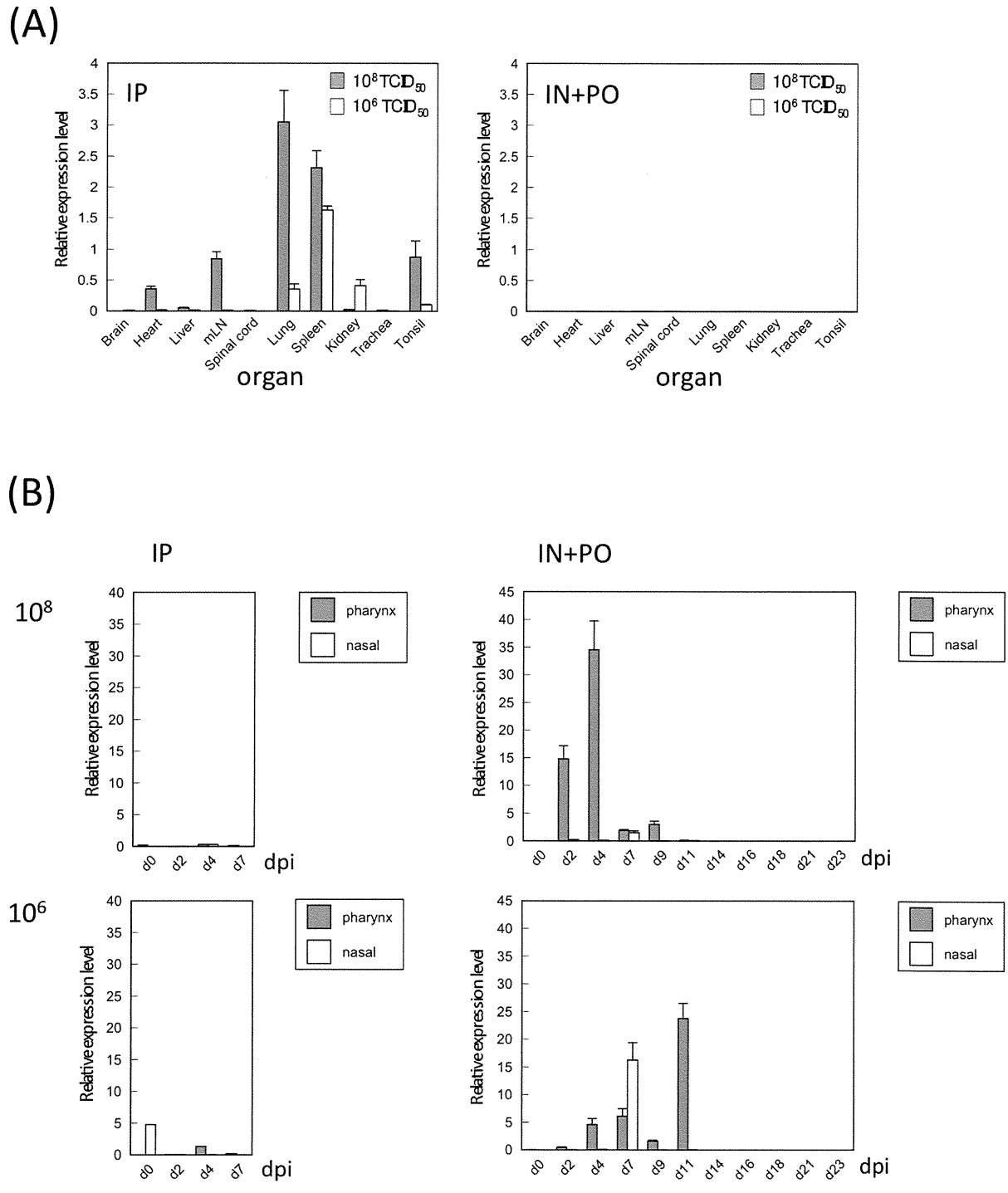

Figure 4. Virus replication in Nipah virus-infected monkeys. Virus replication was determined in tissues (A) and oral and nasal swabs (B) of NiV-infected animals by qPCR. (A) Tissue samples of monkeys infected *via* the intraperitoneal (IP) route were collected at 7 dpi, while samples from monkeys infected *via* intranasal (IN)+per os (PO) route were collected at 24 dpi. (B) Nasal and pharynx swab samples were collected every 2 days. All samples were measured in triplicate, and error bars represent the standard error of the mean (SEM).
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a lethal dose upon challenge, although rMV-Ed-G induced NiV-specific IgG antibody level was low in a small number of hamsters. The antibody response is considered to be an essential component of protection against NiV encephalitis [14,28,29]; however, cellular immunity might play an important role in eradicating NiV infection. We tested two MV vectors, based on our previous

Table 1. Pathological findings in organ samples of NiV infected monkeys.

	IP, 10^6	IP, 10^8	INPO, 10^6	INPO, 10^8
Liver	Congestion, focal necrosis, and slight infiltration of neutrophils in the sinusoids.	Congestion, centrilobular necrosis with hemorrhage	No histopathological changes (None).	None
Heart	None	None	None	None
Kidney	Endothelial syncytia in large to middle sized blood vessels.	Necrosis.	None	None
Spleen	Syncytial cells in germinal center. Follicular necrosis with hemorrhages.	Lymphocyte depletion and necrotic germinal center.	Follicular hyperplasia,	Follicular hyperplasia, VA (-)
Lung	Confluent consolidation with serum protein in alveoli.	Confluent consolidation with serum protein in alveoli.		Focal consolidation with serum protein in alveoli. VA (±, Blood vessels,)
Lymph Node	Lymphocyte depletion	Lymphocyte depletion	None	None, VA (−)
Tonsil	Lymphocyte depletion	Lymphocyte depletion	Follicular hyperplasia,	Follicular hyperplasia, VA (+, Germinal center)
Trachea			None	None
Cerebrum			None	None
Cerebellum			None	None

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experiences where we have observed that rMV-HL-based vaccines sometimes elicit a stronger effect than rMV-Ed-based vaccines. Both rMV-HL-G and rMV-Ed-G induced well protective effect in hamsters against NiV challenge.

