

Partial Deletion of Glycoprotein B5R Enhances Vaccinia Virus Neutralization Escape while Preserving Oncolytic Function

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Vaccinia virus (VV) has been utilized in oncolytic virotherapy, but it risks a host antiviral immune response. VV has an extracellular enveloped virus (EEV) form consisting of a normal virion covered with a host-derived outer membrane that enables its spread via circulation while evading host immune mechanisms. However, the immune resistance of EEV is only partial, owing to expression of the surface protein B5R, which has four short consensus repeat (SCR) domains that are targeted by host immune factors. To engineer a more effective virus for oncolytic virotherapy, we developed an enhanced immune-evading oncolytic VV by removing the SCRs from the attenuated strain LC16mO. Although deletion of only the SCRs preserved viral replication, progeny production, and oncolytic activity, deletion of whole B5R led to attenuation of the virus. Importantly, SCR-deleted EEV had higher neutralization resistance than did B5R-wild-type EEV against VV-immunized animal serum; moreover, it retained oncolytic function, thereby prolonging the survival of tumor-bearing mice treated with anti-VV antibody. These results demonstrate that partial SCR deletion increases neutralization escape without affecting the oncolytic potency of VV, making it useful for the treatment of tumors under the anti-virus antibody existence.

INTRODUCTION

Oncolytic virotherapy is a novel anti-cancer strategy that has shown promising clinical results in the U.S. Food and Drug Administration-approved talimogene laherparepvec. Several viruses have been engineered as next-generation oncolytic agents with their functionalities enhanced via viral recombination,^{1–3} loading of therapeutic factors,⁴ or combination with conventional therapies.^{4,5} However, oncolytic viruses elicit antiviral immune responses in the host that significantly limit their therapeutic potential, especially in reaching remote tumors.^{6–8}

Vaccinia virus (VV) can escape antiviral immunity using a unique infectious form—namely, the extracellular enveloped virus (EEV).⁹

Most progeny virions mature as the normal infectious form, intracellular mature virus (IMV), and are released after host cell death; however, a few are covered with or wrapped in early endosomes or a *trans*-Golgi network, yielding an intracellular enveloped virus (IEV) that is exposed on the cell surface (referred to as a cell-associated virus) via membrane fusion before being released as an EEV through actin polymerization.⁹ EEVs are far less abundant than IMVs, representing <1% of total infectious virions;¹⁰ however, they have advantages in viral dissemination, such as rapid spreading without causing host cell death^{9,11} and efficient entry into target cells independent of cell-signaling mechanisms.^{12,13} In addition, the EEV outer membrane presents host-derived antigens, such as complement control proteins (e.g., cluster of differentiation [CD]46, CD55, and CD59) and major histocompatibility complex class I,^{14,15} which allow the virus to spread systemically via circulation while escaping neutralization by complement factors or anti-VV antibodies.^{15,16}

Some engineered VV strains produce large amounts of EEV (e.g., International Health Department (IHD)-J, vA34R, or WI) due to a point mutation in glycoprotein A34R,¹⁷ making them suitable for oncolytic virotherapy.^{2,3} However, EEV has only partial immune resistance, although it is higher than that of IMV. Of the five EEV outer membrane surface antigens (A33R, A34R, A36R, A56R, and B5R), B5R is the most frequent target of host-neutralizing antibodies.^{18,19} B5R is a 42-kDa envelope glycoprotein containing four short consensus repeat (SCR) domains, whose expression is critical for viral morphogenesis, trafficking, dissemination, and EEV production.^{20–25} However, anti-B5R antibody recognizing the SCR1/SCR2 boundary and/or the B5R stalk region²⁶ induces complement-dependent EEV neutralization.^{27–29}

Received 1 December 2018; accepted 9 May 2019;
<https://doi.org/10.1016/j.omto.2019.05.003>.

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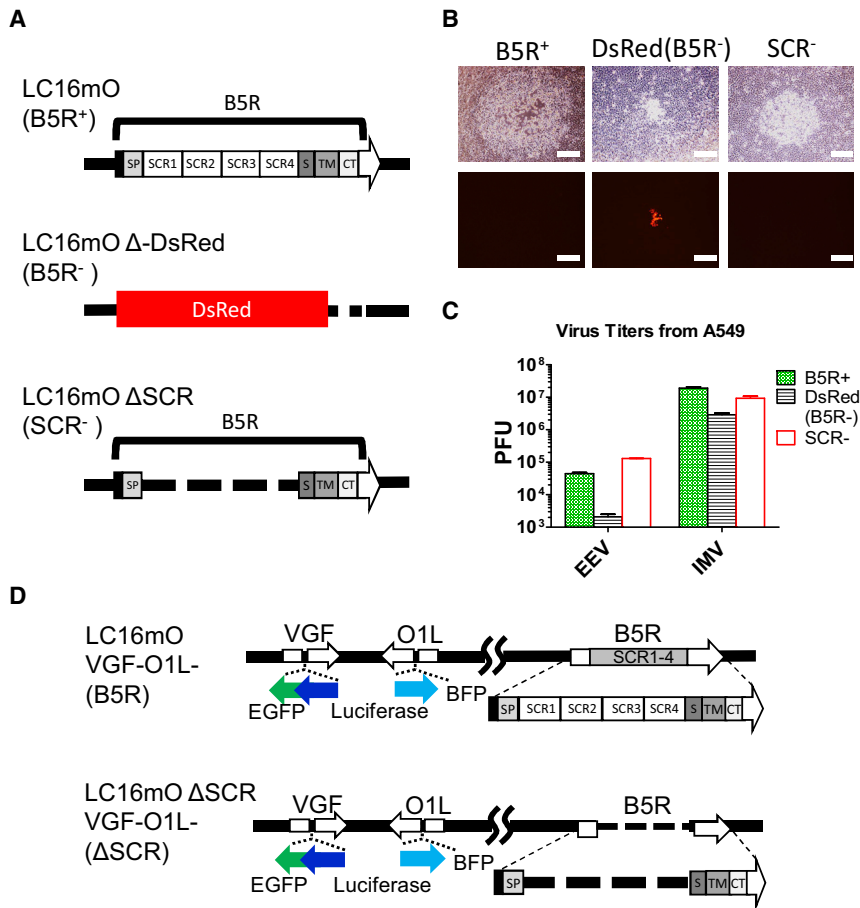


Figure 1. Phenotypic Differences among B5R Recombinant Viruses

(A) Schematic representation of the B5R recombinant VVs LC16mO (B5R⁺), LC16mO Δ -DsRed (B5R⁻), and LC16mO Δ SCR (SCR⁻). (B) RK13 cells were infected with the three B5R recombinant viruses, and viral plaques were photographed 48 h later. Top shows phase-contrast images, and bottom shows fluorescence micrographs of the same fields. Scale bar, 500 μ m. (C) EEV and IMV were recovered from culture supernatants or lysates of A549 lung carcinoma cells infected with the three B5R recombinant viruses at a MOI of 0.1, and they were individually titrated in RK13 cells. Total PFU was calculated from plaque numbers and total volume of viral reagent. Data represent mean \pm SD (n = 3). (D) Schematic representation of the VGF- and O1-deleted virus B5R LC16mO VGF⁻/O1⁻ (B5R) and SCR-deleted virus LC16mO Δ SCR VGF⁻/O1⁻ (Δ SCR).

sults demonstrate that Δ SCR can be an effective agent for cancer treatment, including in patients with pre-existing neutralizing antibodies.

