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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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Running title: **MV V affects MDA5-induced IFN- β induction**

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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 infected 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.

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, A., G., and Fitzgerald, K., A., 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 κ B kinase ϵ (IKK ϵ) and NAK-associated protein 1 (NAP-1) (Sasai et al., 2006a). These complexes then phosphorylate IFN regulatory factor 3 (IRF-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; Platanius, 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 (Naiche 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.

Materials and Methods

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-conjugated secondary antibodies were from Invitrogen Life Technologies. Polyribinosinic/polyribocytidylic acid (polyI:C) was from Amersham Biosciences.

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.

Virus preparation and titration

Nagataha (NV) and Edmonston (ED) starins 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).

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'-GTGATCAATGGCCCAATCC-3'; and for q-PCR human β -actin: 5' -CCTGGCACCCAGCACAAAT-3' and 5'-GCCGATCCACACGGAGTACT-3'; and for q-PCR human IFN- β : 5'-CAATTGCTTGGATTCTTACAAAG-3' and 5'-TATTCAAGCCTCCCATTCATTG-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.

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'-TATAAGCTTACCAGACAAAGCTGGGAATAGAACTTCG-3'/JM403; 5'-CGAAGATATTCTGGGTGTAAGTCTAGTA-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.

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 μ l lysis buffer. The luciferase activity was measured using Dual-Luciferase Reporter assay systems (Promega) and was shown as the means \pm S.D. of three experiments.

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 MgCl₂, 1% NP-40, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na₃VO₄) on ice for 30 min and then centrifuged at 12000 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% polyacrylamid gel Native (Dai-ichi Pure Chemicals). For immunoprecipitation, cells were lysed in the TritonX-100 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1.5 mM MgCl₂, 1% TritonX-100, 10% glycerol, protease inhibitor cocktail, 0.1 mM PMSF, 50 mM NaF, and 1 mM Na₃VO₄) and then centrifuged at 12000 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.

Confocal microscopy

HeLa cells (2.5×10^4 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-conjugated 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 X 63 magnification under an LSM510 META microscope (Zeiss).

Statistical analysis

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

Results

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 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.

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 protein 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 IKK ϵ 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/IKK ϵ 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-mediated 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.

272C is responsible for suppression of MDA5-induced IFN- β promoter activity

To reveal the molecular mechanism that determines whether ED V protein inhibits MDA5-induced IFN- β promoter activity, we compared the amino acid sequence of the ED V protein with that of wild-type V proteins. As shown 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, 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 (17, 18), 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 suppress poly I:C-induced IRF-3 nuclear translocation and interact with MDA5 (Fig. 6B, 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.

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.

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 suppressed 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 (Plaumet 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; Takeushi 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 declared conflict of interest in this study.

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Figure legends

Figure 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.

Figure 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 μ g/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 μ g/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.**

Figure 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, 100ng). After 24 h, cells were lysed and subjected to Western blotting with anti-FLAG antibody and anti- β -actin antibody (internal control).

Figure 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.

Figure 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 $\mu\text{g/ml}$ 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.

Figure 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 $\mu\text{g/ml}$ 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.

Figure 1
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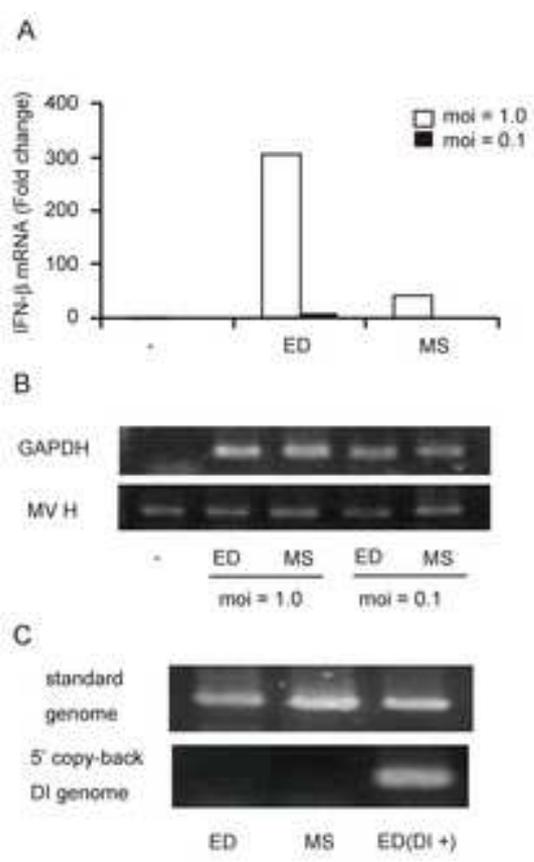