The HL strain is isolated from patient and still possesses weak virulence in monkeys. On the other hand, the Edmonston strain was first licensed vaccine in United States in 1963, and further attenuated vaccine derived from the Edmonston strain is widely adopted in the world. Aiming at early practical use, we tested the recombinant Edomonston vaccine in this monkey study. Vaccinated monkeys did not show any symptoms of NiV infection. We used a lower titer (10^5 $TCID_{50}$) of NiV for challenge in this experiment, to observe symptoms of infected monkeys for a slightly longer period than those with 10^6 $TCID_{50}$. This dose did not induce a lethal pathology, even in unvaccinated individuals. However, histopathological and clinical observations of monkeys indicated that those challenged with NiV did suffer from a severe illness, with unimmunized monkeys found to also have lesions in their brains. The rMV-Ed-G vaccine did completely protect vaccinated monkeys from infection. Further, NiV challenge caused pathological changes in the brain, which has been widely documented in human cases. This observation might be due to slow spreading of the virus in animals challenged with a lower dose.

Table 2. Vaccination with rMV-Ed-G induced well antibody responses in monkeys.

	d0	d7	d14	d21	d28	d35
T+	ND	ND	ND	ND	ND	ND
T+	ND	ND	ND	ND	ND	ND
Ed 8192	ND	ND	6400	3200	1600	3200
Ed 8358	ND	ND	ND	ND	ND	1600

Monkeys were immunized with rMV-Ed-G twice on d0 and d28. Antibody levels were measured by ELISA. Shadowed columns represent the samples which showed positive response. T+: unimmunized. ND: Not detected (<1:100).

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NiV is highly virulent and has a broad host range, causing respiratory and neurological symptoms that often lead to encephalitis. The rate of mortality in humans range from 40–92% [8,32,33]. To date, no vaccine for NiV disease has been

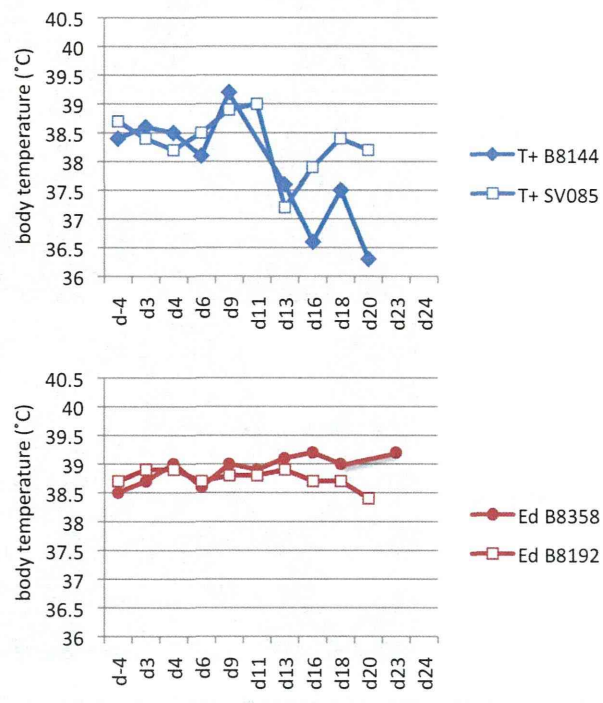

Figure 5. Body temperature of monkeys after NiV challenge. The rectal temperature of unimmunized (upper) monkeys or monkeys immunized with 10^5 $TCID_{50}$ of rMV-Ed-G was recorded from 4 days before the NiV challenge until the end of the experiment. T+ B8144 and T+ SV085 were unimmunized monkeys. Ed B8358 and Ed B8192 were monkeys immunized with rMV-Ed-G before virus challenge.

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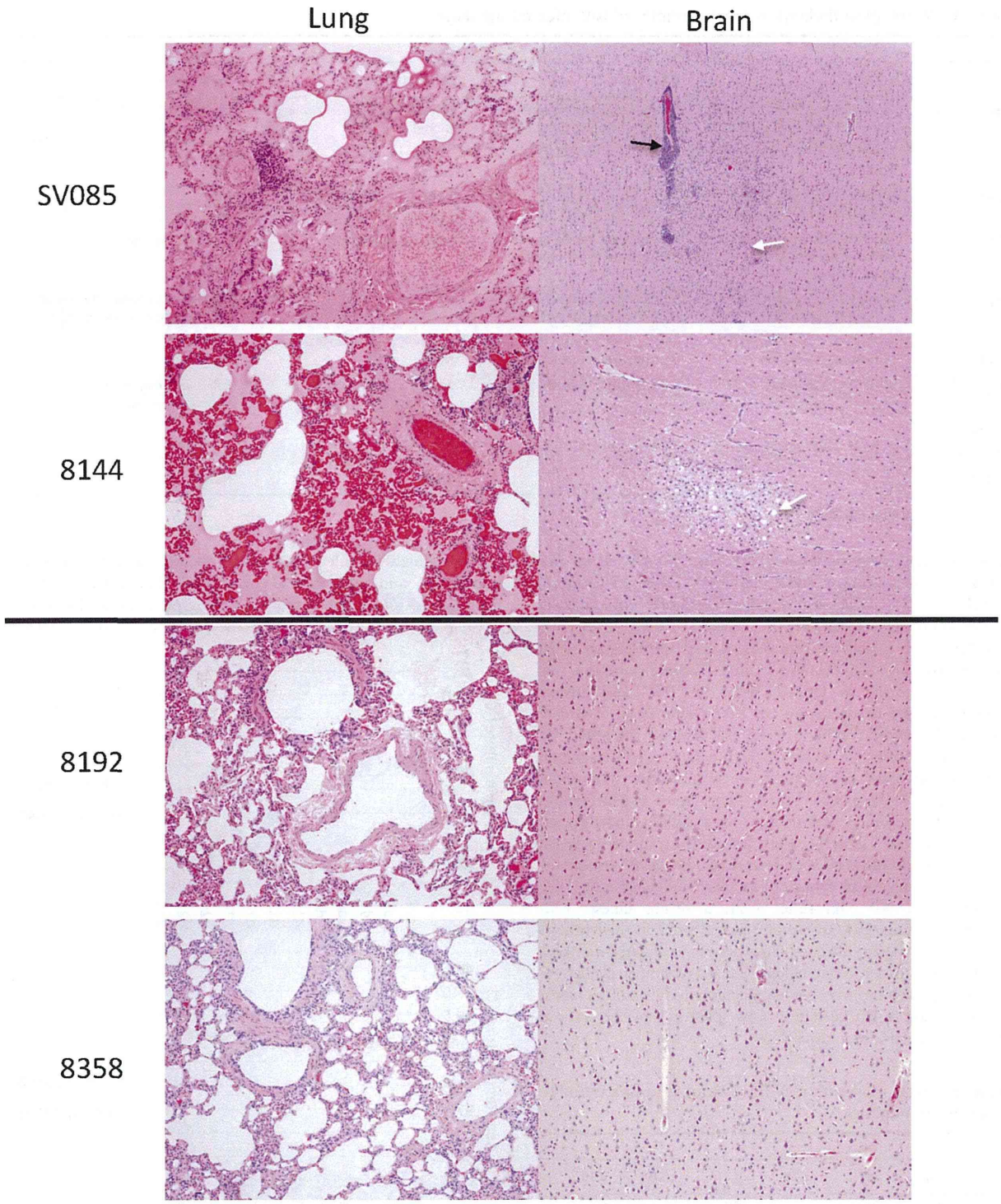

