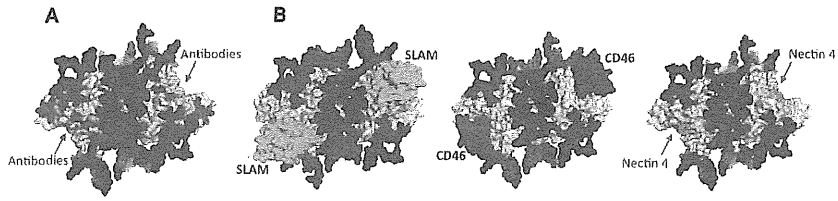

FIGURE 2 | Structural basis of the effectiveness of measles vaccine. **(A)** Epitopes of anti-MV-H monoclonal antibodies (red) on the MV-H homodimer (blue white) with potential N-linked sugars (mesh, black) as viewed downwards from the top. **(B)** Receptor-binding sites on MV-H. Left, crystal structure of MV-H–SLAM (SLAM shown in cyan). Middle, crystal structure of MV-H–CD46 (CD46 in purple). Right, putative nectin-4 binding sites (green), based on site-directed mutagenesis of MV-H.

only a single serotype (Hashiguchi et al., 2011). Interestingly, this receptor-binding area is highly conserved not only among MV strains but also among morbilliviruses, suggesting that this region could serve as a template for a universal vaccine targeting all morbilliviruses (Hashiguchi et al., 2007). The human immunodeficiency virus glycoprotein lacks the similarly conserved region to be targeted by antibodies, which has hampered the vaccine development.

PERSPECTIVE

Recent advances in our understanding of MV-H structures, combined with functional studies, have provided new insights into the mechanism of MV entry. Although the receptor-binding mode of MV-H is different from that of other paramyxovirus HN and G proteins, the overall entry process may be common among paramyxoviruses. MV-H forms tetramer (dimer of dimers), like other HN and G proteins, and we propose a model for fusion triggering, based on the presence of two forms of MV-H tetramer. However, further structural studies of MV-H and MV-F are required to completely understand the whole process of MV entry.

Crystal structures have also illuminated how a sugar-uncovered, exposed area of MV-H is targeted by receptors as well as by neutralizing antibodies. This nicely explains why only a single serotype occurs for MV and why measles vaccine, derived from one strain, has been effective for a long time. It is hoped that better understanding of the MV entry process will not only contribute to our measles eradication effort worldwide, but also allow us to develop effective vaccines for other challenging pathogens.

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Laboratory and Epidemiology Communications

Progress toward Measles Elimination between 2008 and 2010 in the Hokkaido District, Japan

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Measles is an acute and highly contagious disease mainly characterized by high fever, cough, and a maculopapular rash (1). Since the occurrence in Japan of a nation-wide measles epidemic in 2001 (2), measles control efforts have intensified across the county, resulting in a dramatic decrease in total cases. However, a small and local epidemic of measles did occur in 2006 in the Kanto area and expanded to impact the entire country. In the Hokkaido district, the northern islands of Japan, we experienced several local epidemics of measles between 2007 and 2008 (3). A feature of these local epidemics was that the infection mainly targeted both vaccinated and unvaccinated teenagers. This measles epidemic led to a change in the surveillance system used in Japan, requiring mandatory reporting instead of sentinel reporting as of January 1, 2008 (2). Herein, we report the status of measles infections and surveillance systems between 2008 and 2010 in the Hokkaido district.

In 2008, 1,462 measles cases were reported in the Hokkaido district, of which 951 (65%) were diagnosed clinically, 408 (28%) were confirmed by laboratory diagnoses, and 103 (7%) were deemed as "modified" measles (Table 1). Regarding age distribution, the highest peak occurred in patients aged 16 years, and

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patients aged less than 20 years accounted for approximately 70% of all cases. Vaccinated patients numbered 419 (29%), 664 (45%) were unvaccinated, and the vaccination status was unknown for 379 (26%) patients. Fever, rash, and cough were observed in greater than 80% of the patients. Throat swab specimens were obtained from 28 suspected cases, and viral RNA was extracted using a QIAamp Viral RNA Mini Kit (Qiagen, Germantown, Md., USA). A one-step RT-PCR Kit (Qiagen) was used to amplify the measles virus nucleoprotein (N) gene as previously described (3). Measles virus RNA was detected in 20 specimens, and further isolation was conducted on 5 specimens using Vero/hSLAM cells (4). These 20 specimens containing measles virus RNA all belonged to the genotype D5, identical to the virus isolated in other areas of Japan.

A drastic decline in reported cases (17 total) was observed in 2009, and the portion of clinical diagnoses also decreased to 6 cases (35%; Table 1). The measles IgM enzyme immunoassay (EIA) was performed at commercial laboratories on specimens from the remaining 11 cases. However, the EIA values were not reported to the regional Public Health Centers. We did not have the opportunity to perform RT-PCR on any clinical specimens from these reported cases. Only 2 throat swabs were obtained from the other suspected measles cases, and both were negative by RT-PCR.

Table 1. Measles cases by the surveillance system between 2008 and 2010 in the Hokkaido district, Japan

Year	Reported measles cases	% only by clinical diagnosis	Positive no. of RT-PCR/ cases tested[1)]
2008	1,462	65	20/28
2009	17	35	0/2
2010	5	20	2/15

1): "Cases tested" indicates number of clinical specimens obtained as a suspected case at Prefectural and Municipal Public Health Institutes in the Hokkaido district.

The number of reported measles cases further decreased to 5 in 2010 (Table 1). One case was a clinically diagnosed 2-year-old boy, who presented with fever, cough, runny nose, and rash, did not have laboratory confirmation, and had not been vaccinated. Another case was "modified" measles with clinical symptoms that included fever, rash, and positive IgM for measles by EIA performed in a commercial laboratory; however, the IgM EIA results were not reported to the regional Public Health Center. The clinical specimens for these 2 cases were not submitted to Prefectural and Municipal Public Health Institutes for the confirmation of infection. The other 2 were imported measles cases: one (Case no. 2 shown in Table 2) was a Chinese woman aged 28 years with unknown vaccination status visiting Sapporo on May 1, and the other (Case no. 13) was a Japanese woman aged 35 years with a history of no measles vaccination who had traveled to India from November 2 to 10. Clinical specimens were obtained 7 and 11 days after fever onset from the Chinese (throat swab, urine, and whole blood) and Japanese (whole blood and urine) patients, respectively. Measles IgM EIA (Denka Seiken Co., Tokyo, Japan) was performed according to the manufacturer's instructions and EIA index values were highly positive (14.03 and 25.87, respectively). The RT-PCR results for specimens from both patients were all positive. Phylogenetic analyses were performed by direct sequencing of the amplified products of the N gene (450 nucleotides [nt]) as previously described (3). The 2 strains detected (Mvi/Sapporo. JPN/19.10/1 and Mvi/Sapporo. JPN/48.10/1) were identified as H1 (the Chinese patient, GenBank accession no. AB569977) (the Infectious Agents Surveillance Report [IASR] online at http://idsc.nih.go.jp/iasr/rapid/pr3652.html in Japanese) and D4 (the Japanese patient, GenBank accession no. AB605257) (IASR online at http://idsc.nih.go.jp/iasr/rapid/pr3722.html in Japanese), respectively (Fig. 1). Measles virus was successfully isolated from the specimens of the Chinese patient alone using Vero/hSLAM cells. Case

Table 2. Laboratory test results of suspected measles cases performed at Prefectural and Municipal Public Health Institutes in the Hokkaido district, 2010

Case no.	Day after the onset of fever	Age (y)	Sex	Specimen	IgM index value	RT-PCR	Vaccination status
1	1	1	Male	Ts	ND	—	Vaccinated
2	7	28	Female	Ts, U, WB	14.03	H1	Unknown
3	4	1	Male	Ts, U, WB	1.14	—	Vaccinated
4	6	1	Female	Ts, WB	0.8	—	Vaccinated
5	2	32	Female	Ts, WB	0.02	—	Unknown
6	15	51	Male	Ts, WB	4.73	—	Unknown
7	5	4	Male	Ts, WB	0.86	—	Vaccinated
8	4	1	Male	Ts, U, WB	0.05	—	Vaccinated
9	14	0	Male	Ts, WB	0.46	—	Unvaccinated
10	6	28	Female	Ts, U, WB	2.32	—	Vaccinated
11	12	2	Male	S	1.66	—	Vaccinated
12	11	1	Male	Ts, WB	0.06	—	Unvaccinated
13	11	35	Female	U, WB	25.87	D4	Unvaccinated
14	?	?	Female	U	ND	—	Unknown
15	7	30	Female	U	ND	—	Vaccinated

?, unspecified; Ts, throat swab; U, urine; WB, whole blood; S, serum; ND, not done; —, negative; H1, measles virus genotype H1; D4, measles virus genotype D4.

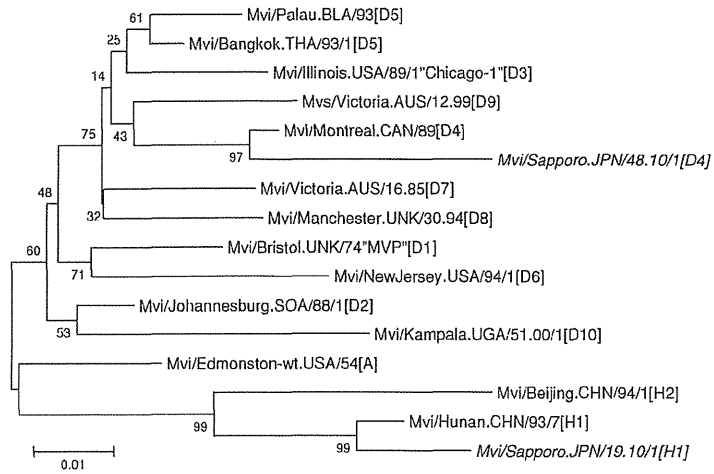

Fig. 1. Phylogenetic tree of 450 nucleotides of N gene of measles virus. Numerals at each branch indicate the bootstrap values of the clusters in percent. The present strains are represented in italics.

no. 10 was a female aged 28 years with "modified" measles and a history of one measles vaccination, who presented with fever and rash and whose blood specimen indicated a measles IgM index value of 2.32 (weakly positive) but was negative for RT-PCR test on measles RNA and negative by IgM and PCR for parvovirus B19, which commonly causes an illness with rash (5). This case was not able to be confirmed by intensive laboratory tests; however, the possibility of true measles could not be ruled out. Furthermore, all other suspected cases were not confirmed by the RT-PCR test. Thus, as described above, only 2 cases could have been classified as laboratory-confirmed ones.

Since the measles epidemic in 2008, the number of reported cases has dramatically declined each year. We had learned of only 5 reported measles cases in the Hokkaido district in 2010, 2 of which were confirmed by laboratory tests and were imported. Therefore, judging from the criterion of measles elimination that indicates an incidence of <1 case of measles confirmed by laboratory or epidemiologic linkage per 1,000,000 population is necessary, while excluding imported cases (6), endemic measles transmission in the Hokkaido district (approximately 5,500,000 population) may be considered as eliminated. With the low number of measles cases occurring due to high vaccination coverage, some laboratory tests, including molecular tests and those that approach virus isolation in ways other than serologic assays, e.g., IgM EIA, may be useful for final diagnosis in suspected measles cases that have low values for IgM EIA, possibly due to cross-reactivity to other viral infections such as parvovirus B19 and human herpes virus type 6 (5). Thus, the use of molecular-based tests, in addition to serologic assays, to confirm measles infections is required. Maintaining high vaccination coverage and using an improved surveillance system that includes viral detection are important for the elimination of measles.