RESULTS

B5R Deletion Affects Vaccinia Viral Phenotype

The effects of B5R mutation were evaluated using three recombinant VVs, namely, B5R-wild-type (WT) LC16mO, B5R-deleted LC16mO Δ -DsRed, and SCR-deleted LC16mO Δ SCR (Figure 1A). B5R deletion affected not only viral pathogenicity but also phenotype, including plaque formation and progeny virus production. The reduction in viral plaque size was proportional to the extent of B5R deletion, with LC16mO Δ SCR generating plaques of an intermediate size between the normal-sized LC16mO plaques and smaller LC16mO Δ -DsRed plaques (Figure 1B). Progeny virus production was markedly reduced by complete deletion of B5R, but not SCR. LC16mO Δ -DsRed showed decreased EEV and IMV production compared to that of LC16mO (Figure 1C); in contrast, progeny virus production was mostly unaltered for LC16mO Δ SCR, but EEV production in A549 lung carcinoma cells was increased.

SCR Deletion Alters EEV Generation without Reducing Normal Progeny Virus Production and Oncolytic Activity

Progeny virus production by B5R-WT and SCR-deleted viruses was evaluated in various human cancer cell lines. VGF and O1 were deleted in both viruses to confer tumor-specific viral replication (Figure 1D), yielding B5R-WT LC16mO VGF⁻/O1⁻ and SCR-deleted LC16mO Δ SCR VGF⁻/O1⁻, which are hereafter referred to as B5R and Δ SCR, respectively. Deletions were achieved by inserting a gene cassette, harboring the firefly luciferase gene fused to EGFP- or blue fluorescent protein (BFP)-encoding genes, into each locus. B5R gene expression was confirmed from those VGF⁻/O1⁻ viruses by RT-PCR (Figure S1A).

Whole B5R deletion drastically reduces viral plaque size, pathogenicity, actin polymerization, and EEV production.^{21,24,25,30} In contrast, partial SCR deletion causes a variety of viral phenotypes, including a mild reduction in plaque size²² and comet-shaped spreading similar to that seen in IHD-J.^{18,22,23,31} Interestingly, SCR deletion reportedly increases EEV production in rabbit kidney RK13 cells,^{22,23} despite the loss of actin polymerization.^{22–24,31} Since SCR domains are responsible for EEV neutralization, their deletion may allow the virus to evade anti-B5R antibody¹⁸ without affecting its capacity for replication or oncolytic activity.

To examine the above possibility, we reinforced an immune-evasive form of oncolytic VV by partially deleting the SCRs. This Δ SCR virus was structured from the low-neurovirulent LC16mO strain isolated from the Lister strain through repeated passaging and selection for temperature sensitivity.^{32,33} Tumor specificity was conferred by limiting viral replication in normal cells via deletion of the two viral mitogen-activated protein kinase-dependent growth factors VV growth factor (VGF) and O1.³⁴ The resultant Δ SCR EEV showed enhanced resistance to anti-VV-neutralizing serum and antibody (Ab) compared to that of VV with intact B5R, while replication, progeny virus production, and oncolytic potency were unaffected. Our re-

Table 1. Ratio of B5R and Δ SCR Progeny Virus Production

Tumor Cell Line		EEV Ratio (Δ SCR:B5R)	IMV Ratio (Δ SCR:B5R)
Type	Name		
Ovarian	A2780	6.333**	1.523
	CaOV3	7.159***	1.657*
	RMG-1	1.825**	0.75
	SKOV3	1.835*	1.161
	AsPC1	0.969	0.837
Pancreatic	BxPC3	0.091***	0.551
	Panc1	0.418**	0.249**
	SW1990	0.952	0.636
	Caco2	0.992	0.968
Colon	HT29	0.229**	0.279**
	LoVo	2.719*	0.654
	SW480	0.254*	0.961
	A549	4.634***	0.425**
Breast	MCF-7	0.913	1.217
	SK-BR-3	1.223	0.664
Hepatocellular	HepG2	3.164**	1.242
Neuroblastoma	SK-N-AS	0.766	0.621
	SK-N-BE	0.335	0.676
Epidermoid	A431	1.132	1.198
	Hep2	0.436*	1.267

Various tumor cell lines were infected with B5R or Δ SCR at an MOI of 0.1. EEVs and IMVs were recovered from the cultures 48 h later, and they were titrated in RK13 cells as described in Figure 1C. The table shows the progeny virus productive ratio of Δ SCR to B5R. Titers are listed in Figure S2. *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t test).

EEV and IMV were recovered from various human cancer cell lines infected with B5R or Δ SCR at an MOI of 0.1, and they were titrated in RK13 cells. EEV or IMV phenotype was distinguished concisely by detecting host cellular CD46 and viral D8L. Both EEV and IMV expressed the viral D8L protein, but host cellular CD46 was presented only in EEV covered with host-derived membrane (Figure S1B). EEV production by B5R and Δ SCR viruses differed in the various human cancer cell lines (Table 1; Figure S2); Δ SCR increased EEV production in all ovarian carcinoma cell lines (A2780, CaOV3, RMG-1, and SKOV3), LoVo colon carcinoma cells, HepG2 hepatocellular carcinoma cells, and A549 cells, but it decreased production in BxPC3 and Panc1 pancreatic cancer cells, HT29 and SW480 colon carcinoma cells, and Hep2 laryngeal carcinoma cells (all p < 0.05). On the other hand, most cell lines showed no differences between IMV production by B5R and Δ SCR viruses, with only four cell lines showing statistically significant differences.

