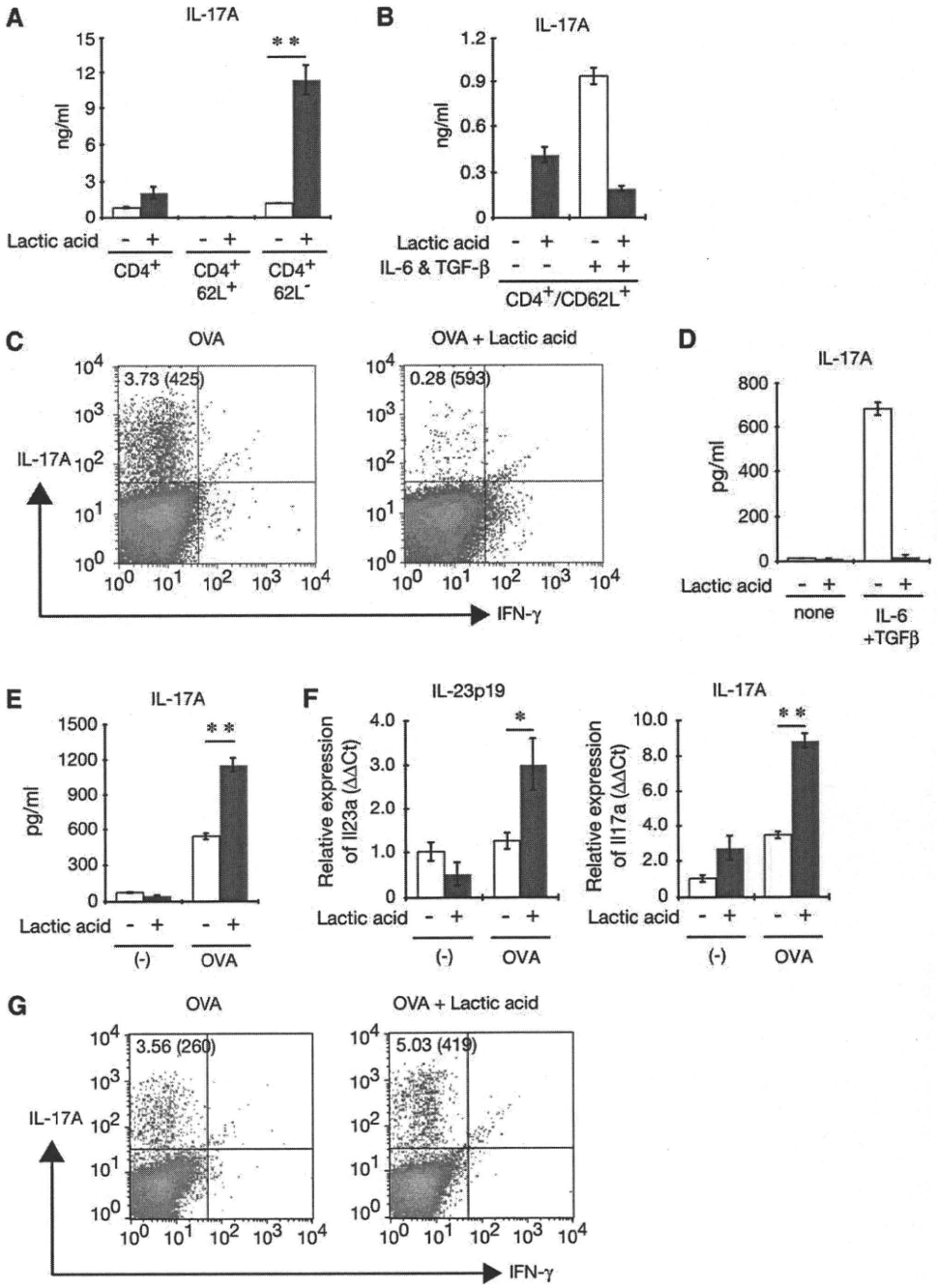


**Fig. 4.** Lactic acid induces IL-17A production from effector/memory $CD4^+$ T and $T_h17$ cells but inhibits differentiation of $T_h17$ from naive T cells. (A) Total $CD4^+$, $CD4^+CD62L^+$ (naive) or $CD4^+CD62L^-$ (effector/memory) T cells derived from OT-II mouse spleens were mixed with $CD11b^+$ cells at a 1:1 ratio and stimulated with OVA peptide in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. Effector/memory $CD4^+$ T cells, but not naive $CD4^+$ T cells, produced IL-17A by lactic acid stimulation. (B and C) In the presence of mouse IL-6 and human TGF-β, purified naive $CD4^+CD62L^+$ T cells were co-cultured with OVA peptide-loaded $CD11b^+$ cells and stimulated with or without lactic acid for 4 days for IL-17A production or 4.5 days for $T_h17$ differentiation. Amounts of IL-17A production are shown in (B). IL-17A-producing $CD4^+$ T cells were stained for CD4, IFN-γ and IL-17A and observed by FACS analysis (C). Plots gated on $CD4^+$ cells are shown. (D) Purified naive T cells were stimulated with plate-coated anti-CD3ε and anti-CD28 antibodies with (right bars) or without (left bars) IL-6 and TGF-β in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. Lactic acid strongly suppressed differentiation into $T_h17$ cells. (E) Naive $CD4^+$ T cells derived from OT-II mice were differentiated into $T_h17$ cells *in vitro*. $T_h17$ cells were mixed with $CD11b^+$ cells at a 1:1 ratio and stimulated with or without OVA peptide in the presence (gray bars) or absence (white bars) of lactic acid for 4 days. (F) $T_h17$ cells were mixed with $CD11b^+$ cells and stimulated, as described above, for 12 h. The relative expressions of IL-23p19 and IL-17A were determined as described in Fig. 1(B). (G) $T_h17$ cells were co-cultured with $CD11b^+$ cells for 4.5 days and observed by FACS analysis. Plots gated on $CD4^+$ cells are shown. Numbers in plots indicate percentages (MFI) of $IL\text{-}17A^+$ cells in total cell populations. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$ and $^{**}P < 0.01$.

inhibited IL-17A production by naive T cells co-stimulated with anti-CD3ε and anti-CD28 antibodies (Fig. 4D). These results suggest that lactic acid negatively regulates the differentiation of naive $CD4^+$ T cells into $T_h17$ cells. However, lactic acid enhanced IL-17A production (Fig. 4E) and the expression of the IL-17A and IL-23p19 transcripts (Fig. 4F) in a co-culture of $CD11b^+$ cells and *in vitro*-differentiated $T_h17$ cells. In addition, $T_h17$ cells that were stimulated with OVA and lactic acid showed 1.4-fold (3.56 to 5.03%) and 1.6-fold increases (260 to 419) in the population of $T_h17$ cells and in the MFI, respectively (Fig. 4G). These data indicate that lactic acid is not involved in $T_h17$ cell differentiation but that it enhances IL-17A production from effector/memory T cells and *in vitro*-differentiated $T_h17$ cells but not naive $CD4^+$ T cells.

### *CD40L–CD40 interactions are involved in the increased production of IL-17A via IL-12/23p40 expression*

In the co-culture system, we hypothesize that $CD4^+$ T cells activated by antigen stimulate $CD11b^+$ cells together with lactic acid, leading to the increased production of IL-17A from $CD4^+$ T cells. Activated $CD4^+$ T cells are known to activate nuclear factor-κB (NF-κB) signaling pathways through CD40L–CD40 interactions, leading to the activation of APCs and the efficient induction of various pro-inflammatory cytokines, including IL-12/23p40 (33–35). First, we examined the effect of CD40L–CD40 interactions on the influence of lactic acid. Anti-CD40L blocking antibodies significantly inhibited the increased expression of IL-17A and IL-12/23p40 induced by lactic acid in co-cultures of $CD11b^+$ and OT-II $CD4^+$ T cells (Fig. 5A). Furthermore, anti-IL-12/23p40 blocking antibodies also inhibited the enhanced IL-17A production by lactic acid (Fig. 5B). These results suggest that CD40L–CD40 interactions are essential for the increased expression of IL-12/23p40 that induces IL-17A production. The ligation of CD40 is known to induce the expression of CD40 itself, as well as pro-inflammatory cytokines (36). To clarify whether the enhanced IL-17A production by lactic acid is mediated by the increased expression of CD40, we examined the induction of CD40 expression using FACS and real-time PCR. However, lactic acid did not increase the expression of CD40 protein (Fig. 5C, left) or CD40 transcript (Fig. 5C, right) by $CD11b^+$ cells in this co-culture system. Furthermore, lactic acid did not elicit the nuclear translocation of NF-κB dimers containing either p65 or p52, which are known to be activated by CD40 ligation (33, 37) (Supplementary

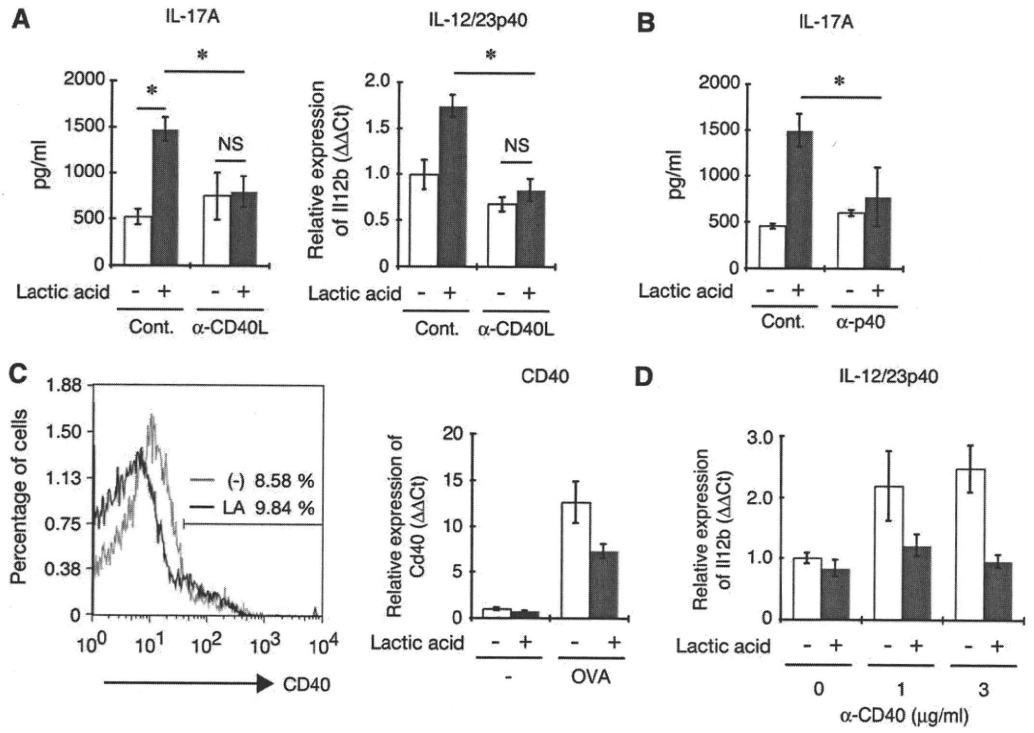


**Fig. 5.** CD40L–CD40 interactions are involved in the increased production of IL-17A via IL-12/23p40 expression. (A) $CD11b^+$ and OT-II $CD4^+$ T cells were co-cultured at a 1:1 ratio and stimulated with (gray bars) or without (white bars) lactic acid in the presence of anti-CD40L (α-CD40L) or isotype control antibodies (Cont.) for 4 days for IL-17A production or 12 h for the relative expression of IL-12/23p40. Anti-CD40L antibodies suppressed the expression of IL-17A and IL-12/23p40 by lactic acid stimulation; NS, not significant. (B) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated as described above in the presence of anti-IL-12/23p40 (α-p40) or isotype control antibodies (Cont.) for 4 days. Anti-IL-12/23p40 also suppressed the production of IL-17A. (C) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated as described above and the expression of CD40 was analyzed by FACS (left) and real-time PCR (right). Cell percentages indicated on the *y*-axis were calculated using FlowJo software (Tree Star Inc., Ashland, OR, USA). $CD40^+$ cell populations in $CD11b^+$ cells stimulated with (9.84%) or without (8.58%) lactic acid (LA) are presented in a representative histogram (left) and relative expression of CD40 transcripts is shown (right). (D) In the presence (gray bars) or absence (white bars) of lactic acid, $CD11b^+$ cells were stimulated for 12 h with anti-CD40 activating antibody (α-CD40) at the indicated concentrations. The relative expression levels of IL-12/23p40 were measured. Stimulation with anti-CD40 antibodies induced the expression of IL-12/23p40, and lactic acid suppressed the induced expression of IL-12/23p40. The data represent mean values ± standard deviation ($n = 3$); $^*P < 0.05$.