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Laboratory and Epidemiology Communications

First Detection of Measles Virus Genotype G3 in a Japanese Woman: an Imported Case

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Since 2007, the number of measles patients in Japan had continued to decrease because of regular and widespread measles immunization program (1). However, 450 cases of measles including the suspected cases were reported in 2010 (1). Epidemiological data suggests that most of these cases were imported into Japan, but domestic cases have also been reported (1). Recent molecular epidemiological studies reported the detection of measles virus (MV) genotypes D3, D4, D5, D9, and H1 in Japan (2–4). The D4 and D9 genotypes have usually been detected in imported cases, while the D3, D5, and H1 genotypes have been detected in domestic cases (2–4). Here, we describe the detection of another genotype, G3, in an imported case of measles in a Japanese woman. To the best of our knowledge, this is the first report on the detection of MV genotype G3 in Japan.

The patient was a 28-year-old Japanese woman who resided in Chiba Prefecture, Japan. She did not have a history of measles and had not been immunized against measles. She had visited Indonesia for 10 days (from January 31 to February 9, 2011) with six colleagues. On February 14, she developed common cold-like symptoms such as cough and shivering, and consulted a local physician, who made a diagnosis of common cold. On February 22, she developed clinical symptoms including high fever (39°C), cough, conjunctivitis, Koplik's spots, and rashes on the face and neck. She then consulted another physician at a general hospital. The physician suspected her to have contracted measles, and suggested that she got admitted to the hospital. Informed consent was obtained, and her whole blood sample was collected on the next day. Viral RNA was extracted from the blood sample using the High Pure Viral RNA Kit (Roche, Indianapolis, Ind., USA), and was suspended in DNase/RNase-free water. Thereafter, reverse transcriptase-polymerase chain reaction (RT-PCR) and nested PCR were performed as previously described (2–4). Amplicons were purified using the High Pure PCR Product Purification Kit (Roche), and the nucleotide sequence was determined using direct se-

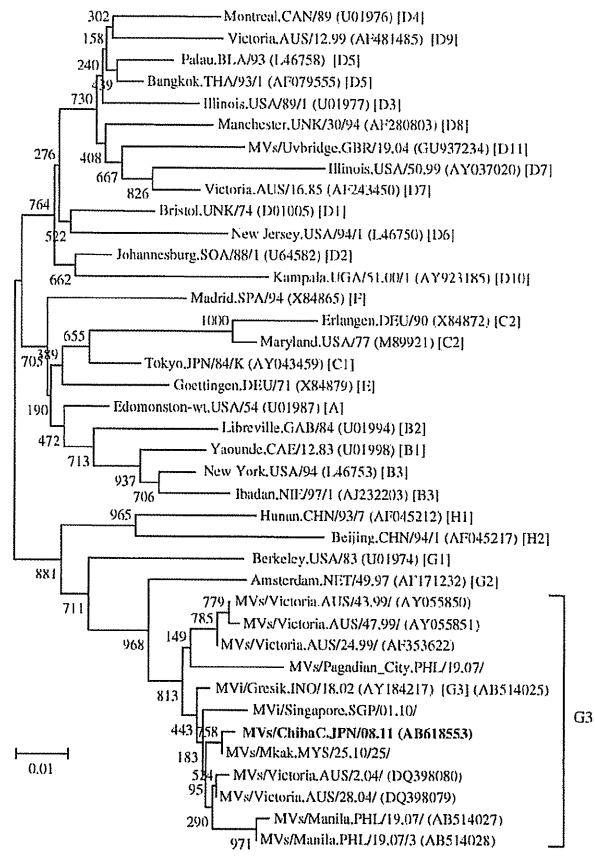

Fig. 1. Phylogenetic tree based on the nucleotide protein (*N*) gene sequences of various strains of the measles virus. The evolutionary distance was calculated using Kimura's two-parameter method, and the tree was plotted using the neighbor-joining method. Numbers at each branch indicate the bootstrap values of the clusters supported by that branch. Genbank accession numbers are given in parentheses. The present strain is represented in bold type.

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quencing method (4). The nucleotide sequence of the partial *N* gene (450 bp) of MVs was analyzed phylogenetically using Molecular Evolutionary Genetics Analysis (MEGA) software (version 4) (2–4). Evolutionary distances were estimated using Kimura's two-parameter method and the phylogenetic tree was constructed using the neighbor-joining (NJ) method (2–4). Reliability of the phylogenetic tree was estimated by 1,000 bootstrap replications.

We constructed a phylogenetic tree based on the *N* gene of the detected MV strain and the reference strains (Fig. 1). The strain was genotyped as MV G3 in the phylogenetic tree. The homology between the reference strain (MVi/Gresik.INO/18.02 [G3], GenBank accession no. AY184217) and the present strain was 99.1% at the nucleotide level and 98.7% at the amino acid level. Epidemiological investigations have not reported occurrence of measles among the patient's family and colleagues.

To the best of our knowledge, this is the first report on MV G3 detection in Japan. The genotype G3 was first detected in Australia and East Timor in 1999 (5). Infection with G3 has not been frequently reported in these countries after 1999. However, this may be attributed to the lack of aggressive MV surveillance in these countries. At present, a small number of the population in Chiba Prefecture may be susceptible to measles because of lack of immunization against the disease. However, as measles is highly contagious in humans (6), and spreads rapidly from one area to another, up-to-date information on the epidemiological status of this disease in our country is needed.

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Conflict of interest None to declare.

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Expression of the Sendai (murine parainfluenza) virus C protein alleviates restriction of measles virus growth in mouse cells

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Measles virus (MV), a human pathogen, uses the signaling lymphocyte activation molecule (SLAM) or CD46 as an entry receptor. Although several transgenic mice expressing these receptors have been generated as small animal models for measles, these mice usually have to be made defective in IFN-α/β signaling to facilitate MV replication. Similarly, when functional receptors are expressed by transfection, mouse cells do not allow MV growth as efficiently as primate cells. In this study, we demonstrate that MV efficiently grows in SLAM-expressing mouse cells in which the Sendai virus (SeV) C protein is transiently expressed. We developed a SLAM-expressing mouse cell line whose genome also encodes the SeV C protein downstream of the sequence flanked with loxP sequences. When this cell line was infected with the recombinant MV expressing the Cre recombinase, the SeV C protein was readily expressed. Importantly, the Cre recombinase-encoding MV grew in this cell line much more efficiently than it did in the parental cell. The minigenome assay demonstrated that the SeV C protein does not modulate MV RNA synthesis. Analyses using the mutant proteins with the defined functional defects revealed that the IFN-antagonist function, but not the budding-accelerating function, of the SeV C protein was critical for supporting efficient MV growth in mouse cells. Our results indicate that insufficient IFN antagonism can be an important determinant of the host range of viruses, and the system described here may be useful to overcome the species barrier of other human viruses.

interferon | innate immunity | paramyxovirus

Measles is an acute contagious human disease characterized by high fever and a maculopapular rash (1). Measles virus (MV), its causative agent, is an enveloped virus classified as a member of the genus *Morbillivirus* in the family *Paramyxoviridae*, the order *Mononegavirales*. The virus has a nonsegmented negative-sense RNA genome that contains six genes encoding the nucleocapsid (N), phospho- (P), matrix, fusion, hemagglutinin (H), and large (L) proteins, respectively. The P gene encodes additional gene products, termed the V and C proteins, via an RNA editing and alternative translational initiation in a different reading frame, respectively (2, 3). The V and C proteins have been shown to counteract host innate immune responses and play important roles in MV virulence in vivo (1, 4). The V protein directly inhibits induction (5) and signaling (6–8) of IFN-α/β, and the C protein suppresses the host IFN response by modulating viral RNA synthesis (9–11).

The human signaling lymphocyte activation molecule (SLAM, also called CD150) acts as a cellular receptor for MV (12), and vaccine strains can use CD46 as an alternative receptor because of the amino acid (aa) changes in their attachment H protein (13, 14). Several transgenic or knock-in mice expressing human SLAM or CD46 have been generated as small animal models for measles (15–25). To facilitate MV replication, these mice usually have to be made defective in IFN signaling (16, 18–20) or used when they are newly born (15, 17). Similarly, when human SLAM or CD46 is expressed by transfection, rodent cells become susceptible to MV, but do not allow MV growth as efficiently as primate cells (14, 26). The restriction of MV growth in rodents may involve blockades at different steps of the virus replication cycle (26, 27).

Sendai virus (SeV, also known as murine parainfluenza virus 1), a member of the genus *Respirovirus* in the family *Paramyxoviridae*, infects epithelial cells in the respiratory tract and causes bronchopneumonia in rodents (28). Like that of MV, its P gene encodes virulence factor proteins in addition to the P protein. These proteins are C′, C, Y1, Y2 (collectively referred to as the C proteins), V, and W proteins. The four C proteins are translated from a +1 reading frame relative to the P reading frame using alternative start codons. V and W mRNAs are produced by insertion of one and two guanine residues at a specific position during transcription, respectively. The SeV C proteins are nonessential for virus replication but play important roles in viral propagation in vivo (29). Several functions of the SeV C protein have been documented. First, it physically associates with signal transducers and activator of transcription (STAT) 1, and inhibits tyrosine phosphorylation of STAT1 and STAT2 and dephosphorylation of STAT1, thereby blocking the IFN signaling pathway (30). Second, it inhibits virus-induced apoptosis, which is probably mediated by the IFN signaling pathway (31). Third, it modulates viral RNA synthesis through its interaction with the viral polymerase L protein (32–34). Fourth, it enhances the SeV budding by recruiting Alix/AIP1, a cellular protein involved in apoptosis and endosomal trafficking, to the plasma membrane (35, 36). In the absence of the C protein, there is a significant delay in the incorporation of SeV structural proteins into progeny virions (37). The C′, Y1, and Y2 proteins share all or some of these functions with the C protein (38, 39). For example, the Y1 protein, which has the common carboxyl terminus with the C protein but is 23 aa shorter in length, does not have the ability to enhance the viral budding (36) but is fully functional in antagonizing the host IFN response and regulating viral RNA synthesis (30, 40).

Mutants of the SeV C protein lacking its different functions have been described. The mutant Cm5 has aa substitutions K151A, E153A, and R154A, whereas Cm* has substitutions K151A, E153K, and R157L (41, 42). These C-protein mutants no longer possess the ability to bind STAT1 and IFN-antagonist activity. The mutant d194 has 10-aa truncation at the carboxyl terminus, and therefore cannot interact with AIP1/Alix (35); this eliminates the budding-accelerating function.

In this study, we addressed why MV cannot grow efficiently in mice possessing functional receptors. To test the hypothesis that

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MV lacks some factors allowing its efficient growth in mouse cells, we examined the effects of the SeV C protein and its mutants on MV growth in mouse cells. We also developed the Cre-loxP expression system for the SeV C protein in which the Cre recombinase-expressing recombinant MV induces the expression of the SeV C protein in infected mouse cells. Our results indicate that insufficient IFN antagonism is responsible for restricted MV growth in mouse cells.