The oncolytic activity of B5R and Δ SCR was compared in human ovarian cancer cell lines, which tend to have high EEV production. Cells were separately infected with EEV from culture supernatant or with whole virus including IMV, and cell viability was evaluated. Viral infection area increased in A2780 and CaOV3 cells upon infec-

tion with Δ SCR-derived EEV (Figure 2A), with a concomitant decrease in viability as compared to cells treated with B5R-derived EEV (Figure 2B). There were no differences in infection area or viability between RMG-1 and SKOV3 ovarian carcinoma cells (Figures 2A and 2B). On the other hand, viral expansion and viability were comparable between B5R and Δ SCR following whole-virus infection (Figures 2C and 2D). These results are consistent with the observed IMV productivity of each virus (Table 1; Figure S2B).

SCR-Deleted EEV Escapes Neutralization by Immunized Serum

Next, to compare the neutralization resistance of EEVs derived from B5R and Δ SCR, we explored anti-B5R Ab (an EEV-neutralizing Ab) from human serum samples. Derived from patients who received smallpox vaccinations, 20 human serum samples were used to calculate 50% neutralization doses (ND₅₀) against vaccinia virus (Table S1). Five of 20 samples showed neutralizing activity, but titers were undetectable in the other 15. The 5 serum samples, 221 (ND₅₀: not detected), 183, 195, 175, and 214 (ND₅₀: 16.09, 50.26, 53.55, and 114.77, respectively), were chosen for detecting the Ab response against whole VV and B5R by ELISA.

The results showed almost no anti-VV and anti-B5R antibodies even in the sample with the highest ND₅₀, 214 (Figures 3A and 3B). This suggests that most patients have no pre-existing antibodies against VV (including its EEV form). Therefore, artificially immunized animal serum was used to explore anti-EEV Abs. The ND₅₀ and ELISA titer were measured in serum derived from cynomolgus monkeys treated with three different doses of VV (high dose = 10⁸ plaque-forming units [PFU], low dose = 10⁷ PFU, and mock = 0 PFU) (Table S2; Figures 3C and 3D). In immunized serum (non-mock treated), neutralization activity and antibody response against whole VV and B5R increased as a function of viral dose (Table S2; Figures 3C and 3D). Thus, the serum had potential for EEV neutralization.

To compare neutralization resistance, monkey serum samples were mixed with progeny virions from B5R or Δ SCR, and GFP expression and cytotoxicity of the escaped viruses were evaluated. Half or nearly all B5R EEV was neutralized by 1% high (001) or low (006) VV dose-immunized serum. In contrast, Δ SCR EEV escaped neutralization at the same concentration of immunized serum (Figure 4A). Quantification of GFP fluorescence revealed that Δ SCR EEV had higher replicative capacity than did B5R EEV, especially when mixed with 0.5% or 1% immunized serum (Figure 4B). Furthermore, cell viability was inversely proportional to viral GFP expression, and it was lower for Δ SCR EEV mixed with 0.5% or 1% high or low VV dose-immunized serum than for B5R EEV (Figure 4C). In IMV neutralization, B5R and Δ SCR were inhibited by almost comparable concentrations of immunized serum (Figure 4D). These findings are consistent with observed trends for viral replication and oncolytic activity; that is, B5R and Δ SCR IMVs showed similar variations in GFP expression (Figure 4E) and infected cell viability (Figure 4F). Thus, SCR deletion affects EEV, but not IMV, and Δ SCR-derived

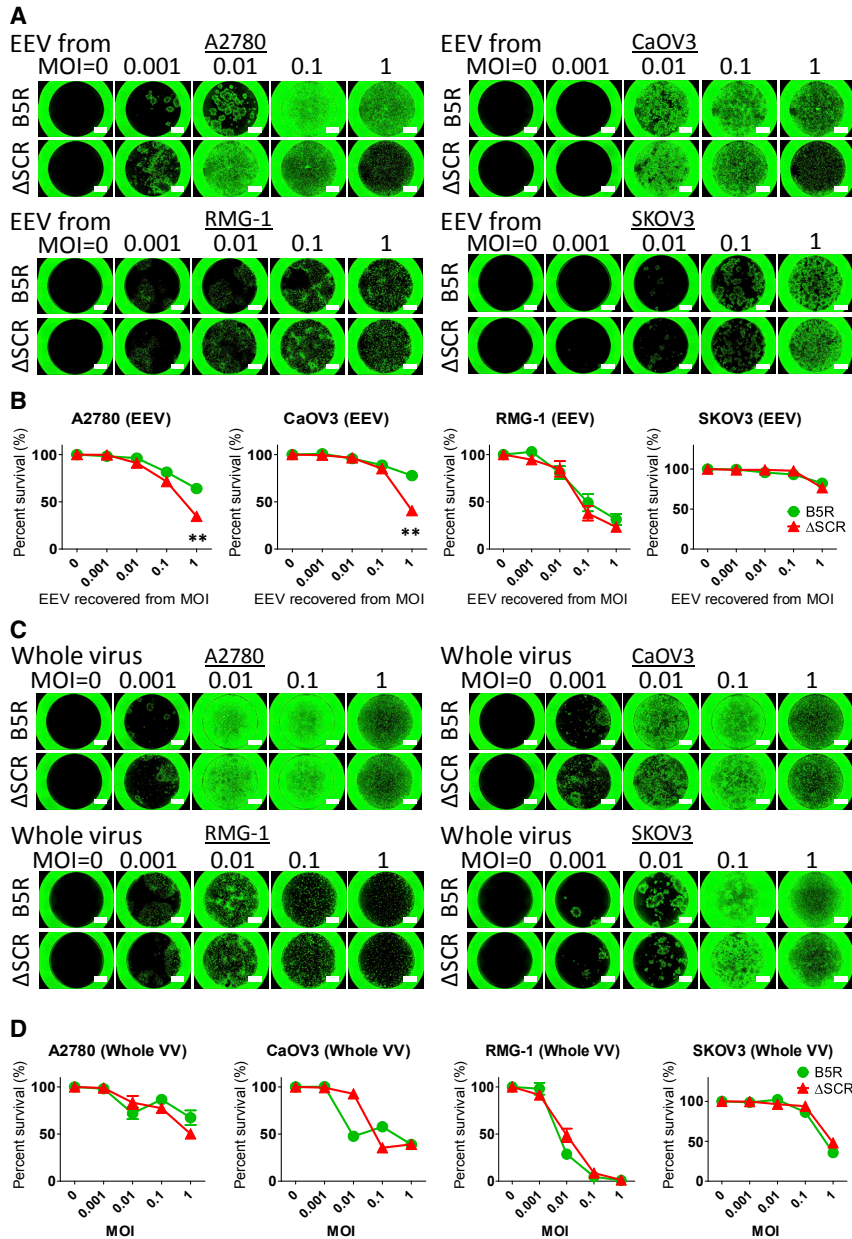


Figure 2. Oncolytic Activity of EEV or IMV Derived from SCR-Deleted VV against Ovarian Cancer Cell Lines

