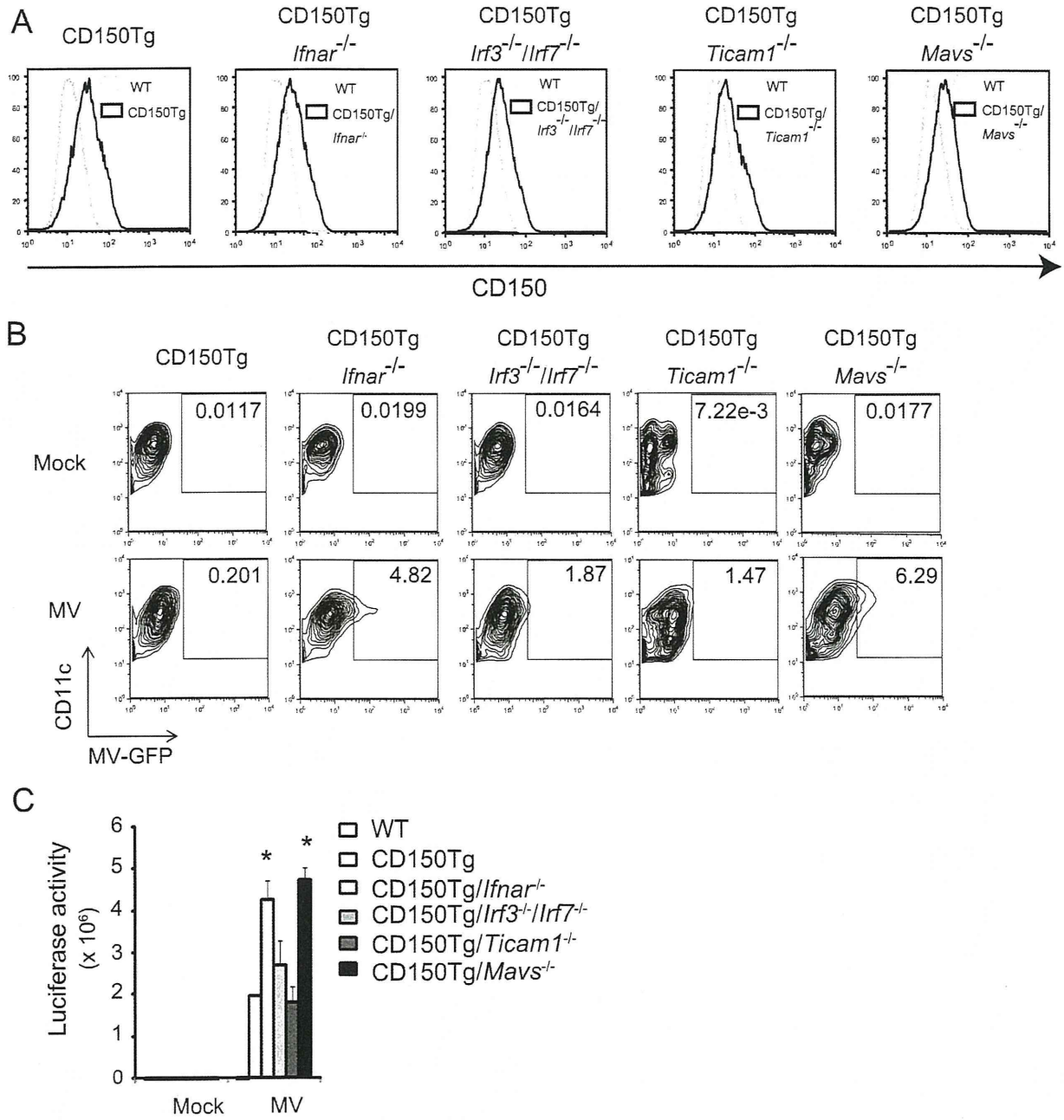

Fig. 1. CD150Tg/*Mavs*$^{-/-}$ BMDCs were permissive to MV infection. (A) Expression levels of human CD150 in BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were measured by FACS. The results are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