Results

Effect of the SeV C Protein on MV Growth in Mouse Cell Lines. We reasoned that MV, a human virus, lacks some factors allowing its efficient growth in mouse cells, which mouse viruses may naturally possess. To test this contention, we examined whether the expression of the SeV C protein, a pleiotropic protein, can alleviate restriction of MV growth in mouse cells. Four cell lines expressing human SLAM were used for this experiment: mouse cell lines NIH 3T3/hSLAM and L/hSLAM and human cell lines HeLa/hSLAM and HEK293/hSLAM. These cell lines were transiently transfected with the expression plasmid encoding the SeV C protein or the empty vector, and then infected with the recombinant MV encoding the reporter *Renilla* luciferase (MV-Luci) (43). Luciferase activities obtained with MV-Luci infection correlate well with levels of MV multiplication (Fig. S1). Transient expression of the SeV C protein resulted in 20- and fivefold increased luciferase activities in NIH 3T3/hSLAM and L/hSLAM cells, respectively, compared with those in cells transfected with the empty vector (Fig. 1*A*). In contrast, luciferase activities were increased only marginally in human cell lines transfected with the SeV C protein (Fig. 1*A*), even though human cell lines produced higher levels of the SeV C protein after transfection than mouse cell lines (Fig. 1*B*).

MV produces C and V proteins analogous to the SeV C protein. To address the possibility that the MV C and V proteins are not sufficiently expressed in MV-infected mouse cells, we examined whether these proteins expressed through transfection can promote MV growth in mouse NIH 3T3/hSLAM cells. Unlike that of the SeV C protein, the plasmid-mediated expression of the MV C or V protein did not increase luciferase activity at all (Fig. 1*C*). These results indicate that the SeV C protein has an ability to support efficient MV growth in mouse cells, whereas the MV C and V proteins do not.

Development of a Cre-loxP–Mediated Expression System for the SeV C Protein. To examine the growth kinetics of MV in the presence of the SeV C protein, we attempted to establish mouse cell lines stably expressing the protein. However, such cell lines were not successfully obtained, presumably because continuous expression of the SeV C protein may be detrimental to cells. Therefore, we decided to develop a Cre-loxP–mediated expression system for the SeV C protein. The DNA fragment encoding the SeV C protein was inserted downstream of the puromycin-resistant gene flanked with loxP sequences in the eukaryotic expression vector pCA7 (44) (Fig. 2*A*). This plasmid, pCA7-loxP-SC, directed the expression of the SeV C protein only when the plasmid encoding the Cre recombinase (pCA7-Cre) was cotransfected (Fig. 2*B*). Although the SeV C gene used in this study can direct the expression of the Y1 and Y2 proteins in addition to the C protein, Western blot analysis revealed that the Y1 and Y2 proteins were expressed in trace amounts, if any. Luciferase activity of MV-Luci–infected NIH 3T3/hSLAM cells was greatly increased when both pCA7-loxP-SC and pCA7-Cre plasmids were cotransfected, whereas that of infected HeLa/hSLAM cells was not (Fig. 2*C*). These results show that the SeV C protein expressed via the Cre-loxP system can enhance MV growth in mouse cells.

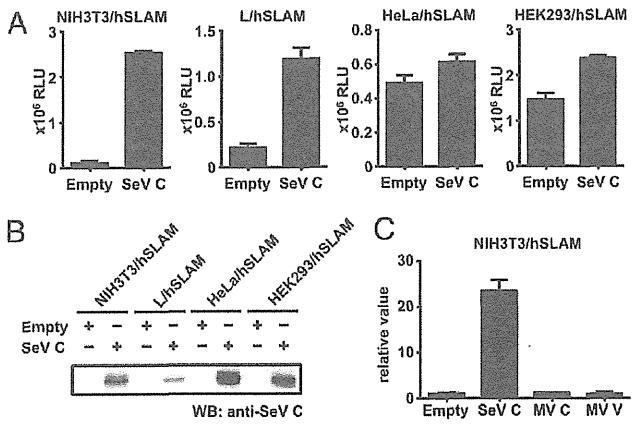

Fig. 1. Effect of the SeV C protein on MV growth in mouse and human cell lines. (*A*) NIH 3T3/hSLAM, L/hSLAM, HeLa/hSLAM, and HEK293/hSLAM cells were transfected with the vector pCA7 (Empty) or the expression plasmid encoding the SeV C protein (SeV C). At 24 h posttransfection, the cells were infected with MV-Luci, and at 48 h postinfection, *Renilla* luciferase activities in the infected cells were measured. RLU, relative light units. (*B*) NIH 3T3/hSLAM, L/hSLAM, HeLa/hSLAM, and HEK293/hSLAM cells were transfected with pCA7 or the SeV C protein expression plasmid. At 24 h posttransfection, the cells were lysed, and lysates were subjected to SDS/PAGE and Western blotting for the detection of the SeV C protein. (*C*) NIH 3T3/hSLAM cells were transfected with pCA7 or expression plasmids encoding the SeV C, MV C, or MV V protein, and were infected with MV-Luci as in *A*. *Renilla* luciferase activities in the infected cells were measured. The luciferase activity in the cells transfected with the empty vector was set to 1.0. Data represent the means ± SD of triplicate samples.

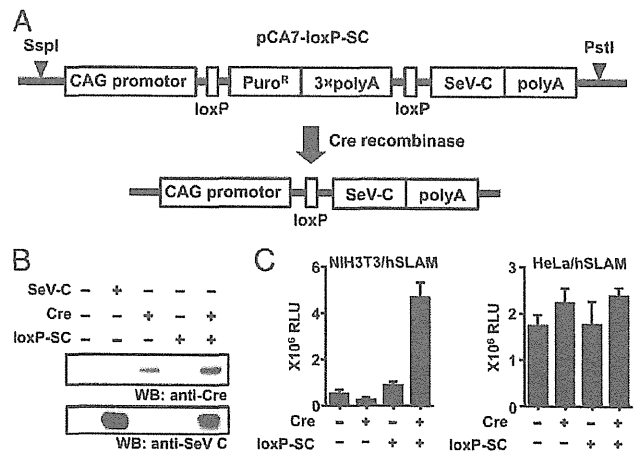

Fig. 2. Development of the Cre-loxP–mediated expression system for the SeV C protein. (*A*) The structure of the expression plasmid pCA7-loxP-SC is shown. CAG promoter, the strong eukaryotic promoter of pCA7. Puro^R, the puromycin resistance gene. polyA, polyA signal sequence. 3× polyA, polyA signal sequences tandem repeated three times. SeV-C, the gene encoding the SeV C protein. In the presence of the Cre recombinase, the DNA fragment between loxP sequences is removed and the SeV C protein is expressed. Filled triangles indicate the restriction enzyme sites used for the generation of 3SC cells (Fig. 4). (*B*) 293T cells were transfected with pCA7-SeV-C, pCA7-Cre, pCA7-loxP-SC, and pCA7 in various combinations. +, the plasmid included for transfection; –, not included. Total amounts of transfected plasmids (500 ng) were adjusted by pCA7. At 48 h posttransfection, the cells were lysed and the lysates were subjected to SDS/PAGE and Western blotting for the detection of the Cre recombinase and SeV C protein. (*C*) NIH 3T3/hSLAM and HeLa/hSLAM cells were transfected with plasmids as in *B*. At 24 h posttransfection, these cells were infected with MV-Luci, and at 48 h postinfection, *Renilla* luciferase activities in the infected cells were measured. Data represent the means ± SD of triplicate samples.

Generation of Recombinant MVs Possessing a Transcriptional Unit of the Cre Recombinase. To make this Cre-loxP expression system for the SeV C protein more usable, we generated recombinant MVs possessing a transcriptional unit of the Cre recombinase by reverse genetics. The Cre recombinase gene was inserted at two different positions of the MV genome (in front of the N gene for the recombinant MV-Cre and between the H and L genes for the recombinant MV-addCre) so that optimal expression levels of the protein could be achieved (Fig. 3*A*). HeLa/hSLAM cells were infected with MV-Cre, MV-addCre, or MV expressing the enhanced GFP (MV-EGFP) (45), and the proteins from infected cells were analyzed by SDS/PAGE and Western blotting (Fig. 3*B*). The MV N protein was expressed comparably in cells infected with the three recombinant MVs, indicating that these viruses grew at similar levels in HeLa/hSLAM cells. EGFP encoded in the first transcriptional unit of MV-EGFP and MV-addCre was also expressed comparably in cells infected with these two viruses. In contrast, the Cre recombinase was expressed in cells infected with MV-Cre ~3.5 times higher than it was in those infected with MV-addCre, consistent with the 3′ to 5′ decreasing gradient of viral transcripts levels common to the order *Mononegavirales* (28, 46). Growth kinetics of these recombinant MVs was analyzed in African green monkey Vero/hSLAM cells (47). All these viruses grew similarly until 48 h postinfection and reached their peak titers at 48 or 72 h postinfection (Fig. 3*C*). At later time points, MV-Cre grew even better than MV-EGFP. The results indicate that the expression of the Cre recombinase does not greatly affect MV replication in permissive primate cells.

Growth of Cre-Recombinase–Expressing MV in Mouse Cells in Which the SeV C Protein Is Expressed via the Cre-loxP System. To generate mouse cells in which the SeV C protein is expressed via the Cre-loxP system, NIH 3T3/hSLAM cells were transfected with the DNA fragment containing the SeV C gene produced by digesting pCA7-loxP-SC with SspI and PstI (Fig. 2*A*), and a stable clone named 3SC was obtained by selecting transfected cells in culture medium containing 2 μg/mL of puromycin. NIH 3T3/hSLAM and 3SC cells were infected with MV-EGFP and MV-Cre, and protein expressions in them were examined at 36 h postinfection. The SeV C protein was detected only in 3SC cells infected with MV-Cre (Fig. 4*A*). Accordingly, the MV N protein was expressed at a higher level in MV-Cre–infected 3SC cells, compared with 3SC cells infected with MV-EGFP or NIH 3T3/hSLAM cells infected with either of these viruses. Importantly, phosphorylation of STAT1 was also suppressed in MV-Cre–infected 3SC cells (Fig. 4*A*), consistent with the IFN-antagonist function of the SeV C protein (48).

Growth kinetics of MV-EGFP was almost identical in NIH 3T3/hSLAM and 3SC cells (Fig. 4*B*), indicating that 3SC cells do not have any adventitious changes affecting MV replication (other than the presence of the SeV C gene). In contrast, MV-Cre and MV-addCre grew much more efficiently in 3SC cells than they did in NIH 3T3/hSLAM cells. The peak titers of MV-Cre and MV-addCre were about 50- and 100-times higher in 3SC cells than those in NIH 3T3/hSLAM cells, respectively. Furthermore, the peak titer of MV-Cre in 3SC cells was 20-times higher than that of MV-EGFP (2×10^5 pfu/mL vs. 10^4 pfu/mL). It was notable that MV-Cre and MV-addCre continued to replicate in 3SC cells until the end of the assay (infectious centers were still present among normal-appearing cells at 120 h postinfection), whereas growth of MV-EGFP declined rapidly after it had reached the peak (at 48 h postinfection) and the virus titer became below the detection level at 96 h postinfection (when there were only normal-appearing cells with no infectious centers).