(A) Human ovarian cancer cell lines were treated with EEV from culture medium recovered from ovarian cancer cells infected with B5R or Δ SCR at an MOI of 0, 0.001, 0.01, 0.1, or 1, and they were photographed under a fluorescence microscope 5 days later. Scale bars, 1,500 μ m. (B) Viability of ovarian cancer cells described in (A) after EEV infection. Data are presented as percent survival of mock-infected cells. ** $p < 0.01$ (unpaired t test). (C) Human ovarian cancer cell lines were infected with whole-B5R or Δ SCR virus (IMV) at an MOI of 0, 0.001, 0.01, 0.1, or 1, and they were photographed as described in (A). (D) Viability of IMV-infected ovarian cancer cells shown in (C), evaluated as described in (B). Data in (B) and (D) are presented as means \pm SD ($n = 3$).

was observed by fluorescence microscopy. B5R EEV showed slight resistance to anti-virus antibody and complement alone, but it was strongly suppressed by the combination of $>0.2\%$ antibody and 3% complement (Figure 5A). Δ SCR EEV showed higher immune resistance than did B5R EEV, escaping mixtures of up to 0.5% antibody and 10% complement. Accordingly, Δ SCR EEV showed higher GFP than B5R expression when mixed with antibody and complement (Figure 5B). Similar results were obtained using RMG-1 (Figure S3A) and A2780 (data not shown) cells as EEV-producing and -infecting cells, respectively. Δ SCR EEV decreased the viability of RMG-1 cells more than did B5R EEV when mixed with antibody and 3%–10% complement (Figure S3B).

Neither IMV escaped anti-virus antibody and complement alone or in combination (Figure 5C). Both B5R and Δ SCR IMVs were strongly inhibited by $>0.2\%$ antibody and/or 3% complement. There was no difference in

EEV has higher neutralization resistance than does B5R EEV against whole serum derived from immunized monkeys.

SCR-Deleted VV Escapes Complement-Dependent EEV Neutralization

EEV is neutralized by anti-B5R antibody combined with complement,^{27–29} we therefore mixed EEV and IMV derived from B5R and Δ SCR with rabbit anti-VV antibody (immunoglobulin G [IgG] fraction, anti-B5R antibody was detected by ELISA; data not shown) and rabbit complement separately. These mixtures were used to infect SKOV3 cells, and the replication of active virus

viral GFP expression between B5R and Δ SCR IMVs (Figure 5D). These results suggest that EEV neutralization depends on the combination of antibody and complement, and SCR-deleted VV efficiently escapes their neutralizing activity.

SCR Deletion Enhances the Oncolytic Activity of EEV in the Presence of Anti-virus Antibody

The neutralization resistance and oncolytic activity of EEVs were examined in a mouse model of anti-VV antibody-induced peritoneal dissemination. Athymic nude mice bearing A2780 ovarian cancer cells were injected with anti-VV antibody (IgG fraction) derived