Figure 1 is available at *International Immunology* Online). Although the stimulation of CD11b$^+$ cells by anti-CD40 antibodies increased the expression of IL-12/23p40 in a dose-dependent manner, lactic acid suppressed the CD40-dependent induction of IL-12/23p40 (Fig. 5D). These results indicate that, in addition to CD40L–CD40 interactions, further stimulations by activated T cells are required for the increased IL-17A production by lactic acid via IL-12/23p40 expression. In contrast, CD40L–CD40 interactions were not involved in the increased expression of IL-23p19 by lactic acid (Supplementary Figure 2A and B is available at *International Immunology* Online).

### *The involvement of an IL-23-independent pathway in the increased production of IL-17A*

We previously showed that increased production of IL-17A from CD4$^+$ T cells is induced through the enhanced production of IL-23 by lactic acid (21). Because we showed that IL-12/23p40 was essential for enhanced IL-17A production in Fig. 5(C), we predicted that IL-12/23p40 was required to form a heterodimer with IL-23p19. To clarify the involvement of IL-23 in the co-culture of CD11b$^+$ and CD4$^+$ T cells, we first examined the effect of anti-IL-23p19 blocking antibodies on the increased production of IL-17A by lactic acid. Anti-IL-23p19 antibodies reproducibly, but only partially, inhibited the IL-17A production in total splenocytes as previously described (Fig. 6A, left panel) (21). However, in the co-culture of CD11b$^+$ and CD4$^+$ T cells, anti-IL-23p19 antibodies hardly affected IL-17A production (Fig. 6A, right panel). Two other antibodies, goat anti-IL-23p19 and rat monoclonal anti-IL-23 receptor, also failed to suppress the increased IL-17 production (Supplementary Figure 3 is available at *International Immunology* Online). Although the IL-23 that was secreted from J774.1 cells stimulated with TLR ligands and lactic acid was detected by using commercially available ELISA kits (Invitrogen-Biosource cytokines & signaling) (21), IL-23 production was not detectable in the co-culture of CD11b$^+$ and CD4$^+$ T cells, and in total splenocytes, without TLR ligand stimulation (<7.8 pg ml$^{-1}$, data not shown).

We next examined the effect of IL-12 family cytokines sharing the IL-12/23p40 subunit on this enhancement. In the presence of IL-23, IL-12p70 or the IL-12p40 homodimer, CD11b$^+$ and CD4$^+$ T cells were stimulated by OVA peptide with or without lactic acid (Fig. 6B and C). Stimulation by IL-23 increased IL-17A production in the absence of lactic acid in a dose-dependent manner and lactic acid further enhanced the increased production of IL-17A by IL-23 (Fig. 6B). IL-17A production by lactic acid in the absence of exogenous IL-23 was also significantly higher than after treatment with 0.25 ng ml$^{-1}$ IL-23 ($P < 0.01$). In contrast, the

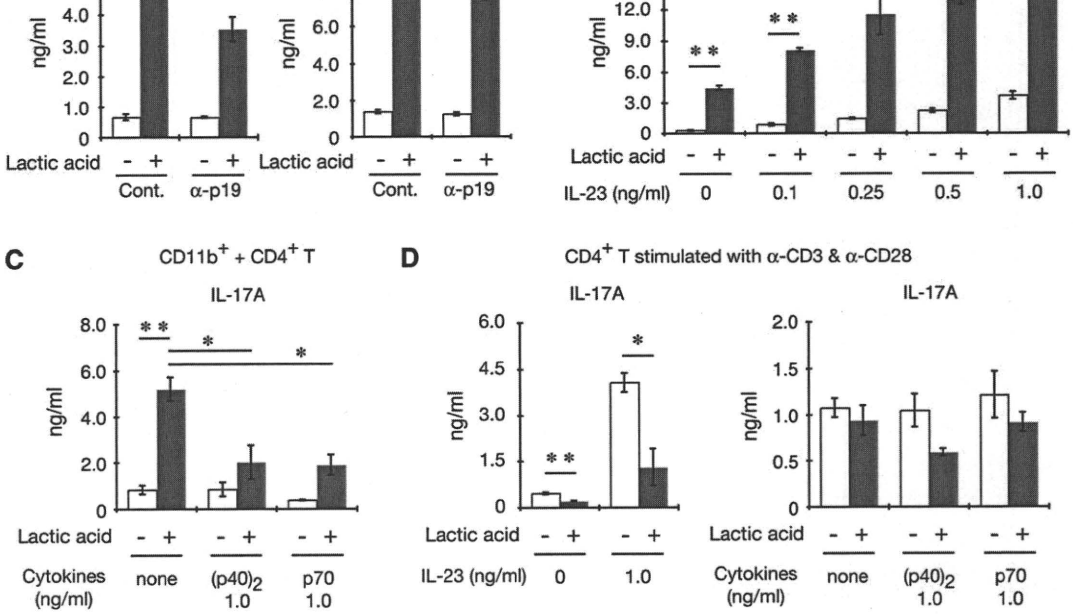


**Fig. 6.** The activation of IL-23-dependent and -independent pathway by lactic acid. (A) The effect of anti-IL-23 antibodies on the increased production of IL-17A in total OT-II splenocytes (left) or the co-culture of CD11b$^+$ and OT-II CD4$^+$ T cells (right). In the presence of anti-IL-23p19 (α-p19) or control antibodies (Cont.), cells were stimulated with OVA peptide and lactic acid (gray bars) or OVA peptide alone (white bars) for 4 days. (B and C) The effect of exogenous cytokines sharing the IL-12/IL-23p40 subunit on the increased production of IL-17A in the co-culture of CD11b$^+$ and OT-II CD4$^+$ T cells. In the presence of IL-23 (B), IL-12p70 (p70) or the IL-12p40 homodimer [$(p40)_2$] (C) at the indicated concentration, cells were stimulated with OVA peptide and lactic acid (gray bar), or OVA peptide alone (white bar) for 4 days. (D) In the presence of IL-23, IL-12p70 or the IL-12p40 homodimer, OT-II CD4$^+$ T cells alone stimulated with plate-coated anti-CD3ε and anti-CD28 antibodies were treated with lactic acid. At day 4, IL-17A production was measured. The data represent mean values ± standard deviation ($n = 3$); *$P < 0.05$ and **$P < 0.01$.

IL-12p40 homodimer and IL-12p70 remarkably suppressed the production of IL-17A in the presence of lactic acid (Fig. 6C). However, IL-23 increased IL-17A production from $CD4^+$ T cells activated by plate-coated anti-CD3ε and anti-CD28 antibodies, but lactic acid inhibited its production. The IL-12p40 homodimer and IL-12p70 did not affect IL-17A production from $CD4^+$ T cells activated by plate-coated anti-CD3ε and anti-CD28 antibodies (Fig. 6D). Therefore, in addition to increased IL-23 production, lactic acid may induce the expression of unidentified cytokines that share IL-12/23p40 or other factors involved in the enhanced production of IL-17A.

### *Enhanced IL-23p19 and IL-17A expression by lactic acid is independent of MyD88, CARD9 and IL-1β*

It is known that the MyD88, CARD9 (caspase-recruitment domain 9) and NALP3 (NACHT-, LRR- and pyrin-domain

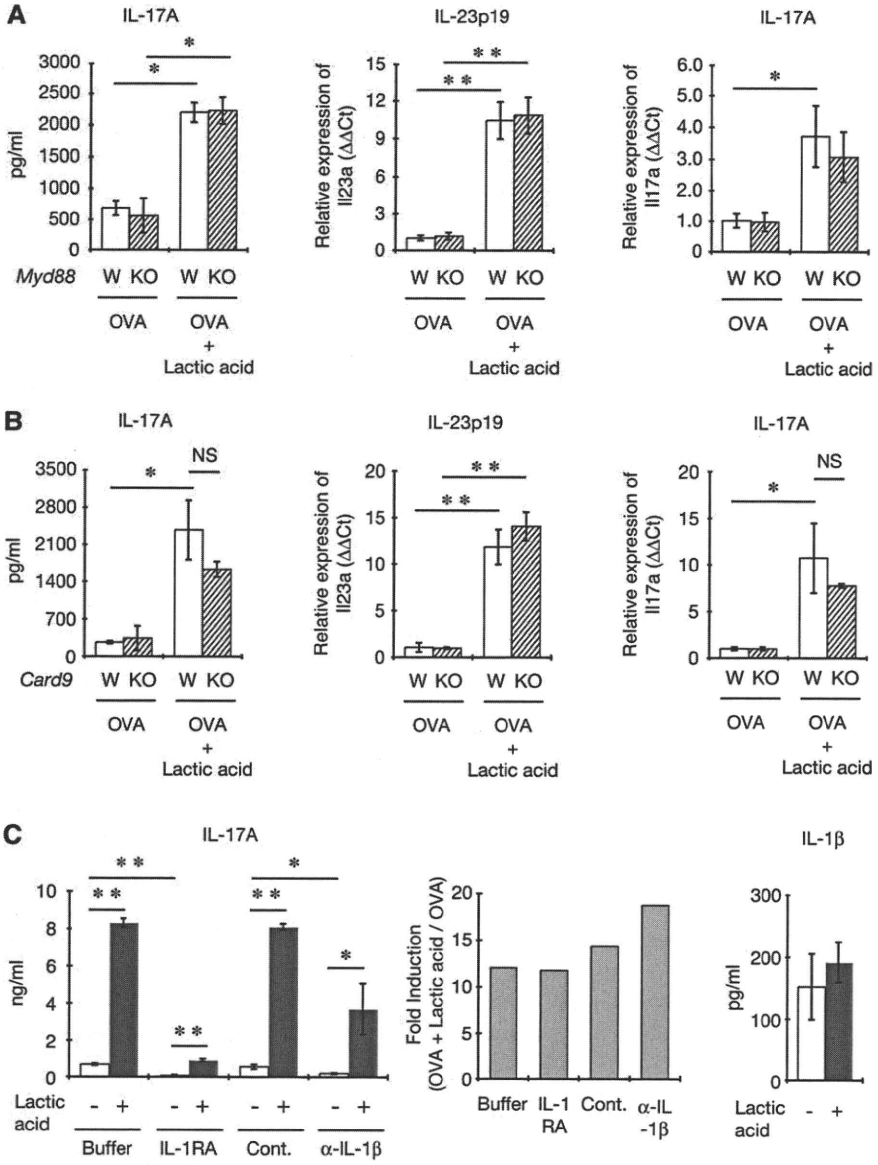


**Fig. 7.** Lactic acid enhances IL-17A production independently of MyD88, CARD9 and IL-1β. (A) and (B) $CD11b^+$ cells derived from *MyD88* (*Myd88*$^{-/-}$) (A), *Card9* (*Card9*$^{-/-}$) (B) deficient mice (KO, hatched bars) or their littermate controls (W, white bars) were mixed with OT-II $CD4^+$ T cells at a 1:1 ratio and stimulated with OVA peptide in the presence or absence of lactic acid for 4 days for IL-17A production or 12 h for the relative expression of IL-23p19 and IL-17A transcripts. *Myd88*$^{-/-}$ and *Card9*$^{-/-}$ $CD11b^+$ cells showed enhanced expression of IL-23p19 and IL-17A in the presence of lactic acid; NS, not significant. (C) $CD11b^+$ and OT-II $CD4^+$ T cells were stimulated with OVA peptide alone (white bars) or OVA peptide plus lactic acid (gray bars) in the presence of IL-1RA, anti-IL-1β (α-IL-1β) or isotype control (Cont.) antibodies for 4 days (left panel). Buffer (PBS containing 0.1% BSA) indicates control for IL-1RA. Fold induction by lactic acid in left panel was calculated relative to OVA peptide alone (middle panel). Although IL-1RA and anti-IL-1β antibodies suppressed the total amount of IL-17A produced when stimulated with OVA peptide alone or OVA peptide plus lactic acid, enhanced IL-17A production by lactic acid was hardly impaired. IL-1β production, when incubated with buffer, is shown in right panel. The data represent mean values ± standard deviation ($n$ = 3); $^*P < 0.05$ and $^{**}P < 0.01$.

containing protein 3)-inflammasome pathways are activated by intrinsic stimuli from damaged cells as well as external pathogens and that they play roles in the induction of inflammation and inflammatory diseases. To determine whether lactic acid is involved in these signaling pathways, we examined CD11b$^+$ cells derived from *Myd88* and *Card9* knockout mice splenocytes. In co-cultures with OT-II CD4$^+$ T cells, enhanced expression of IL-23p19 and IL-17A after lactic acid stimulation was not impaired in CD11b$^+$ cells from *Myd88* (Fig. 7A) and *Card9* (Fig. 7B) knockout mice. The activation of the NALP3-inflammasome pathway by molecules secreted from damaged cells such as ATP (5, 6) mediates the processing of caspase-1 to convert pro-IL-1β into its active form, IL-1β. Secreted IL-1β is involved in the induction of IL-17A expression and the maintenance of $T_h17$ cells (38–40). To examine the effect of IL-1β on enhanced IL-17A production by lactic acid, we stimulated CD11b$^+$ and CD4$^+$ T cells with OVA peptide and lactic acid in the presence of IL-1RA or anti-IL-1β antibodies (α-IL-1β) (Fig. 7C, left panel). IL-1RA and anti-IL-1β antibodies potently inhibited IL-17A production when stimulated with OVA peptide alone or with OVA peptide plus lactic acid. However, the relative activity induced by lactic acid was still elevated in the presence of IL-1RA (12.1-fold for Buffer and 11.8-fold for IL-1RA) or anti-IL-1β antibodies (14.4-fold for Control and 18.7-fold for anti-IL-1β antibodies) (Fig. 7C, middle panel). Furthermore, even when we observed the remarkable enhancement of IL-17A production, we detected only a slight or no increase in IL-1β transcription and IL-1β secretion by lactic acid (Figs 1C and 7C, right panel). These data suggest that IL-1β is involved in IL-17A production (38–40) but plays no significant role in enhanced IL-17A expression by lactic acid.