Figure 6. Histopathology of monkey tissues. Lung and brain samples from unvaccinated monkeys (T +B8144, T+ SV085) and vaccinated monkeys (Ed B8358, Ed 8192) were stained with hematoxylin and eosin. 100× magnification. The lungs of T+ B8144 and T+ SV085 showed severe congestion, infiltration of neutrophils and accumulation of blood plasma in the alveoli. Their brains showed perivascular cuffing (black arrow; SV085) and an accumulation of glial (white arrow; SV 085) and foam cells (white arrow in; B8144) in the cerebral cortex. No lesions were observed in tissues from Ed B8358 or Ed B8192.
doi:10.1371/journal.pone.0058414.g006

developed that is both safe and protective in humans. Our recombinant MV-Ed-G vaccine has the potential to elicit long-term immunity against both MV and NiV in children and adults located in endemic areas. Therefore we believe it is an effective vaccine candidate for human use. We were only able to use two monkeys for vaccination in this study, as the costs of non-human primate and spaces for animal experimentation in BSL4 facility were prohibitive. Further studies in greater number of monkeys will be necessary to validate the safety and efficacy of our vaccine candidate.

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Author Contributions

Conceived and designed the experiments: MY CK. Performed the experiments: MY MCGC FI MI NN FJ. Analyzed the data: MY HS CK. Contributed reagents/materials/analysis tools: MI HS HR. Wrote the paper: MY CK.

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ORIGINAL ARTICLE

Measles virus selectively blind to signaling lymphocyte activation molecule as a novel oncolytic virus for breast cancer treatment

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Oncolytic viruses hold much promise as novel therapeutic agents that can be combined with conventional therapeutic modalities. Measles virus (MV) is known to enter cells using the signaling lymphocyte activation molecule (SLAM), which is expressed on cells of the immune system. Although human breast cancer cell lines do not express SLAM, we found that a wild-type MV (HL strain) efficiently infected various breast cancer cell lines, causing cell death. Based on this finding, we used reverse genetics to generate a recombinant MV selectively unable to use SLAM (rMV-SLAMblind). The rMV-SLAMblind lacked infectivity for SLAM-positive lymphoid cells, while retaining oncolytic activity against breast cancer cells. We showed that, unlike the MV vaccine strains, rMV-SLAMblind used PVRL4 (polio virus receptor-related 4) as a receptor to infect breast cancer cells and not the ubiquitously expressed CD46. Consistent with this, rMV-SLAMblind infected CD46-positive primary normal human cells at a much-reduced level, whereas a vaccine strain of the Edmonston lineage (rMV-Edmonston) efficiently infected and killed them. The rMV-SLAMblind showed antitumor activity against human breast cancer xenografts in immunodeficient mice. The oncolytic activity of rMV-SLAMblind was significantly greater than that of rMV-Edmonston. To assess the *in vivo* safety, three monkeys seronegative for MV were inoculated with rMV-SLAMblind, and no clinical symptoms were documented. On the basis of these results, rMV-SLAMblind could be a promising candidate as a novel oncolytic virus for breast cancer treatment.

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Keywords: breast cancer; measles virus; rMV-SLAMblind; virotherapy

INTRODUCTION

Breast cancer is the most prevalent cancer in the world and the leading cause of cancer-related deaths in women.[1] Despite recent improvements in detection and treatment, when metastasis occurs it is generally difficult to treat by conventional therapies.[2] Therefore, the development of new therapeutic strategies is clearly needed.

Oncolytic virotherapy holds much promise as a novel strategy for cancer treatment that can be combined with conventional therapies. Currently, a wide variety of viruses from many virus families are being evaluated as oncolytic agents both at the pre-clinical and clinical level.[3] Measles virus (MV, genus Morbillivirus, family Paramyxoviridae) is an enveloped virus with a non-segmented, negative-strand RNA genome.[4] In contrast to retroviruses and some DNA viruses, MV replicates entirely in the cytoplasm[4] and the risk of viral sequences integrating into host chromosomal DNA is eliminated. Recently, live attenuated MV Edmonston vaccine strain derivatives have been investigated as potential oncolytic agents for various types of cancer.[5–8]

Three cellular proteins have been identified as MV receptors. Signaling lymphocyte activation molecule (SLAM) is predominantly expressed on cells of the immune system.[9–11] SLAM serves as a receptor for both wild-type and vaccine strains of MV. CD46 is ubiquitously expressed on all human cells except erythrocytes.[12,13] CD46 serves as a receptor only for MV vaccine strains. Polio virus receptor-related 4 (PVRL4) has recently been identified as an epithelial cell receptor for both wild-type and vaccine strains of MV.[14,15] PVRL4 is a member of adhesion receptors of the immunoglobulin superfamily and is normally localized to adherens junctions together with cadherins. It is mainly expressed in the placenta and slightly in the trachea.[16] Recent studies have shown that PVRL4 is not necessary for systemic spread and virulence of MV but is important for virus shedding into the airways.[15,17] In addition, it has been reported to be a tumor cell marker for breast, lung and ovarian cancers.[18–20]

MV has two envelope glycoproteins, the hemagglutinin (H) and fusion (F) proteins. The H protein interacts directly with the cellular receptors and triggers the F protein to execute membrane fusion.[4] In previous studies, H protein amino acids essential for interaction with its receptors have been identified, and the introduction of amino-acid substitutions at those positions resulted in the generation of recombinant MVs selectively blind to each receptor.[17,21]

In this study, we found that a wild-type MV HL strain[22] grew efficiently, resulting in cell death, in various breast cancer cell lines that did not express SLAM. Based on this finding, we generated a recombinant MV selectively blind to SLAM (rMV-SLAMblind) and investigated its potential as a novel therapeutic agent against breast cancer. rMV-SLAMblind decreased the viability of breast cancer cell lines, but did not affect the viability of SLAM-positive lymphoid cells. We showed that rMV-SLAMblind used PVRL4 as a receptor and not CD46, and infected CD46-positive normal human cells at a very low level. rMV-SLAMblind showed a greater oncolytic activity than that of a vaccine strain of the Edmonston

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lineage (rMV-Edmonston) in tumor-bearing mice. rMV-SLAMblind was found to be attenuated in measles-naïve monkeys. Our data suggest that rMV-SLAMblind may be a promising candidate therapeutic agent for breast cancer.

