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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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Running title: **MAVS-mediated IFN- β restricts MV infection**

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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 BMDC produce initial type I interferon (IFN) has not been elucidated in MV infection. RNA virus infection usually allows cells to generate double-stranded RNA and induce activation of IRF3/7 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 BMDC 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*^{-/-} BMDC 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 interferon-regulatory factors (IRF) and induction of type I IFN, which initiates potent antiviral responses (Honda et al., 2006; Rathinum & 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 & Akira, 2006; Matsumoto et al., 2011). Each virus species has its own strategy to circumvent IFN induction, thereby successfully replicating in host cells.

Measles virus (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 interferon (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 (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 was 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

(Sigma) 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 antibody and streptavidin beads (Miltenyi Biotec) (typically > 90% purity) (Akazawa et al., 2007). For coculture experiment, 2 x 10⁵ CD4⁺ T cells and 1 x 10⁴ 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 x 10⁵ CD4⁺ T cells were cultured with the plate bound anti-CD3 antibody (0 - 1 µg/ml) for 48 hours.

2.3. Virus

Vero/CD150 was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum and antibiotics. MV323, 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). MV323 luciferase was kindly gifted from Dr. M. Takeda (Department of Virology III, National Institute of Infectious Disease, Tokyo, Japan) (Takeda et al., 2007). MV323-luciferase and MV 323-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 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 x 10⁴ to 2 x 10⁵) 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 MV323-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 BMDC transfer, CD150Tg/*Mavs*^{-/-} BMDCs were infected with MV (MOI = 0.25) or mock for 24 hrs. 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 Transcription 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.

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 Cytotfix/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 hours 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.

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). MV-luciferase BMDCs were infected with MV-luciferase at MOI of 0.25 for 24 hours 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*.

3.2. Type I IFN induction rendered CD150Tg/*Irf3*^{-/-}/*Irf7*^{-/-} 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 (Marie 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 □□□□ tly 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 2B), MV was able to infect CD150Tg/*Mavs*^{-/-} BMDCs. Moreover, type I IFN expression in response to MV infection depends on the MAVS pathway in BMDCs.

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 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).

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 CD4⁺, CD8⁺, CD11c⁺ and CD19⁺ cells. 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 hrs 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- κ B (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- κ B signaling pathway in West Nile virus infection, the case being not only for DC (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 BMDC transfer. It will be of interest to determine whether the results are reproducible in other DC subsets in the mouse MV-infection model.

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

Recently a study reported that IL-10 is the cause of MV-induced immunosuppression in MV-infectious model mice (Koga et al., 2010). However, during MV infection, both the cells which produce IL-10 and the induction mechanism of IL-10 in these cells have yet to be elucidated. In this report, we showed that CD4⁺ T cells are one of the cell types that produce IL-10 in response to MV infection both *ex vivo* and *in vitro*. MV-infected BMDCs induce IL-10- and IFN- γ -producing CD4⁺ T cells, but not Treg cells. Previous reports showed that T regulatory (Tr1) cells became IL-10 and IFN- γ producing CD4⁺ T cells (Vieira et al., 2004; Roncarolo et al., 2006),

and that Tr1 cells in concert with IL-10-producing DCs were indispensable for a high level of IL-10 (Roncarolo et al., 2006). However, in Fig. 4A, IL-10 was neither produced in BMDCs nor up-regulated in mouse sera irrespective of MV-infection. It is CD4⁺ T cells that produce IL-10 in response to MV and CD3 stimulation (Fig. 4B).

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

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

possible mechanisms by which MV induces immune modulation.

Conflict of interest

There is no declared conflict of interest in this study.

Acknowledgements

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Footnotes

Key words: innate immunity; dendritic cells; type I interferon; mitochondrial antiviral signaling protein (MAVS); measles virus

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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Figure legends

Figure 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 hrs 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 hrs 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 v.s. MV-infected knockout BMDCs.

Figure 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 hours 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. * $p < 0.05$, v.s. mock-infected. (B) At 24 hours 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. * $p < 0.05$, v.s. 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 $\mu\text{g/ml}$). At 24 hrs 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 hrs. 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 luciferase 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 \pm SD of three independent samples. * $p < 0.05$.