Figure 1

Figure 2
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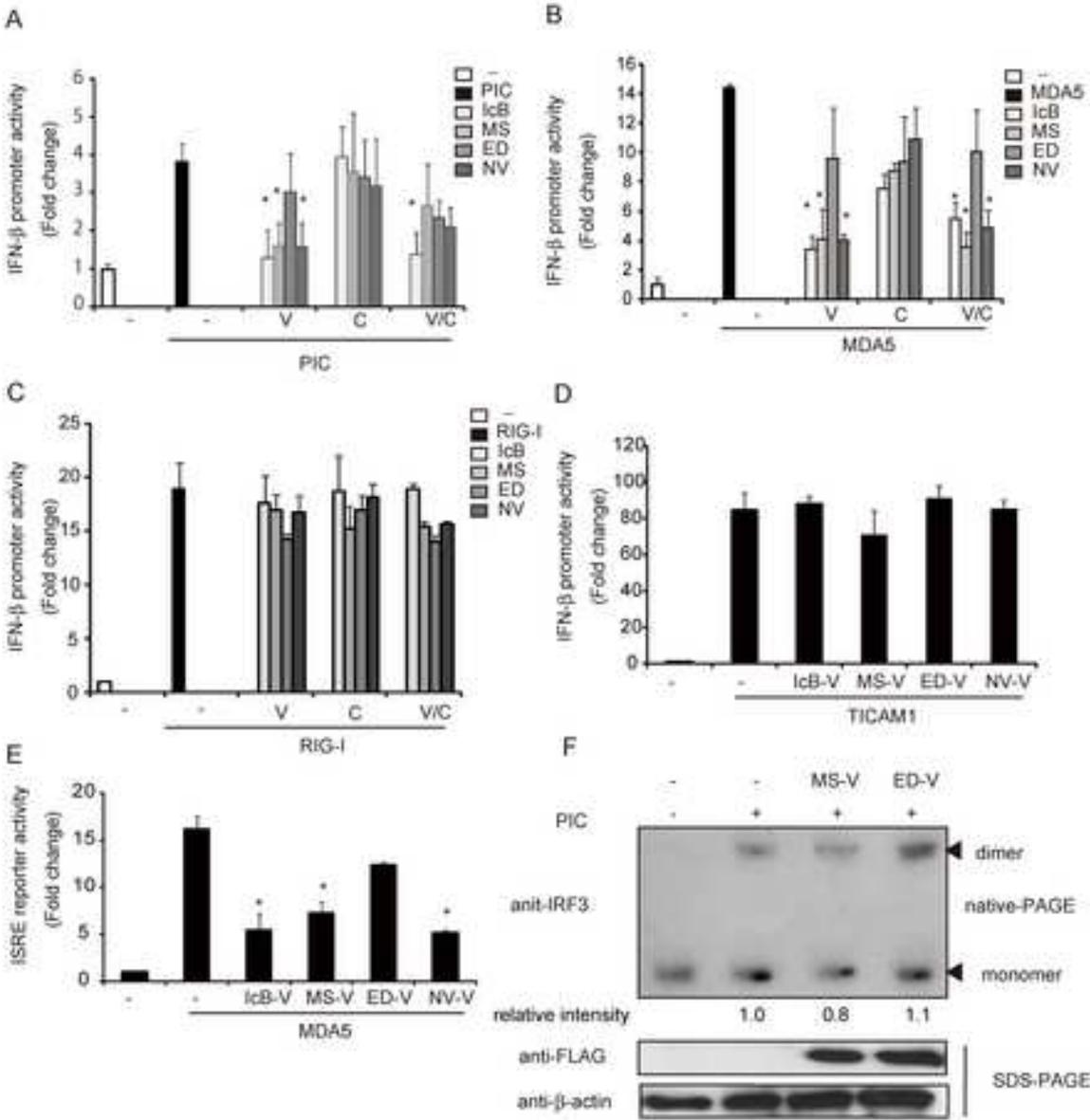