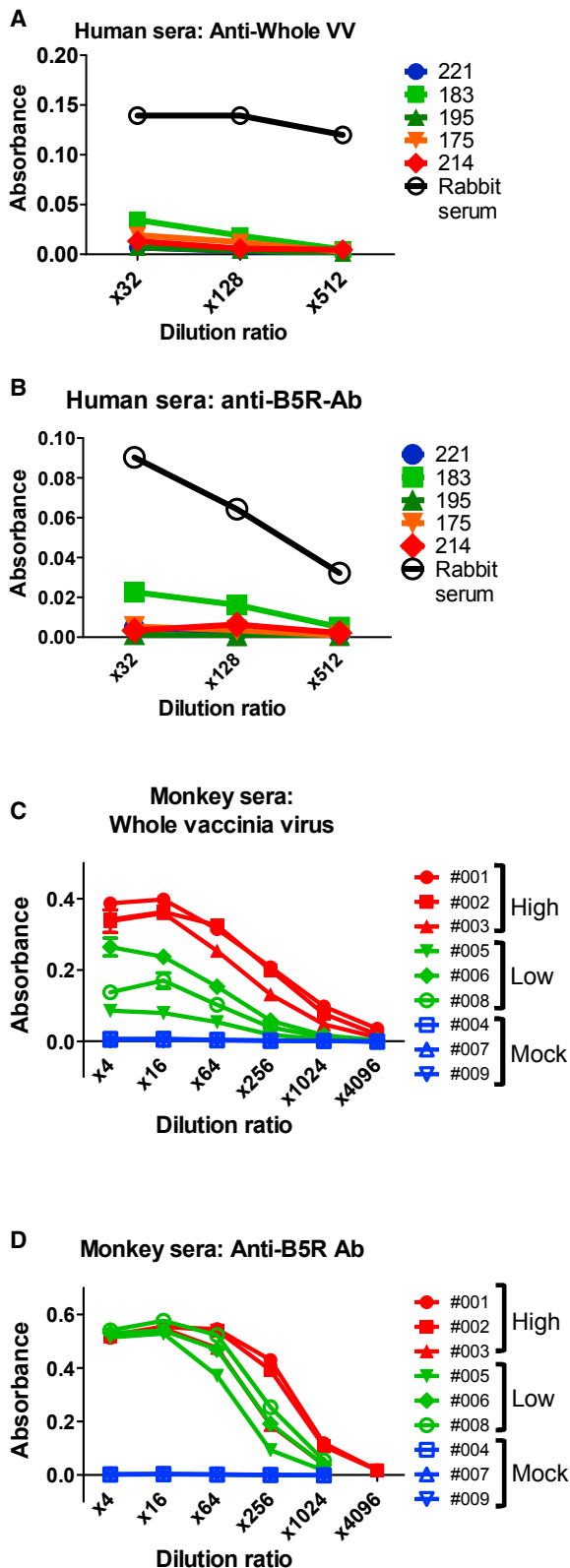


Figure 3. Detection of Anti-VV and -B5R Antibody in Serum

(A) Whole-VV antigen-coated wells were incubated with 4-fold serial dilutions ($\times 32$, $\times 128$, $\times 512$) of 5 human sera (patients 221, 183, 195, 175, and 214) or control immunized rabbit IgG. Antibody response was detected by measuring optical density at 405 nm (OD_{405}) after incubation with horseradish peroxidase-conjugated rabbit anti-human IgG (SouthernBiotech) and substrate. (B) Purified B5R protein-coated wells were incubated with the same serial dilutions of human serum and secondary antibody shown in (A), and antibody response was detected as described above. (C) Whole-VV antigen-coated wells were incubated with 4-fold serial dilutions ($\times 4$ – $4,096$) of serum from monkeys immunized with high (001, 002, and 003) or low (005, 006, and 008) doses of VV or from mock-immunized monkeys (004, 007, and 009). Antibody response was detected after incubation with horseradish peroxidase-conjugated goat anti-monkey IgG (Abcam) and substrate. (D) Purified B5R protein-coated wells were incubated with the same serial dilutions of monkey serum shown in (C), and antibody response was detected as described above. Data in (A)–(D) are presented as means \pm SD ($n = 2$).

from immunized rabbit or PBS, followed by intraperitoneal injection of B5R or Δ SCR EEV. Viral replication and tumor growth were evaluated based on firefly luciferase (Fluc) and *Renilla* luciferase (Rluc) expression, respectively.

Fluc luminescence reflecting viral distribution was reduced by antibody pretreatment on day 3 after virus injection; however, on day 7, Δ SCR EEV showed higher replication than did B5R EEV in the presence of antibody (Figure 6A, middle). On the other hand, the level of Rluc luminescence in tumors, which reflected tumor growth, was similar among mice before virus injection (Figure 6A, top), but it disappeared on day 8 after injection upon the administration of either B5R or Δ SCR EEV without antibody pretreatment. In contrast, in the presence of antibody, tumors disappeared in half of Δ SCR EEV-treated mice, while they only disappeared in a single B5R EEV-treated mouse (Figure 6A, bottom).

Quantitative analysis of viral Fluc luminescence revealed that the replication kinetics of both EEVs were similar in the absence of antibody (Figure 6B). When antibody was present, the peak intensity of viral Fluc signals was delayed 2 days by Δ SCR EEV and 5 days by B5R EEV (Figure 6B). Δ SCR EEV showed higher replication than did B5R EEV on day 7 after virus injection with antibody pretreatment ($p < 0.05$). Quantification of tumor Rluc luminescence indicated that the signal in mice without antibody treatment was almost completely abolished by B5R and Δ SCR EEVs. In contrast, Δ SCR EEV suppressed tumor signals in antibody-treated mice to levels in mice without antibody treatment, whereas the anti-cancer effect of B5R EEV was strongly inhibited by antibody treatment (Figure 6C). The survival of mice was prolonged by injection with B5R and Δ SCR EEVs without anti-VV antibody treatment as compared to survival after mock treatment (Figure 6D). Meanwhile, Δ SCR EEV prolonged the survival of mice with and without antibody treatment, although antibody treatment reduced survival in B5R EEV-treated mice (Figure 6D). The log rank test showed that the B5R EEV-antibody combination prolonged survival compared to that in mice treated with PBS-antibody ($p = 0.0041$); however, Δ SCR EEV-antibody had an even greater effect ($p = 0.0291$). Thus, B5R and Δ SCR EEVs have comparable therapeutic efficacies in the absence of