RESULTS

Efficient growth of wild-type MV in SLAM-negative breast cancer cell lines and generation of rMV-SLAMblind

Wild-type MV HL strain efficiently infected and killed various human breast cancer cell lines including MCF7, MDA-MB-453 and SKBR3 cells (Figure 1a, upper panels). Wild-type MVs are known to use SLAM as the main receptor,[11] which is expressed in cells of the immune system. However, RT-PCR (reverse transcription PCR) and flow cytometric analysis indicated that these breast cancer cell lines were SLAM-negative (Figures 1b and c). Thus, MV infection was occurring via a SLAM-independent mechanism. This finding suggested the possibility that a recombinant MV selectively unable to use SLAM would lose cytotoxicity to SLAM-positive immune cells, while retaining the oncolytic activity against breast cancer cells. Using a reverse genetics system for HL strain,[23] we generated SLAM-blind recombinant MVs by introducing a single amino-acid substitution[21] (R533A) into the H protein open reading frame (Figure 1d). The SLAM-blind recombinant MV containing the enhanced green fluorescent protein (EGFP) gene (rMV-EGFP-SLAMblind) infected SLAM-positive B95a cells at a very low efficiency, whereas it infected breast cancer cell lines as efficiently as the parental rMV-EGFP (Figure 1a). Growth kinetics of rMV-SLAMblind were compared with those of the parental virus (rMV) and a vaccine strain of the Edmonston lineage (rMV-Edmonston) in MCF7 cells (Figure 1e). The rMV-SLAMblind grew more slowly than rMV, but the maximum titer was similar. The growth speed

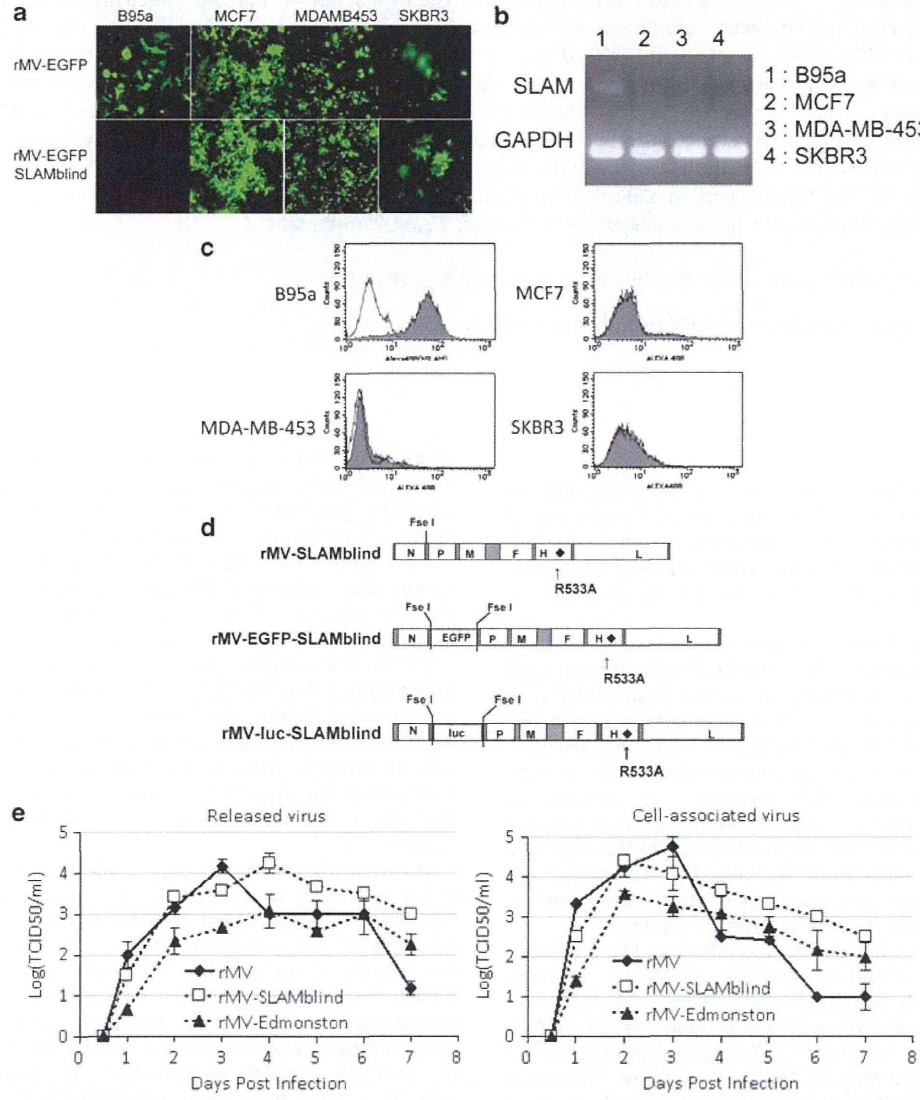

Figure 1. SLAM-independent infection of MV and generation of rMV-SLAMblind. (**a**) Cells infected with rMV-EGFP or rMV-EGFP-SLAMblind at an MOI of 0.01 and photographed at 3 (B95a) or 5 d.p.i. (the others). Magnification: × 100. (**b**) SLAM and GAPDH mRNA expression levels in SLAM-positive B95a cells and breast cancer cells assessed by standard RT-PCR. GAPDH served as a loading control. (**c**) Cells incubated with anti-SLAM MAb (gray histogram) or isotype control (white histogram) followed by incubation with Alexa-488-conjugated secondary antibody and analysis by flow cytometry. (**d**) Schematic representation of SLAM-blind recombinant MVs. The diamond represents R533A substitution. The EGFP gene or the firefly luciferase gene (*luc*) was inserted between the N and P genes. (**e**) MCF7 cells were infected with rMV, rMV-SLAMblind or rMV-Edmonston at an MOI of 0.01, and infectious titers in culture medium (released virus) and cells (cell-associated virus) were determined at various time points.

and the maximum titer of rMV-Edmonston were lower than those of rMV-SLAMblind.

