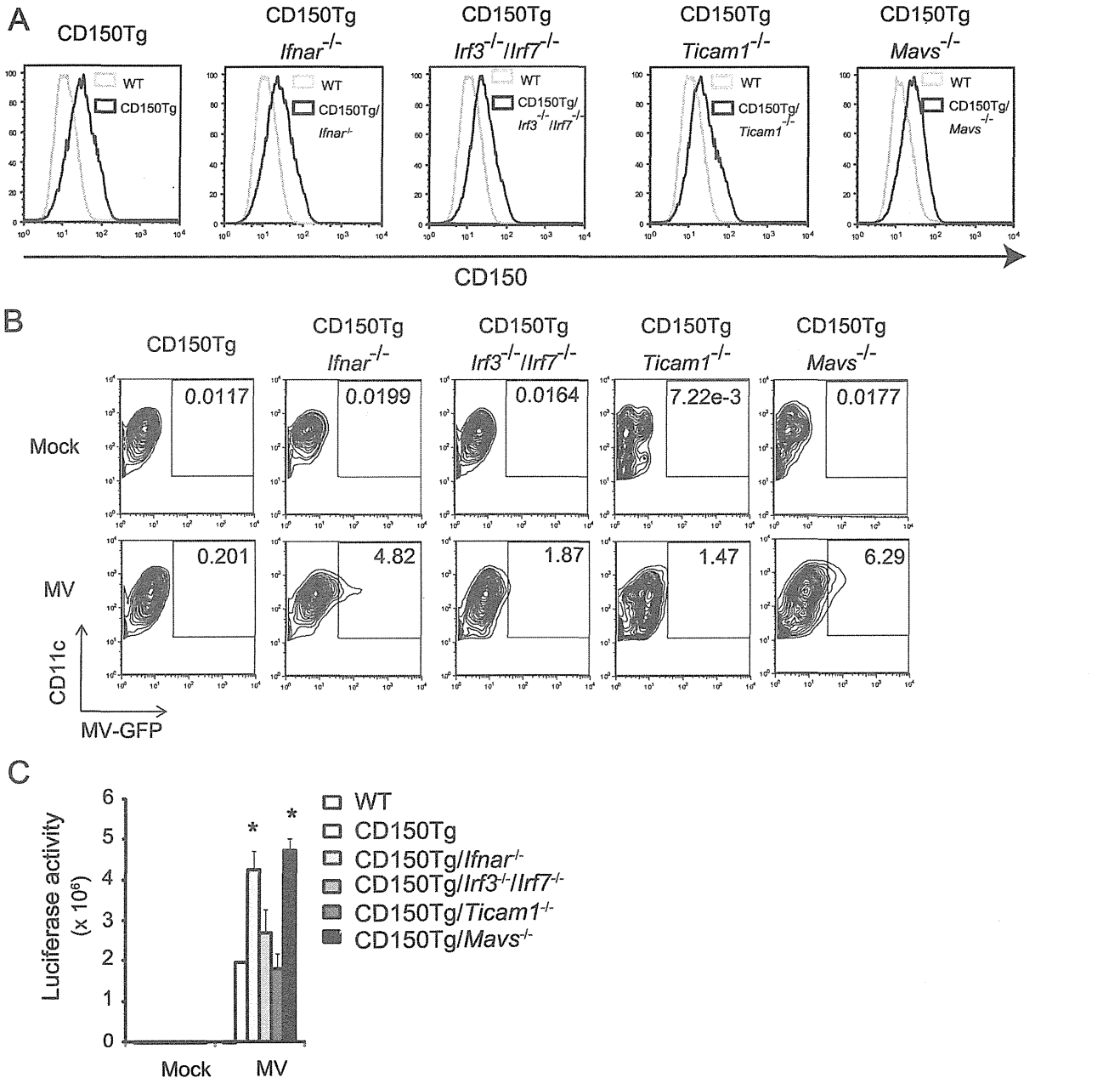

Fig. 1. CD150Tg/*Mavs*$^{-/-}$ BMDCs were permissive to MV infection. (A) Expression levels of human CD150 in BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were measured by FACS. The results are 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.