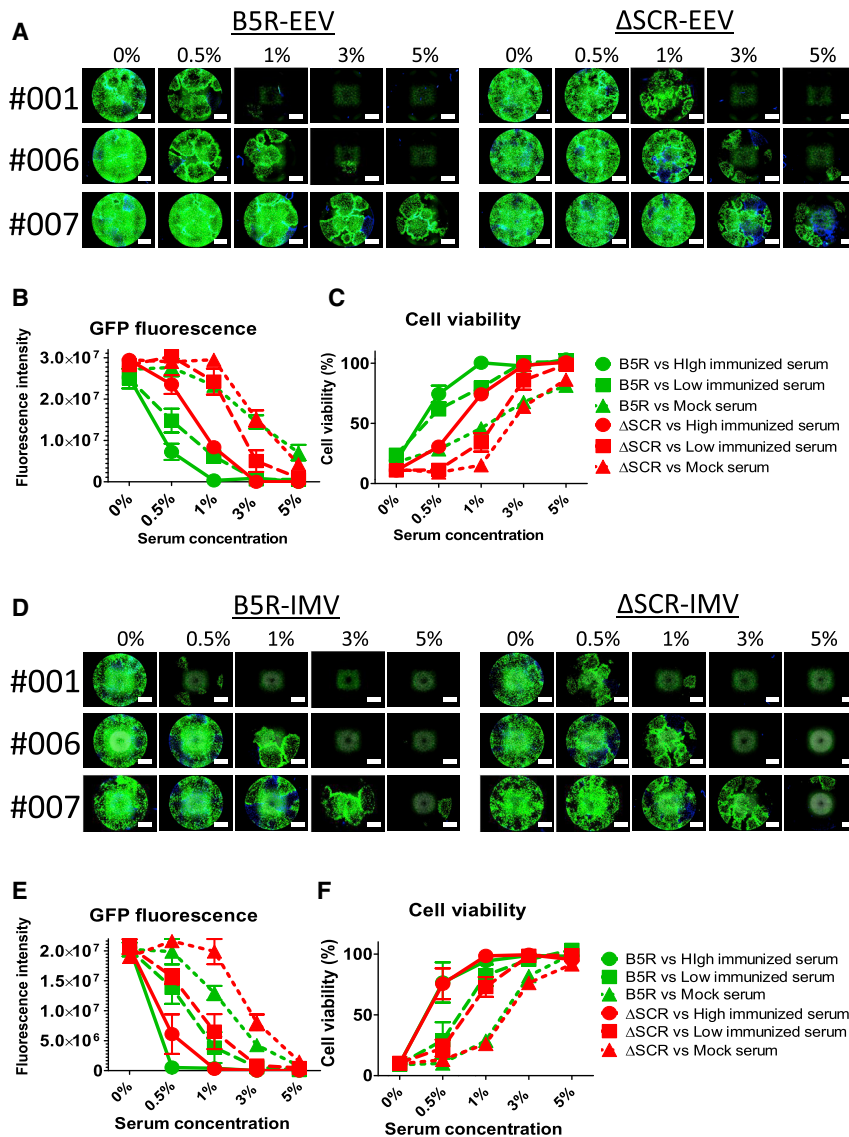


Figure 4. Neutralization Resistance of EEV or IMV against Immunized Monkey Serum

(A) EEV in the supernatant of RMG-1 cells infected with B5R or Δ SCR was mixed with 0%, 0.5%, 1%, 3%, and 5% serum from monkeys immunized with a high (001: 1×10^8 PFU by intravenous injection) or low (006: 1×10^7 PFU by intravenous injection) dose of VV or from a mock-immunized (007) monkey. The mixtures were used to infect RMG-1 cells, which were imaged by phase-contrast and fluorescence microscopy 120 h later. Scale bar, 1,500 μ m. (B) Quantification of GFP fluorescence shown in (A). (C) Viability of infected cells described in (A) expressed as percent cell survival of mock-infected cells. B5R and Δ SCR EEVs differed in terms of cytolytic activity when mixed with 0.5% (high dose, $p = 0.0042$ and low dose, $p = 0.0012$; unpaired t test) or 1% (high dose, $p = 0.0007$ and low dose, $p = 0.0044$; unpaired t test) serum from immunized monkeys. (D) IMV in the lysate of RMG-1 cells infected with B5R or Δ SCR was used to infect RMG-1 cells as described (A). Images were acquired 120 h later. (E) Quantification of GFP fluorescence in (D). (F) Viability of cells in (D). Data in (B), (C), (E), and (F) are presented as means \pm SD ($n = 3$).

potently inhibits viral delivery to remote organs,^{3,6-8} virus injection is basically limited to the intratumoral route. Although various strategies have been developed to escape antiviral immunity, such as carrier-cell therapy,^{5,7} lipid coating,³⁷ and immunosuppression,^{6-8,36} there are none based on viruses alone.

In this study, we enhanced the ability of an immune-resistant form of VV to escape host anti-virus antibody by partially deleting the antigenic SCR region of the viral glycoprotein B5R. Whole-B5R deletion significantly reduced viral plaque size as well as both EEV and IMV production (Figures 1B and 1C). SCR-deleted virus (Δ SCR) had an intermediate plaque size between those of whole-B5R deletion and WT viruses (Figure 1B), which is consistent with a previous report.²² However, SCR deletion had almost no effect on IMV production (Figure 1C; Figure S2B) or oncolytic activity compared to levels after infection by the WT virus (B5R) (Figures 2C and 2D), and it tended to increase EEV production, especially in ovarian cancer cell lines such as A2780 and CaOV3 (Table 1; Figure S2A), which showed decreased viability with increasing EEV titer (Figure 2B). These symptoms suggest the potential of EEV utilization because of the lower productivity of the WT virus.

The effect of Δ SCR on EEV production varied among tumor cell types, suggesting the influence of host cell factors on EEV morphogenetic processes, such as viral entry, transport, wrapping, exposure, or egress. In contrast, IMV formation was unaffected, as

anti-virus antibody, whereas Δ SCR EEV is more potent when antibody is present.

DISCUSSION

The balance of antitumor and antiviral immunity is the most important determinant of the efficacy of oncolytic virotherapy; the efficacy is enhanced by the former and inhibited by the latter. Antitumor immunity can be enhanced by several strategies, including the loading of immunotherapeutic agents (e.g., cytokines, chemokines, and co-stimulation molecules) and/or combined immune-checkpoint blockade.⁴ Pre-existing antiviral immunity does not necessarily inhibit the viral therapeutic effect, especially in the case of direct intratumoral injection.³⁵ However, there is evidence that the oncolytic effect of therapeutic viruses is enhanced by immunosuppression.^{6,8,36} Furthermore, since pre-existing immunity

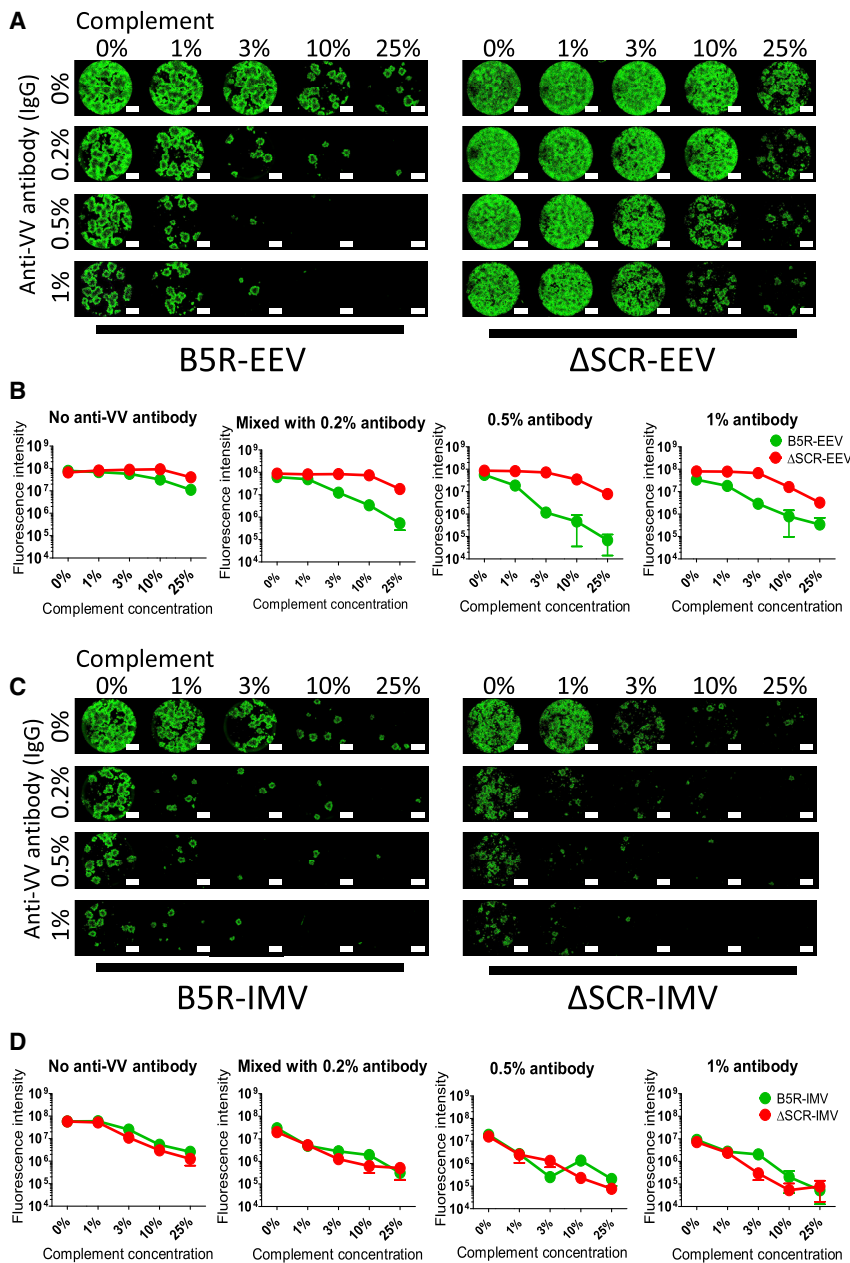


Figure 5. Neutralization Resistance of SCR-Deleted EEV or IMV against Anti-virus Antibody and Complement