## Discussion

This study analyzed the induction mechanism of IL-17A by lactic acid without TLR ligands using splenocytes of OT-II transgenic mice. It is postulated that lactic acid enhances the antigen-dependent production of IL-17A via three steps: Step 1, through CD4$^+$ T cell activation by APCs in an antigen-dependent manner; Step 2, through activation of APCs by activated CD4$^+$ T cells and lactic acid; and Step 3, through activation of the IL-17A promoter in CD4$^+$ T cells stimulated by APCs. Using fractionated splenocytes, this study showed that monocytes/macrophages and effector/memory CD4$^+$ T cells are essential for the enhanced activity of antigen-dependent production of IL-17A by lactic acid. Lactic acid suppresses the proliferation of both IL-17A- and IFN-γ-producing CD4$^+$ T cells but maintains the phenotype of IL-17A-producing cells. Furthermore, CD40L–CD40 interaction was found to be essential for co-stimulation of CD11b$^+$ cells together with lactic acid in Step 2, leading to IL-12/23p40 production. IL-12/23p40 production is necessary to up-regulate IL-17A production, but known cytokines containing IL-12/23p40, such as IL-23, IL-12 and the IL-12/23p40 homodimer, were not involved in this up-regulation in the co-culture of CD4$^+$ T and CD11b$^+$ cells. Therefore, a new cytokine containing IL-12/23p40 may be one of the molecules that enhance IL-17A production by effector/memory CD4$^+$ T cells after lactic acid stimulation in Step 3. While IL-1β induction plays an important role in IL-17A up-regulation in both the presence and absence of lactic acid in Step 3, IL-1β was not involved in the enhancement of IL-17A production by lactic acid.

In splenocytes, CD11b$^+$ and F4/80$^+$ cells most significantly stimulated IL-17A production from CD4$^+$ cells by lactic acid. Likewise, BMDMs differentiated by treatment with M-CSF potently up-regulated IL-17A. Therefore, monocytes/macrophages were activated by lactic acid and were involved in amplifying IL-17A expression. Although DCs generally have higher antigen-presenting abilities, these studies demonstrated that CD11b$^-$CD11c$^+$ and F4/80$^-$CD11c$^+$ cells in splenocytes had less stimulatory activity than CD11b$^+$ and F4/80$^+$ cells. BMDCs strongly induced IL-17A production from CD4$^+$ T cells, but lactic acid only slightly enhanced the IL-17A expression mediated by BMDCs (data not shown). These results suggest that lactic acid acts mainly on monocytes/macrophages, despite their weak antigen presenting activity. In support of these findings, it is well known that tumor-associated macrophages often infiltrate tumor microenvironments to support tumor proliferation and progression (41, 42). Therefore, tumor-secreted lactic acid could act on macrophages that induce chronic inflammation in tumors.

In this study, we also clarified the effects of lactic acid on IL-17A-producing cells. Lactic acid enhanced IL-17A production from CD4$^+$CD62L$^-$ effector/memory T cells and $T_h17$ cells, but not naive CD4$^+$ T cells. Meanwhile, lactic acid strongly inhibited differentiation of $T_h17$ cells from naive T cells in response to IL-6 and TGF-β. Furthermore, lactic acid increased the proportion of IL-17A-producing cells and the MFI of IL-17A but suppressed the proliferation of both IL-17A- and IFN-γ-producing cells. The Kreutz group also reported that lactic acid suppresses the proliferation of human cytotoxic T cells and the production of IFN-γ and IL-2 in these T cells upon antigen stimulation (24, 25). Therefore, lactic acid acts on monocytes/macrophages to maintain only the cells that are involved in the induction of inflammation, to induce specific pro-inflammatory cytokines such as IL-17A and IL-21 and to negatively regulate the $T_h1$ and cytotoxic T cells that are involved in anti-tumor immunity.

It has been reported that cell–cell contacts through CD40L–CD40 interactions are necessary for the activation of APCs by CD4$^+$ T cells to induce IL-12/23p40 expression (34, 35). We verified that the CD40L–CD40 interaction is essential for the IL-12/23p40 expression that induces increased IL-17A production by CD4$^+$ T cells. However, lactic acid did not enhance either NF-κB signaling pathways or the expression of IL-12/23p40 elicited by CD40 ligation. Therefore, we predict that lactic acid does not directly modify the CD40 signaling pathway. The expression of IL-12/23p40 was also slightly enhanced in the presence of lactic acid (Fig. 5A) (21), but the enhanced expression of IL-12/23p40 by lactic acid was not always detected even when increased IL-17A production was induced (data not shown). We predicted that IL-23 would induce elevated IL-17A production in response to a lactic acid-induced increase in IL-23p19, as previously described (21). However, anti-IL-23p19 and anti-IL-23 receptor antibodies did not suppress increased

IL-17A production in the co-culture system of $CD4^+$ T and $CD11b^+$ cells. Furthermore, IL-23 production was not detectable in this co-culture system (<7.8 pg $ml^{-1}$). Lactic acid induced a higher level of IL-17A than 0.25 ng $ml^{-1}$ exogenous IL-23, even when IL-23 induced by lactic acid was not detected. These results indicate the involvement of another IL-12/23p40-containing factor that can induce IL-17A production independent of IL-23. However, the IL-12p40 homodimer and IL-12p70, which also share the IL-12/23p40 subunit, suppressed IL-17A production. Therefore, an additional unidentified cytokine containing IL-12/23p40 may act directly on effector/memory $CD4^+$ T cells to stimulate increased IL-17A production. The IL-12p40 homodimer and IL-12p70 may block the binding of the new IL-12/23p40-containing cytokine to their shared receptor IL-12Rβ1. Lactic acid may induce the enhanced expression of a subunit of the cytokine that contains IL-12/23p40, leading to increased IL-17A production. Alternatively, the new cytokine may act on $CD11b^+$ cells to stimulate the expression of accessory molecules that cooperate with TCR–MHC interactions, but not in increased IL-17A production. The expression of these accessory molecules may be enhanced by lactic acid stimulation. Furthermore, Lactic acid also induced the expression of IL-21 produced by $T_h17$ cells, which increases their population through an autocrine or paracrine feedback loop (13). Therefore, other factors that mediate the increased expression of IL-21 by stimulation of lactic acid are likely involved in the increased proportion of cells that express IL-17A.

It is known that several signaling pathways are activated upon stimulation with intrinsic inflammatory ligands. We analyzed whether lactic acid is involved in the activation of three of these known pathways: MyD88, CARD9 and NALP3. The results indicated that the elevated activity induced by lactic acid was independent of the MyD88 and CARD9 signaling pathways. Recently, Samuvel *et al.* (43) reported that lactate enhances TLR4 signaling via MD-2 expression in human U937 histiocyte cells. However, MyD88, which is an adaptor molecule of TLR4, was not involved in the higher expression of IL-23p19 and IL-17A in our system. In addition, lactic acid did not enhance the NF-κB signaling pathway in J774.1 cells co-stimulated with TLR ligands (21). Although it has been reported that the Syk-CARD9 pathway is involved in the differentiation of IL-17A-producing $CD4^+$ cells (44), the enhancement of IL-17A production by lactic acid was unaffected in a co-culture with $CD11b^+$ cells derived from *Card9* knockout mice. To test whether lactic acid participates in the NALP3-inflammasome pathway, we examined the expression of IL-1β. IL-1β was produced after stimulation with the OVA peptide alone and the addition of lactic acid only slightly enhanced or left unchanged its levels of expression. The IL-1 signaling pathway regulates $T_h17$ cell differentiation and maintains the expression of IL-17A in $T_h17$ cells (40). The inhibition of IL-1 signaling by IL-1RA or anti-IL-1β antibodies strongly suppressed IL-17A production, stimulated either with the OVA peptide plus lactic acid or with the peptide alone (Fig. 7C). However, the production of IL-17A was still significantly enhanced by lactic acid even in the presence of either IL-1RA or anti-IL-1β antibodies. Therefore, this result suggests that IL-1β stimulation is important for the strong induction of IL-17A but that it acts independently of the lactic acid signaling pathway.

Recently, it has been reported that GPR81, a G-protein coupled receptor (GPCR) expressed in adipocytes, is a sensor for lactate that mediates an anti-lipolytic effect (45–47). Surprisingly, the half-maximal effective concentration for L-lactate to activate GPR81 is remarkably high (~5 mM) compared with values for ligands of other typical GPCRs, which are in the nanomolar range. This value is almost equivalent to that required to induce activity in our system. However, for a number of reasons, we predict that other molecules would sense lactic acid in macrophages and that these molecules induce the increase of IL-23p19 expression and IL-17A production. First, GPR81 is specifically expressed in adipose tissue but not the spleen (46). Second, neutralized lactate and sodium lactate did not enhance the promoter activity of the human IL-23p19 gene (21) or the expression of IL-23p19 and IL-17A in a co-culture of $CD11b^+$ and $CD4^+$ T cells, whereas the lactate anion acts on GPR81 under neutral conditions (46). Third, although GPR81 negatively controls adenylate cyclase activity, lactic acid did not modulate the enhancer activity of cyclic adenosine 3',5'-monophosphate - responsive elements stimulated by the TLR2 ligand in J774.1 cells (data not shown). Therefore, we predict that lactic acid is co-transported with protons into cells via MCTs and recognized by an intracellular molecule that activates monocytes, leading to increased IL-17A production from $CD4^+$ T cells. In this study, lactic acid was shown to act mainly on monocytes/macrophages rather than DCs. Therefore, we speculate that the intracellular lactic acid sensor is specifically expressed in monocytes/macrophages. Because we observed the enhancement of IL-23p19 promoter activity in J774.1 cells but not in RAW264 cells, the lactic acid sensor may be deficient in RAW264 cells. In contrast, the Kreutz group recently reported that lactic acid inhibits glycolytic flux and export of lactate, resulting in suppression of TNF secretion from monocytes (48). Furthermore, inhibition of hexokinase by 2-deoxyglucose also suppressed the secretion of TNF. Thus, the expression of IL-23p19 and other molecules induced by lactic acid may also depend on blocking glycolytic flux. Future studies will help to further elucidate the unique molecular mechanisms controlled by lactic acid in the IL-23-dependent and -independent pathways to increase IL-17A production.

It is known that tumor cells secrete a variety of factors including cytokines, chemokines and damaged cellular components to induce local inflammation around tumors (1, 41, 42). The increase of lactic acid production in tumors is a common feature due to the Warburg effect (22, 49). We and another group reported that lactic acid suppresses the proliferation of T cells and the production of IFN-γ, which mediates anti-tumor activities (24, 25), but enhances the IL-23-dependent and -independent IL-17 pathways that mediate chronic inflammation in our studies (21). Furthermore, in human carcinomas and mouse tumor models, higher proportions of $T_h17$ cells are recruited to the tumor microenvironment than the tumor-draining lymph nodes and peripheral blood (50). Therefore, lactic acid may be an important pro-inflammatory mediator that supports tumor progression. In fact, it has been reported that high concentrations of lactate

in solid tumors, such as cervical carcinoma and head and neck cancers, are associated with higher frequencies of distant metastasis and poor prognosis (23). Studies have also shown that the inhibition of lactic acid production in tumors reduces tumor growth (51). Therefore, modulation of the lactic acid signaling pathway may become an attractive target for treating many solid tumors. Agents that target this pathway could suppress chronic inflammation and instead induce anti-tumor immunity, as well as inhibit tumor growth.