Figure 2

Figure 3
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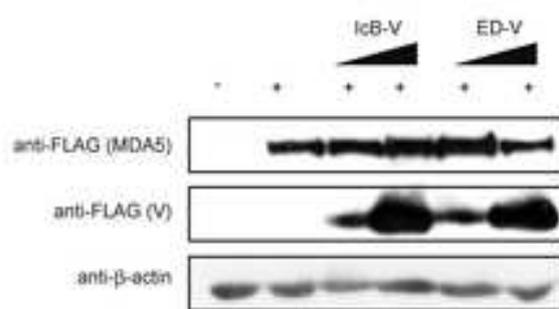


Figure 3

Figure 4
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MD-V 1  MARRQARHYVYKGLRCLTRALKARFIOGLAIUENANAAMEIIEHWPOQRATCKEERAGGSGL 60
IC-B-V 1  MARRQARHYVYKGLRCLTRALKARFIOGLAVSRANAAMEIIEHWPOQRATCKEERAGGSGL 60
HV-V 1  MARRQARHYVYKGLRCLTRALKARFIOGLAIUENANAAMEIIEHWPOQRATCKEERAGGSGL 60
ED-V 1  MARRQARHYVYKGLRCLTRALKARFIOGLAIUENANAAMEIIEHWPOQRATCKEERAGGSGL 60
*****
MS-V 61  SKPCLEAIGSTRGGAPRIHQQSGRSDDDDTETLGIENRNLQASSTGLQCYVYVCHSDEAV 120
IC-B-V 61  SKPCLEAIGSTRGGAPRIHQQSGRSDDDDTETLGIENRNLQASSTGLQCYVYVCHSDEAV 120
HV-V 61  SKPCLEAVGSTRGGAPRIHQQSGRSDDDDTETLGIENRNLQASSTGLQCYVYVCHSDEAV 120
ED-V 61  SKPCLEAIGSTRGGAPRIHQQSGRSDDDDTETLGIENRNLQASSTGLQCYVYVCHSDEAV 120
*****
MD-V 121  KFIQQADSIMVQSGLDGDSSTLQGGERSRSDVDVIGDFOTRQYAITDRGSAPIEMGFRAE 180
IC-B-V 121  KFIQQADSIMVQSGLDGDSSTLQGGERSRSDVDVIGDFOTRQYAITDRGSAPIEMGFRAE 180
HV-V 121  KFIQQADSIMVQSGLDGDSSTLQGGERSRSDVDVIGDFOTRQYAITDRGSAPIEMGFRAE 180
ED-V 121  KFIQQADSIMVQSGLDGDSSTLQGGERSRSDVDVIGDFOTRQYAITDRGSAPIEMGFRAE 180
*****
MS-V 191  DVVTARQGEIHLLRLQSRGNTPELIGKTLNVFPPFQGRAGASTPTIEKIDHREISLIM 240
IC-B-V 191  DVVTARQGEIHLLRLQSRGNTPELIGKTLNVFPPFQGRAGASTPTIEKIDHREISLIM 240
HV-V 191  DVVTARQGEIHLLRLQSRGNTPELIGKTLNVFPPFQGRAGASTPTIEKIDHREISLIM 240
ED-V 191  DVVTARQGEIHLLRLQSRGNTPELIGKTLNVFPPFQGRAGASTPTIEKIDHREISLIM 240
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MD-V 241  NGDEVFIDHWCHMCEVTLQTIKASCTGSRFVCEQCRDTOTGVOTRIWYHLESLIPE 299
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HV-V 241  NGDEVFIDHWCHMCEVTLQTIKASCTGSRFVCEQCRDTOTGVOTRIWYHLESLIPE 299
ED-V 241  NGDEVFIDHWCHMCEVTLQTIKASCTGSRFVCEQCRDTOTGVOTRIWYHLESLIPE 299
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Figure 4