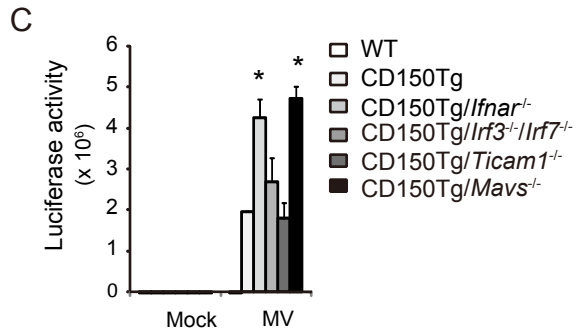
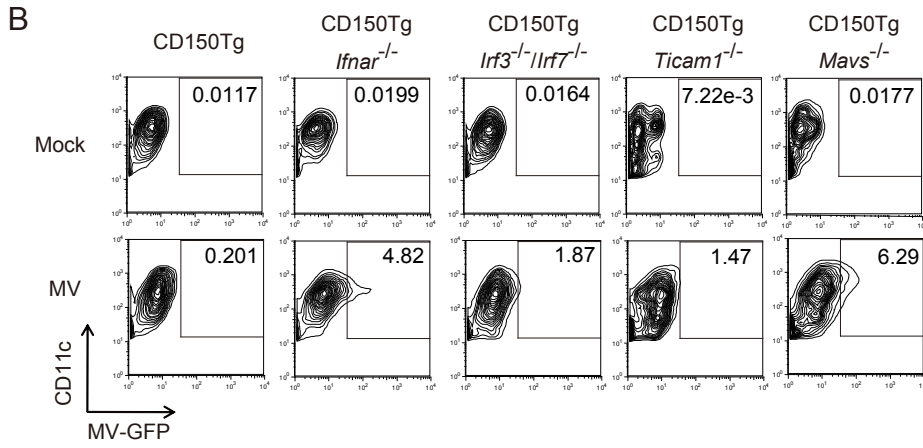
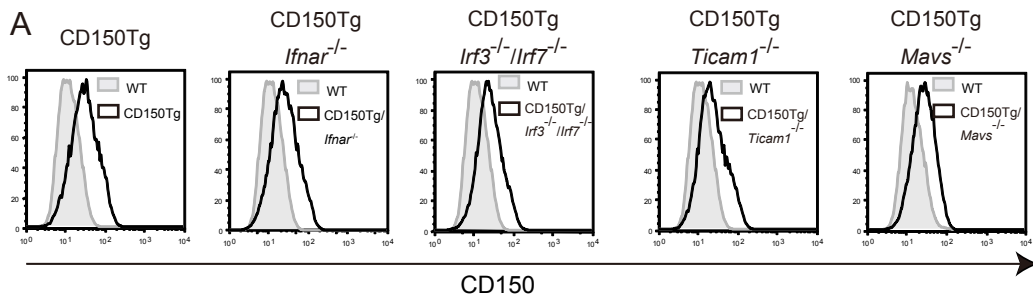
Figure 3. MV-infected BMDCs induced IL-10 and IFN- γ producing CD4⁺ T cells.