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

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

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

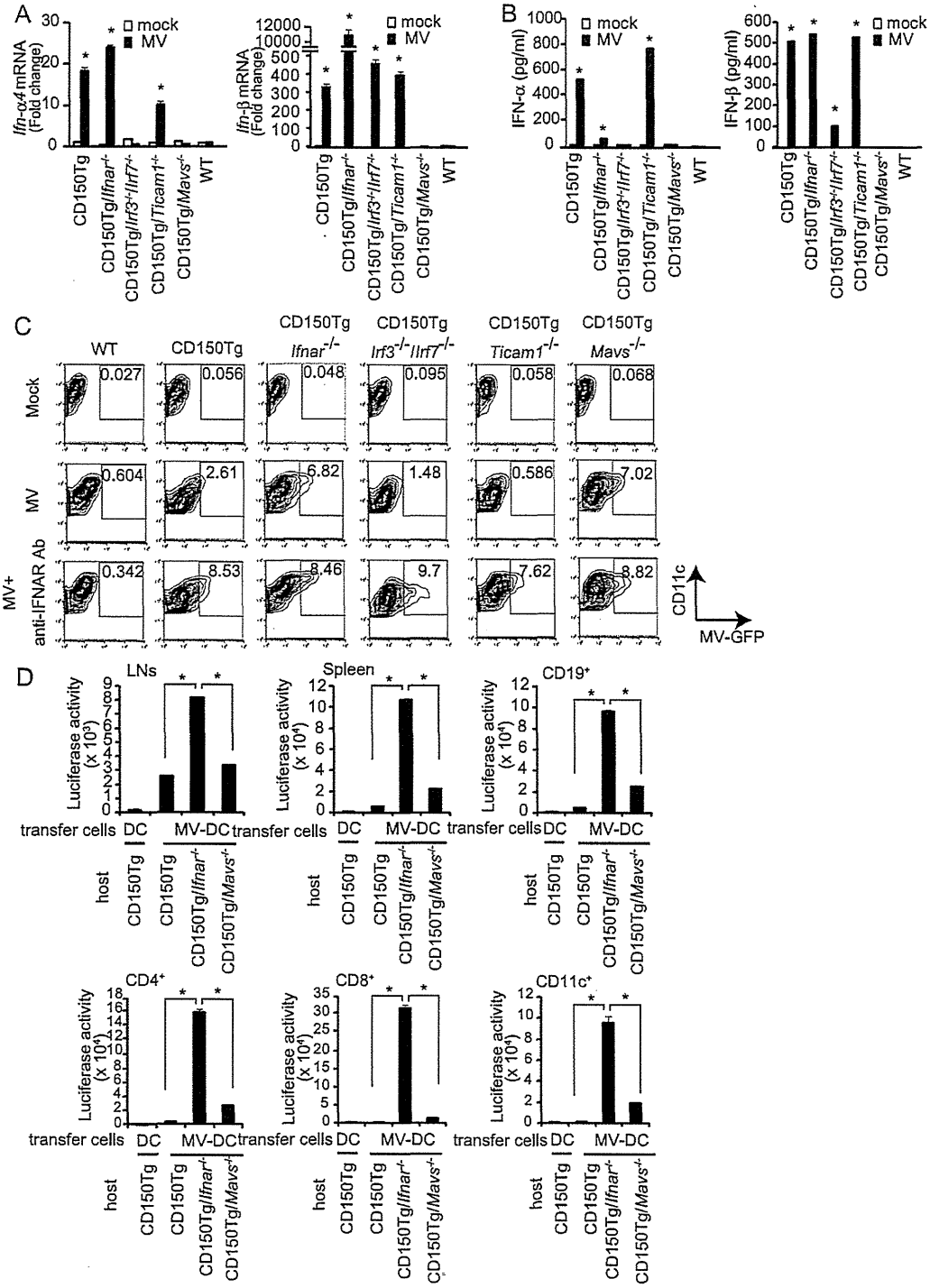

Fig. 2. MV infection did not induce type I IFN in CD150Tg/*Mavs*$^{-/-}$ BMDCs. BMDCs derived from WT, CD150Tg, CD150Tg/*Ifnar*$^{-/-}$, CD150Tg/*Irf3*$^{-/-}$/*Irf7*$^{-/-}$, CD150Tg/*Ticam1*$^{-/-}$ and CD150Tg/*Mavs*$^{-/-}$ mice were infected with MV-GFP (MOI = 0.25) or mock infected. (A) At 24 h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