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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

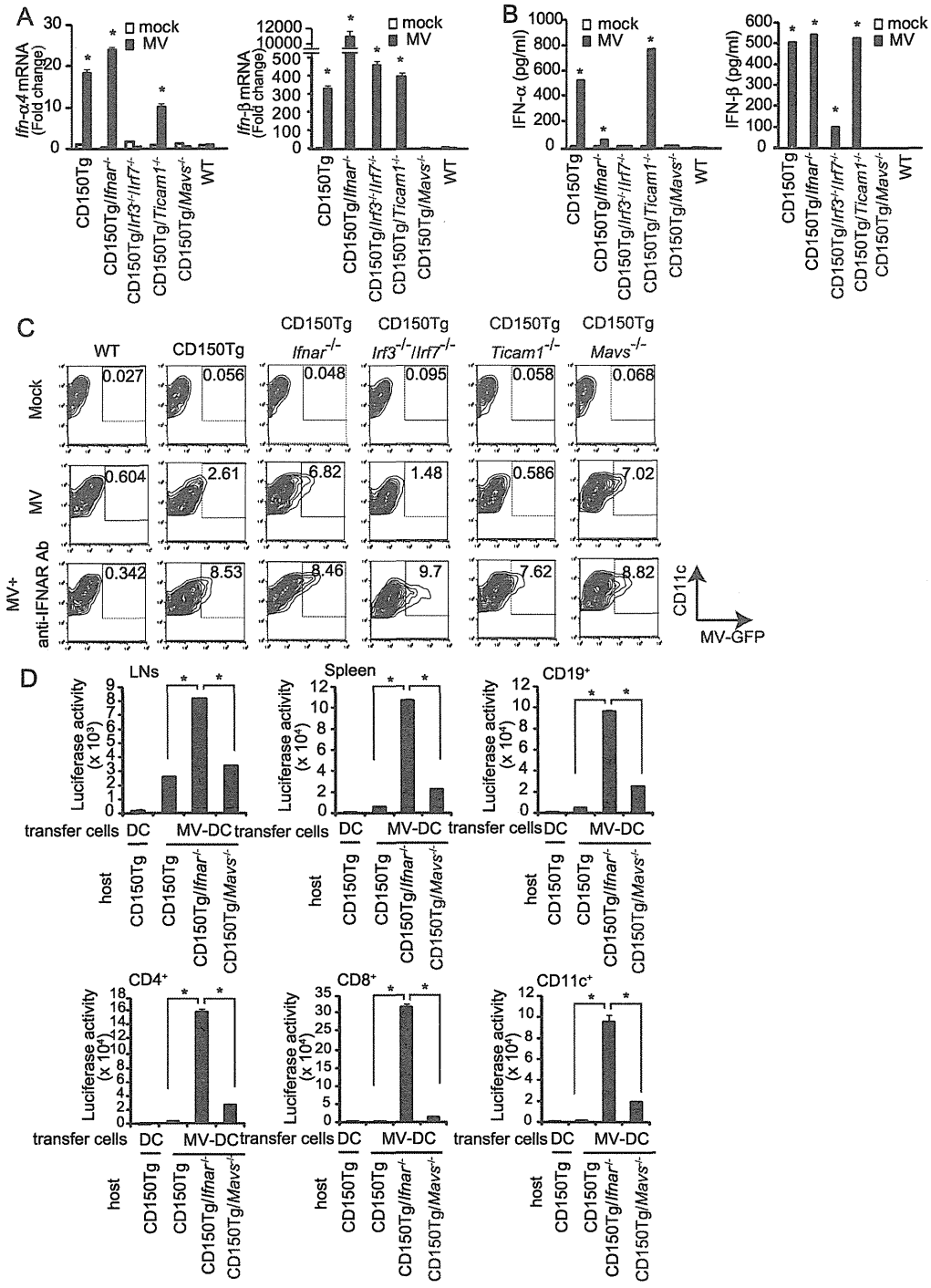

Fig. 2. MV infection did not induce type I IFN in CD150Tg/*Mavs*$^{-/-}$ BMDCs. BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-GFP (MOI = 0.25) or mock infected. (A) At 24 h 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