rMV-SLAMblind uses PVRL4 and not CD46 to infect breast cancer cells

In addition to SLAM, CD46 and PVRL4 have been identified as receptors for MV. CD46 is a receptor only for MV vaccine strains. PVRL4 has recently been identified as a receptor[14,15] for both wild-type and vaccine strains of MV. Both molecules were expressed on the surface of breast cancer cell lines (Figure 2a). Therefore, we performed an infection inhibition assay using anti-CD46 and anti-PVRL4 antibodies (Figure 2b). The anti-CD46 antibody did not inhibit infection of breast cancer cells with rMV-EGFP and rMV-EGFP-SLAMblind, whereas the anti-PVRL4 antibody almost completely inhibited infection. The infection of breast cancer cells with rMV-Edmonston was not inhibited by either antibody alone, consistent with the fact that MV vaccine strains use both CD46 and PVRL4 as receptors. Vero cells, which are CD46-positive and PVRL4-negative,[14,15] were efficiently infected only by

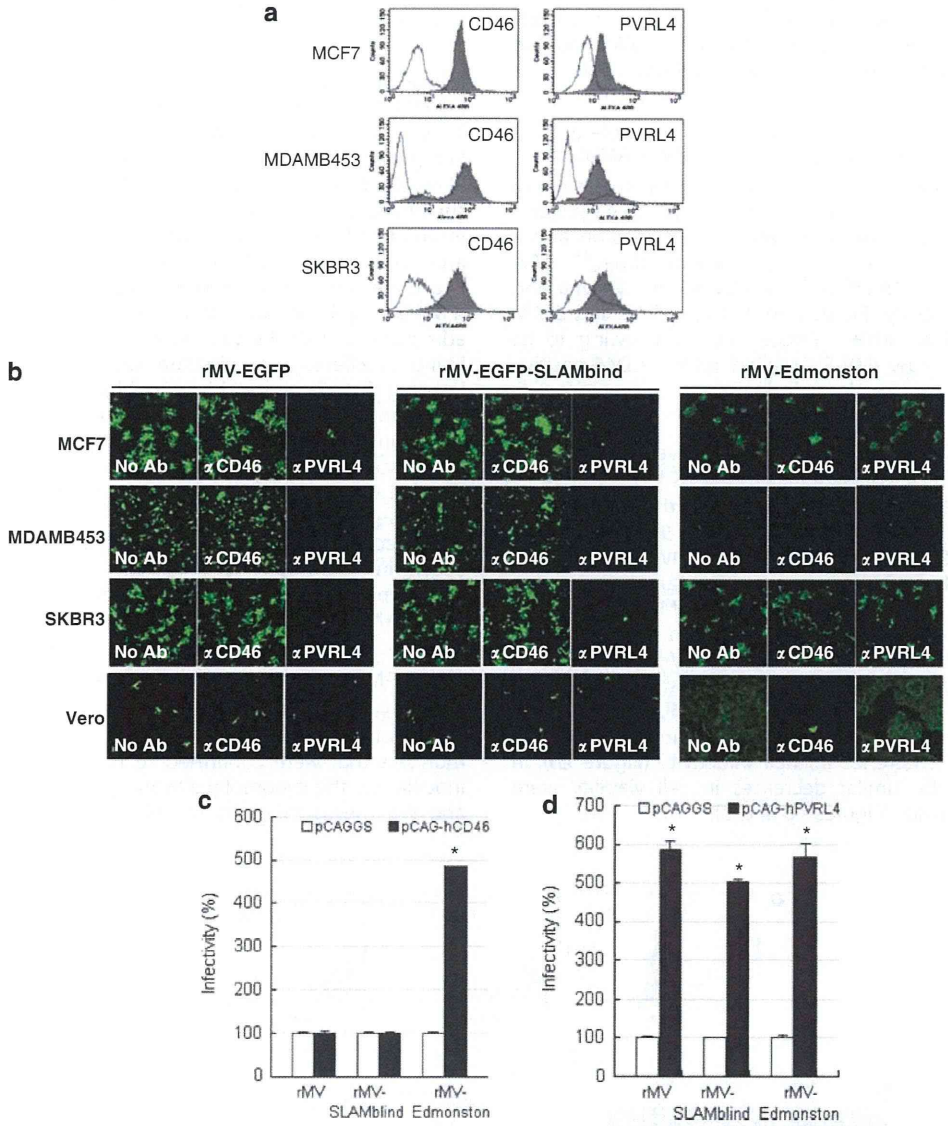

Figure 2. PVRL4 and not CD46 is involved in the infection of breast cancer cell lines with rMV-SLAMblind. (**a**) Surface expression of CD46 and PVRL4 in breast cancer cell lines analyzed by flow cytometry. (Left) Cells incubated with anti-CD46 mouse MAb (gray histogram) or isotype control (white histogram) followed by incubation with Alexa-488-conjugated goat anti-mouse antibody. (Right) Cells incubated with anti-PVRL4 goat polyclonal antibody (gray histogram) or isotype control (white histogram) followed by incubation with Alexa-488-conjugated rabbit anti-goat antibody. (**b**) Cells were pretreated with anti-CD46 or anti-PVRL4 antibody and infected with rMV-EGFP, rMV-EGFP-SLAMblind or rMV-Edmonston at an MOI of 0.1. Cells were incubated in medium with or without antibody and were photographed at 2 (Vero) or 3 d.p.i. (the others). Cells infected with rMV-Edmonston were immunostained using anti-N rabbit polyclonal antibody. Magnification: × 100. (**c**) CHO-K1 cells were transfected with pCAG-hCD46 or the empty vector (pCAGGS). After 2 days, they were infected with rMV, rMV-SLAMblind or rMV-Edmonston at an MOI of 1 and incubated in the presence of fusion inhibitory peptide. At 2 d.p.i., cells were fixed and immunostained using anti-N MAb and the number of infected cells was counted. Infectivity in cells transfected with pCAGGS was set as 100%. Error bars indicate s.e. $^*P<0.001$ versus pCAGGS. (**d**) BHK cells were transfected with pCAG-hPVRL4 or pCAGGS. After two days, they were infected with rMV, rMV-SLAMblind or rMV-Edmonston at an MOI of 0.1 and incubated in the presence of fusion inhibitory peptide. At 2 d.p.i., infectivity was determined as described in (**c**). $^*P<0.001$ versus pCAGGS.

rMV-Edmonston and infection was almost completely inhibited by anti-CD46 antibody. In addition, the infection efficiency of rMV-Edmonston in MDA-MB-453 cells was lower than that of rMV-EGFP and rMV-EGFP-SLAMblind.