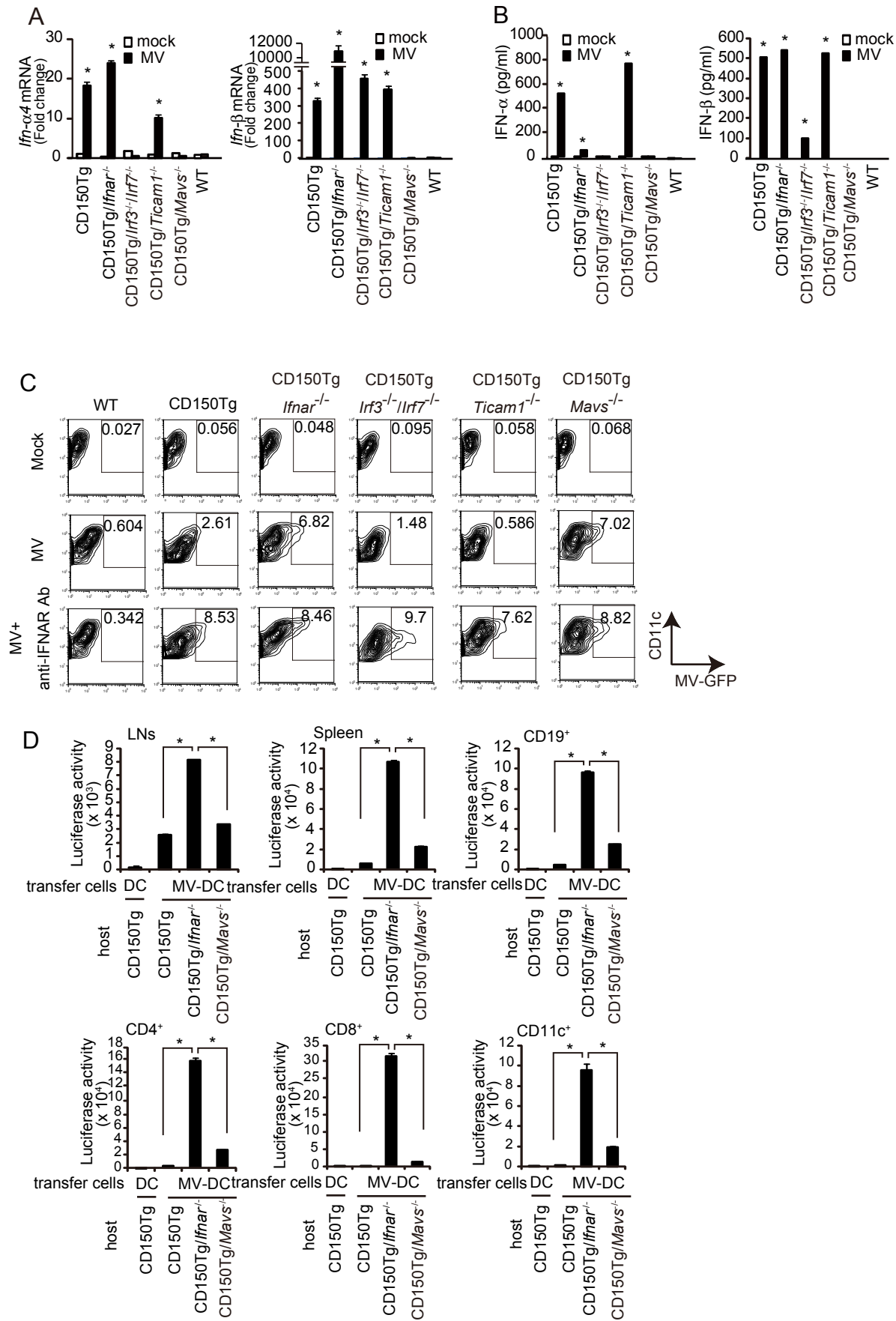
CD150Tg/*Ifnar*^{-/-} BMDCs were infected with MV (MOI = 0.25) or mock for 24 hrs. Naïve CD4⁺CD25⁻ T cells (2×10^5) isolated from WT mice were cocultured with 1×10^4 BMDCs in the presence or absence of 0.1 $\mu\text{g/ml}$ of the anti-CD3 antibody. (A) At 6 days after coculture, cells were stained with 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 \pm 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 \pm SD of three independent samples. * $p < 0.05$. (D) At 4 days after