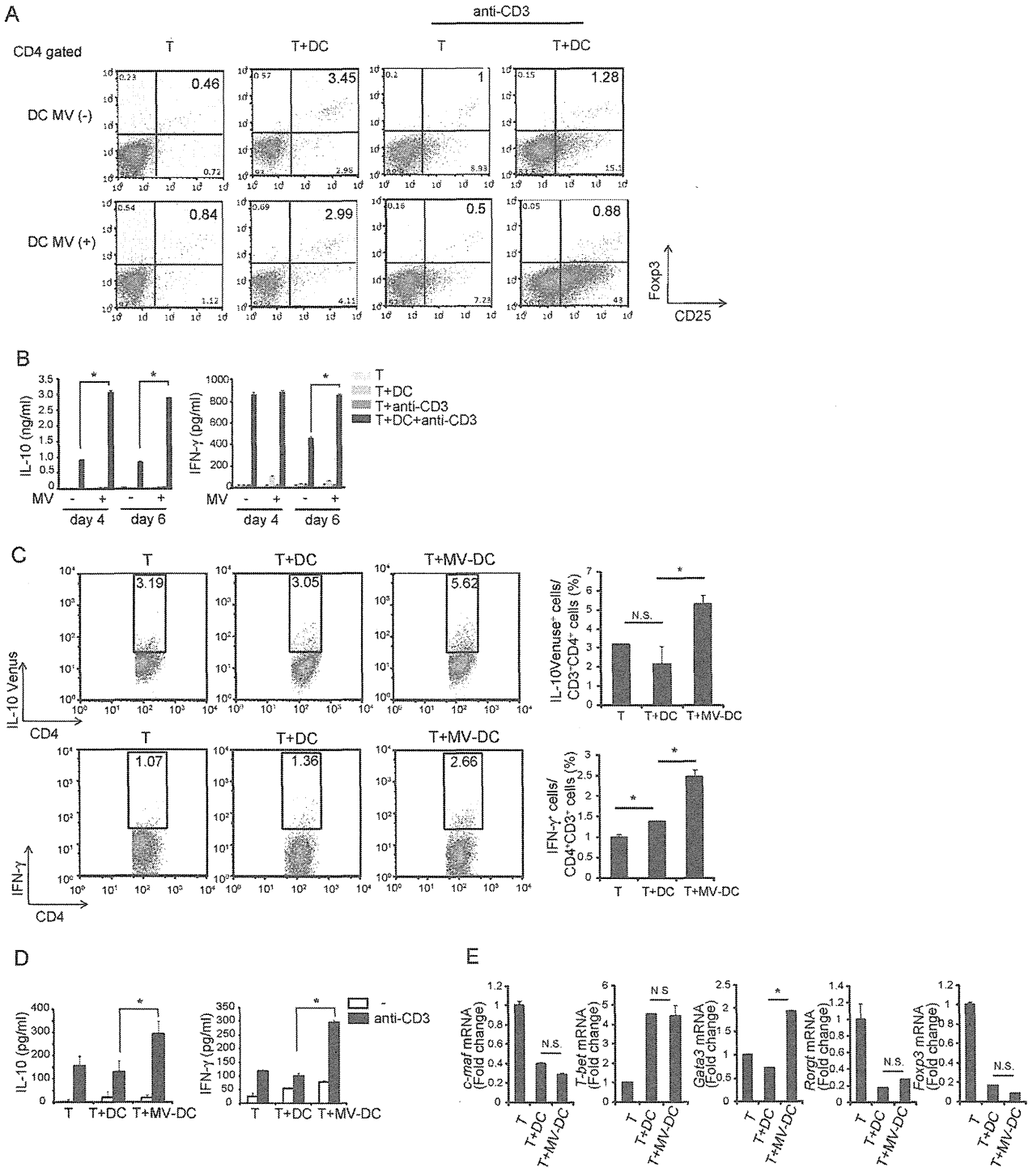

Fig. 3. MV-infected BMDCs induced IL-10 and IFN-γ producing CD4+ T cells. CD150Tg/*Ifnar*$^{-/-}$ BMDCs were infected with MV (MOI = 0.25) or mock for 24 h. Naïve CD4+CD25− T cells (2×10^5) isolated from WT mice were cocultured with 1×10^4 BMDCs in the presence or absence of 0.1 μg/ml of the anti-CD3 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, *$p < 0.05$.