Role of IFN-Antagonist Function of the SeV C Protein in Supporting Efficient MV Growth in Mouse Cells. The SeV C protein has multiple functions to promote virus replication. To determine which function of the SeV C protein is critical for supporting efficient MV growth in mouse cells, the mutants lacking its different functions were studied.

NIH 3T3/hSLAM cells were transfected with various amounts (25, 100, and 400 ng) of expression plasmids encoding the wild-type C protein, Y1 protein, or mutants described above. At 48 h posttransfection, part of the respective samples was examined by SDS/PAGE and Western blot analysis, and the remainder was infected with MV-Luci. All of the proteins had comparable levels of expression, except d194, which was detected at a lower level than others (Fig. 5*A*). The expression of the C or Y1 protein resulted in increased luciferase activities in a dose-dependent manner, as measured at 48 h postinfection (Fig. 5*B*). In contrast, the expression of the Cm5, Cm*, or d194 protein did not lead to the enhancement of the reporter gene expression. Because the Y1 protein, which does not promote viral budding, effectively enhanced MV growth in mouse cells, we suspected that d194 fails to inhibit the IFN-signaling pathway, which we confirmed in L929 cells (Fig. S2). The minimal amount (25 ng) of the expression plasmid used was sufficient for the SeV C protein to increase the reporter gene expression (Fig. 5*B*). The expression levels of Cm5, Cm*, and d194 proteins as produced with 400 ng each of expression plasmids were higher than that of the SeV C protein as produced with 25 ng of the expression plasmid (Fig. 5*A*). Thus, we concluded that these three mutant proteins cannot enhance MV growth in mouse cells.

The SeV C protein is also known to have the ability to modulate SeV RNA synthesis (32–34). To examine the possible effect of the SeV C protein on MV RNA synthesis, the minigenome assay was performed. Consistent with previous reports (10, 11, 49, 50), the inclusion of the MV C protein in the assay reduced the expression

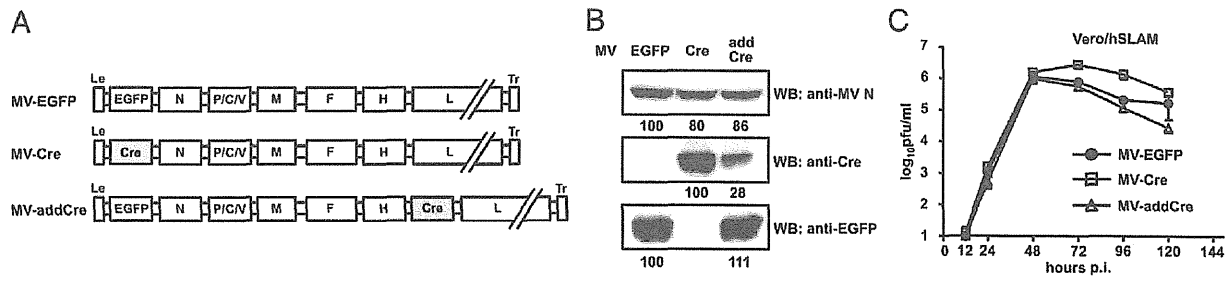

Fig. 3. Generation of recombinant MVs possessing a transcriptional unit of the Cre recombinase. (*A*) Schematic diagram of the recombinant MV genomes. (*B*) HeLa/hSLAM cells were infected with the recombinant MVs at an MOI of 0.1. At 48 h postinfection, the cells were lysed, and the lysates were subjected to SDS/PAGE and Western blotting for the detection of the MV N protein, Cre recombinase, and EGFP. The signal intensities are indicated below the respective bands. Those of Cre recombinase and EGFP were normalized by the intensity of the corresponding N protein. The values in MV-EGFP– or MV-Cre–infected cells were set to 100%. (*C*) Vero/hSLAM cells were infected with the recombinant MVs at an MOI of 0.01. The virus titers were determined at various time points after infection.

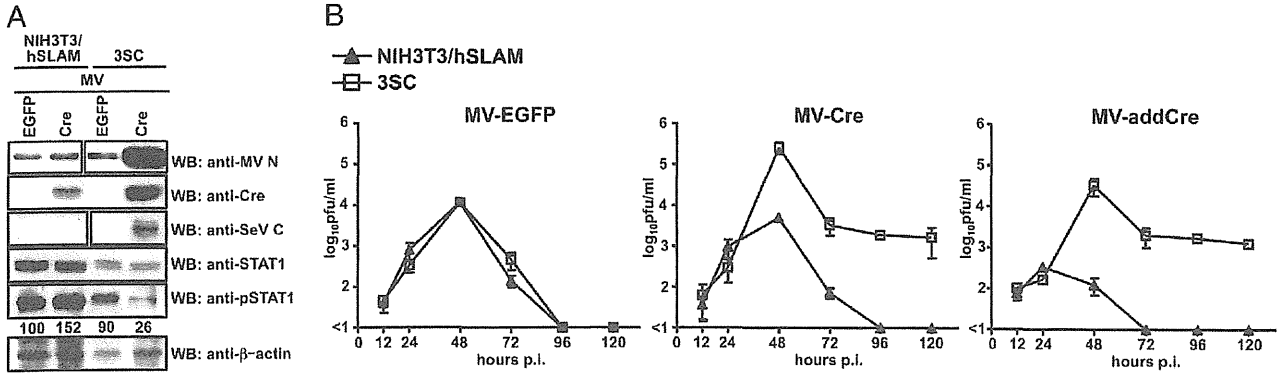

Fig. 4. Growth of Cre-recombinase–expressing MV in mouse cells in which the SeV C protein is expressed by the Cre-loxP system. (*A*) NIH 3T3/hSLAM and 3SC cells were infected with MV-EGFP or MV-Cre at an MOI of 0.5. At 36 h postinfection, the infected cells were lysed, and the lysates were subjected to SDS/PAGE and Western blotting for the detection of the MV N protein, Cre recombinase, SeV C protein, STAT1, phosphorylated STAT1 (pSTAT1), and β-actin. Indicated below pSTAT1 bands are the signal intensities as normalized by that of the corresponding STAT1. The value in NIH 3T3/hSLAM cells infected with MV-EGFP was set to 100%. (*B*) NIH 3T3/hSLAM and 3SC cells were infected with MV-EGFP, MV-Cre, or MV-addCre at an MOI of 0.05. The virus titers were determined at various time points after infection.

level of the reporter gene (Fig. 5*C*). The SeV C protein did not inhibit MV RNA synthesis, but rather slightly enhanced the minigenome expression (Fig. 5*C*).

Taken together, these results indicate that IFN-antagonist activity of the SeV C protein, but not its ability to promote viral budding or modulate viral RNA synthesis, is critical for supporting efficient MV growth in mouse cells. The importance of IFN antagonism in enhancing MV growth in mouse cells was also revealed by using the influenza virus NS1 protein (Fig. S3). The MV V protein is known to block IFN signaling (6–8), like the SeV C protein. Therefore, we examined whether the MV V protein can inhibit IFN signaling in mouse cells. Although the SeV C protein inhibited IFN signaling in both human 293T and mouse L929 cells, the MV V protein was found to do so in human cells, but not in mouse cells (Fig. 5*D*).

Discussion

In the present study, we have shown that the SeV C protein has an ability to enhance MV growth in SLAM-expressing mouse cells, and that its IFN-antagonist function is critical for this ability. We initially directed our attention to the SeV C protein because it plays important roles in SeV propagation in mice (42). Among multiple functions of the SeV C protein, IFN antagonism has been well investigated. The carboxyl terminal half of the SeV C protein is sufficient to inhibit IFN action (40), and only 14-aa truncation from the carboxyl terminus of the protein diminishes this function (51). In this study, we demonstrated that the mutant d194 that cannot interact with Alix/AIP1 and facilitate viral budding (35) also fails to inhibit the IFN-signaling pathway and enhance MV replication in mouse cells. Thus, 10-aa truncation in the carboxyl terminus not only prevents the SeV C protein from promoting viral budding, but also from counteracting the host IFN response. The SeV C protein can reduce viral RNA synthesis through its binding with the viral RNA polymerase L protein (32, 33) and regulate the balance between sense and antisense viral genome RNAs (34). However, our results indicated that the SeV C protein could not reduce the reporter gene expression in the MV minigenome assay. Therefore, enhancement of MV growth in mouse cells is unlikely to be caused by the ability of the SeV C protein to modulate viral RNA synthesis.

We showed that transient expression of the SeV C protein did not result in increased reporter gene expression in MV-Luci–infected human cells. Does this mean that the SeV C protein cannot antagonize the human IFN response? Many groups have shown that the SeV C protein is capable of inhibiting IFN action in human cell lines (30); we also demonstrated that it is indeed the case (Fig. 5*D*). Therefore, it is likely that the SeV C protein

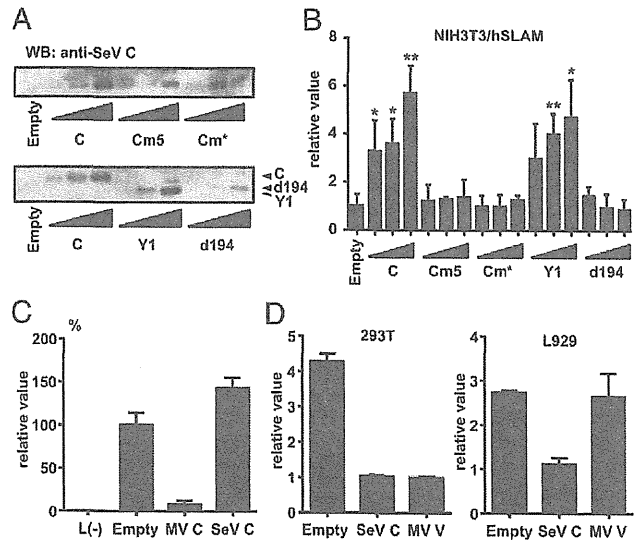

Fig. 5. Role of IFN-antagonist function of the SeV C protein in supporting efficient MV growth in mouse cells. (*A* and *B*) NIH 3T3/hSLAM cells were transfected with various amounts (25, 100, and 400 ng) of expression plasmids encoding wild-type or mutant SeV C proteins. Total amounts of transfected plasmids were adjusted by the empty vector pCA7. At 48 h posttransfection, part of the respective samples were lysed and subjected to SDS/PAGE and Western blotting for the detection of the SeV C protein (*A*), and the rest of the samples were infected with MV-Luci (*B*). At 48 h postinfection, *Renilla* luciferase activities in the infected cells were measured. The luciferase activity in the cells transfected with the empty vector was set to 1.0. Data represent the means ± SD of triplicate samples. $*P < 0.05$, $**P < 0.01$ (significant differences from the sample transfected with the empty vector, based on a *t* test). (*C*) The empty vector pCA7 or expression plasmid encoding the MV C or SeV C protein was included in the MV minigenome assay. The L protein expression plasmid was omitted from the transfection mixtures for negative control [L (–)]. The luciferase activity in the cells transfected with pCA7 was set to 100%. Data represent the means ± SD of triplicate samples. (*D*) Human 293T and mouse L929 cells were transfected with pISRE-Luc and pRL-TK, together with pCA7 (Empty) or the expression plasmid encoding the SeV C or MV V protein. The cells were treated with IFN-α for 8 h and the relative luciferase activities were determined as described in *Materials and Methods*. Data represent the means ± SD of triplicate samples.

does not exhibit the enhancing effect in human cells because MV can effectively circumvent the human IFN response through the actions of its C and V proteins. On the other hand, transient expression of the MV C or V protein did not result in increased MV growth in mouse cells. Our results indicate that unlike SeV, which can antagonize the IFN response in both mouse and human cells, MV can do so only in human cells. MV must have evolved such that it can counteract the IFN response in humans, its natural host species. To what extent its IFN antagonists V, C, and P proteins can interact with their target protein counterparts in mouse cells remain to be defined. Thus, in addition to cellular receptors, the ability to counteract the host IFN response is an important determinant for the host range of MV (15, 18–20, 23). Other host factors may also play roles in determining the host range. This question can be answered by examining whether mice supplied with a receptor and the SeV C protein reproduce human disease after MV infection.