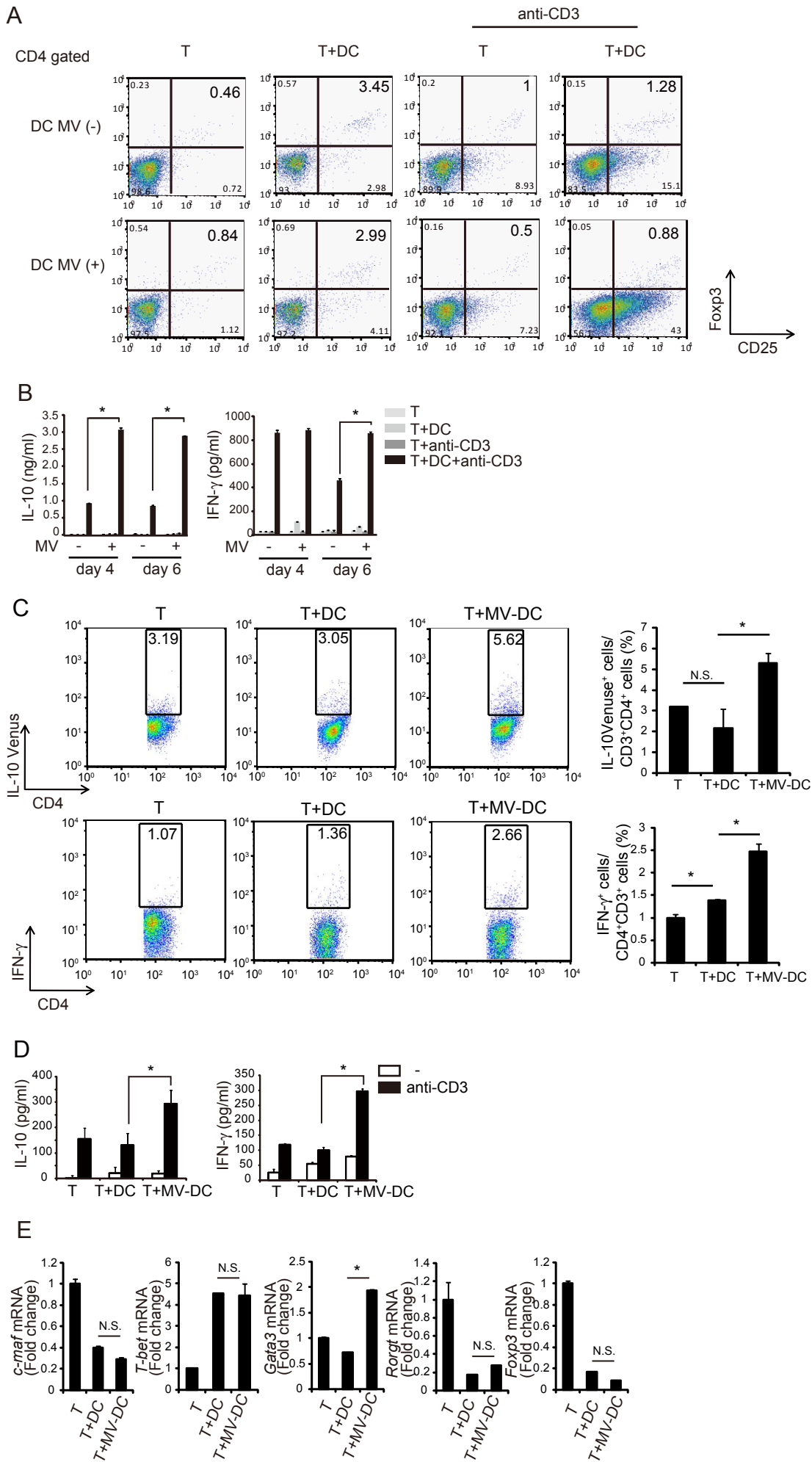
the coculture, cells were collected and washed twice. Two x 10⁵ cells were restimulated with the anti-CD3 plate-bound antibody for 3 days. At 3 days after restimulation, IL-10 and IFN- γ in the culture supernatants were measured by ELISA. The data are the means \pm SD of three independent samples. * $p < 0.05$. (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 \pm SD of three independent samples. N.S.; not significant, * $p < 0.05$.