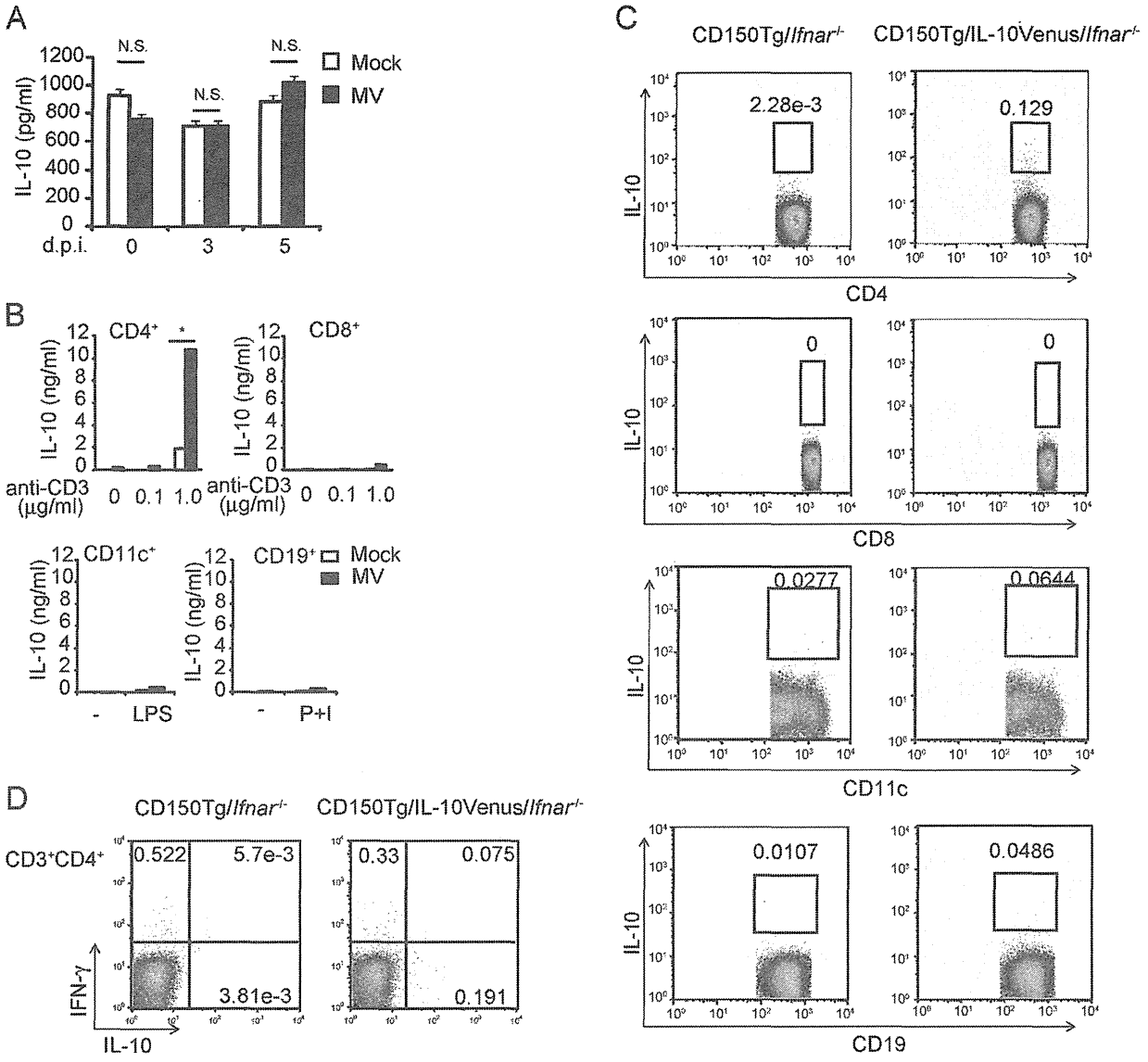

Fig. 4. CD4⁺ T cells produced IL-10 *ex vivo*. (A) CD150Tg/*Ifnar*$^{-/-}$ mice were infected i.p. with 1×10^6 pfu MV-GFP or mock. At the indicated days after infection, IL-10 production in sera was measured by ELISA. The data are the means ± SD of three independent samples. N.S.; not significant. (B) 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.

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The MyD88 Pathway in Plasmacytoid and CD4$^+$ Dendritic Cells Primarily Triggers Type I IFN Production against Measles Virus in a Mouse Infection Model

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Infection by measles virus (MV) induces type I IFN via the retinoic acid–inducible gene I/melanoma differentiation–associated gene 5/mitochondrial antiviral signaling protein (MAVS) pathway in human cells. However, the in vivo role of the MAVS pathway in host defense against MV infection remains undetermined. CD150 transgenic (Tg) mice, which express human CD150, an entry receptor for MV, with the disrupting IFNR gene (*Ifnar*$^{-/-}$), are susceptible to MV and serve as a model for MV infection. In this study, we generated CD150Tg/*Mavs*$^{-/-}$ mice and examined MV permissiveness compared with that in CD150Tg/*Ifnar*$^{-/-}$ mice. MV replicated mostly in the spleen of i.p.-infected CD150Tg/*Ifnar*$^{-/-}$ mice. Strikingly, CD150Tg/*Mavs*$^{-/-}$ mice were not permissive to MV in vivo because of substantial type I IFN induction. MV barely replicated in any other organs tested. When T cells, B cells, and dendritic cells (DCs) isolated from CD150Tg/*Mavs*$^{-/-}$ splenocytes were cultured with MV in vitro, only the DCs produced type I IFN. In vitro infection analysis using CD150Tg/*Mavs*$^{-/-}$ DC subsets revealed that CD4$^+$ and plasmacytoid DCs, but not CD8α^+ and