It seems to be straightforward to generate the recombinant MV possessing the SeV C gene and infect mouse cell lines or genetically modified mice expressing receptors with this virus. However, we did not attempt to do it. As mentioned above, the SeV C protein has the ability to inhibit both human and mouse IFN responses, and there is a potential risk that such a recombinant MV will be more virulent for humans than field-circulating MVs. We should keep this in mind even though, in human cell lines, expression of the SeV C protein did not greatly enhance MV growth. Accordingly, we developed the Cre-loxP–mediated expression system for the SeV C protein. Our intention was to make infected cells more permissive to MV rather than to make the virus more virulent. In mouse cells infected with the Cre recombinase-expressing MV, the enzyme catalyzes the recombination at loxP sequences, thereby allowing the SeV C protein to be expressed, which will in turn lead to efficient MV growth. On the other hand, uninfected mouse cells do not express the SeV C protein, and their IFN system remains intact. In this way, we should be able to study MV infection in mice without totally compromising the IFN system. Generation of transgenic mice stably expressing the SeV C protein has not been reported like stable cell lines, probably because of the deleterious effect of its continuous expression. Because human cell lines stably expressing the protein have been obtained (40, 51), the effect may be specific to mice. Our present data warrant the generation of a new mouse model for MV infection, based on our conditional Cre-loxP expression system, and this approach is now under way. Furthermore, this system is readily applicable to other human viruses, such as hepatitis C virus, that do not grow well in immunocompetent mice (52).

Materials and Methods

For MV minigenome assay, monolayers of VV5-4 cells (53) cultured in 24-well cluster plates were infected with vTF7-3 (54) at a multiplicity of infection (MOI) of 0.5, and then transfected with the MV minigenome plasmid p18MGFLuc01 encoding the firefly luciferase gene and support plasmids expressing the MV N, P, and L proteins, respectively (55), together with pCA7 or pCA7-SeV-C or pCA7-MV-C (10), using Lipofectamine 2000 reagent (Invitrogen Life Technologies). At 48 h posttransfection, enzymatic activities of the luciferase were measured. To perform reporter assay for the inhibition of IFN-α signaling, cells were transfected with pISRE-Luc (56) and pRL-TK (Promega), together with pCA7, pCA7-SeV-C, or pCA7-MV-VΔC (10), using Lipofectamine 2000. At 36 h posttransfection, the medium was changed to one supplemented, or not, with 1,000 IU/mL IFN-αA/D (Sigma). After a further 8 h of incubation, the relative luciferase activities were determined by dividing the firefly luciferase activity of IFN-α–treated cells by that of untreated cells. Transfection efficiencies of different samples were normalized against the *Renilla* luciferase activity, which is regulated by the herpes simplex virus thymidine kinase promoter, and therefore unaffected by IFN treatment. Detailed materials and methods are described in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank A. Kato, K. Komase, C. Meno, B. Moss, M. B. A. Oldstone, K. Ozato, and T. Yagi for providing reagents; and E. Ono and C. Meno for invaluable discussions and technical support. This work was supported by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

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Supporting Information

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SI Materials and Methods

Cells and Viruses. NIH 3T3, L, HeLa, HEK293, and Vero cells constitutively expressing human signaling lymphocyte activation molecule (SLAM) (NIH 3T3/hSLAM, L/hSLAM, HeLa/hSLAM, HEK293/hSLAM, and Vero/hSLAM, respectively) (1, 2) were maintained in DMEM (Invitrogen) supplemented with 7.5% FBS and 500 μg/mL G418. 293T and L929 cells were maintained in DMEM supplemented with 7.5% FBS. VV5-4 cells (a derivative of CHO cells) were maintained in RPMI medium 1640 (MP Biomedicals) supplemented with 7.5% FBS. Recombinant measles virus (MV) based on the virulent IC-B strain were generated using cloned cDNAs and vTF7-3 (a gift from B. Moss, National Institutes of Health, Bethesda, MD), a vaccinia virus encoding the T7 RNA polymerase, as described previously (3, 4).

Antibodies. Rabbit polyclonal antibodies against the Cre recombinase, STAT1 and phosphorylated STAT1, and mouse monoclonal antibodies against EGFP (JL-8) and β-actin (C-2) were purchased from EMD chemicals Inc., Santa Cruz Biotechnology, Cell Signaling Technology, TAKARA BIO Inc., and Santa Cruz Biotechnology, respectively. The serum of a patient with subacute sclerosing panencephalitis containing high-level antibodies against MV proteins including the nucleocapsid (N) protein (5) and rabbit serum against the recombinant SeV C protein (6) were kindly provided by M. B. A. Oldstone, The Scripps Research Institute, La Jolla, CA and A. Kato, National Institute of Infectious Diseases, Tokyo, Japan, respectively.

Plasmids. p(+)MV323-EGFP and p(+)MV323-Luci were described previously (7, 8). p(+)MV323-Cre was generated by replacing the transcriptional unit of EGFP of p(+)MV323-EGFP with that of the Cre recombinase fused with the nuclear localization signal in its amino terminus. p(+)MV323-EGFP-addCre was generated by replacing the PΔC and C genes of p(+)MV323-PΔC-addC (9) with the intact P gene and the sequence encoding the Cre recombinase fused with nuclear localization signal in its amino terminus, respectively. The recombinant MVs generated from p(+)MV323-EGFP, p(+)MV323-Luci, p(+)MV323-Cre, and p(+)MV323-EGFP-addCre were designated MV-EGFP, MV-Luci, MV-Cre, and MV-addCre, respectively. Expression plasmids pCA7-SeV-C, pCA7-Cre, and pCA7-NS1 were generated by inserting the DNA fragments encoding the SeV C protein, Cre recombinase (gifts from A. Kato, National Institute of Infectious Diseases, Tokyo, Japan and T. Yagi, Osaka University, Osaka, Japan, respectively), and the NS1 protein from the influenza A virus PR8 strain into the expression vector pCA7. pCA7-SeV-Cm5, pCA7-SeV-Cm*, pCA7-SeV-Y1, and pCA7-SeV-C-d194 were generated by cloning into the pCA7 vector DNA fragments encoding mutant SeV C proteins with amino acid (aa) substitutions K151A, E153A, and R154A, with aa substitutions K151A, E153K, and R157L, with amino-terminal truncation of 23 aa and with carboxyl-terminal truncation of 10 aa, respectively. The expression plasmids pCA7-IC-C and pCA7-IC-VΔC encoding the MV C and V proteins, respectively, were reported previously (10). pCA7-IC-VΔC does not direct the production of the C protein. To generate pCA7-loxP-SC, the plasmid encoding the puromycin resistance gene with the polyA signal sequence flanked by loxP sequences (a gift from C. Meno, Kyushu University, Fukuoka, Japan) was used. Two SV40 polyA signal sequences were tandemly inserted downstream of the original polyA signal to prevent readthrough transcription. The plasmids used for the MV minigenome assay [p18MGFLuc01 and support plasmids expressing the MV N, phospho- (P), and large (L) proteins] were kindly provided by K. Komase, National Institute of Infectious Diseases, Tokyo, Japan. The IFN-α/β inducible plasmid pISRE-Luc (a gift from K. Ozato, National Institute of Child Health and Human Development, Bethesda, MD) has three tandem repeat sequences of the IFN-stimulated response element followed by the firefly luciferase gene. The plasmid pRL-TK has the herpes simplex virus thymidine kinase promoter followed by the *Renilla* luciferase gene.

Virus Titration. Monolayers of Vero/hSLAM cells in 12-well cluster plates were infected with serially diluted virus samples. After 1 h of incubation at 37 °C, the virus samples were removed, and the cells were overlaid with DMEM containing 2% FBS and 0.75% agarose. At 6 d postinfection, the numbers of pfu were counted.

Comparison of Luciferace Activities with Viral Multiplication. Subconfluent NIH 3T3/hSLAM cells in six-well cluster plates were mock-treated or treated with IFN-αA/D (Sigma) at the concentration of 10, 100, or 1,000 units/mL for 24 h. The cells were infected with MV-Luci at a multiplicity of infection (MOI) of 0.05. At 48 h postinfection, *Renilla* luciferace activities in part of the samples were measured using a *Renilla* luciferase assay system (Promega) and a luminometer Mithras LB 940 (Berthold Technologies). The rest of the samples were scraped into medium, and their viral titers were determined by plaque assay.

Measurement of MV Growth. Subconfluent monolayers of NIH 3T3/hSLAM, L/hSLAM, HeLa/hSLAM, or HEK293/hSLAM cells in 12-well cluster plates were transfected with 1 μg of pCA7 or pCA7-SeV-C using the lipofectamine 2000 reagent. In other experiments, NIH 3T3/hSLAM cells in 24-well cluster plates were transfected with 0.5 μg of pCA7, pCA7-SeV-C, pCA7-MV-C, or pCA7-MV-VΔC; NIH 3T3/hSLAM and HeLa/hSLAM cells with the total 250 ng of pCA7-Cre, pCA7-loxP-SC, and pCA7 in various combinations (total amounts of transfected plasmids were adjusted by pCA7); NIH 3T3/hSLAM cells with various amounts (25, 100, and 400 ng) of expression plasmids encoding wild-type or mutant SeV C proteins (total amounts of transfected plasmids were adjusted to 400 ng by pCA7); NIH 3T3/hSLAM cells with 0.5 μg of pCA7, pCA7-SeV-C, or pCA7-NS1. At 24 or 48 h posttransfection, these cells were infected with MV-Luci at an MOI of 0.05, and at 48 h postinfection, enzymatic activities in infected cells were measured using a *Renilla* luciferase assay system and a luminometer Mithras LB 940.

Growth Kinetics. Monolayers of Vero/hSLAM, NIH 3T3/hSLAM, and 3SC cells on six-well cluster plates were infected with MV-EGFP, MV-Cre or MV-addCre at an MOI of 0.01 (Vero/hSLAM) or 0.05 (NIH 3T3/hSLAM and 3SC) and cultured in 2 mL of medium. At various time intervals, the infected cells were scraped into the medium and the viral titer was determined by plaque assay.