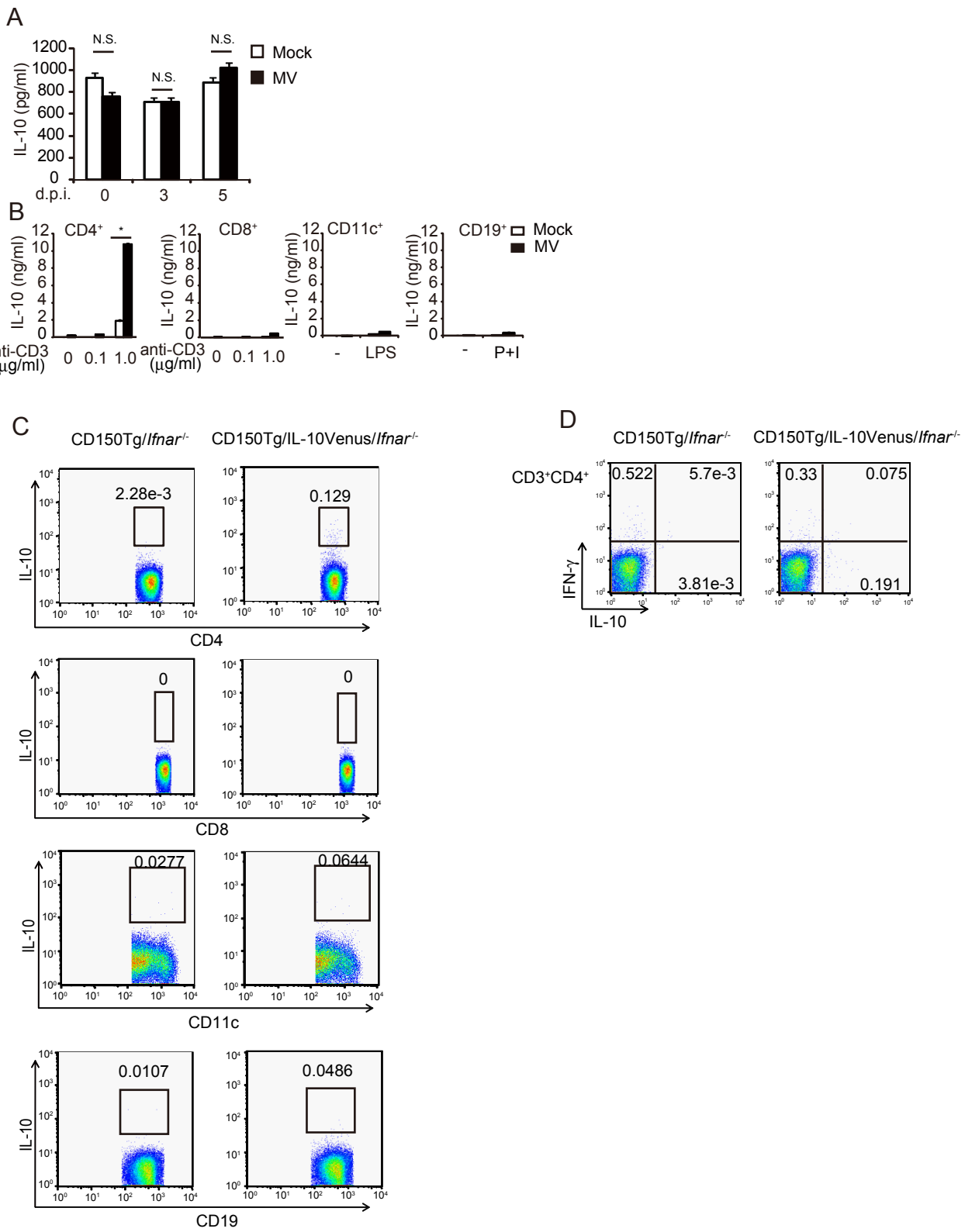
Figure 4. CD4⁺ T cells produced IL-10 *ex vivo*.

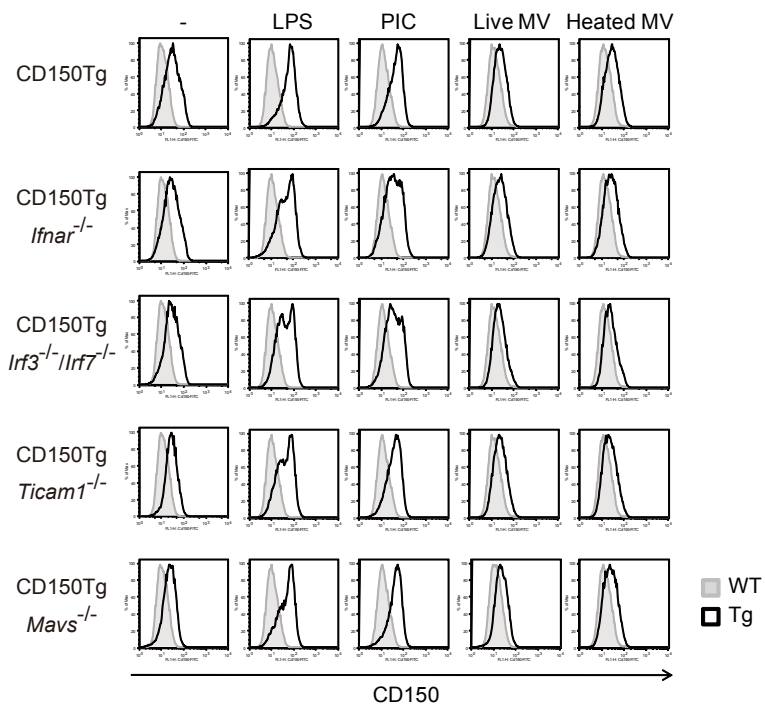
(A) CD150Tg/*Ifnar*^{-/-} mice were infected i.p. with 1 x 10⁶ pfu MV-GFP or mock. At the indicated days after infection, IL-10 production in sera was measured by ELISA. The data are the means \pm SD of three independent samples. N.S.; not significant. (B) CD150Tg/*Ifnar*^{-/-} mice were infected i.p. with 1 x 10⁶ 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 μ g/ml), LPS (100 ng/ml) or PMA (1 μ g/ml) plus ionomycin (1 μ g/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 \pm SD of three independent samples. * $p < 0.05$. (C, D) CD150Tg/*Ifnar*^{-/-} and CD150Tg/IL-10 Venus/*Ifnar*^{-/-} mice were infected with MV (1 x 10⁶ 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.