CD8α^-CD4$^-$ double negative DCs, were exclusively involved in type I IFN production in response to MV infection. Because CD150Tg/*Mavs*$^{-/-}$ mice turned permissive to MV by anti-IFNAR Ab, type I IFN produced by CD4$^+$ DCs and plasmacytoid DCs plays a critical role in antiviral protection for neighboring cells expressing IFNAR. Induction of type I IFN in these DC subsets was abolished by the MyD88 inhibitory peptide. Thus, production of type I IFN occurs via the MyD88-dependent and MAVS-independent signaling pathway during MV infection. *The Journal of Immunology*, **2013, 191: 4740–4747.**

Type I IFNs (IFN-α/β) are crucial for protection against viral infections (1). Viral RNA is detected by cytosolic RNA sensors and induces expression of type I IFN (2). Extracellular dsRNA of a virus product is detected by the endosomal TLR3, whereas intracellular dsRNA is sensed by the retinoic acid–inducible gene I (RIG-I) and the melanoma differentiation–associated gene 5 (MDA5) (3). Upon recognizing dsRNA, TLR3 recruits the Toll/IL-1R (TIR) homology domain–containing adaptor molecule 1 (TICAM-1, also referred to as TRIF) and induces type I IFN production (4, 5). The RIG-I–like receptors (RLRs), RIG-I and MDA5, signal via the mitochondrial antiviral signaling protein (MAVS; also known as VISA, Cardif, or IPS-1) and also induce type I IFN expression (6). Knocking out these adaptor molecules results in failure to activate the transcription factors IFN regulatory factor (IRF)-3 and IRF-7, leading to an incompetence in type I IFN production and antiviral host defense (3, 7, 8). Type I IFN induction following the recognition of measles virus (MV) RNA is dependent on the RIG-I/MDA5-MAVS pathway in human epithelial cell lines (9, 10). However, the role of the RIG-I/MDA5-MAVS pathway during in vivo MV infection remains undetermined.