Western Blot Analysis. Western blot analysis was performed as described previously (11). The membranes were incubated with appropriate antibody, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Invitrogen Life Technologies), anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc.), or anti-human IgG antibodies (EY Laboratories). The Chemi Lumi One Super reagent (Nacalai Tesque) was used to elicit chemiluminescent signals, and the signals on the membranes were detected and visualized using a VersaDoc 3000 imager (Bio-Rad).

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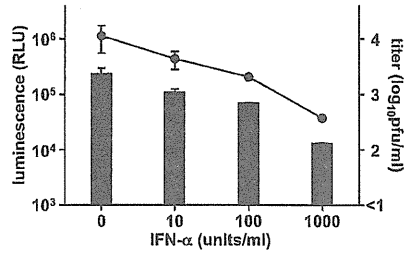

Fig. S1. Comparison of luciferace activities with viral titers. NIH 3T3/hSLAM cells were mock-treated or treated with IFN-α at the concentration of 10, 100, or 1,000 units/mL for 24 h. The cells were infected with MV-Luci, and at 48 h postinfection, *Renilla* luciferace activities (left *y* axis, filled bars) and viral titers (right *y* axis, filled circles) of the respective samples were determined. Data represent the means ± SD of triplicate samples.

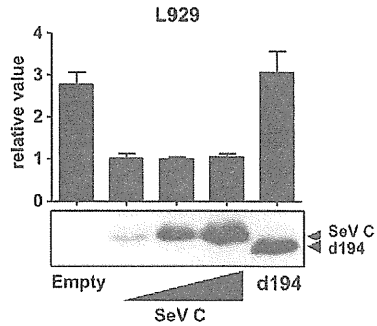

Fig. S2. IFN-antagonist activities of the SeV C and d194 proteins. Mouse L929 cells were transfected with pISRE-Luc and pRL-TK, together with pCA7 (Empty), various amounts (25, 100, and 400 ng) of the expression plasmid encoding the SeV C protein, or 400 ng of that encoding d194. Total amounts of transfected plasmids were adjusted by pCA7. At 36 h posttransfection, part of the respective samples were lysed and subjected to SDS/PAGE and Western blotting for the detection of the SeV C and d194 proteins. The cells were treated with IFN-α for 8 h, and the relative luciferase activities were determined as described in *Materials and Methods*. Data represent the means ± SD of triplicate samples.

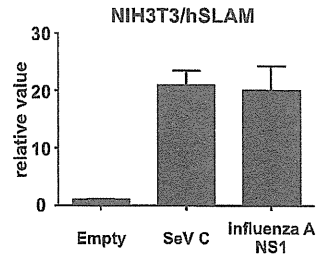

Fig. S3. Effects of the SeV C and influenza A virus NS1 proteins on MV growth in mouse cells. NIH 3T3/hSLAM cells were transfected with pCA7 or expression plasmids encoding the SeV C or influenza A virus NS1 protein. At 24 h posttransfection, the cells were infected with MV-Luci, and at 48 h postinfection, *Renilla* luciferase activities in the infected cells were measured. The luciferase activity in the cells transfected with the empty vector was set to 1.0. Data represent the means ± SD of triplicate samples.

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Measles Virus V Protein Inhibits NLRP3 Inflammasome-Mediated Interleukin-1β Secretion▽

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Inflammasomes are cytosolic protein complexes that stimulate the activation of caspase-1, which in turn induces the secretion of the inflammatory cytokines Interleukin-1β (IL-1β) and IL-18. Recent studies have indicated that the inflammasome known as the NOD-like-receptor-family, pyrin domain-containing 3 (NLRP3) inflammasome recognizes several RNA viruses, including the influenza and encephalomyocarditis viruses, whereas the retinoic acid-inducible gene I (RIG-I) inflammasome may detect vesicular stomatitis virus. We demonstrate that measles virus (MV) infection induces caspase-1-dependent IL-1β secretion in the human macrophage-like cell line THP-1. Gene knockdown experiments indicated that IL-1β secretion in MV-infected THP-1 cells was mediated by the NLRP3 inflammasome but not the RIG-I inflammasome. MV produces the nonstructural V protein, which has been shown to antagonize host innate immune responses. The recombinant MV lacking the V protein induced more IL-1β than the parental virus. THP-1 cells stably expressing the V protein suppressed NLRP3 inflammasome-mediated IL-1β secretion. Furthermore, coimmunoprecipitation assays revealed that the V protein interacts with NLRP3 through its carboxyl-terminal domain. NLRP3 was located in cytoplasmic granular structures in THP-1 cells stably expressing the V protein, but upon inflammasome activation, NLRP3 was redistributed to the perinuclear region, where it colocalized with the V protein. These results indicate that the V protein of MV suppresses NLRP3 inflammasome-mediated IL-1β secretion by directly or indirectly interacting with NLRP3.

Measles is an acute contagious disease that remains a major cause of childhood mortality worldwide, especially in developing countries (6). Measles virus (MV), a member of the family *Paramyxoviridae*, is an enveloped virus with a nonsegmented single-stranded negative-sense RNA genome (15). Upon MV infection, cells produce alpha/beta interferon (IFN-α/β) following recognition of the virus by RNA helicases encoded by retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) (22, 43) or Toll-like receptor (TLR) 7 (51). MV encodes phosphoprotein (P) and V and C proteins, which can counteract the host IFN response (11, 34, 36, 54, 64). Among these viral proteins, the V protein is the most versatile antagonist. It blocks IFN-α/β signal transduction in infected cells (9, 11, 13, 36, 38, 47, 59, 64) and inhibits TLR7-mediated IFN-α production in human plasmacytoid dendritic cells (42). Furthermore, the MV V protein, like that of other paramyxoviruses, interacts with MDA5 and inhibits its function with respect to IFN induction (2). The N-terminal domain of the V protein (Vn), which is common to the P and V proteins, has been shown to interact with STAT1 (signal transducer and activator of transcription 1) and Jak1, which are involved in IFN signaling (9), whereas the unique cysteine-rich C-terminal domain of the V protein (Vc) was found to interact with MDA5 (39), STAT2 (47), and IκB kinase α (IKKα), which is involved in the activation of interferon-regulatory factor 7 through TLR7 (42). Furthermore, Vc interacts with the NF-κB subunit p65 (RelA) to suppress NF-κB activity (53). The mechanism by which the MV C protein acts as an IFN antagonist is not well understood, but it probably suppresses IFN induction by regulating viral RNA synthesis (34).

Secretion of the inflammatory cytokines interleukin-1β (IL-1β) and IL-18 is tightly regulated by the inflammasomes known as caspase-1-activating molecular complexes (32). Recognition of pathogens by pattern recognition receptors such as TLRs induces pro-IL-1β and pro-IL-18 in the cytoplasm. The activation of the inflammasomes leads to the conversion of procaspase-1 to caspase-1, which cleaves procytokines into mature IL-1β and IL-18, which are then secreted. The best-characterized inflammasome is the NOD-like receptor (NLR)-family pyrin domain-containing 3 (NLRP3, also known as Nalp3 or cryopyrin) inflammasome, which can be activated by a wide range of stimuli, such as endogenous danger signals from damaged cells, bacterial components, and environmental irritants (52).

Infection by RNA viruses such as influenza virus (1, 19, 20, 61), encephalomyocarditis virus (EMCV) (44, 45), and vesicular stomatitis virus (VSV) (45) also stimulates NLRP3, which then recruits the apoptosis-associated speck-like protein containing a caspase recruitment domain (ASC) and procaspase-1, forming the NLRP3 inflammasome. How RNA viruses are recognized by NLRP3 is not well-known, but in the case of influenza virus, the proton-selective ion channel M2 protein, not genomic RNA, has been shown to stimulate the NLRP3 inflammasome pathway (20). One study reported that infection with VSV or transfection with 5′-triphosphate RNA activates

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the RIG-I inflammasome (44). Furthermore, AIM2 (absent in melanoma 2) binds to double-stranded DNA (dsDNA) and engages the adaptor protein ASC to form a caspase-1-activating inflammasome (7, 12, 18, 50). Experiments using AIM2-deficient mice revealed that AIM2 is essential in regulating caspase-1-dependent maturation of IL-1β and IL-18 in response to dsDNA, as well as DNA viruses such as vaccinia virus and mouse cytomegalovirus (49).

In the present study, we showed that MV induces secretion of IL-1β in human macrophage-like cells. By generating NLRP3 and RIG-I knockdown cells, we also demonstrated that MV activates the NLRP3 inflammasome, which results in caspase-1-mediated maturation of IL-1β. Furthermore, our results indicate that the MV V protein has the ability to interact with NLRP3 through its C-terminal domain, thereby inhibiting NRLP3 inflammasome-mediated IL-1β secretion.

MATERIALS AND METHODS

Cells and viruses. Vero cells constitutively expressing human signaling lymphocyte activation molecule (SLAM; Vero/hSLAM cells) (37) and HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Wako Pure Chemical Industry) supplemented with 7.5% (vol/vol) fetal bovine serum (FBS). PLAT-gp cells (a gift from M. Shimojima and T. Kitamura) containing the retroviral *gag* and *pol* genes (33) were maintained in DMEM supplemented with 7.5% FBS and blasticidin (10 μg/ml; Invitrogen). Human monocytic THP-1 cells were cultured in RPMI 1640 medium (Wako Pure Chemical Industry) containing L-glutamine (2 mM; Nacalai Tesque), 2-mercaptoethanol (50 μM; Nacalai Tesque), and 10% (vol/vol) FBS. For macrophage differentiation, THP-1 cells were treated with phorbol 12-myristate 13-acetate (PMA) (0.5 μM; Sigma) at 37°C for 3 h. Cell surface expression of SLAM on THP-1 cells was examined by flow cytometry analysis using an anti-SLAM monoclonal antibody IPO-3 (Cayman Chemical). IC323-EGFP (17) and MV-ΔV (22) are enhanced green fluorescent protein (EGFP)-expressing recombinant viruses based on a wild-type IC-B strain of MV. MV-ΔV was generated by introducing four nucleotide substitutions into the region corresponding to the RNA-editing motif of the P gene of IC323-EGFP. All four substitutions were synonymous in the reading frame of the P protein. Viruses were titrated on Vero/hSLAM cells. UV inactivation was performed by exposing viruses to 1.0 J of UV light/cm^2 with a Stratalinker UV cross-linker (Stratagene).

Plasmid constructions. pCA7-Flag-MDA5 was generated by inserting Flag-tagged MDA5 from pEF-Flag-MDA5 (34) into the expression vector pCA7 (58). The cDNA for human NLRP3 was purchased from the National Institute of Technology and Evaluation, Biological Resource Center, Japan. The cDNAs encoding human ASC, procaspase-1, and pro-IL-1β were obtained by reverse transcription of total RNA from lipopolysaccharide (LPS)-treated THP-1 cells, followed by PCR using specific primers. These cDNAs were cloned into pCA7 (pCA7-NLRP3, pCA7-ASC, pCA7-procaspase-1, and pCA7-pro-IL-1β) or pCA7-Flag to produce Flag-tagged proteins (pCA7-Flag-NLRP3 and pCA7-Flag-ASC). pCAG-HA-IC-V and pCAG-HA-IC-Vn were generated by inserting the DNA fragments encoding the hemagglutinin (HA)-tagged full-length V protein from the IC-B strain of MV and the truncated V protein containing only the N-terminal 231 residues (36) into pCAGGS (35), respectively. pCA7-HA-orange-Vc, pCA7-HA-orange-Vc(C272R), pCA7-IC-V, and pCA7-EGFP were generated by inserting the DNA fragments encoding the HA-tagged kusabira orange-fused C-terminal 69 residues of the V protein (orange-Vc), orange-Vc with the C272R substitution [orange-Vc(C272R)], the full-length V protein, and EGFP into pCA7, respectively.