## Supplementary data

Supplementary data are available at *International Immunology* Online.

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# Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-β-inducing potential

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ABSTRACT

Laboratory-adapted and vaccine strains of measles virus (MV) induce type I interferon (IFN) in infected cells to a far greater extent than wild-type strains. We investigated the mechanisms for this differential type I IFN production in cells infected with representative MV strains. The overexpression of the wild-type V protein suppressed melanoma differentiation-associated gene 5 (MDA5)-induced IFN-β promoter activity, while this was not seen in A549 cells expressing CD150 transfected with the V protein of the vaccine strain. The V proteins of the wild-type also suppressed poly I:C-induced IFN regulatory factor 3 (IRF-3) dimerization. The V proteins of the wild-type and vaccine strain did not affect retinoic acid-inducible gene 1 (RIG-I)- or toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1)-induced IFN-β promoter activation. We identified an amino acid substitution of the cysteine residue at position 272 (which is conserved among paramyxoviruses) to an arginine residue in the V protein of the vaccine strain. Only the V protein possessing the 272C residue binds to MDA5. The mutation introduced into the wild-type V protein (C272R) was unable to suppress MDA5-induced IRF-3 nuclear translocation and IFN-β promoter activation as seen in the V proteins of the vaccine strain, whereas the mutation introduced in the vaccine strain V protein (R272C) was able to inhibit MDA5-induced IRF-3 and IFN-β promoter activation. The other 6 residues of the vaccine strain V sequence inconsistent with the authentic sequence of the wild-type V protein barely affected the IRF-3 nuclear translocation. These data suggested that the structural difference of vaccine MV V protein hampers MDA5 blockade and acts as a nidus for the spread/amplification of type I IFN induction. Ultimately, measles vaccine strains have two modes of IFN-β-induction for their attenuation: V protein mutation and production of defective interference (DI) RNA.



## 1. Introduction

Innate immunity is the first line of defense against virus infection, and the most powerful antiviral agent possessed by the host immune system is interferon (IFN). Expression of type I IFN in host cells induces a set of IFN-inducible genes which efficiently suppress viral replication and spread (Pichlmair and Reis, 2007). Host cells usually terminate virus replication in response to IFN induction. Recent studies elucidated the mechanism by which type I IFN is induced and found that it senses virus patterns such as 5′-triphosphate (5′-3P) and stem-loops or double-stranded RNA (dsRNA) (Takeuchi and Akira, 2008). dsRNA specifically is present in several forms: viral genomes, single-stranded RNA virus replication intermediates, DNA virus symmetrical transcription products, defective viral particles and debris from lysed cells (Bowie and Fitzgerald, 2007). These viral products all present patterns that activate the IFN system. In fact, extracellular dsRNA is sensed by endosomal Toll-like receptor 3 (TLR3), and intracellular dsRNA is detected by cytoplasmic RNA helicase retinoic acid-inducible gene 1 (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (Takeuchi and Akira, 2008). TLR3 recruits the adaptor, toll-IL-1R homology domain-containing adaptor molecule 1 (TICAM-1, also named TRIF) (Oshiumi et al., 2003). RIG-I and MDA5 signal through IFN-β promoter stimulator 1 (IPS-1). These adaptor molecules activate kinase TANK-binding kinase I (TBK1), inhibitor of κB kinase ε (IKKε) and NAK-associated protein 1 (NAP-1) (Sasai et al., 2006a). These complexes then phosphorylate IFN regulatory factor 3 (IRF-

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3) and IRF-7, promoting their dimerization, nuclear translocation and transcription of IFN-stimulated genes (ISGs), such as ISG56, as well as IFN and other cytokines (Medzhitov, 2007; Platanias, 2005). On the other hand, secreted IFNs bind to the IFN-α/β receptor on the surface of adjacent cells and activate the Janus kinase–signal transducer and activator of transcription (JAK/STAT) signaling pathway, which amplifies IFN induction and stimulates transcription of a variety of antiviral genes (Samuel, 2001). Many viruses encode specific proteins to inhibit IFN induction or the JAK/STAT pathway (Katze et al., 2002; Sen, 2001). The V protein of measles virus (MV) blocks the IFN-inducing pathway mediated by MDA5 and the JAK/STAT pathway (Ohno et al., 2004; Nakatsu et al., 2008). The C protein of MV acts as a regulator of viral RNA synthesis, thereby acting indirectly to suppress IFN induction (Nakatsu et al., 2008).

It has been reported that wild-type measles strains barely induce type I IFN (Naniche et al., 2000; Shingai et al., 2007). The levels of IFN protein or mRNA are lower than the detection limit in cells infected with wild-type MV, while higher levels of IFN are detectable in cells infected with vaccine strains. Although the mechanism behind the strain-to-strain differences in IFN-inducing potential remain unclear, an early report suggested that a laboratory strain, strain Edmonston (ED), possesses a unique V protein with low suppression of IFN-α/β receptor (IFNAR)-amplifiable IFN induction (Ohno et al., 2004). We previously reported that vaccine/laboratory strains harbor defective interference (DI) RNA which activates RIG-I and/or MDA5. Type I IFN is efficiently yielded by DI RNA during viral RNA replication (Shingai et al., 2007). We found that the majority of measles vaccine and laboratory-adapted strains possess DI RNA. However, the IFN-inducibility of attenuated MV strains does not always correlate with the presence of DI RNA. Therefore, the mechanisms by which the primary IFN-inducing activity by RIG-I/MDA5 is impaired during wild-type measles infection still remain unexplained.

In this study, using wild-type and DI-negative attenuated measles strains, we investigated the predominate mechanisms that act on the host IFN system to modulate IFN production. We identified amino acid differences between the V proteins of the attenuated ED strain and wild-type MV, and found that the cysteine residue at position 272 (272C) was required for suppression of MDA5-induced type I IFN production.

## 2. Materials and methods

### 2.1. Cell culture and reagents

The human lung epithelial cell line (A549), A549/CD150, African green monkey kidney cell line (Vero), Vero/CD150 and HEK293FT cells were maintained in DMEM supplemented with 10% heat-inactivated FCS and antibiotics (Tanabe et al., 2003). HeLa cells were cultured in Eagle's MEM with 10% heat-inactivated FCS and L-glutamine. For establishing CD150-expressing A549 and Vero cell lines, pCNX2-huCD150 was introduced into cell lines using FugeneHD (Roche) according to the manufacturer's protocol. Twenty-four hours after transfection, the neomycin analog G418 (Sigma–Aldrich) was added to the medium at the final concentration of 1.4 mg/ml or 0.6 mg/ml for Vero or A549 cells. During selection, G418-containing medium was changed once every 4 days. G418-resistant, stably transfected clones were propagated for the study of surface expression of CD150 by flow cytometer. The following antibodies were obtained commercially: anti-FLAG (Sigma–Aldrich); anti-Myc (Santa Cruz); anti-IRF-3 (IBL). Alexa Fluor 488- and Alexa Fluor 568-conjugtaed secondary antibodies were from Invitrogen Life Technologies. Polyriboinosinic/polyribocytidylic acid (polyI:C) was from Amersham Biosciences.

### 2.2. Plasmids

Complementary DNAs of human TICAM-1, MDA5, RIG-I, V and C were cloned in our laboratory by RT-PCR and ligated into the cloning site of the expression vector, pEF-BOS, pcDNA4 Myc-HisA and pCMV10-FLAG (Funami et al., 2008). Mutations were introduced by site-directed mutagenesis using PCR. All constructs were confirmed by sequencing.

### 2.3. Virus preparation and titration

Nagataha (NV) and Edmonston (ED) strains were obtained from Dr. S. Ueda (the Research Institute for Microbial Diseases, Osaka University, Osaka, Japan) and University of Washington (Seattle, WA), respectively. Ichinose (IC)-B was provided from Dr. F. Kobune (National Institutes of Health, Tokyo, Japan) (Kubune et al., 1990). Masusako (MS) was propagated in our laboratory (Kurita-Taniguchi et al., 2000; Murabayashi et al., 2002). NV, ED and MS strains were maintained in Vero/CD150 cells in our laboratory (Shingai et al., 2007). IC-B strain was maintained in B95a cells. Virus titer was determined as PFUs on Vero/CD150 and the multiplicity of the infection (MOI) of each experiment was calculated based on this titer (Kubune et al., 1990).

### 2.4. 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 following oligonucleotides were used for human GAPDH: 5′-TCCACCACCCTGTTGCTGTA-3′ and 5′-ACCACAGTCCATGCCATCAC-3′; and for MV-H: 5′-CCCTTATCAACGGATGATCC-3′ and 5′-GTGATCAATGGCCCCAATCC-3′; and for q-PCR human β-actin: 5′-CCTGGCACCCAGCACAAT-3′ and 5′-GCCGATCCACACGGAGTACT-3′; and for *q*-PCR human IFN-β: 5′-CAATTGCTTGGATTCCTACAAAG-3′ and 5′-TATTCAAGCCTCCCATTCAATTG-3′. IFN-β mRNA were normalized to β-actin and fold inductions of transcripts were calculated using the ddCT method relative to unstimulated HeLa cells.

### 2.5. RT-PCR amplification of cDNA from 5′ copy-back DI RNAs

We modified the RT-PCR amplification protocol of Calain et al. (1992), where the copy-back DI RNAs were amplified using two set of MV primers (for 5′ copy-back DIs, JM396; 5′-TATAAGCTTACCAGACAAAGCTGGGAATAGAAACTTCG-3′/JM403; 5′-CGAAGATATTCTGGTGTAAGTCTAGTA-3′, and for standard genome, JM396/JM402; 5′-TTTATCCAGAATCTCAARTCCGG-3′) (Sidhu et al., 1994; Whistler et al., 1996). Viral RNA from the culture supernatant was extracted with QIAamp Viral RNA Mini kit (Qiagen). Total RNA from viral-infected cells was extracted with TRIzol Reagent 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 PCR-amplified products were confirmed by sequencing.

### 2.6. Reporter gene assay

Cells were seeded onto 24-well plates and transfected with various amounts of expression vectors, the reporter gene, and the phRL-TK control plasmid using FuGene HD (Roche) according to the manufacturer's instructions. After 24 h, the cells were harvested in 100 μl lysis buffer. The luciferase activity was measured using Dual-

Luciferase Reporter assay systems (Promega) and was shown as the means ± S.D. of three experiments.

### 2.7. Native PAGE, SDS-PAGE, Western blotting, and immunoprecipitation assay

Cells were solubilized in the lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% NP-40, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM $Na_3VO_4$) on ice for 30 min and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were separated by SDS-PAGE, and the gel was transferred onto polyvinylidene difluoride membranes. The membranes were then blocked with Tris-buffered saline (TBS) pH 8.0 containing 5% skim milk, immunoblotted with specific antibodies, and visualized with the appropriate horseradish peroxidase-conjugated secondary antibodies using the ELC plus Western Blotting Detection System (Amersham Pharmacia). For detection of IRF3-dimerization, whole cell extracts were subjected to 7.5% polyacrylamide gel Native (Daiichi Pure Chemicals). For immunoprecipitation, cells were lysed in the Triton X-100 lysis buffer (50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1.5 mM $MgCl_2$, 1% Triton X-100, 10% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM $Na_3VO_4$) and then centrifuged at 12,000 × *g* for 10 min at 4 °C. The supernatants were incubated with anti-Myc antibody and protein G-Sepharose (Amersham Pharmacia) for overnight at 4 °C. The immunoprecipitates were collected by centrifugation, washed 4 times in the lysis buffer, and then analyzed by SDS-PAGE.

### 2.8. Confocal microscopy

HeLa cells ($2.5 \times 10^4$ cells/well) were plated on a micro cover glass (Matsunami Glass) in 12-well plate. The following day, cells were transfected with the indicated plasmids using FuGENE HD (Roche). The total amounts of DNA were kept constant by adding empty vector. After 24 h, cells were fixed in acetone and blocked in PBS containing 1% BSA and then labeled with the indicated primary antibodies for 1 h at room temperature. Alexa Fluor 488- or Alexa Fluor 594-conjugtaed secondary antibodies were used for the visualizing proteins detected by the primary antibodies. For nucleus staining, cells were treated with DAPI in PBS. After all staining procedures were finished, micro cover glasses were mounted onto a slide glass using PBS containing 2.3% DABCO and 50% glycerol. Cells were visualized at 63× magnification under an LSM510 META microscope (Zeiss).