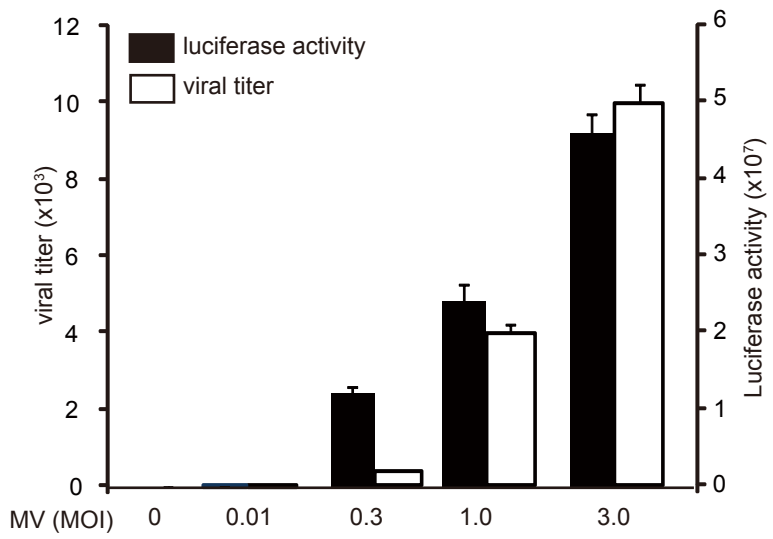


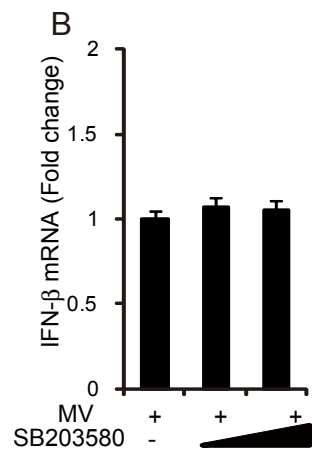
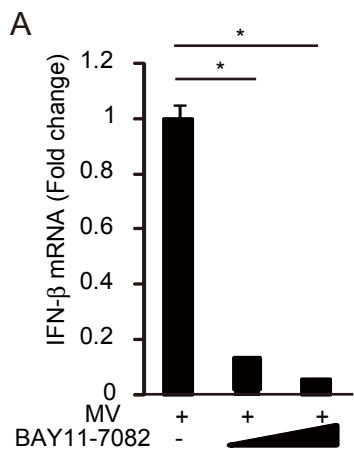


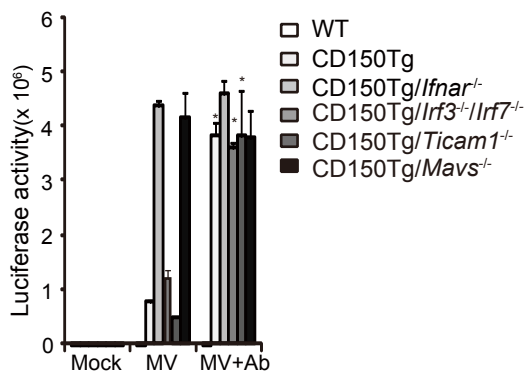


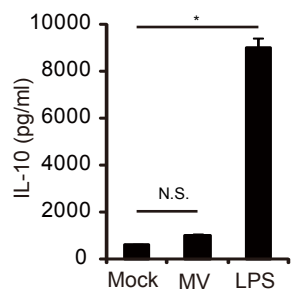


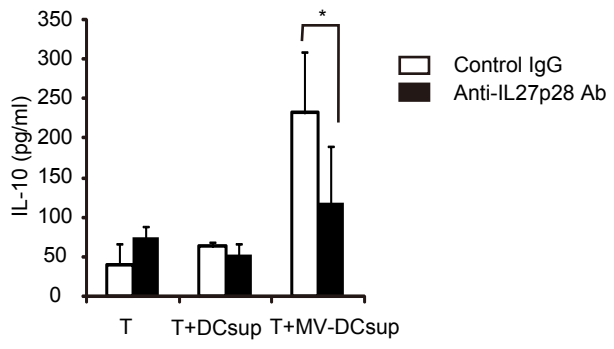












Supplemental Table 1. Oligonucleotides sequences of primers for real-time PCR.