Figure 5
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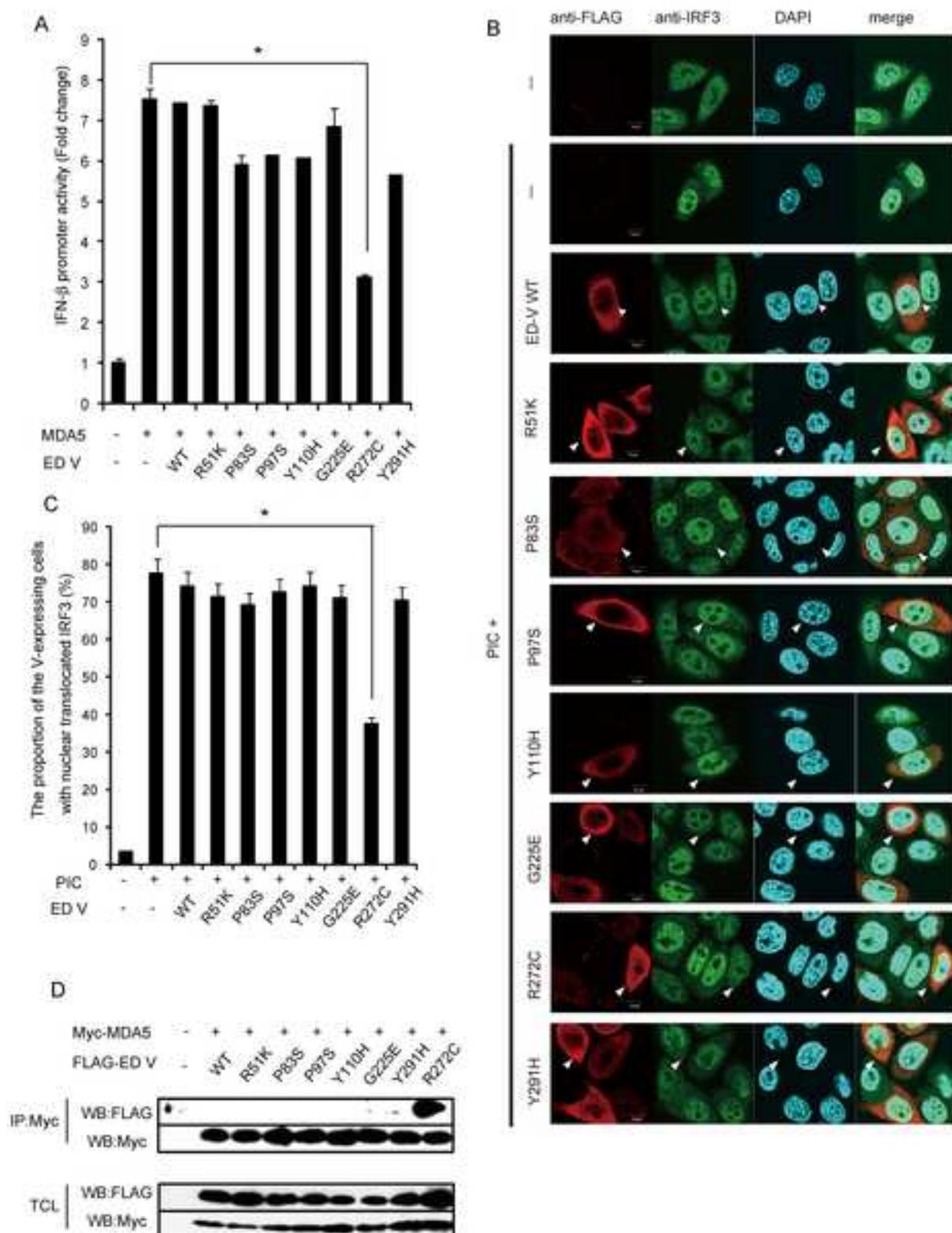