### 2.9. Statistical analysis

The statistical significance was analyzed using Student's *t*-test. *p* values $<0.05$ were considered significant.

## 3. Results

### 3.1. Laboratory adapted strain ED induces IFN-β mRNA in A549/CD150 cells

We tested whether MV induced the expression of IFN-β mRNA in infected A549/CD150 cells and found that laboratory-adapted strain ED induced IFN-β mRNA expression, whereas IFN-β mRNA was virtually undetectable in wild-type strain MS-infected cells (Fig. 1A). To confirm the efficiency of virus infection, we measured MV-H mRNA levels by RT-PCR (Fig. 1B). The MV-H mRNA level in MS-infected cells was comparable to that found in ED-infected cells. Our previous report showed that DI RNA in MV isolates is a crucial determinant for high IFN induction (Shingai et al., 2007). However, no amplifiable 5′ copy-back DI RNA was detected in the MV culture supernatants (Fig. 1C), suggesting that the ED and MS strains used

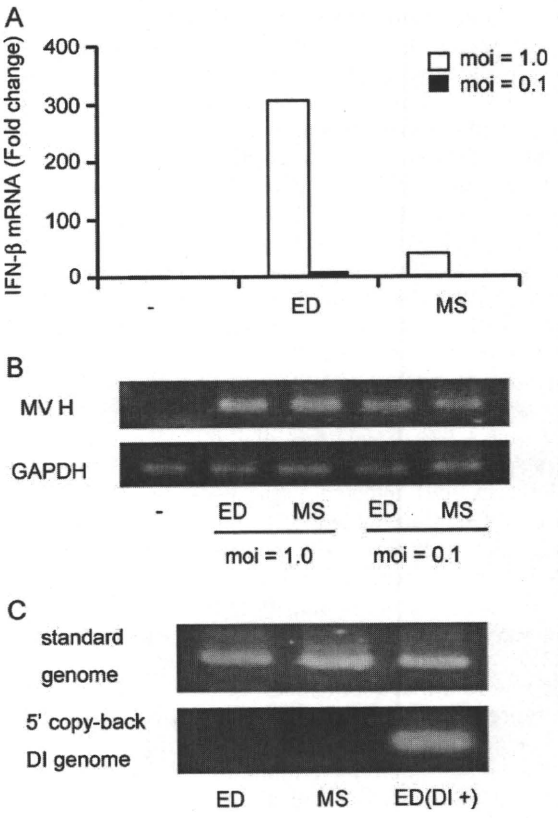


**Fig. 1.** ED strain induces IFN-β mRNA expression in the infected cells. (A) A549/CD150 cells were infected with mock, ED (vaccine strain) or MS (wild-type strain) at MOI = 0.1 or 1. After 12 h, RNA samples were collected and mRNAs of IFN-β and β-actin were measured by real-time PCR. The value for IFN-β mRNA expression was normalized to that of β-actin mRNA. Fold induction against control medium is shown. (B) MV-H mRNA level was determined by RT-PCR. (C) RT-PCR amplification of 5′ copy-back DI RNA from MV culture supernatants. RT-PCR was performed using standard genome-specific primers or DI-specific primers.

in this study, do not contain 5′ copy-back DI RNA. Thus, in this DI RNA-negative ED strain, a factor other than DI RNA is implicated in the induction of IFN-β mRNA.

### 3.2. The ED-V protein barely suppresses MDA5-induced IFN-β promoter activity

To explain the differential type I IFN-inducing abilities of ED versus wild-type strains, we transfected cDNAs encoding MV proteins into A549/CD150 cells, established in our laboratory (Tanabe et al., 2003). In these pilot studies, we found that expression of MV V protein suppresses IFN-β promoter activation, as reported by other groups (Nakatsu et al., 2008; Ohno et al., 2004; Takeuchi et al., 2003). We then focused on the function of the V and C proteins of various MV strains. The V and C proteins of MV are not essential products (Radecke and Billeter, 1996) but play important roles in MV virulence (Patterson et al., 2000). The V protein has been shown to inhibit IFN induction via binding to MDA5 (Childs et al., 2007, 2009). On the other hand, the C protein does not block the IFN-inducing pathway, but affects infectivity by acting as a regulator of viral RNA synthesis (Nakatsu et al., 2008). When A549/CD150 cells were stimulated with polyI:C or transfected with RIG-I or MDA5, efficient IFN-β promoter activation was detected using a reporter assay (Fig. 2A–C). Using this assay, we examined the effects of the transfected V and/or C proteins on IFN-β promoter activity. PolyI:C-induced IFN-β promoter activation was inhibited by the V protein expressed by wild-type strains, MS and IC-B, and an attenuated NV strain, which possesses DI RNA (Shingai et al., 2007). The ED-V pro-

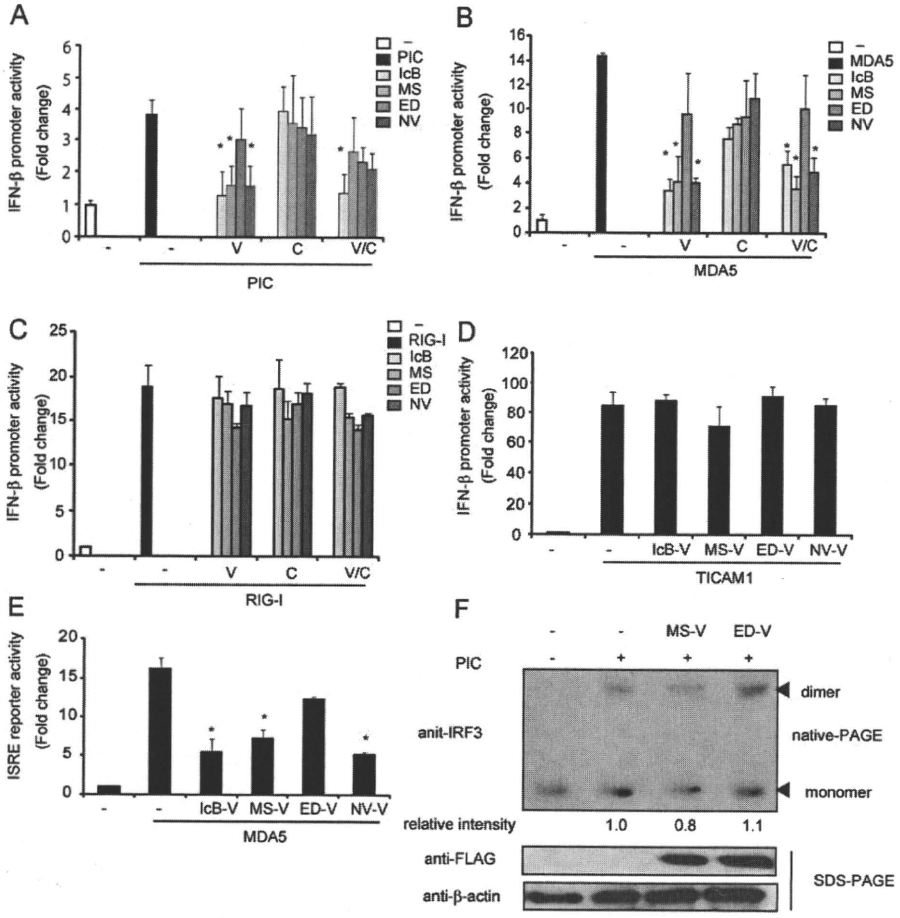


**Fig. 2.** Strain-to-strain difference in V protein function for MDA5-induced IFN-β promoter activation. (A) A549 cells in 24-well plates were transfected with pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the IFN-β promoter reporter (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the cells were stimulated with 50 μg/ml polyI:C for 6 h, and then the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 (100 ng, B), pEF-BOS RIG-I (100 ng, C), pEF-BOS TICAM1 (100 ng, D), pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the IFN-β promoter reporter (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the luciferase reporter activity was measured. (E) A549 cells in 24-well plates were transfected with pCMV10-MV-V (100 ng) and pCMV10-MV-C (100 ng) together with the ISRE luciferase gene (100 ng) and phRL-TK (50 ng). Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. $^*p < 0.05$. (F) HeLa cells transfected with pCMV10 FLAG-MV-V (100 ng). After 24 h, cells were stimulated with 10 μg/ml polyI:C for 1 h and then lysed with native-PAGE lysis buffer or SDS-PAGE lysis buffer. For native-PAGE, the cell lysates were subjected to native-PAGE and immunoblotted with anti-IRF-3 antibody. For SDS-PAGE the cell lysates were subjected to SDS-PAGE and immunoblotted with anti-FLAG antibody or anti-β-actin (internal control). The band intensity was quantified by NIH Image J and relative band intensity was shown. The results were reproducible in three additional experiments.

tein barely suppressed polyI:C-induced IFN-β promoter activation (Fig. 2A). None of the C proteins analyzed affected IFN-β promoter activation.

PolyI:C is regarded as an analog of viral dsRNA and activates TLR3 in the endosomes and RIG-I/MDA5 in the cytoplasm. TLR3 recruits TICAM-1 while RIG-I and MDA5 recruit IPS-1 as adaptors. The two pathways converged upon NAP1, which assembles IKKε and TBK1 to activate IRF-3 and promote induction of IFN-β (Sasai et al., 2006b). Production of a trace amount of IFN-β results in amplified production of type I IFN via the IFNAR pathway, as controlled by the ISRE promoter (Takaoka and Yanai, 2006). To reveal the target pathway inhibited by the V protein of wild-type MV, we examined whether the wild-type MV V proteins block IFN-β induction in cells containing overexpressed MDA5, RIG-I or TICAM-1 (Fig. 2B–D). The V proteins of strains MS and IC-B inhibited MDA5-induced IFN-β and ISRE promoter activation but barely affected RIG-I and TICAM-1-induced IFN-β induction (Fig. 2B–E). It is notable that in our setting, V proteins of various MV strains did not suppress RIG-I-mediated activation of IFN-β promoter (Fig. 2C). These data suggested that the V proteins of wild-type strains suppress the MDA5 pathway for type I IFN induction while the C proteins barely affect MDA5-, RIG-I- and TICAM-1-dependent IFN-β transcription. Under these conditions, only the V protein of strain ED abrogates the inhibitory function of MDA5 in both IFN-β and ISRE reporters.

IRF-3 activation in the cytoplasm occurs via C-terminal phosphorylation of IRF-3 by the TBK1/NAP1/IKKε complex. These modifications promote IRF-3 homodimerization and the subsequent nuclear import of these molecules (Medzhitov, 2007; Platanias, 2005). In our studies for detection of IRF-3 dimer formation, although the V protein of the wild-type strain suppressed polyI:C-induced IRF-3 dimerization, the ED-V protein hardly inhibited polyI:C-induced IRF-3 dimerization (Fig. 2F). These data suggested that the V protein of wild-type strains inhibited polyI:C-induced IFN-β induction via the suppression of MDA5-meditaed IRF-3 activation. To exclude the possibility that the MV-V protein causes MDA5 degradation, we confirmed the MDA5 protein level by Western blotting (Fig. 3). The MDA5 protein levels in the MS-V or ED-V transfected cells were comparable to those found in untreated cells.