(A) EEV in the culture supernatant of SKOV3 cells infected with B5R or ΔSCR was mixed with 0%, 0.2%, 0.5%, or 1% rabbit anti-VV-immunized serum (IgG fraction) and/or 0%, 1%, 3%, 10%, or 25% rabbit complement. The mixture was used to infect SKOV3 cells, which were photographed under a fluorescence microscope 96 h after infection. Scale bar, 1,500 μm. (B) Quantification of GFP fluorescence described in (A) as a function of serum (IgG) concentration. (C) IMV produced from lysates of SKOV3 cells infected with B5R or ΔSCR was mixed and used for SKOV3 cell infection as described (A). Images were acquired 96 h later. (D) Quantification of GFP fluorescence in (C). Data in (B) and (D) are presented as means ± SD (n = 3).

gress by detaching from the cell surface due to loss of interaction between the luminal domain of B5R and the plasma membrane, similar to SCR4-mutated (B5R^{P189S}) VV.⁴¹ Thus, cells with high EEV productivity may support viral wrapping and/or transport out of the cell in the case of ΔSCR.

There are several factors that may facilitate the processes described above. For example, RAB1A—a member of the Ras oncogene family—mediates trafficking of matured virions to the wrapping site.⁴² During the wrapping process, VV utilizes retrograde transport factors in the Golgi-associated transport pathway, such as syntaxin 6 and vacuolar protein sorting 52 homolog.^{43,44} In addition, wrapped virions (IEV) employ microtubule transport to reach the cell membrane through recruitment of kinesin-1 by the IEV-associated protein A36R.^{45–47} The kinesin-1 component KIF5B is associated with lung cancer prognosis,⁴⁸ and other kinesin family members have also been implicated in the progression of various malignancies, including ovarian cancer.^{49–51} Microtubule dynamics are important not only to tumor growth

evidenced by the comparable levels of IMV productivity of B5R and ΔSCR. B5R regulates several aspects of morphogenesis; however, EEV entry requires an intact B5R stalk region,³⁸ while mature virion transfer and accumulation in the wrapping region depend on the transmembrane domain and cytoplasmic tail of B5R, respectively, but not on the SCRs.³⁹ A major function of the SCRs is to mediate switching from microtubule transport to actin-based motility during EEV exposure at the cell surface.⁴⁰ SCR4 is the region responsible for this activity, but the ΔSCR virus maintained or increased EEV productivity in various tumor cell lines (>70% of cells, as shown in Table 1). ΔSCR EEV may readily

but also to drug resistance, especially to those used to treat ovarian cancers, as these mainly target the tumor cell cytoskeleton.^{52,53}

As for preexisting anti-virus antibodies, we explored anti-VV and -B5R (EEV-specific) antibodies from human and immunized monkey serum. Interestingly, there were almost no VV- or B5R-specific antibodies in the vaccinated human serum (Figures 3A and 3B). This result is consistent with past clinical reports of systemic VV treatment, which resulted in comparable therapeutic effects regardless of base neutralizing titer or vaccination history.^{54,55} This suggests that most patients have few pre-existing antibodies that neutralize the