MV, of the genus *Morbillivirus* from the Paramyxoviridae family, is a highly pathogenic, nonsegmented negative single-stranded RNA virus that causes respiratory distress and immunosuppression in humans (11). Wild-type strains of MV enter cells via human CD150, which is also referred to as signaling lymphocyte activation molecule (12), and human poliovirus receptor–like protein 4 (13, 14). Expression of these receptors is restricted either to activated lymphocytes, dendritic cells (DCs), and macrophages for CD150 or to the basolateral surface of epithelial cells for PVRL4 (15). Among these cell populations, CD11c$^+$ DCs and alveolar macrophages (AMs) are reported to be the first target cells of early-phase MV infection in the CD150 transgenic (Tg) mouse model (16, 17) and in nonhuman primates (18, 19). Moreover, DCs are found to be involved in pathogenesis and immunosuppression during and after acute MV infection (20, 21). However, it is unclear how DCs and macrophages recognize MV RNA to produce type I IFN.

Human CD150Tg mice, which are slightly permissive to MV, are used to study host responses against MV infection in vivo (16, 22–24). CD150Tg/*Ifnar*$^{-/-}$ mice, which are generated by crossing CD150Tg mice with *Ifnar*$^{-/-}$ mice, are susceptible to MV infection

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Abbreviations used in this article: AM, alveolar macrophage; BMDC, bone marrow–derived DC; cDC, conventional DC; DC, dendritic cell; DN, double negative; IRF, IFN regulatory factor; LN, lymph node; MAVS, mitochondrial antiviral signaling protein; MDA5, melanoma differentiation–associated gene 5; MOI, multiplicity of infection; MV, measles virus; MyD88, myeloid differentiation factor 88; pDC, plasmacytoid DC; PDCA-1, pDC Ag 1; RIG-I, retinoic acid–inducible gene I; RLR, RIG-I–like receptor; Tg, transgenic; TICAM-1, Toll/IL-1R homology domain–containing adaptor molecule 1; TIR, Toll/IL-1R.

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and serve as a useful mouse model (16, 24). In the current study, using the CD150Tg mouse model in combination with *Mavs*$^{-/-}$, *Irf3*$^{-/-}$/*Irf7*$^{-/-}$, and *Ticam1*$^{-/-}$ mice, we found that CD150Tg/*Mavs*$^{-/-}$ mice were not permissive to MV in vivo, whereas CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$ mice were permissive. Furthermore, CD150Tg/*Mavs*$^{-/-}$ plasmacytoid DCs (pDCs) and CD4$^+$ DCs produced type I IFN in response to MV infection in vitro. Analysis using the myeloid differentiation factor 88 (MyD88) inhibitory peptide and MyD88$^{-/-}$ mice revealed that type I IFN production in these DC subsets was dependent on the MyD88 pathway. To our knowledge, this is the first study to show that type I IFN induction in MV-infected mouse DCs depends on the MyD88 pathway. The properties of the MV-permissive mouse DC subsets may be crucial for ensuring immune response, including immunosuppression during MV infection.

Materials and Methods

Mice

All mice were backcrossed to C57BL/6 mice more than eight times before use. CD150Tg (16), *Ticam1*$^{-/-}$ (25), and *Mavs*$^{-/-}$ (26) mice were generated in our laboratory. *Irf3*$^{-/-}$ and *Irf7*$^{-/-}$ mice were provided by Dr. T. Taniguchi (University of Tokyo, Tokyo, Japan). *Myd88*$^{-/-}$ mice were provided by Drs. K. Takeda and S. Akira (Osaka University, Osaka, Japan). 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 wk of age. This study was carried out in strict accordance with the recommendations in the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University. All mice were used according to the guidelines of the Institutional Animal Care and Use Committee of Hokkaido University, which approved this study as no.13-0024. All inoculation and experimental manipulation were performed with the animals under anesthesia that was induced and maintained with pentobarbital sodium, and all efforts were made to minimize suffering.