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

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

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

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

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

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

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

4. Discussion

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

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

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

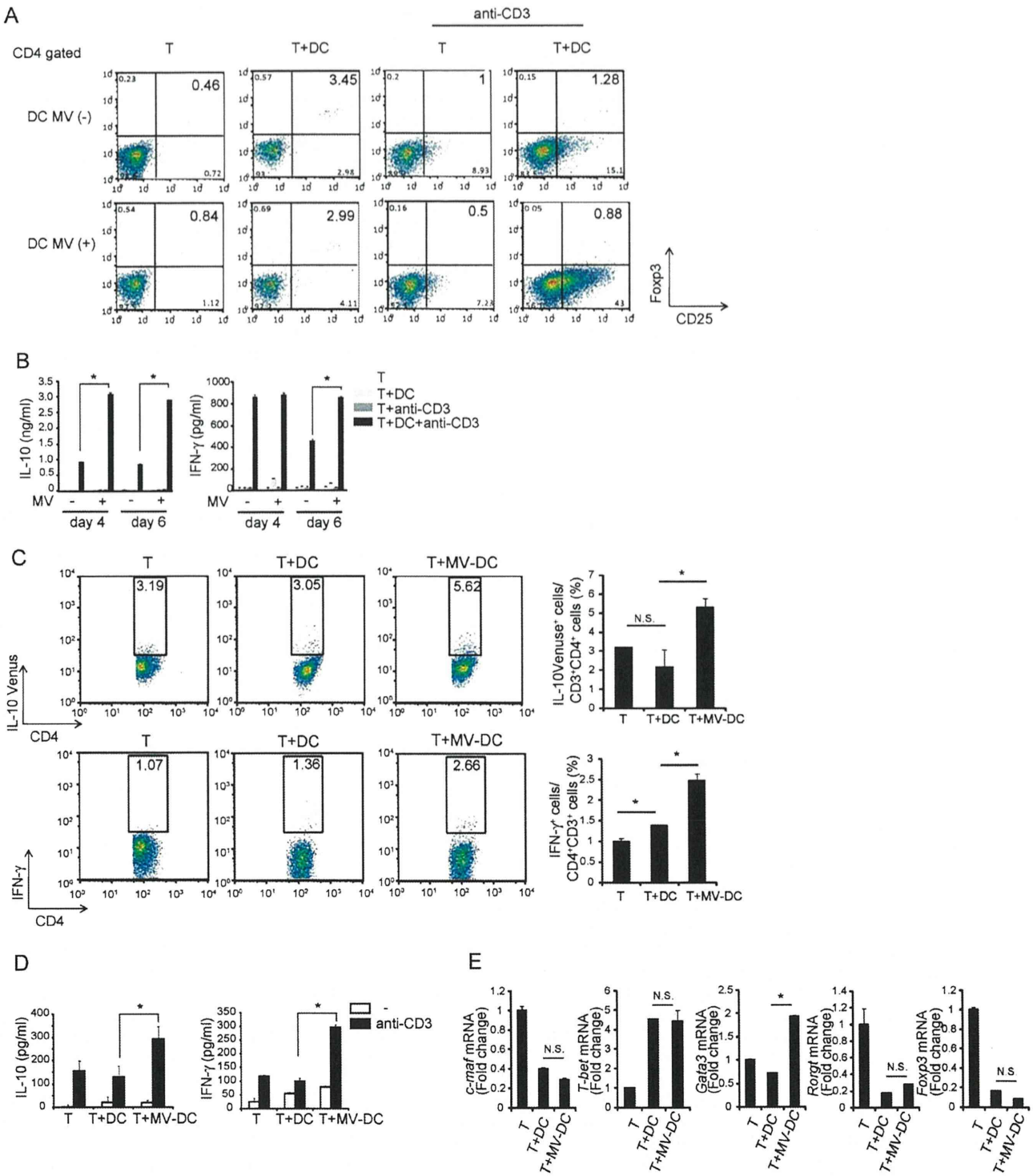

Fig. 3. MV-infected BMDCs induced IL-10 and IFN-γ producing CD4+ T cells. CD150Tg/*Ifnar*$^{-/-}$ BMDCs were infected with MV (MOI = 0.25) or mock for 24 h. Naïve CD4+CD25− T cells (2×10^5) isolated from WT mice were cocultured with 1×10^4 BMDCs in the presence or absence of 0.1 μg/ml of the anti-CD3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