Figure 5

Figure 6
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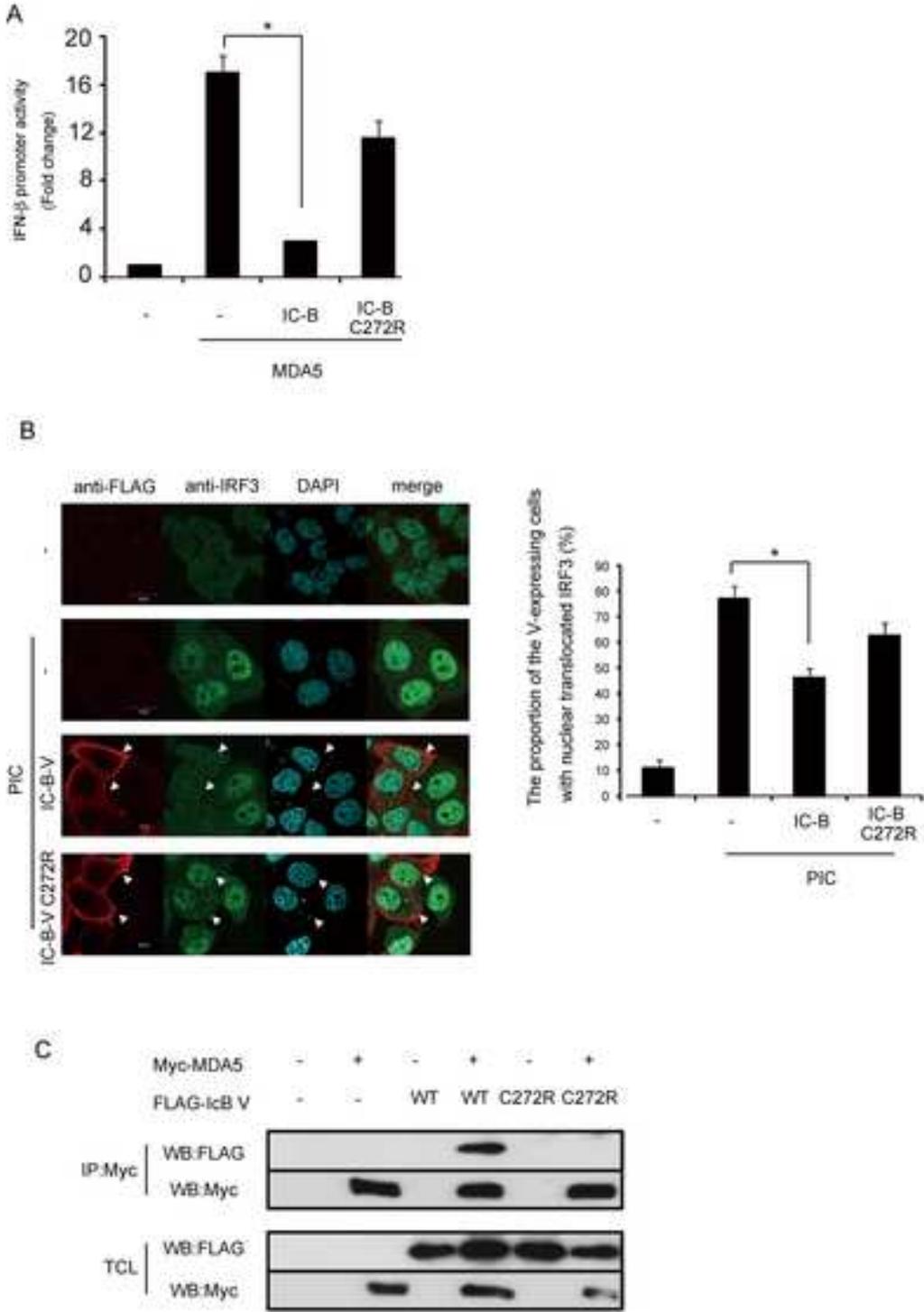


Figure 6