To examine whether CD46 and PVRL4 act as entry receptors, we transfected plasmids encoding human CD46 (pCAG-hCD46) or human PVRL4 (pCAG-hPVRL4) into cells non-susceptible to MV, such as CHO-K1 and BHK cells. The infectivity of rMV-Edmonston was higher in cells transfected with pCAG-CD46 than in those transfected with the empty vector (pCAGGS), but the infectivity of rMV-SLAMblind and the parental virus did not differ between the two groups (Figure 2c). In contrast, all viruses showed a higher infectivity in cells transfected with pCAG-hPVRL4 than in control cells (Figure 2d). These results indicated that rMV-SLAMblind and the parental virus used PVRL4 as a receptor and not CD46.

CD46-positive normal human cells, which are susceptible to rMV-Edmonston, have a low susceptibility for rMV-SLAMblind

CD46 is ubiquitously expressed, and normal human cells such as normal human dermal fibroblasts (NHDFs) are CD46-positive (Figure 3a). In contrast to infection with rMV-Edmonston and a previously reported oncolytic MV Edmonston strain,[24] rMV-SLAMblind infected few NHDF cells, producing no syncytia and not affecting their viability (Figures 3b and c). PVRL4 and SLAM were not expressed in NHDFs (Figure 3a). Thus, owing to its restricted receptor usage, rMV-SLAMblind infects CD46-positive normal human cells at a very low efficiency.

rMV-SLAMblind lacks cytotoxicity for SLAM-positive lymphoid cells while retaining oncolytic activity against breast cancer cells

We infected B95a cells with either rMV-SLAMblind or the parental virus (rMV) at a multiplicity of infection (MOI) of 1 and measured cell viability after infection. In contrast to rMV, rMV-SLAMblind did not affect the viability of B95a cells (Figure 4a). However, the viability of breast cancer cell lines infected with rMV-SLAMblind at the same dose declined rapidly after infection (Figure 4b). In addition, we compared the cytotoxicity of rMV-SLAMblind and rMV-Edmonston in the breast cancer cell lines at an MOI of 0.1. The rMV-SLAMblind decreased the viability of MDA-MB-453 cells more efficiently than did rMV-Edmonston (Figure 4d). This was probably due to the difference in their infectivity (Figure 2b). In MCF7 and SKBR3 cells, similar decreases in cell viability were observed for both viruses (Figures 4c and e).

rMV-SLAMblind shows greater oncolytic activity than rMV-Edmonston in human breast cancer xenografts

rMV-SLAMblind and rMV-Edmonston were administered intratumorally to severe combined immune deficiency mice bearing subcutaneous MCF7 xenografts. Administration of both viruses (three doses of 10^5 $TCID_{50}$ (50% tissue culture infectious dose)) caused suppression of tumor growth, but rMV-SLAMblind caused a greater degree of suppression than rMV-Edmonston (Figure 5a). In MDA-MB-453 xenografts, intratumoral administration of both viruses (two doses of 10^6 $TCID_{50}$) also caused suppression of tumor growth, but rMV-SLAMblind suppressed tumor growth earlier than rMV-Edmonston (Figure 5b). SKBR3 cells were unable to form tumors in mice (data not shown).

A luciferase-expressing SLAM-blind recombinant MV localizes and persists within human breast cancer xenografts

To visualize virus localization in xenografted mice, we used a SLAM-blind recombinant MV expressing firefly luciferase (rMV-luc-SLAMblind; Figure 1d). MDA-MB-453 cells were implanted subcutaneously in 11 nude mice. Six mice received a single intratumoral administration of 10^6 $TCID_{50}$ of rMV-luc-SLAMblind and five mice received medium only (medium control). A further five nude mice were implanted with phosphate-buffered saline (PBS)/Matrigel mixture without cells and were subcutaneously administered with the virus at the same site (tumor-free control). Using D-luciferin as a substrate, we performed bioluminescence imaging (BLI). Strong and localized luminescence was detected in *the xenografted mice administered with the virus (Figure 6a)* at least until 21 days post infection (d.p.i.) (Figure 6d). No luminescence was detected from either of the control groups (Figures 6b and c). Moreover, we performed combined bioluminescence and magnetic resonance imaging (MRI) at 10 d.p.i., as described previously.[25] The BLI/MRI fusion image of one mouse is shown in Figure 6e. Virus localization observed with BLI merged with tumor localization observed with MRI. These results demonstrated that virus replication was localized within the tumor.

rMV-SLAMblind is attenuated in monkeys

To assess the *in vivo* safety, we subcutaneously inoculated 10^6 $TCID_{50}$ of rMV-SLAMblind into one cynomolgus and two rhesus monkeys that were confirmed to be seronegative for MV. After inoculation, the cynomolgus monkey was monitored for 1 month and the rhesus monkeys for 14 days. No clinical symptoms of

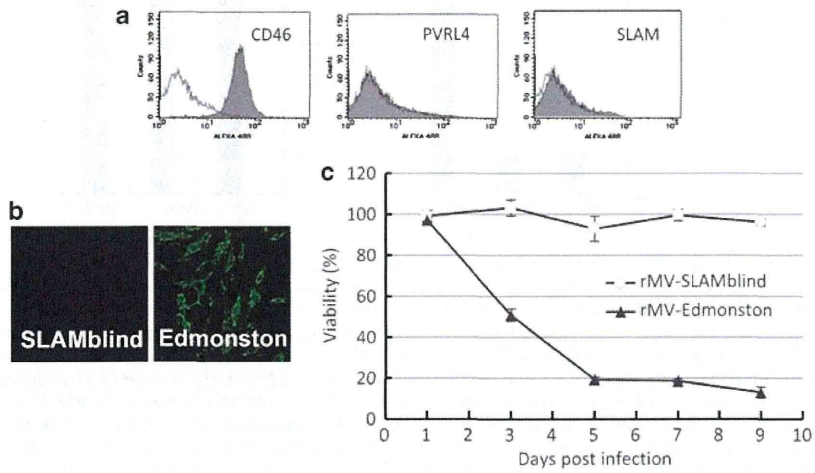

Figure 3. Infection of CD46-positive normal human cells. (**a**) Surface expression of CD46, PVRL4 and SLAM in NHDFs analyzed by flow cytometry as described in Figures 1**c** and 2**a**. (**b**, **c**) NHDFs were infected with rMV-SLAMblind or rMV-Edmonston at an MOI of 1. (**b**) Cells fixed and immunostained with anti-N MAb at 3 d.p.i. Magnification: × 100. (**c**) Cell viability measured at each time point by WST-1 assay.