Virus and cell culture

Vero/CD150 cells were maintained in DMEM supplemented with 10% heat-inactivated FBS and antibiotics. IC323, corresponding to the IC-B strain of MV (27), was recovered from the plasmid p(+)MV323 encoding the antigenomic IC-B sequence (28). IC323-Luci (MV-luciferase), which expresses the reporter *Renilla* luciferase from the first gene position of the MV genome, was a kind gift from Dr. Y. Yanagi (Kyushu University, Fukuoka, Japan) (29). MV-luciferase was maintained in Vero/CD150 cells (30). Virus titer was determined as PFUs on Vero/CD150, and the multiplicity of infection (MOI) of each experiment was calculated based on this titer (27). Splenic CD19$^+$, CD4$^+$, CD8$^+$ cells, and CD11c$^+$ DCs were isolated using anti-CD19, anti-CD4, anti-CD8, and anti-CD11c MACS beads (Miltenyi Biotec). Splenic CD8α$^+$ DCs, CD4$^+$ DCs, and double negative (DN) DCs were isolated using CD8α$^+$ or CD4$^+$ DC isolation kits (Miltenyi Biotec) according to the manufacturer's instructions. For isolation of pDCs and conventional DCs (cDCs), spleens were treated with 400 IU Mandle U/ml collagenase D (Roche) at 37°C for 25 min in HBSS (Sigma-Aldrich). EDTA was added, and the cell suspension was incubated for an additional 5 min at 37°C. After removal of RBCs with ammonium chloride–potassium lysis buffer, CD11c$^+$ DCs were isolated using CD11c MACS beads. MACS-sorted DCs were stained with anti-CD11b–FITC, anti-pDC Ag 1 (PDCA-1)–PE (eBioscience), and anti-CD11c–allophycocyanin (BioLegend) and sorted using a FACSAria II (BD). The purity of sorted cells was > 98%.

FACS analysis

For pDC staining, splenocytes were stained with anti-CD11c–allophycocyanin (BioLegend), anti–PDCA-1–PE (BioLegend), and anti-human CD150–FITC (eBiosciences). For CD4$^+$ DCs, CD8$^+$ DCs, and DN DCs staining, splenocytes were stained with anti-CD11c–allophycocyanin (BioLegend), anti-CD4–PerCP (BioLegend), anti-CD8–PE (BioLegend), and anti-human CD150–FITC. For B cell staining, splenocytes were stained with anti-B220–allophycocyanin (BioLegend), anti-CD19–PE (BioLegend), and anti-human CD150–FITC. For T cell staining, anti-CD3–allophycocyanin (BioLegend), anti-CD4–PerCP, anti-CD8–PE, and anti-human CD150–FITC were used. Fluorescence intensity of CD150 was measured by flow cytometry. For TLR7 intracellular staining in pDCs, DCs were stained with anti-TLR7–FITC (IMGENEX), anti-CD11c–allophycocyanin, and anti–PDCA-1–PE using the BD Cytofix/Cytoperm Kit (BD Biosciences). For TLR7 intracellular staining in CD4$^+$, CD8$^+$, and DN DCs, DCs were stained with anti-TLR7–FITC (IMGENEX), anti-CD11c–allophycocyanin, anti-CD4–PerCP, and anti-CD8–PE, using the BD Cytofix/Cytoperm Kit (BD Biosciences). Stained cells were analyzed by flow cytometry.