Gene symbol	Forward primer sequence (5'-3')	Reverse primer sequence (5'-3')
<i>β-actin</i>	ttgcagctccttcggtgc	tcgtcatccatggcgaact
<i>Ifn-β</i>	ccagctccaagaaaggacga	cgccctgtaggtgaggtgat
<i>Ifn-α4</i>	ctgctggctgtgaggacatact	aggcacagaggctgtgtttctt
<i>c-Maf</i>	ttcctctcccgaattttca	actagcaagcccactcagga
<i>T-bet</i>	caaccagcaccagacagaga	ccacatccacaaacatcctg
<i>Gata3</i>	cttatcaagccaagcgaag	cattagcgttcctcctccag
<i>Rorgt</i>	ggaggtgaccagctaccaga	ttggcaaactccaccacata
<i>Foxp3</i>	gtcatggtggcaccgtctg	ggcatccacagtggagagct

Supplemental Figure Legends

Supplemental Figure 1. CD150 expression in BMDCs.

BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*^{-/-}, CD150Tg/*Irf3*^{-/-}/*Irf7*^{-/-}, CD150Tg/*Ticam1*^{-/-} and CD150Tg/*Mavs*^{-/-} mice were stimulated with 100 ng/ml LPS or 50 µg/ml PolyI:C for 24 hrs. BMDCs were infected with MV or heated-MV (at 50 °C for 30 min) for 24 hrs. Expression levels of human CD150 in BMDCs were measured by FACS. One representative experiment of two is shown.

Supplemental Figure 2. Luciferase activity was correlated with viral titer obtained from MV-luciferase-infected cells.

Vero/SLAM cells were infected with MV-luciferase at the indicated MOI for 1 hrs. After infection, cells were washed with PBS and cultured with medium. After 24 hrs, luciferase activity and viral titer were measured. The data are shown as the means ± SD of three independent samples.

Supplemental Figure 3. Type IFN induction in CD150Tg/*Irf3*^{-/-}/*Irf7*^{-/-} BMDCs were inhibited by NF-κB inhibitor.

CD150Tg/*Irf3*^{-/-}/*Irf7*^{-/-} BMDCs were treated with 5 µM or 10 µM BAY11-7082 (NF-κB inhibitor, A) or BS203580 (ATF2 inhibitor, B). After 30 min, BMDCs were stimulated with MV (MOI = 0.25) for 8 hrs. *Ifn-β* mRNA expression was determined by real-time PCR. The data are shown as the means ± SD of three independent samples. * $p < 0.05$.

Supplemental Figure 4. CD150Tg/*Irf3*^{-/-}/*Irf7*^{-/-} BMDCs were not permissive to MV infection. BMDCs were infected with MV-luciferase (MOI = 0.25) or mock in the

presence or absence of an anti-IFNAR antibody (10 µg/ml). At 24 hrs after infection, the luciferase activity was measured. The data are the means ± SD of three independent samples. * $p < 0.05$, v.s. MV-infected KO BMDCs.

Supplemental Figure 5. IL-10 was not induced in MV-infected CD150Tg/*Ifnar*^{-/-} BMDCs. CD150Tg/*Ifnar*^{-/-} BMDCs were stimulated with MV (MOI = 0.25) or 100 ng/ml LPS for 24 hrs. IL-10 in the culture supernatants was detected by ELISA. Data are shown as means ± SD of three independent samples. N.S.; not significant, * $p < 0.05$.

Supplemental Figure 6. IL-10 production in CD4⁺ T cells were suppressed by blocking of IL-27p28.

CD150Tg/*Ifnar*^{-/-} BMDCs were infected with MV (MOI = 0.25) or mock for 24 hrs. Naïve CD4⁺CD25⁻ T cells (2×10^5) isolated from WT mice were cultured with supernatants of MV- or mock-infected BMDCs in the presence of control IgG or anti-IL-27p28 antibody for 3 days. IL-10 in the culture supernatants were determined by ELISA. Data are shown as means ± SD of three independent samples. * $p < 0.05$.