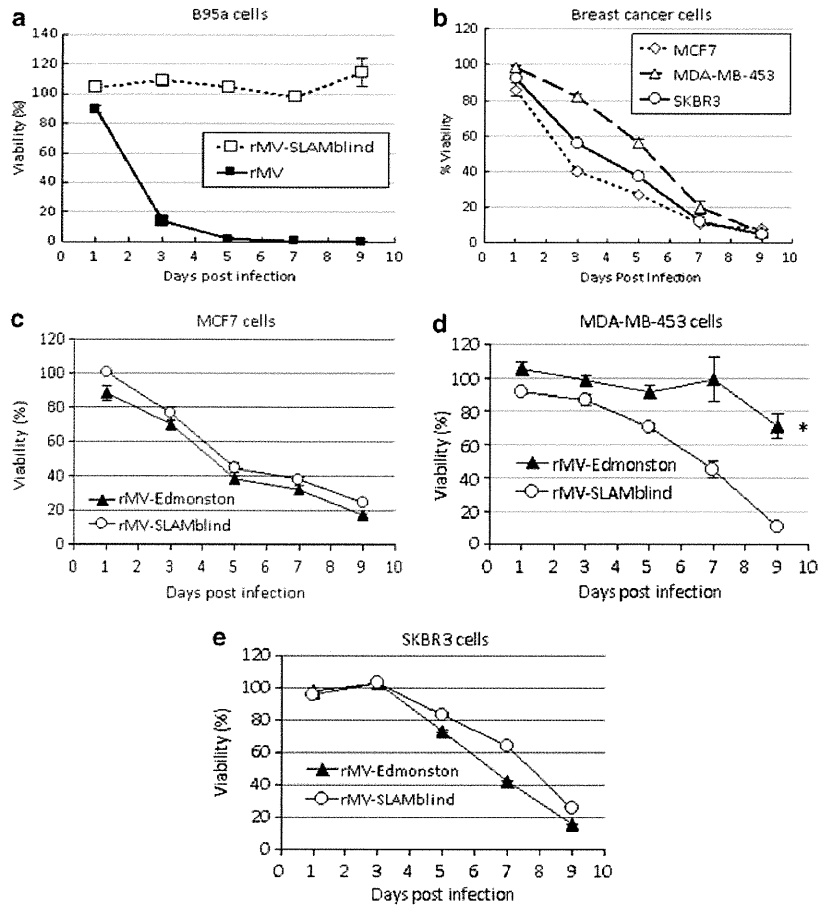

Figure 4. *In vitro* cytotoxicity of rMV-SLAMblind. Cell viability was measured at each time point by WST-1 assay. (**a**) B95a cells infected with rMV-SLAMblind or rMV at an MOI of 1. (**b**) Breast cancer cells infected with rMV-SLAMblind at an MOI of 1. (**c**–**e**) Breast cancer cells infected with rMV-SLAMblind or rMV-Edmonston at an MOI of 0.1. Error bars indicate s.e. $^*P<0.05$ versus rMV-SLAMblind.

measles including anorexia, diarrhea and rash were observed in any of the monkeys (Figure 7a). No meaningful effects on body weight were seen (Figure 7b). Virus levels in peripheral blood mononuclear cells (PBMCs) were below the lower detection limits (Figure 7a) and lymphocyte numbers were not decreased after virus inoculation (Figure 7c). Although a transient increase in neutrophil number was observed at 0 d.p.i. in the cynomolgus monkey, it was regarded as irrelevant because the blood had been collected before virus inoculation. These results are in contrast to those reported for the parental wild-type MV HL strain and other wild-type MV strains, which cause typical clinical symptoms of measles, viremia and lymphopenia in infected monkeys.[22,26–29] These results show that rMV-SLAMblind is attenuated *in vivo*.

DISCUSSION

To develop an oncolytic MV for potential clinical use, we generated rMV-SLAMblind, and demonstrated that it was attenuated in monkeys. Previous studies of patients and infected monkeys have indicated that the distribution of SLAM is well correlated with sites of MV spread *in vivo*,[30] and SLAM-positive T and B lymphocytes are the major target of MV.[31] It has been suggested that most MV pathology, including lymphopenia and immunosuppression, can be explained by the utilization of SLAM by MV. Therefore, a recombinant MV unable to use SLAM would be attenuated. This prediction was confirmed by the result that no clinical evidence of disease was documented in monkeys infected with rMV-SLAMblind (Figure 7), proving that efficient SLAM recognition was necessary for virulence. It has previously been reported that a recombinant wild-type MV IC-B strain, with an R533A substitution introduced into the H protein, was attenuated in rhesus monkeys.[32] The SLAM-blind IC-B virus did not cause measles-like symptoms in any of the six inoculated monkeys, although low-level viremia was detected in only one. These data may support our results because the IC-B strain genome has a >99.7% nucleotide homology with that of the HL strain. Moreover, we note that rMV-SLAMblind has two nucleotide mutations introduced to change an arginine to alanine. Thus, it is considered to be a safer virus than those with single nucleotide mutations.

Our data show that both rMV-SLAMblind and the parental MV HL strain (rMV) use PVRL4 and not CD46 as a receptor. The MV HL strain is a wild-type strain isolated from blood leukocytes of a measles patient using marmoset lymphoblastoid B95a cells, in which it was propagated. Thus, it is consistent with previous findings that wild-type MV strains cannot use CD46 as a receptor,[30] but can use PVRL4.[14,15] CD46 is frequently overexpressed in tumors[33] and MV Edmonston vaccine strain derivatives preferentially kills cells with high CD46 density.[34] Therefore, it has been investigated as a therapeutic agent against various types of cancer. However, our results indicated that the use of CD46 was not required for the oncolytic activity of