Knockdown of genes using shRNA. Using the pRS-U6/puro vector (OriGene), plasmids pRS-shNLRP3, pRS-shRIG-I and pRS-shEGFP were constructed. They expressed short hairpin RNAs (shRNAs) targeting human NLRP3, human RIG-I, and EGFP mRNAs, respectively. Target sequences were designed using BLOCK-iT RNAi Designer (Invitrogen) or had been described previously (22, 63): 5′-GGA GAG ACC TTT ATG AGA AAG-3′ for NLRP3, 5′-GCC AGA ATG TTA GTG AGA ATT-3′ for RIG-I, and 5′-GGC ACA AGC TGG AGT ACA ACT-3′ for EGFP. To generate shRNA-expressing retroviruses, PLAT-gp cells in 10-cm dishes were transfected with 20 μg of each shRNA-expressing plasmid and 2 μg of pCVSV-G, which encodes the VSV G protein (55) using PEI-Max (Polysciences, Inc.). Culture medium was replaced with fresh medium 6 h later, and supernatants containing retroviruses were collected at 48 h post-transfection. To generate THP-1 cells constitutively expressing shRNA targeting NLRP3, RIG-I, and EGFP mRNAs, respectively, 4×10^5 THP-1 cells in 200 μl complete medium were centrifuged with each shRNA-expressing retrovirus (200 μl) containing Polybrene (10 μg/ml) at 370 × *g* at room temperature for 90 min. Then, 24 h later, the transduction was repeated to enhance virus infection, and cells were incubated for a further 24 h in the presence of Polybrene. Cells were cultured for 2 to 3 weeks in medium containing puromycin (0.5 μg/ml) to kill nontransduced cells.

Generation of cells stably expressing the MV V protein. To generate the retrovirus expressing the MV V protein, the cDNA encoding the V protein of the IC-B strain of MV was cloned into pMXs-GFP (a gift from M. Shimojima and T. Kitamura) (27), producing pMX-V. PLAT-gp cells were transfected with 20 μg of pMX-V and 2 μg of pCVSV-G using PEI-Max, and the retrovirus was recovered. Cells in 24-well plates were centrifuged with 200 μl of medium containing the retrovirus expressing the MV V protein and Polybrene (10 μg/ml) at 370 × *g* for 90 min at room temperature. At 24 h after transduction, culture medium was replaced with complete RPMI 1640 medium containing puromycin (0.5 μg/ml). A single clone was isolated from puromycin-resistant clones that formed colonies in methylcellulose medium. As a control, cells stably expressing EGFP were also generated as above.

MV infection. Cells were infected with MV at a multiplicity of infection (MOI) of 0.1 or influenza virus PR8 at an MOI of 2 for 2 h at 37°C, washed with phosphate-buffered saline (PBS), and cultured with complete RPMI medium for 24 to 48 h. To inhibit caspase-1 activation, cells were pretreated with 5 μM Ac-YVAD-CHO (Bachem) for 30 min. The cells were then infected with MV in the presence of Ac-YVAD-CHO for 2 h at 37°C, washed with PBS, and cultured with complete RPMI medium containing Ac-YVAD-CHO.

Measurement of IL-1β and pro-IL-1β. Cell-free supernatants were collected at 24 to 48 h postinfection, at 24 h following transfection with poly(dA · dT) (Invitrogen) or at 24 h after stimulation with LPS plus ATP. The supernatants were analyzed for the presence of IL-1β using an enzyme-linked immunosorbent assay (ELISA) utilizing paired antibodies (eBiosciences) (20). To measure intracellular pro-IL-1β, cells were lysed by repeated cycles of freezing and thawing in PBS containing 2% FBS, and the lysates were analyzed by ELISA (44).

Reconstitution of the NLRP3 inflammasome in HEK293T cells. HEK293T cells grown to approximately 60% confluence in 24-well plates were transfected with 15 ng of pCA7-NLRP3, 5 ng of pCA7-ASC, 5 ng of pCA7-procaspase-1, 150 ng of pCA7-pro-IL-1β, and either 600 ng of pCA7-IC-V or 600 ng of pCA7-EGFP using PEI-Max. Cell-free supernatants were collected 24 h after transfection and analyzed for IL-1β by ELISA. Under this condition, exclusion of any one of the expression plasmids encoding ASC, procaspase-1, or pro-IL-1β led to no production of IL-1β (data not shown).

Coimmunoprecipitation and Western blot analyses. Subconfluent monolayers of HEK293T cells in six-well plates were transfected with 6 μg of pCA7-Flag-NLRP3, 2 μg of pCA7-Flag-ASC, or 6 μg of pCA7-Flag-MDA5 together with 2 μg of pCAG-HA-IC-V, 2 μg of pCAG-HA-IC-Vn, 2 μg of pCA7-HA-orange-Vc, or 2 μg of pCA7-HA-orange-Vc(C272R). At 48 h posttransfection, the cells were washed with PBS and lysed in 1 ml of coimmunoprecipitation buffer [50 mM Tris (pH 8.0), 280 mM NaCl, 0.5% NP-40, 0.2 mM EDTA, 2 mM EGTA, 10% glycerol, 1 mM dithiothreitol (DTT)] (29) containing protease inhibitors (Sigma). The lysates were centrifuged at 20,630 × *g* for 90 min at 4°C. A small amount (50 μl) of each supernatant was mixed with sodium dodecyl sulfate (SDS) loading buffer (50 mM Tris [pH 6.8], 100 mM DTT, 2% SDS, 0.1% bromophenol blue, 10% glycerol) and boiled for 5 min. The rest of the supernatant was incubated for 90 min at 4°C with protein A-Sepharose (GE Healthcare AB), which had been pretreated with an anti-Flag (F1804; Sigma) or anti-HA (sc-7392; Santa Cruz) antibody for 90 min at 4°C. Complexes with the Sepharose were obtained by centrifugation and washed three times with coimmunoprecipitation buffer. The polypeptides in the precipitated complexes were fractionated by SDS-polyacrylamide gel electrophoresis (PAGE) using 10 to 15% polyacrylamide gel and electroblotted onto polyvinylidene difluoride (PVDF) membranes (Hybond-P; Amersham Biosciences). The membranes were incubated with anti-Flag (F7425; Sigma) or anti-HA (561; Medical & Biological Laboratories Co.) antibody, followed by incubation with horseradish peroxidase-conjugated anti-rabbit IgG (Invitrogen) for detection of the Flag-tagged or HA-tagged proteins.

To detect the cleaved form of IL-1β, the supernatant was incubated for 90 min at 4°C with Dynabeads Pan Mouse IgG (Invitrogen), which had been pretreated with a mouse monoclonal antibody against human IL-1β (clone 8516.311; R&D Systems, Inc.) for 90 min at 4°C. Complexes with the beads were obtained using magnets and washed with 1× RIPA buffer (150 mM NaCl, 0.1% SDS, 1% deoxycholate, 1% Triton X-100, and 10 mM Tris [pH 7.4]) (29). For immuno-

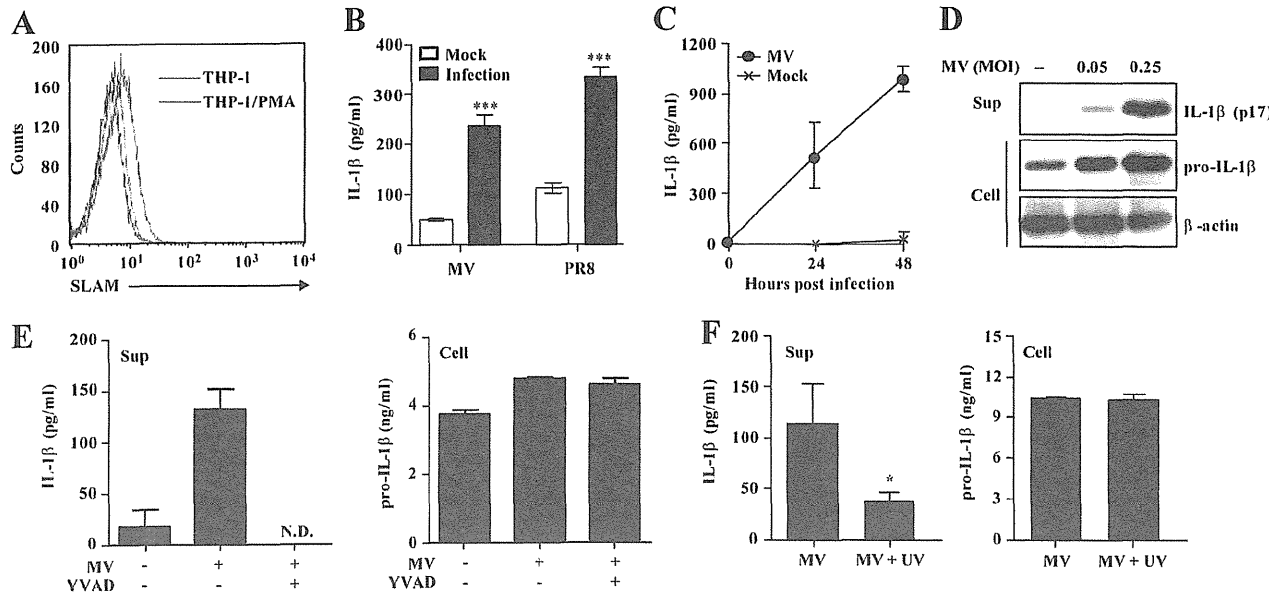

FIG. 1. Caspase-1-dependent IL-1β production by MV-infected THP-1 cells. (A) THP-1 and THP-1/PMA cells were stained with anti-SLAM antibody IPO-3 or mouse IgG1 control antibody (THP-1 cells, shaded histogram), followed by staining with fluorescein isothiocyanate-labeled anti-mouse IgG. (B and C) THP-1/PMA cells were infected with MV or influenza virus. Cell-free supernatants were collected at 24 h postinfection (B) or at the indicated time points (C) and analyzed for IL-1β using an ELISA. (D) Immunoblot analysis of the mature (p17) form of IL-1β in the supernatants (Sup) and pro-IL-1β in cell extracts (Cell) of the cells infected with MV at the indicated MOIs. (E) The effect of YVAD-CHO on IL-1β and pro-IL-1β production was determined in THP-1/PMA cells at 24 h after MV infection. N.D., not detected. (F) THP-1/PMA cells were inoculated with live or UV-inactivated MV. Cell-free supernatants and cell lysates were collected at 24 h postinfection and analyzed for IL-1β by ELISA. Values are means and standard deviations from triplicate samples. The data shown are representative of three experiments. *, $P < 0.05$; ***, $P < 0.001$.

blotting, a mouse anti-human IL-1β monoclonal antibody (MAB201; R&D) was used in Can Get Signal solution 1 (Toyobo) followed by a horseradish peroxidase-conjugated secondary antibody diluted in Can Get Signal solution 2. To detect human NLRP3, RIG-I, β-actin, and MV V protein, cells were lysed in 1× RIPA buffer. The lysates were subjected to SDS-PAGE under reducing conditions and subsequently blotted using the antibodies to the following proteins: for human NLRP3, AG-20B-0014-C100 (AdipoGen); for human RIG-I, 54285 (AnaSpec); for β-actin, sc-8432 (Santa Cruz); and for MV V protein, rabbit antiserum (34). The PVDF membranes were treated with Chemi-Lumi One Super (Nacalai Tesque) to elicit chemiluminescent signals, and the signals were detected and visualized using a VersaDoc 3000 imager (Bio-Rad).