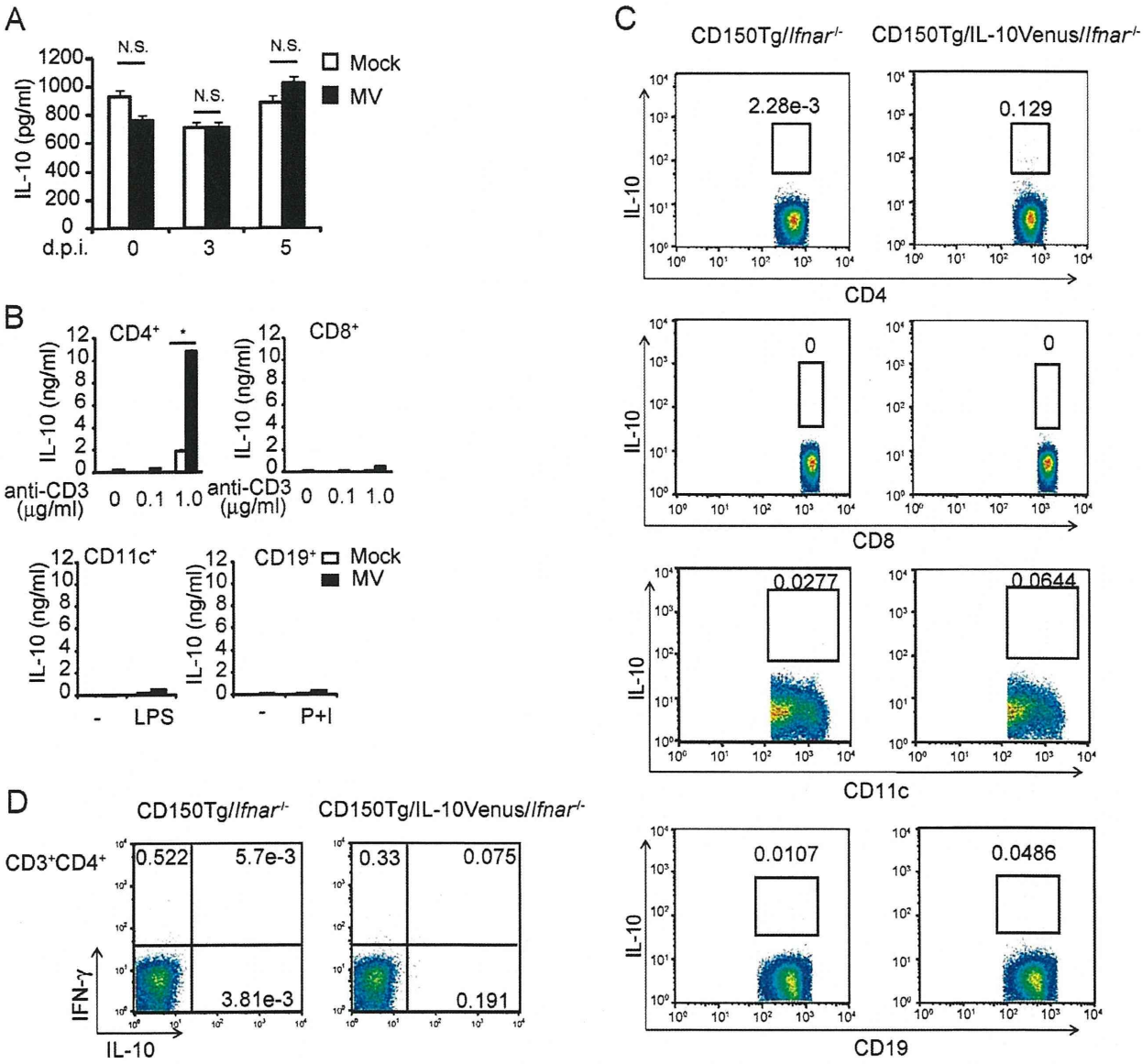

Fig. 4. CD4+ T cells produced IL-10 *ex vivo*. (A) CD150Tg/*Ifnar*$^{-/-}$ mice were infected i.p. with 1×10^6 pfu MV-GFP or mock. At the indicated days after infection, IL-10 production in sera was measured by ELISA. The data are the means ± SD of three independent samples. N.S.; not significant. (B)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

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

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

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

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

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

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

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

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

Conflict of interest

There is no declared conflict of interest in this study.

Acknowledgements

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CHAPTER TEN

Assessment of the Toll-Like Receptor 3 Pathway in Endosomal Signaling

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Contents

Abstract

The innate immune system plays key roles in antimicrobial responses by developing the pattern-recognition receptors that recognize microbial components. The endosomal Toll-like receptors (TLRs) and cytosolic RIG-I-like receptors (RLRs) both recognize viral nucleic acids and are essential for antiviral immunity. Recent evidence suggests that compartmentalization of the receptors, and also their adaptor molecule, is important for discrimination between self and nonself and for distinct innate immune signals. TLR3 is a type I transmembrane protein that localizes in the endosomal membrane in myeloid dendritic cells (DCs) and fibroblasts/epithelial cells. TLR3 recognizes

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extracellular viral double-stranded RNA (dsRNA) and the synthetic dsRNA, poly(I:C). On recognition of dsRNA in the endosomes, TLR3 oligomerizes and induces type I interferon and proinflammatory cytokine production via an adaptor molecule, TICAM-1 (also known as TRIF). Additionally, the TLR3 signal in DCs triggers gene transcription required for DC maturation and the activation of natural killer cells and cytotoxic T lymphocytes. Remarkably, it has been reported that extracellular dsRNA is also recognized by cytosolic RLR. Making a distinction between TLR3-mediated endosomal signaling and RLR-mediated signaling is key to understanding the role of these receptors in innate immunity.