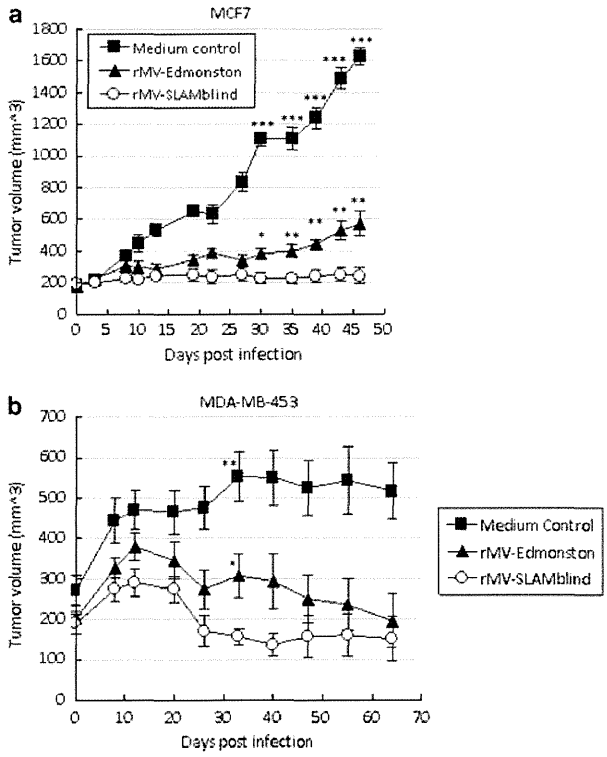

Figure 5. *In vivo* oncolytic activity of rMV-SLAMblind. Tumor growth curves of subcutaneous MCF7 xenografts (**a**) and MDA-MB-453 xenografts (**b**) intratumorally injected with rMV-SLAMblind, rMV-Edmonston or control medium. Error bars indicate s.e. $*P<0.05$, $**P<0.01$, $***P<0.00001$ versus rMV-SLAMblind.

rMV-SLAMblind against the breast cancer cell lines. In fact, CD46 is ubiquitously expressed by all normal human cells except erythrocytes,[35] and an MV Edmonston strain previously used for oncolysis was reported to efficiently infect and kill NHDFs,[24] producing syncytia. In contrast, rMV-SLAMblind infected them minimally (Figure 3). Therefore, rMV-SLAMblind, which is both SLAM- and CD46-blind, may have an important advantage in terms of improved specificity for PVRL4-positive breast cancer cells. Moreover, the previously reported MV Edmonston strain for breast cancer treatment[7] has an unmodified H protein, which can interact with all known MV receptors: SLAM, CD46 and PVRL4.

Previous studies have shown that PVRL4 is highly expressed in tumors of breast origin but scarcely expressed in normal tissues except placenta.[16,20,36] In breast tumor samples, PVRL4 expression was shown to be negatively correlated with luminal-like markers and positively with basal-like markers and HER2.[20,36] In addition, increased PVRL4 expression was strongly correlated with increased grade, increased tumor size, increased lymph nodes infiltration and reduced survival.[36] Thus, it is possible that rMV-SLAMblind could be effective against aggressive types of breast cancer. Furthermore, because PVRL4 has been shown to be upregulated in lung and ovarian cancers,[18,19] rMV-SLAMblind may also be useful for their treatment.

The rMV-SLAMblind showed greater oncolytic activity than did rMV-Edmonston. In MDA-MB-453 cells, oncolytic activity of rMV-SLAMblind was greater *in vitro* and *in vivo* (Figures 4d and 5b). However, in MCF7 cells, rMV-SLAMblind showed *in vitro* cytotoxicity similar to that of rMV-Edmonston, but was more effective for tumor growth suppression *in vivo* (Figures 4c and 5a). This might have been due to differences between the *in vitro* culture conditions and the *in vivo* environment, where factors such as stromal architecture and surrounding innate immune system could limit virus spread. Previous comparative studies between wild-type and Edmonston vaccine strains have indicated important properties of wild-type MVs, including evasion of the intracellular innate immune response[37,38] and enhanced virus replication.[39] These factors could account for the enhanced oncolytic activity of rMV-SLAMblind. Analyses of the underlying mechanisms are currently ongoing.

The rMV-Edmonston used in this study was not identical to the Edmonston strain derivative previously used for breast cancer treatment (MV-CEA).[7] MV-CEA had an additional transcription unit encoding carcinoembryonic antigen (CEA) as a trackable marker of viral gene expression. In addition, we found that the plasmid p(+)MV2A, from which we rescued rMV-Edmonston, had 12 nucleotide and 5 amino-acid differences from the reported sequence data for the infectious complementary DNA (cDNA) clone of the Edmonston B strain (GenBank accession no. Z66517) (see Materials and methods for details). Therefore, in terms of oncolytic activity we cannot directly compare our data with those of MV-CEA. However, these differences do not affect our conclusion that rMV-SLAMblind has improved tumor specificity, because all strains of the Edmonston lineage use CD46.

A major obstacle for MV-based vectors is the presence of pre-existing anti-MV antibodies in patients, which can reduce therapeutic efficacy. A previous study has demonstrated that, when administered intratumorally, the efficacy of oncolytic MV is not compromised by the presence of passively transferred anti-MV antibody in a mouse model.[40] This means that, once virus reaches the tumor, regression may take place even in the presence of antibodies. Although the final goal is to eradicate metastasized cancer cells by intravenous virus administration, the treatment of localized tumor with intratumoral virus administration is a logical first step. We are now exploring strategies to evade neutralization of intravenously administered virus by preexisting antibodies. One possible strategy is to hide virus antigens from antibodies by using cells as delivery vehicles.[41] In this strategy, the body's cells are infected *in vitro* and then administered back systemically, which would carry the oncolytic virus to target tumor cells. Recent studies have shown that cells infected with oncolytic MV can deliver viruses to tumor sites and prolong survival of tumor-bearing mice with pre-existing anti-measles antibodies.[42,43]

In a previous study, virus replication and fusogenic activity in PVRL4-expressing cells was enhanced by introducing a single N481F or N481Y substitution into the H protein of a wild-type IC-B stain, without affecting SLAM or CD46-dependent cell–cell fusion.[44] The H protein of HL strain also has asparagine at position 481. Thus, by introducing the same substitution, we can probably enhance the oncolytic activity of rMV-SLAMblind without affecting its receptor usage. Moreover, H proteins of MV strains of the Edmonston lineage already have tyrosine at position 481, and thus this strategy is not applicable.

In conclusion, we have generated rMV-SLAMblind and demonstrated that it had oncolytic activity against breast cancer xenografts and was attenuated in monkeys. The rMV-SLAMblind did not interact with CD46 and showed greater oncolytic activity than that of rMV-Edmonston. These results point to the potential of rMV-SLAMblind as a novel oncolytic virus for breast cancer treatment, which warrants further investigation.

MATERIALS AND METHODS

Cells

B95a, CHO-K1, Vero and 293 cells have been described previously.[45,46] SKBR3 and MCF7 human breast cancer cells (obtained from the Cell Resource Center for the Biomedical Research Institute of Development, Aging and Cancer, Tohoku University, Miyagi, Japan) were maintained in RPMI medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (FBS). MDA-MB-453 human breast cancer cells (ATCC, Manassas, VA, USA) were maintained in L-15 medium (Sigma-Aldrich,