### *3.3. 272C is responsible for suppression of MDA5-induced IFN-β promoter activity*

To reveal the molecular mechanism that determines whether MV V protein inhibits MDA5-induced IFN-β promoter activity, we compared the amino acid sequence of the ED V protein with that of

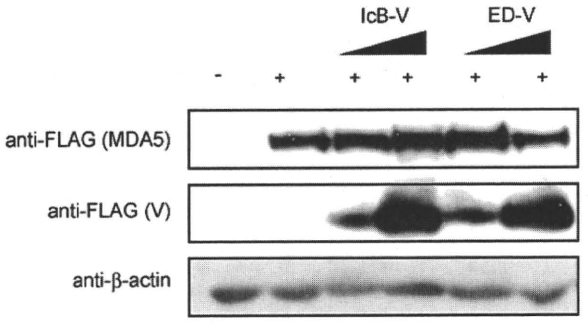


**Fig. 3.** Forced expression of V protein did not affect the expression level of MDA5 protein. HEK 293FT cells were transfected with pEF-BOS FLAG-MDA5 (100 ng) and pCMV10 FLAG-MV-V (10 ng, 100 ng). After 24 h, cells were lysed and subjected to Western blotting with anti-FLAG antibody and anti-β-actin antibody (internal control).

wild-type V proteins. As shown in Fig. 4, we identified 7 amino acid substitutions (51R, 83P, 97P, 110H, 225G, 272R and 291H) in the ED V protein. These conversions are ED strain V-specific, since the authentic V sequence is conserved in other strains. We then constructed R51K, P83S, P97S, H110Y, G225E, R272C and H291Y mutants of ED V protein and examined the effects of these mutants on MDA5-induced IFN-β promoter activity (Fig. 5A). As shown in Fig. 5A, only R272C mutant of ED V protein suppressed MDA5-activated IFN-β promoter. Next, we examined whether the V protein inhibited polyI:C-induced IRF-3 nuclear translocation. Although WT ED V protein did not inhibit polyI:C-induced IRF-3 nuclear translocation, overexpression of R272C mutant suppressed IRF-3 nuclear translocation (Fig. 5B and C). R51K, P83S, P97S, H110Y, G225E and H291Y mutants did not affect IRF-3 nuclear translocation. Since previous reports have shown that the V proteins of paramyxoviruses interacted with MDA5 to inhibit MDA5 activity and suppress IFN-β induction (Childs et al., 2007, 2009), we examined the interaction between MDA5 and the V proteins by immunoprecipitation. As expected, only R272C mutant interacted with MDA5, whereas WT ED V and the other mutants did not bind MDA5 (Fig. 5D). These data suggest that the arginine at position 272 in ED V protein is responsible for insuppressible activity of MDA5-induced IFN-β promoter activation. The cysteine residue at position 272 of V protein is conserved among paramyxoviruses. To clarify that 272C is important for suppressive activity of WT V protein, we examined effects of IC-B V C272R mutant on MDA5-induced IFN-β promoter activity. As shown in Fig. 6A, although IC-B V protein suppressed IFN-β promoter activity, C272R mutant was not able to inhibit IFN-β promoter activation. Similarly, C272R mutant did not suppressed poly I:C-induced IRF-3 nuclear translocation and interact with MDA5 (Fig. 6B and C). These data infer that the 272C residue of V protein is crucial for interacting with MDA5 and suppressing IRF-3 activation, which reasons that ED V strains fail to interact with MDA5.