1. INTRODUCTION

The innate immune system senses microbial infection using pattern-recognition receptors and signals to activate innate and adaptive antimicrobial immunity (Akira, Uematsu, & Takeuchi, 2006; Janeway & Medzhitov, 2002). The endosomal Toll-like receptors (TLRs 3, 7, 8, and 9) and cytoplasmic RIG-I-like receptors (RLRs) both recognize viral nucleic acids, playing essential roles in protection against viral infection (Diebold, 2008; Yoneyama & Fujita, 2010). TLR3 has been functionally identified as a sensor for viral double-stranded RNA (dsRNA) and the synthetic dsRNA analog polyriboinosinic–polyribocytidylic acid (poly(I:C)) using TLR3-deficient mice or an anti-human TLR3 blocking antibody (Alexopoulou, Holt, Medzhitov, & Flavell, 2001; Matsumoto, Kikkawa, Kohase, Miyake, & Seya, 2002). On recognition of dsRNA in the endosomes, TLR3 oligomerizes and induces type I interferon (IFN-α/β) and proinflammatory cytokine production from host cells via Toll–IL-1 receptor (TIR)-domain-containing adaptor molecule-I (TICAM-1, also known as TRIF) (Oshiumi, Matsumoto, Funami, Akazawa, & Seya, 2003; Yamamoto et al., 2003). Additionally, activation of TLR3 in myeloid dendritic cells (DCs) leads to the maturation of DCs, which activates natural killer (NK) cells and cytotoxic T lymphocytes (CTLs). Because TLR3 signals effectively induce cellular immunity, TLR3 ligands, such as poly(I:C), are considered to be promising adjuvants for cancer and infectious disease vaccines (Seya & Matsumoto, 2009). However, when dsRNA is added to cells extracellularly *in vitro* or administered to mice, it also activates cytosolic dsRNA sensors, including RIG-I and MDA5, in addition to TLR3, inducing type I IFNs and proinflammatory cytokines via adaptor protein MAVS (also known as IPS-1, Cardif, and VISA) (Kato et al., 2006; Kawai et al., 2005; Meylan

et al., 2005; Seth, Sun, Ea, & Chen, 2005; Xu et al., 2005). Thus, it is important to assess which pathway(s) contributes to dsRNA-induced cellular responses in various cells or situations. In this chapter, we focus on the TLR3–TICAM-1 pathway and describe methods used for studying TLR3-mediated signaling.

2. ANALYSES OF TLR3 EXPRESSION AND LOCALIZATION

TLR3 is a type I transmembrane protein and is expressed in fibroblasts, epithelial cells of various tissues, and neural cells, including neurons, astrocytes, and microglia. Among immune cells, only myeloid DCs and macrophages express TLR3 (Muzio et al., 2000; Visintin et al., 2001). Among myeloid DC subsets, TLR3 is highly expressed in professional antigen-presenting DCs, including mouse $CD8\alpha^+$ DCs and human CD141 $(BDCA3)^+$ DCs (Jelinek et al., 2011; Jongbloed et al., 2010). Unlike other nucleic acid-sensing TLRs, the subcellular localization of TLR3 depends on the cell type; human fibroblasts, macrophages, and some epithelial cell lines express TLR3 in both the cell surface and the endosomal membrane, while myeloid DCs express it in early endosomes (Matsumoto et al., 2003, 2002). Cell-surface TLR3 appears to recognize extracellular dsRNA, because an anti-human TLR3 mAb (TLR3.7) partially inhibited poly(I:C)-induced IFN-β production by fibroblasts (Matsumoto et al., 2002). However, TLR3-mediated signaling is initiated from endosomal compartments in either type of cell, requiring endosomal maturation. The expression and subcellular localization of TLR3 are assessed by flow cytometric and immunofluorescent analyses with an anti-TLR3 mAb (Funami et al., 2004, 2007) (Fig. 10.1).

2.1. Flow cytometric analysis

2.1.1 Cell-surface TLR3 (human cells)

1. All procedures are carried out at 4 °C.
2. Harvest and wash cells ($\sim 10^5$–10^6 cells/sample) three times with FACS buffer (PBS, 0.5% BSA, 0.1% NaN_3).
3. Add 50 μL of anti-human TLR3 mAb (e.g., TLR3.7) or isotype control Ab (10 μg/mL in FACS buffer) together with human IgG (for blocking Fc receptors, final conc 0.1 mg/mL) and incubate for 0.5–1.0 h.
4. Wash cells three times with FACS buffer.