Experimental infection and luciferase assay

Mice were infected i.p. with MV-luciferase at the indicated doses. For in vivo blockade of the type I IFNR, mice were i.p. injected with 2.5 mg MAR1-5A3, a mAb against IFNAR-1 (BioLegend), 1 d prior to infection. Tissues were collected from the mice at different time points, and the efficiency of infection was measured by luciferase assay. Cells (1×10^7) from various tissues were harvested in 100 μl lysis buffer. The amount of protein in each lysate was determined by bicinchoninic acid assay. Luciferase assay was performed using a Dual-Luciferase Reporter Assay System (Promega), and luciferase activity was read using a Lumat LB 9507 (Berthold Technologies). To measure the efficiency of in vitro infection, cells (5×10^4–4×10^5) were harvested in 25 μl lysis buffer for luciferase assay. For MyD88 inhibition assay, cells were pretreated with 50 μM MyD88 inhibitory peptide (RQIKIWFQNRRMKWKK-RDVLPGTCVNS-NH2; InvivoGen) or the control peptide (RQIKIWFQNRRMKWKK-SLHGRGDPMEAFII-NH2; InvivoGen) for 6 h, and then cells were infected with MV. MyD88 inhibitory peptide contains a sequence from the MyD88 TIR homodimerization domain (RDVLPGT) preceded with a protein transduction sequence (RQIKIWFQNRRMKWKK) derived from antennapedia, which enables the peptide to translocate through the cell membrane. For intratracheal infection with MV, mice were anesthetized and injected with MV-luciferase (8×10^5 PFU/50 μl in PBS) intratracheally. At 3 d after inoculation, mice were sacrificed and perfused with PBS containing 10 mM EDTA from the right ventricle. Lung lobes were isolated, and collagenase buffer [150 U/ml collagenase D (Roche), 10 μg/ml DNase I (Takara), and 5% FCS in RPMI 1640 medium] was injected into the lobes, using a 27-gauge needle. The lobes were then shredded into small pieces and incubated at 37°C for 45 min. During the last 5 min, EDTA was added at 10 mM. Any remaining small pieces were dispersed by passage in and out through a 20-gauge needle, and the suspension was passed through nylon mesh to remove debris. A single-cell suspension was prepared after RBC lysis. A total of 2×10^6 cells were harvested in 100 μl lysis buffer for luciferase assay.

ELISA

Culture supernatants of cells ($1–5 \times 10^5$) seeded on 96-well plates were collected and analyzed for cytokine levels, using ELISA. ELISA kits for mouse IFN-α and IFN-β were from PBL Biomedical Laboratories. Assays were performed according to the manufacturer's instructions.

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 a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's instructions. Real-time PCR was performed using a StepOne Real-Time PCR System (Applied Biosystems). The following oligonucleotides were used for *β-actin*: 5′-TTTGCAGCTCCTTCGTTGC-3′ and 5′-TCGTCATCCATGGCGAACT-3′; for *Ifn-β*: 5′-CCAGCTCCAAGAAAGGACGA-3′ and 5′-CGCCCTGTAGGTGAGGTTGAT-3′; for *Ifn-α4*: 5′-CTGCTGGCTGTGAGGACATACT-3′ and 5′-AGGCACAGAGGCTGTGTTTCTT-3′; for *Ifit1*: 5′-TGTGCTGAGATGGACTGTGAG-3′ and 5′-TTTCTGGCTCCACTTTCAGAG-3′; for *Cxcl10*: 5′-GTGTTGAGATCATTGCCACGA-3′ and 5′-GCGTGGCTTCACTCCAGTTAA-3′; for *Tlr7*: 5′-GTATGCCGCCAAATCTAAAG-3′ and 5′-GGCTGAGGTCCAAAATTTCC-3′; and for *MV-P*: 5′-cTGCATCACGCAGTGTAATC-3′ and 5′-CTGGTGGAACTTGGCAAGATC-3′. Levels of target mRNAs were normalized to *β-actin*, and fold-induction of transcripts was calculated using the ddCT method relative to unstimulated cells.

Statistical analyses

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

Results

CD150Tg/Mavs$^{-/-}$ *mice are not permissive to MV*

To quantitate the efficiency of MV infection, we used a recombinant MV-luciferase that expresses the reporter *Renilla* luciferase from the insert of the MV genome (29). CD150 expression levels did