## 4. Discussion

In this study, we demonstrated that the V protein of MV strain ED neither interacted with MDA5 nor suppressed MDA5-induced IRF-3 activation. A C272R mutation in the cysteine-rich region of wild-type V protein rendered the V protein IFN-insuppressible and the R272C conversion in ED strains conferred an IFN-suppressive function on the V protein. The V protein targets MDA5 and V proteins possessing the 272C residue co-precipitate with MDA5 by immunoprecipitation. Only V proteins possessing the 272C residue accelerate nuclear translocation of IRF-3. Based on the results of our reporter assay, V protein does not affect TICAM-1- or RIG-I-induced IFN-β promoter activation. Hence, the 272C residue is crucial for the V protein to block MDA5 function and MDA5 is the molecule which V protein targets for inhibition of the initial induction of IFN-β. For this reason, the ED strain used in this study allowed infected cells to induce IFN-β mRNA even in the absence of DI RNA. A previous report showed that the V protein of Sendai virus binds MDA5 via the cysteine-rich region which is conserved among paramyxoviruses (Childs et al., 2009). Accordingly, we found that the MV V protein interacted with MDA5 via the cysteine-rich region.

```
MS-V     1 MAEEQARHVKNGLECIRALKAEPIGSLAIGEAMAAWSEISDNPGQERATCKEEKAGGSGL 60
IC-B-V   1 MAEEQARHVKNGLECIRALKAEPIGSLAVEEAMAAWSEISDNPGQDRATCKEEEAGSSGL 60
NV-V     1 MAEEQARHVKNGLECIRALKAEPIGSLAIGEAMAAWSEISDNPGQERATCKEEKAGGSGL 60
ED-V     1 MAEEQARHVKNGLECIRALKAEPIGSLAIEEAMAAWSEISDNPGQERATCREEKAGSSGL 60
           ****************************: ***************:****:**:**.***

MS-V    61 SKPCLSAIGSTEGGAPRIRGQGSGESDDDTETLGIPSRNLQASSTGLQCYYVYDHSGEAV 120
IC-B-V  61 SKPCLSAIGSTEGGAPRIRGQGSGESDDDAETLGIPSRNLQASSTGLQCYHVYDHSGEAV 120
NV-V    61 SKSCLSAVGSTEGGAPRIRGQGSGESDDDTETLGIPSRNLQASSTGLQCYYVYDHSGEAV 120
ED-V    61 SKPCLSAIGSTEGGAPRIRGQGPGESDDDAETLGIPPRNLQASSTGLQCHYVYDHSGEAV 120
           **.****:**************.******:******.************:::*********

MS-V   121 KGIQDADSIMVQSGLDGDSTLSGGDNESENSDVDIGEPDTEGYAITDRGSAPISMGFRAS 180
IC-B-V121 KGIQDADSIMVQSGLDGDSTLSGGDDESENSDVDIGEPDTEGYAITDRGSAPISMGFRAS 180
NV-V   121 KGIQDADSIMVQSGLDGDSTLSGGDNESENSDVDIGEPDTEGYAITDRGSAPISMGFRAS 180
ED-V   121 KGIQDADSIMVQSGLDGDSTLSGGDNESENSDVDIGEPDTEGYAITDRGSAPISMGFRAS 180
           *************************:************************************

MS-V   181 DVETAEGGEIHELLRLQSRGNTLPKLGKTLNVPPPPDPGRASASETPIKKGHRREISLIW 240
IC-B-V181 DVETAEGGEIHELLKLQSRGNNFPKLGKTLNVPPPPNPSRASTSETPIKKGHRREIGLIW 240
NV-V   181 DVETAEGGEIHELLRLQSRGNTFPKLGKTLNVPPPPDPGRASTSETPIKKGHRREISLIW 240
ED-V   181 DVETAEGGEIHELLRLQSRGNNFPKLGKTLNVPPPPDPGRASTSGTPIKKGHRREISLIW 240
           **************:******.:**************:*. **:* ***********.***

MS-V   241 NGDRVFIDRWCNPMCSKVTLGTIRARCTCGECPRVCEQCRTDTGVDTRIWYHNLPEIPE 299
IC-B-V241 NGDRVFIDRWCNPMCSKVTLGTIRARCTCGECPRVCEQCRTDTGVDTRIWYHNLPEIPE 299
NV-V   241 NGDRVFIDRWCNPMCSKVTLGTIRARCTCGECPRVCEQCRTDTGVDTRIWYHNLPEIPE 299
ED-V   241 NGDRVFIDRWCNPMCSKVTLGTIRARCTCGERPRVCEQCRTDTGVDTRIWHHNLPEIPE 299
           ****************************** *****************:********
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**Fig. 4.** Comparison of the ED V protein amino acid sequences with various MV strain. Several point mutations were found in the ED-V protein. Underline shows the conserved-Cys-rich region. Arrow heads show mutations in ED V protein.

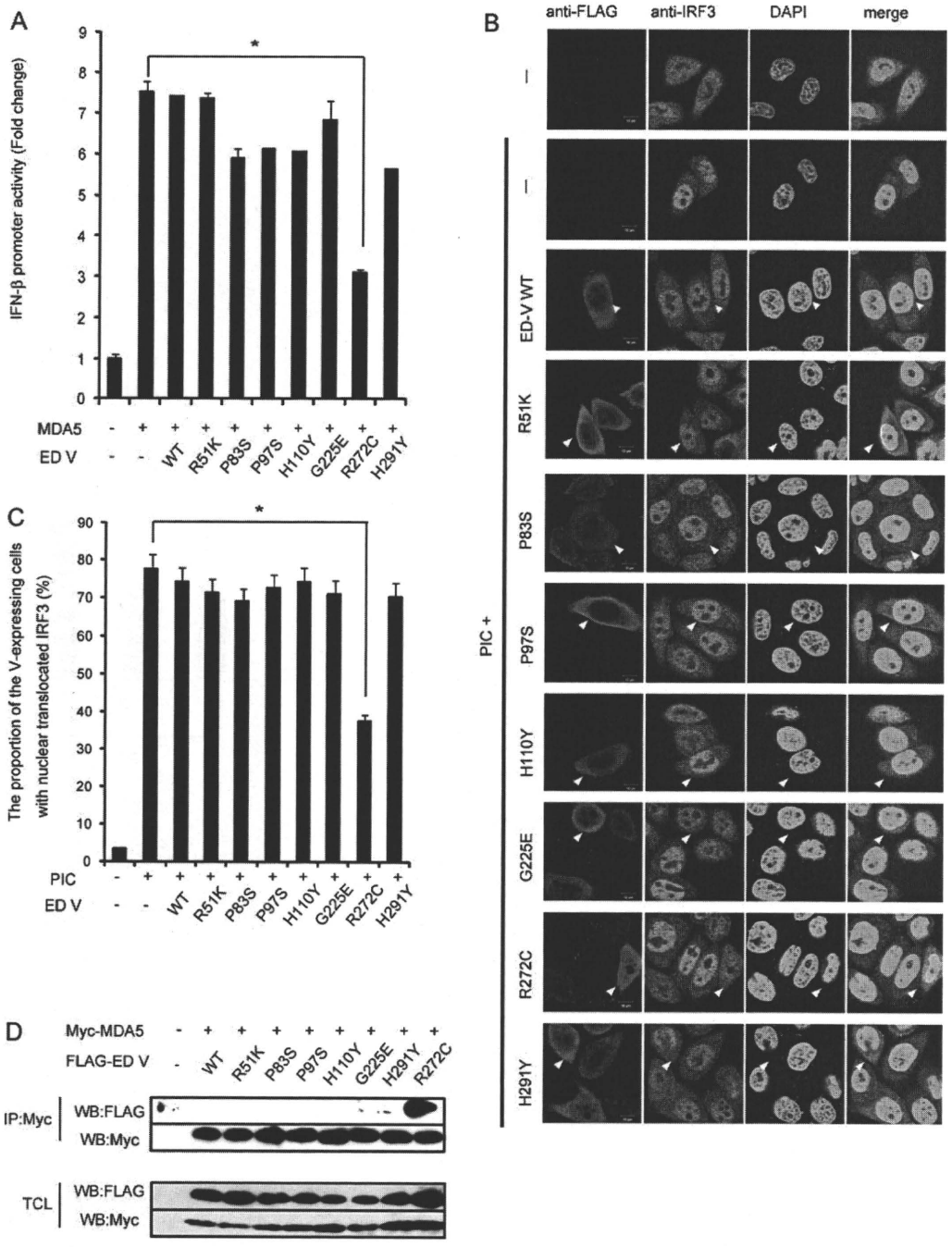


**Fig. 5.** 272C is a critical residue for suppression of MDA5-induced IFN-β promoter activity. (A) A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 and pCMV10-MV-V together with the IFN-β promoter reporter and phRL-TK. Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. *$p < 0.05$. (B) HeLa cells were transfected with various pCMV10 ED-V plasmids. After 24 h, the cells were stimulated with 10 μg/ml polyI:C for 1 h, fixed and stained with anti-IRF-3 and anti-FLAG antibodies (V protein), and visualized with either Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. The same slide was also treated with DAPI for the staining of nuclei. Arrow heads show V-expressing cells. (C) The number of the V-expressing cells with nuclear translocated IRF3 (see panel B) were counted. The results are shown by the proportion of the V-expressing cells with nuclear translocated IRF3 ($n = 50$). The average proportions from three independent assays are shown. *$p < 0.05$. (D) HEK293FT cells were transfected with pcDNA4 Myc-MDA5 and pCMV10 FLAG-MV-V with mutations. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc or anti-FLAG antibodies. An aliquot of each total cell lysate (TCL) was immunoblotted with either anti-Myc or anti-FLAG antibodies.

Childs et al. (2009) reported that the V protein of paramyxovirus specifically inhibited activation of the MDA5 pathway, but not the RIG-I pathway, by specifically binding to the helicase domain of MDA5 and hindering MDA5 from recruiting dsRNA. Consistent with their report, the V protein thus blocks sensing dsRNA via MDA5 to disassemble oligomerization of MDA5. These results infer that the IFN-inducible properties of the laboratory-adapted ED strain were largely attributable to the aberrance of the function of the V protein by introduction of the C272R mutation. We only regret that we could not detect the complex of endogenous MDA5 and MV V in this study since resting cells express only a trace amount of MDA5 (Yoneyama et al., 2005).

Ohno et al. (2004) showed that the 110Y and 272C residues of the V protein were responsible for the suppression of IFN-α and IFN receptor signaling using HEK293 transfectants. In contrast, we clarified that C272R mutant but H110Y mutant of ED V protein sup-

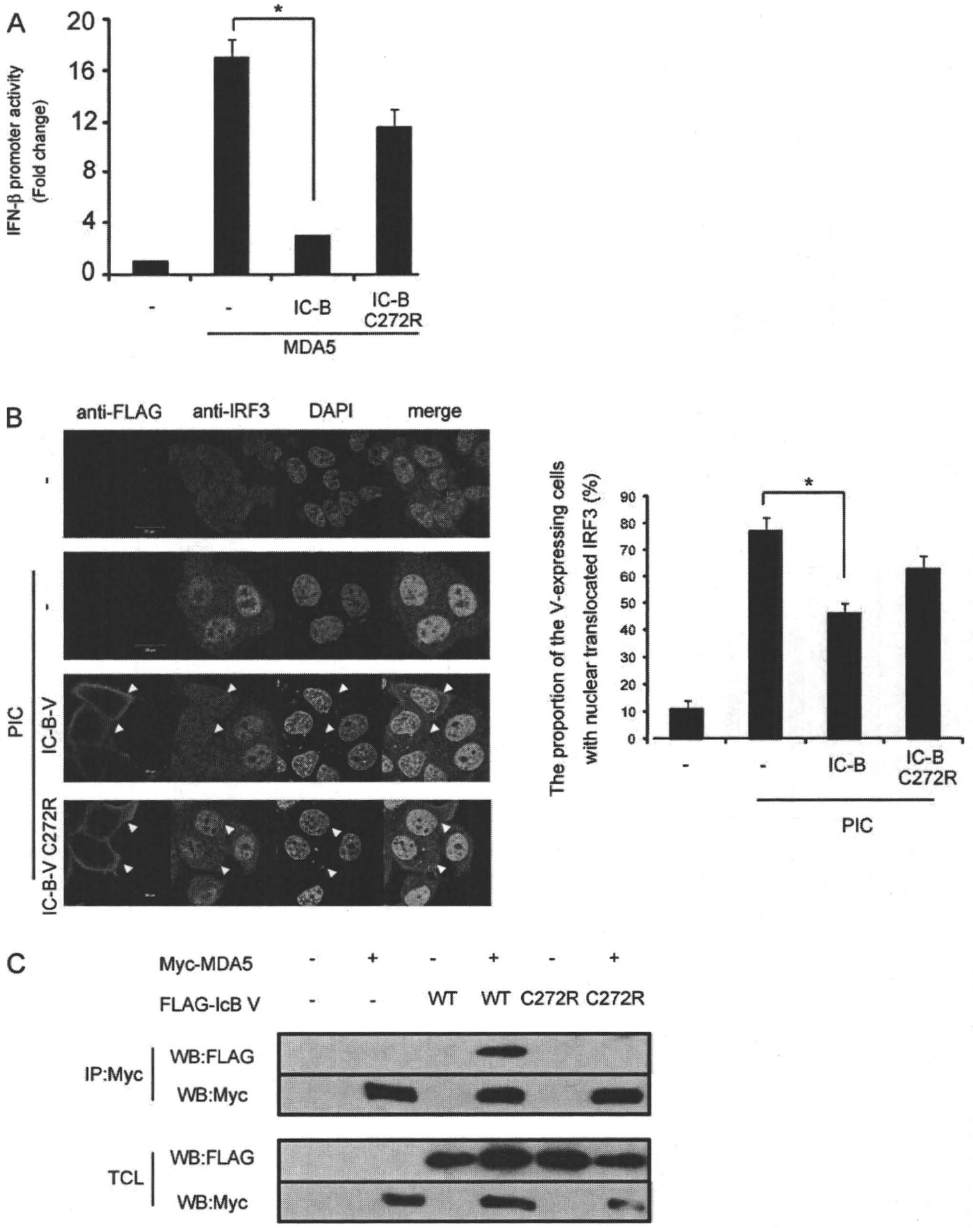


**Fig. 6.** 272C is important for suppressive activity of WT V protein. (A) A549 cells in 24-well plates were transfected with pEF-BOS FLAG-MDA5 and various pCMV10-IC-B-V plasmids together with the IFN-β promoter reporter and phRL-TK. Twenty-four hours after transfection, the luciferase reporter activity was measured. The average activities from three independent assays are shown as fold induction. *$p < 0.05$. (B) HeLa cells were transfected with various pCMV10 ED-V plasmids. After 24 h, the cells were stimulated with 10 μg/ml polyI:C for 1 h, fixed and stained with anti-IRF-3 and anti-FLAG antibodies (V protein), and visualized with either Alexa Fluor 488- or Alexa Fluor 594-conjugated secondary antibodies. The same slide was also treated with DAPI for the staining of nuclei. Arrow heads show V-expressing cells. Right panel shows the proportion of the V-expressing cells with nuclear translocated IRF3. (C) Immunoprecipitation assay in 293T cells. Cells were transfected with pcDNA4 Myc-MDA5 and pCMV10 FLAG-MV-V. After 24 h, the cells were lysed, immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-Myc or anti-FLAG antibodies. An aliquot of each total cell lysate (TCL) was immunoblotted with either anti-Myc or anti-FLAG antibodies.

pressed IFN-β promoter activity in the MDA5 pathway. Hence, the tyrosine at position 110 is responsible only for blocking the IFN amplification pathway via IFN-α/β receptor (IFNAR). On the other hand, the cysteine residue at position 272 is important for inhibiting both MDA5-induced IFN-β transactivation and IFNAR amplification loop. The V protein of strain ED is unable to block not only MDA5 but also the IFNAR amplification pathway, thereby ED-based vaccine strains would be able to induce type I IFN. Consistent with this possibility, Ikegame et al. (2010) reported the participation of MDA5 in MV-mediated IFN induction and MV growth promotion using RIG-I-silenced cells and V protein-deficient MV strains. In fact, the V proteins of ED and wild-type strains play no role in blocking the downstream of TBK1 for IFN-β reporter activation (data not shown). However, we wonder if the viruses produce sufficient amounts of long dsRNA (>40 bp in length, enough to be detected by J2 mAb) to be recognized by MDA5 in an early step of infection, i.e. before the production of V protein. Since RIG-I recognizes 5′-3P-ssRNA or short dsRNA, the RIG-I pathway is thought to be predominantly involved in IFN induction in MV-infected cells (Plumet et al., 2007; Shingai et al., 2007). Detailed analysis will be required to elucidate the predominant usage of RIG-I or MDA5 for type I IFN induction in cells infected with a variety of viruses. Why MV blocks MDA5 but not RIG-I activity and which viral products specifically recognize and bind MDA5 are questions that remained to be answered.

The C protein of MV plays an important role in inhibiting the JAK-STAT pathway of IFNAR signaling (Shaffer et al., 2003), and also acts

as a regulator of viral RNA synthesis, thereby indirectly suppressing IFN induction (Nakatsu et al., 2006, 2008; Takeuchi et al., 2005). MV mutants that fail to express the C protein allow infected cells to generate dsRNA (Ikegame et al., 2010), suggesting that the C protein may also function in controlling the generation of long dsRNA. In this study, we observed that the forced expression of C protein did not affect polyI:C-, RIG-I- and MDA5-induced IFN-β reporter activity and there were no significant amino acid changes in this protein among wild-type and vaccine strains (data not shown). C protein appears neither to directly affect the IFN-inducing pathways, nor to be responsible for the IFN-induction of vaccine strains. An interesting issue is the relationship between activation of the MDA5 pathway by MV vaccine strains and the limited production of long dsRNA due to the function of the C protein.

In conclusion, our data suggest that the C272R mutation in the V protein in MV strains is a major cause of insuppressible IFN production in a certain case of MV infection and that the 272C residue of the V protein is responsible for the MDA5-blocking ability of wild-type MV. Although RIG-I recognizes MV products including DI RNA or 5′-3P-ssRNA, the initial response of MDA5 also acts as a cause for amplifying type I IFN production, at least in some vaccine strains.

## Conflict of interest

There is no conflict of interest in this study.

## Acknowledgements

We are grateful to Drs. A. Watanabe, H.H. Aly, H. Shime, J. Kasamatsu, S. Yamazaki, and T. Ebihara for invaluable discussions. This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture, the Ministry of Health, Labor, and Welfare of Japan, and by the NorthTec Foundation, the Yakult Foundation, Waxman Foundation and the Akiyama Life Science Foundation. Financial support by the Sapporo Biocluster "Bio-S" the Knowledge Cluster Initiative of the MEXT, and the Program of Founding Research Centers for Emerging and Reemerging Infectious Diseases, MEXT are gratefully acknowledged.

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Addendum

# Addendum to "Strain-to-strain difference of V protein of measles virus affects MDA5-mediated IFN-β-inducing potential" [Mol. Immunol. 48(4) (2011) 497–504]

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ABSTRACT

Measles virus (MV) V protein blocks type I IFN signaling in MV-infected cells. Previous studies suggested that some MV strains could release the V protein-mediated type I IFN suppression in affected cells by two distinct modes: V protein mutation and production of DI RNA. These two modes of type I interferon regulation involves the IPS-1 (MAVS, Cardif, VISA) pathway (Takaki et al., 2011. Mol. Immunol. 48(4), 497–504). We add the comment to this previous issue that the release of the V protein-mediated suppression of type I IFN occurs only by a laboratory-adapted strain of Edmonston.

*Dear Editor,*

Recently, we received a comment that the C272R MV strain we used in our recent publication in Molecular Immunology (Takaki et al., 2011) is a recombinant Edmonston (ED) strain, thus not a representative ED vaccine strain. We searched the NCBI database (www.ncbi.nih.gov/nuccore/1041617) for Edmonston strain and found that this comment is right. The clone we used is very similar to that deposited in the bank as Measles virus (strain Edmonston B) RNA (infectious cDNA clone), GenBank: Z66517.1. The ED vaccine lineage was described in Parks et al. (2001). According to the sequence definition, this strain may be originated from the reported recombinant virus of an ED infectious strain. As we did not mention in the paper (Takaki et al., 2011) that this ED strain is originated from the vaccine or reverse-transcribed ED, we describe this addendum to indicate that the ED strain we used is a laboratory-adapted strain. We appreciate the comment raised anonymously by a reader, presumably a virologist, who pointed us to the difference between the vaccine strain and laboratory-adapted strain.