Immunofluorescence staining. Cells were fixed and permeabilized with PBS containing 2.5% formaldehyde and 0.5% Triton X-100. The fixed cells were washed with PBS and then incubated with antibodies against NLRP3 (AG-20B-0014-C100) and MV V protein for 1 h at room temperature, followed by incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG (heavy plus light chain [H+L]; Invitrogen) and Alexa Fluor 594-conjugated donkey anti-rabbit IgG (H+L; Molecular Probes) for 1 h at room temperature. The stained cells were observed using a confocal microscope (Radiance 2100; Bio-Rad).

Statistics. Data were analyzed for statistical significance using a two-tailed Student *t* test. *P* values less than 0.05 were considered statistically significant.

RESULTS

MV induces IL-1β in PMA-stimulated THP-1 cells. The human monocytic cell line THP-1 is differentiated into macrophage-like cells by stimulation with PMA (40, 62). THP-1 cells had little expression of SLAM, the principal cellular receptor for MV (60), but the SLAM expression level was elevated after stimulation with PMA (Fig. 1A). Infection of PMA-stimulated THP-1 cells (THP-1/PMA cells) with MV(IC323-EGFP) induced IL-1β secretion in the supernatant at 24 h postinfection, similar to that seen with the PR8 strain of influenza virus (1) (Fig. 1B). Secretion of IL-1β in MV-infected THP-1/PMA cells was increased in a time-dependent (Fig. 1C) and MOI-dependent (Fig. 1D) manner. Western blot analysis demonstrated that the p17 subunit, the mature processed form of IL-1β, was secreted in the supernatant (Fig. 1D). Furthermore, YVAD-CHO, a specific inhibitor of caspase-1, suppressed IL-1β production in MV-infected THP-1/PMA cells without affecting the amounts of pro-IL-1β in the cytosol (Fig. 1E). In order to examine whether viral replication is required to induce IL-1β, we infected THP-1/PMA cells with UV-treated or untreated MV. Infection with UV-treated MV failed to induce IL-1β secretion (Fig. 1F), indicating that viral particles or genomic RNA are not sufficient to stimulate IL-1β secretion. Together, these results indicate that infection with live MV causes caspase-1-dependent IL-1β secretion in THP-1/PMA cells.

MV activates the NLRP3 inflammasome. To evaluate individual contributions of NLRP3 and RIG-I inflammasomes to the induction of IL-1β after MV infection, we generated THP-1 cells stably expressing shRNA targeting human NLRP3 or RIG-I mRNA. Western blot analysis confirmed knockdown of NLRP3 and RIG-I in respective shRNA-expressing THP-1/PMA cells at 24 h after LPS stimulation (Fig. 2A). Expression levels of NLRP3 and RIG-I in knockdown cells were 16% and 18% (after normalization using expression levels of β-actin), compared with those in control cells, respectively. As expected, IL-1β secretion induced with LPS (an inducer of pro-IL-1β) plus ATP (an NLRP3 agonist), was significantly reduced in NLRP3 knockdown cells but not in RIG-I knockdown cells (Fig. 2B). NLRP3 knockdown but not RIG-I knockdown THP-

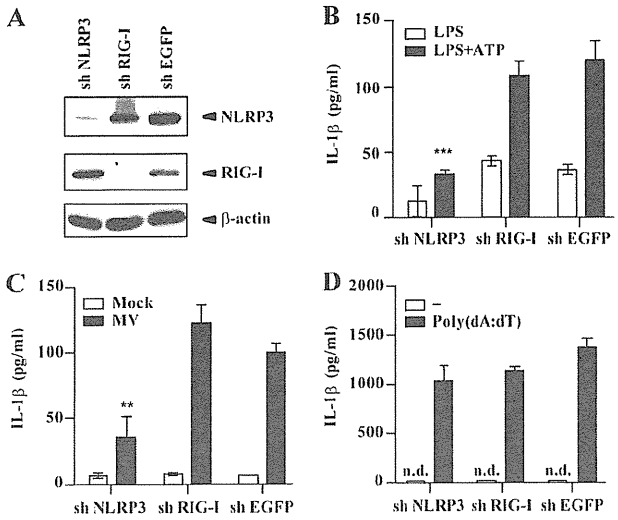

FIG. 2. NLRP3 inflammasome activation by MV. (A) Expression levels of NLRP3 and RIG-I in PMA-stimulated THP-1 cells expressing shRNA targeting NLRP3, RIG-I, or EGFP mRNAs were examined by Western blot analysis at 24 h after LPS (1 μg/ml) stimulation. β-Actin was used as an internal control. (B to D) PMA-stimulated THP-1 cells expressing shRNA targeting mRNAs of NLRP3, RIG-I, or EGFP were treated with LPS (5 ng/ml) or LPS (5 ng/ml) plus ATP (5 mM) (B), MV (C), or poly(dA · dT) (400 ng/ml) (D). Cell-free supernatants were collected 24 h after treatment and analyzed for IL-1β by ELISA. n.d., not detected. Values are means and standard deviations from triplicate samples. The data shown are representative of at least three experiments. **, $P < 0.01$; ***, $P < 0.001$.

1/PMA cells secreted a smaller amount of IL-1β in the supernatant at 24 h after MV infection than THP-1/PMA cells expressing a control shRNA (shEGFP) (Fig. 2C). Reduction of IL-1β production was not due to a general defect in the cytokine response of NLRP3 knockdown cells. After dsDNA-dependent activation of the AIM2 inflammasome, they produced a level of IL-1β comparable to that seen with control and RIG-I knockdown cells (Fig. 2D). These results indicate that the NLRP3 inflammasome, but not the RIG-I inflammasome, is involved in MV-induced IL-1β production in THP-1/PMA cells.

MV V protein inhibits MV-induced IL-1β secretion. The MV V protein counteracts the IFN-α/β-induced antiviral activity through several different mechanisms (2, 9, 11, 38, 47, 59, 64). The inflammasomes, along with the IFN system, play an important role in innate immunity, and we suspected that the V protein may also possess the activity to block inflammasome activation. To test this hypothesis, two sets of experiments were performed. First, we examined the effect of the V protein on IL-1β secretion of HEK293T cells transiently transfected with plasmids encoding components of the NLRP3 inflammasome: NLRP3, ASC, procaspase-1, and pro-IL-1β (10). In this experiment, HEK293T cells were used, as they are human cells that can be transfected at a high efficiency. Furthermore, HEK293T cells are deficient in endogenous ASC and NLRP3 (5). Reconstitution of the NLRP3 inflammasome resulted in IL-1β secretion by HEK293T cells, in which NLRP3 was absolutely required (Fig. 3A). Inclusion of the MV V protein inhibited IL-1β secretion by ~30%. Second, we established THP-1 cells

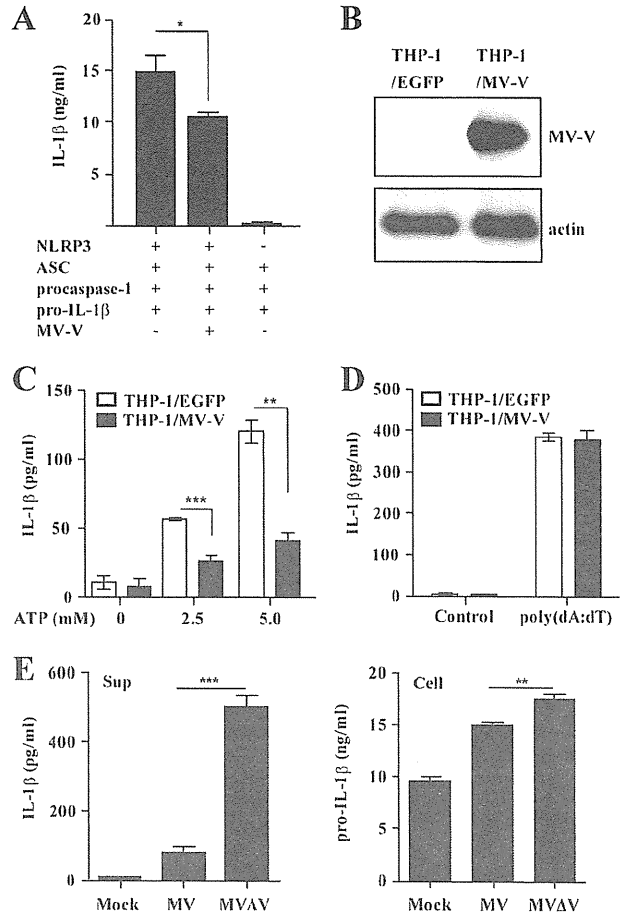

FIG. 3. Inhibition of NLRP3 inflammasome-mediated IL-1β secretion by the MV V protein. (A) HEK293T cells were transfected with expression plasmids encoding NLRP3, ASC, procaspase-1, and pro-IL-1β, together with the expression plasmid encoding the MV V protein or control plasmid (−). Cell-free supernatants were collected 24 h after transfection, and analyzed for IL-1β by ELISA. (B) Expression of the V protein in THP-1 cells stably expressing the MV V protein or EGFP was examined by Western blot analysis. (C and D) PMA-stimulated THP-1 cells stably expressing the MV V protein or EGFP were stimulated with LPS (5 ng/ml) plus ATP (0, 2.5, or 5 mM) (C) or poly(dA · dT) (400 ng/ml) (D). (E) THP-1/PMA cells were infected with wild-type MV or MVΔV virus. Cell-free supernatants (Sup) and cell lysates (Cell) were collected at 24 h postinfection and analyzed for IL-1β and pro-IL-1β, respectively, by ELISA. Values are means and standard deviations from triplicate samples. The data shown are representative of three experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

stably expressing the MV V protein (THP-1/MV-V cells) (Fig. 3B). THP-1/MV-V/PMA cells secreted significantly smaller amounts of IL-1β in response to LPS plus ATP than control cells (THP-1/EGFP/PMA cells) (Fig. 3C), indicating that the V protein blocks NLRP3 inflammasome activation. In contrast, IL-1β production in response to poly(dA · dT) was comparable between THP-1/MV-V/PMA and control cells (Fig. 3D), indicating that the IL-1β secretion pathway, including the level of pro-IL-1β, is not generally affected in THP-1/MV-V/PMA cells. Thus, the inhibitory activity of the V protein appears to be specific for the NLRP3 inflammasome.