The aim of our study (Takaki et al., 2011) was to clarify the pathway of IFN induction in MV strains with different sequences. I believe that the reader and I agreed that the C272R mutation of MV V protein fails to block MDA5 and thereby up-regulates type I IFN induction. This C272R strain appeared to be derived from the ED strain with V protein mutation (Combredet et al., 2003). The V protein mutation may tend to occur in ED laboratory-adapted strains through adaptation to Vero cells which lack antiviral type I IFN response. Ohno et al. (2004) first provided the data on V protein mutation facilitating IFN alpha-dependent IFN-signaling in a MV strain of a C272R mutant, named Edtag. This means that Edtag C272R mutation releases the block of the IFNAR-STAT1/2/IRF-9 pathway by MV V protein, allowing IFN-alpha amplification (Taniguchi and Takaoka, 2002). On the other hand, Takaki's report here (Takaki et al., 2011) indicates that failure of the C272R mutant to sufficiently block MV-mediated IFN induction is rooted in the uncontrolled IPS-1 pathway, which leads to enhanced IRF-3 nuclear translocation.

Shingai et al. (2007) previously reported that defective interference (DI) RNA produced in MV-replicating cells causes accelerated MV-mediated induction of type I IFN. DI RNA acts on the cytoplasmic sensors, RIG-I/MDA5, leading to activation of the IPS-1 pathway (Yoneyama and Fujita, 2007) Studies by Ohno and Shingai revealed that MV laboratory-adapted strains could up-regulate IFN induction in affected cells by two distinct modes: V protein mutation (Ohno et al., 2004) and production of DI RNA (Shingai et al., 2007). Takaki's data (Takaki et al., 2011) further demonstrated that both modes of C272R mutation and DI RNA production involve the IPS-1 pathway, not only the IFNAR pathway. This is just the conclusion this paper wanted to emphasize.

Takaki's results were recently confirmed in a report by Haralambieva et al. (2010). The main conclusion of our study is thus confirmed in both innate immunity and virology laboratories. We think this is a good example of how progress in the field of viral immunity will be attained by promoting communication between the fields of virology and innate immunology.

## Correction

Abstract near to the last conclusion: the structural difference of laboratory-adapted MV V protein hampers MDA5 blockade and acts as a nidus for the spread/amplification of type I IFN induction.

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# Raftlin Is Involved in the Nucleocapture Complex to Induce Poly(I:C)-mediated TLR3 Activation*[S]

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**The double-stranded RNA analog, poly(I:C), extracellularly activates both the endosomal Toll-like receptor (TLR) 3 and the cytoplasmic RNA helicase, melanoma differentiation-associated gene 5, leading to the production of type I interferons (IFNs) and inflammatory cytokines. The mechanism by which extracellular poly(I:C) is delivered to TLR3-positive organelles and the cytoplasm remains to be elucidated. Here, we show that the cytoplasmic lipid raft protein, Raftlin, is essential for poly(I:C) cellular uptake in human myeloid dendritic cells and epithelial cells. When Raftlin was silenced, poly(I:C) failed to enter cells and induction of IFN-β production was inhibited. In addition, cellular uptake of B-type oligodeoxynucleotide that shares its uptake receptor with poly(I:C) was suppressed in Raftlin knockdown cells. Upon poly(I:C) stimulation, Raftlin was translocated from the cytoplasm to the plasma membrane where it colocalized with poly(I:C), and thereafter moved to TLR3-positive endosomes. Thus, Raftlin cooperates with the uptake receptor to mediate cell entry of poly(I:C), which is critical for activation of TLR3.**

Polyriboinosinic:polyribocytidylic acid (poly(I:C)),[2] a synthetic double-stranded RNA (dsRNA), has been used as a potent type I interferon (IFN-α/β) inducer in both *in vitro* and *in vivo* studies since the discovery of anti-viral activity of type I IFNs (1–3). Many types of cells including fibroblasts, epithelial cells, and myeloid dendritic cells (DCs), produce IFN-β upon stimulation with poly(I:C). Studies have demonstrated that extracellular poly(I:C) is recognized by Toll-like receptor (TLR) 3 and cytoplasmic RNA helicase, melanoma differentiation-associated gene 5 (MDA5), and induces innate immune responses including the production of type I IFNs and inflammatory cytokines (4–8). More recently, experimental evidence has accumulated that poly(I:C) acts as an adjuvant that enhances antibody production, natural killer cell activation, and cytotoxic T lymphocyte induction through the activation of TLR3 and/or MDA5 (9–15).

Human TLR3 localizes to the endosomal compartments in myeloid DCs, whereas it localizes to both the cell surface and endosomes of fibroblasts, macrophages, and epithelial cells (5, 16, 17). TLR3 signaling arises from an intracellular compartment in both cell types and requires endosomal maturation. After dsRNA recognition, endosomal TLR3 recruits an adaptor molecule, *i.e.* Toll-IL-1 receptor domain-containing adaptor molecule-1 (TICAM-1, also called TRIF) that activates the NF-κB, IRF-3, and AP-1 transcription factors, leading to IFN-β production (18, 19). Also, extracellular poly(I:C) is sensed by MDA5 in the cytoplasm, resulting in the activation of IRF-3 and NF-κB via the mitochondrial outer membrane protein IPS-1 (also called MAVS, Cardif, or VISA) (20–23). However, the mechanism by which poly(I:C) is delivered from the extracellular fluid to the intracellular dsRNA sensors remains unresolved.

A recent study showed that CD14 directly binds to poly(I:C) and mediates poly(I:C) cellular uptake (24). Bone marrow-derived macrophages from CD14-deficient mice exhibited impaired, but not completely diminished, responses to poly(I:C). Also, a class A scavenger receptor was identified as a cell surface receptor for poly(I:C) in human epithelial cells, although the response of poly(I:C) was only partially impaired in scavenger receptor A-deficient mice (25). These results suggest that an unidentified cell surface molecule mediates cell entry of poly(I:C). Indeed, we and others demonstrated that poly(I:C) is internalized into CD14-negative human myeloid DCs and HEK293 cells via clathrin-dependent endocytosis, and B- and C-type oligodeoxynucleotides (ODNs) share the uptake receptor with poly(I:C) (26–28).

In this study, we isolated poly(I:C)-binding proteins from CD14-negative cell lysates by sequential affinity chromatography with poly(U)- and poly(I:C)-Sepharose and subjected them to mass spectrometric analysis. Among the proteins identified, we selected several proteins that exhibited a transmembrane domain or a membrane-anchoring motif and examined whether they were involved in poly(I:C)-induced TLR3-mediated signaling. We found that Raftlin, a major lipid raft protein

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S3 and Figs. S1–S4.

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[2] The abbreviations used are: poly(I:C), polyriboinosinic:polyribocytidylic acid; 4F2, 4F2 cell-surface antigen heavy chain; DCs, dendritic cells; BMDC, bone marrow-derived DC; CTXB, cholera toxin subunit B; MDA5, melanoma differentiation-associated gene 5; MβCD, methyl-β-cyclodextrin; MoDC, monocyte-derived immature DC; ODN, oligodeoxynucleotide; TICAM-1, Toll-IL-1 receptor-containing adaptor molecule-1; TLR, Toll-like receptor.

expressed by B cells, plays a critical role in poly(I:C) cellular uptake in human myeloid DCs and epithelial cells.

## EXPERIMENTAL PROCEDURES

*Cell Culture and Reagents*—Human B cell lines Raji, BALL-1, and Namalwa were obtained from the Riken Cell Bank (Tukuba, Japan) and maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS (BioSource Intl., Inc.) and antibiotics. HEK293 cells were obtained from Sumitomo Pharmaceuticals Co., Ltd. (Osaka, Japan) and maintained in Dulbecco's modified Eagle's medium low glucose (Invitrogen) supplemented with 10% heat-inactivated FCS and antibiotics. HeLa cells were kindly provided by Dr. T. Fujita (Kyoto University) and maintained in Eagle's minimal essential medium (Nissui, Tokyo, Japan) supplemented with 1% L-glutamine and 10% heat-inactivated FCS. Human monocyte-derived immature DCs (MoDCs) were generated from $CD14^+$ monocytes by culturing for 6 days in the presence of 500 units/ml of granulocyte-macrophage colony-stimulating factor and 100 units/ml of IL-4 (PeproTech). Bone marrow-derived DCs (BMDCs) were prepared as described (10). Polymyxin B, 4′,6-diamidine-2′-phenylindole dihydrochloride (DAPI), saponin, and methyl-β-cyclodextrin (MβCD) were purchased from Sigma. Poly(I:C) was from Amersham Biosciences, FITC-labeled ODN2006 was from InvivoGen, Alexa Fluor 488/cholera toxin subunit B (CTXB) and Alexa Fluor 568/transferrin were from Molecular Probes. MALP-2 was obtained from Biosynthesis (Nagoya, Japan). In addition, the following antibodies were used in this study: anti-dsRNA mAb (K1) (BioLink), anti-β actin mAb (Sigma), anti-clathrin heavy chain mAb (TD.1) (Santa Cruz Biotechnology), anti-Rab5 mAb (Abcam), anti-LAMP1 (H4A3) (BioLegend), HRP-conjugated secondary Abs (BIOSOURCE), FITC-labeled goat anti-mouse IgG (American Qualex), and Alexa Fluorp®-conjugated secondary antibodies (Invitrogen). Anti-human Raftlin polyclonal antibody was prepared as described (29). Anti-human TLR3 mAb (clone TLR3.7) was generated in our laboratory (5). Texas Red-labeled poly(I:C) was prepared using the 5′ EndTag™ Nucleic Acid Labeling System (Vector Laboratories, Burlingame, CA) according to the manufacturer's instructions.

*Mice*—Raftlin$^{-/-}$ mice were provided by Dr. A. Yoshimura (Keio University). Mice were maintained under specific pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine. Animal experiments were performed according to the guidelines established by the Hokkaido University Animal Care and Use Committee.

*Plasmids*—The cDNA fragment encoding the ORF of human TLR2 or TLR3 was amplified by RT-PCR from total RNA prepared from MoDCs, and was ligated into the cloning site of the expression vector pEF-BOS, a gift from Dr. S. Nagata (Kyoto University) (5). Complementary DNA for human Raftlin was generated by PCR from cDNA derived from Raji cells using specific primers (forward primer, 5′-CTCGAGGCCGCCACCATGGGTTG-3′; reverse primer, 5′-GGATCCTTGTTTTCTTCAACCGTACCAAGCTC-3′), and was ligated into the cloning site of the expression vector pEYFP-N1 (C-terminal yellow fluorescent protein (YFP) tag, Clontech).

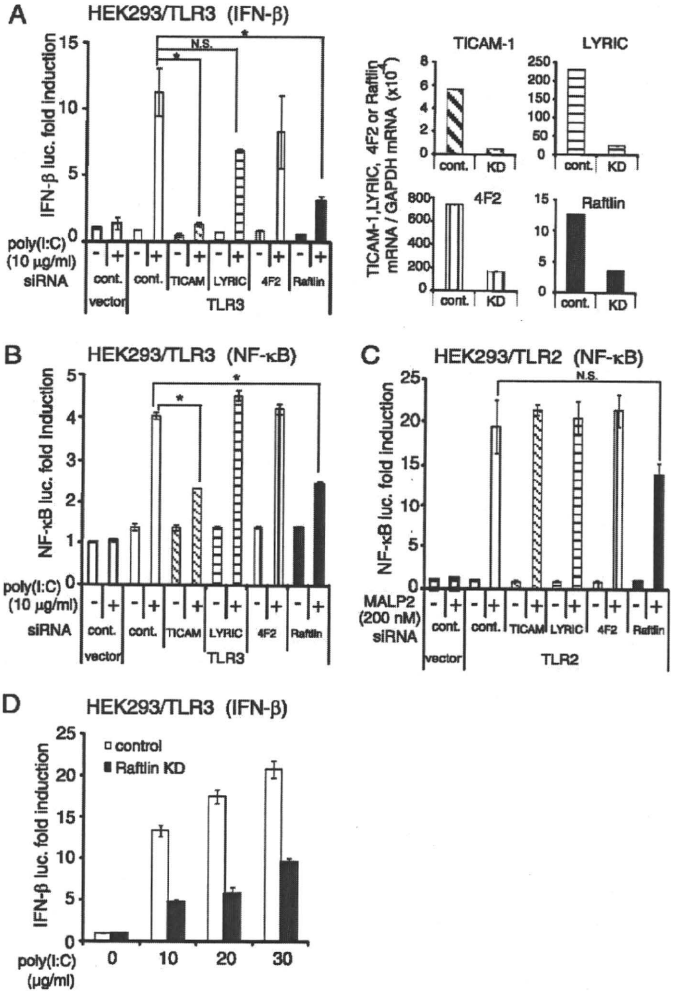


FIGURE 1. **Raftlin participates in poly(I:C)-induced TLR3-mediated signaling.** HEK293 cells were transfected with the indicated siRNAs (20 pmol) together with the expression vector for human TLR3 (*A, B,* and *D*), human TLR2 (*C*), or empty vector and reporter plasmid. Forty-eight hours after transfection, cells were washed and stimulated with 10–30 μg/ml of poly(I:C) or 200 nM MALP-2. After 6 h, the luciferase reporter activities were measured and expressed as fold-induction relative to the activity of unstimulated vector-transfected cells. Representative data from a minimum of three separate experiments are shown (mean ± S.D.). In each experiment, knockdown (*KD*) efficiency was assessed 48 h after transfection by qPCR. Expression of each gene was normalized to GAPDH mRNA expression. As shown in the *right-hand panels* of *A*, expression of the indicated genes is efficiently silenced (knockdown efficiency: TICAM-1, 91.4%; LYRIC, 89.5%; 4F2, 77.4%; Raftlin, 71.8%). *, $p < 0.05$ (*t* test).

*Isolation of Poly(I:C)-binding Proteins*—Raji cells ($1 \times 10^{10}$) were washed twice with Dulbecco's phosphate-buffered saline, frozen and thawed three times in Dulbecco's phosphate-buffered saline ($5 \times 10^7$/ml), and centrifuged at 20,000 × *g* for 10 min. Cell pellets were lysed in lysis buffer (1% Nonidet P-40 in buffer A (20 mM Tris-HCl, pH 7.4, 140 mM NaCl, 25 mM iodoacetamide, 10 mM EDTA, 2 mM PMSF and protease inhibitor mixture)) for 20 min at room temperature. After centrifugation at 10,000 × *g* for 10 min, supernatants were filtrated with Minisalt GF (Zartorius stedim, Japan) and sequentially applied to Sepharose, poly(U)-Sepharose, and poly(I:C)-Sepharose equilibrated with binding buffer (0.2% Nonidet P-40 in buffer A). The poly(I:C)-binding molecules were eluted from poly(I:C)-Sepharose with elution buffer (1.4 M NaCl in washing buffer) after being washed with washing buffer (10 mM CHAPS in buffer A). The eluates were