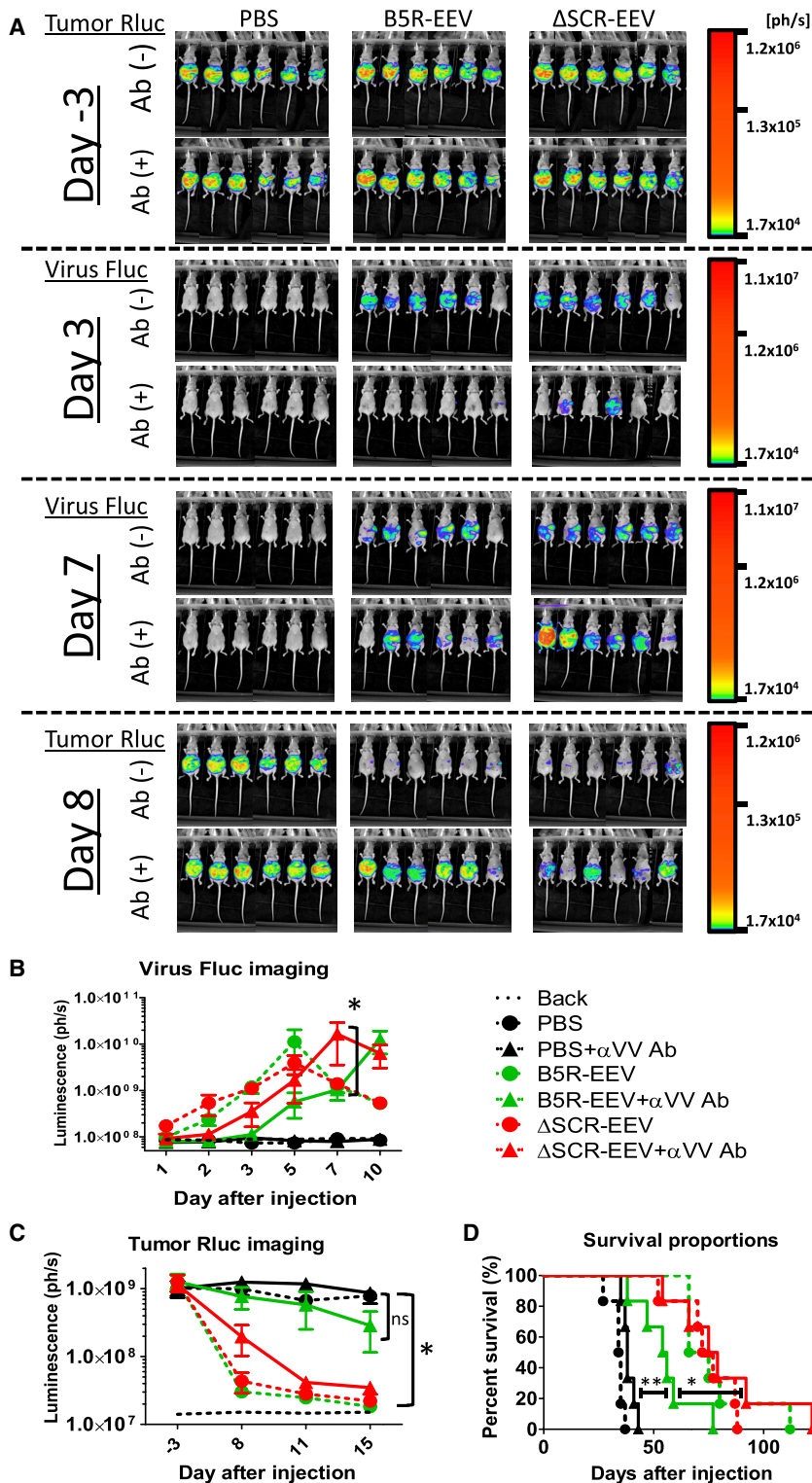


Figure 6. SCR-Deleted EEV Maintains Oncolytic Activity *In Vivo* in the Presence of Vaccinia-Neutralizing Antibodies

(A) *In vivo* bioluminescence imaging of tumor burden and viral replication. Athymic nude mice with peritoneal dissemination of A2780 cells expressing Rluc were inoculated with 100 μ L rabbit anti-VV-immunized serum (IgG fraction) before intraperitoneal injection of B5R EEV or Δ SCR EEV derived from the supernatant of infected A2780 cells. Tumor Rluc was detected on days -3 and 8, and viral Fluc was detected on days 3 and 7 after virus injection (n = 6). (B) Quantification of viral Fluc luminescence after virus injection. Solid and dashed lines represent serum (IgG)-treated and untreated cells, respectively. *p < 0.05 (two-way ANOVA). (C) Quantification of tumor Rluc luminescence before and after virus injection. Data in (B) and (C) are presented as means \pm SD (n = 6). *p < 0.05 (two-way ANOVA). (D) Survival curves of mice in (A)–(C) generated by Kaplan-Meier analysis. Treatment with Δ SCR EEV + α VV Ab (IgG) prolonged survival compared to PBS + α VV Ab (p = 0.0005, log rank test) and B5R EEV + α VV Ab (p = 0.0291, log rank test).

virus sufficiently, likely due to the end of smallpox vaccination in the 1980s.

On the other hand, both anti-VV and -B5R antibodies were clearly detected in immunized monkey serum in proportion to vaccine dosage (Figures 3C and 3D). The levels of VV- and B5R-specific antibodies were measured on days 1, 3, 7, 20, and 30 after virus injection, and very high levels of antibody titers were induced after day 20 (data not shown). This was consistent with the previous finding that anti-VV antibodies are elicited in human cancer patients within 1 month after virus treatment.^{55–58} Furthermore, the elicitation of anti-VV antibodies strongly decreases the viral titers in blood following repeated intravenous VV injections in cancer patients.⁵⁹ Therefore, immune resistance and oncolytic activity of VV were examined by mixing the viruses with monkey serum. High- and low-VV dose-immunized monkey sera contained anti-B5R antibody (Figure 3D) and efficiently neutralized B5R EEV (Figures 4A and 4B). On the other hand, Δ SCR EEV escaped neutralization and retained its oncolytic activity when mixed with either high- or low-dose-immunized serum (Figures 4A–4C). The former more potently neutralized EEV and IMV than did the latter, according to the recorded ELISA titers against B5R.

EEV neutralization strongly depends on anti-B5R antibody and complement, but SCR deletion enhanced EEV escape upon exposure to those neutralizers (Figures 5A and 5B). Rabbit anti-VV antibody (anti-B5R antibody) or complement alone does not neutralize EEV efficiently, but their combination markedly enhanced neutralizing activity against EEV. B5R EEV exhibited greater immune resistance than did B5R IMV (Figures 5A and 5C), although some residual antigenicity was neutralized by the antibody-complement combination. However, Δ SCR EEV showed the greatest resistance (Figures 5A and 5B), even in the presence of an antibody-complement mixture.

The enhancement of neutralization resistance was confirmed in anti-virus antibody-treated mice, in which Δ SCR EEV promoted viral replication to a greater extent than B5R EEV (Figure 6B). Furthermore, Δ SCR EEV decreased tumor burden and prolonged survival in these mice irrespective of the presence of antibody (Figures 6C and 6D), since a 100 μ L volume of anti-virus antibody is equivalent to approximately 0.5% of total murine blood. These data were corroborated by the results of the *in vitro* neutralization test. This xenograft model used here is artificial, because it utilizes immunodeficient mice and a xenogeneic rabbit anti-VV antibody. This model only simulates the *in vitro* conditions in the presence of anti-virus antibodies, which cannot be used to examine the immunogenicity of SCR-deleted EEV against the actual vaccinated situation. However, rabbit antibodies (including anti-B5R antibody) exhibit activity of complement-dependent EEV neutralization (Figures 5A and 5B) similar to human antibodies.²⁸ SCR-deleted EEVs escaped neutralization and showed a higher therapeutic effect than did B5R EEV. Therefore, higher antibody resistance and maintenance of oncolytic effect by SCR deletion can be expected in humans with pre-existing anti-VV (anti-B5R) antibodies.

The other EEV-associated protein, A33R, has also been reported to generate EEV-neutralizing antibodies in humans and rabbits.²⁷ Rabbit anti-VV antibody comprises anti-A33R antibody (manufacture verified). However, SCR-deleted EEV clearly enhanced their antibody resistance through only B5R modification (Figures 5 and 6). Anti-B5R antibody is strongly elicited after vaccination in humans, compared with antibodies targeting other EEV-associated antigens (including A33R).¹⁹ As for VV vaccination, B5R protein has a higher protective potential for lethal VV challenge than does A33R.⁶⁰ Considering this, EEV neutralization is strongly dependent on B5R-specific antibody. On the other hand, SCR-deleted EEV did not exhibit perfect resistance to antibody and complement, especially when mixed with higher concentrations (Figure 5A). This might be the result of A33R-specific antibody.

B5R-specific antibody elicitation is strongly reflected in the presence of raised anti-VV antibody titers.¹⁹ Therefore, anti-B5R antibody is a good indicator not only for EEV protection but also for whole-VV vaccination. As for escaping neutralization by oncolytic VVs, the barrier of anti-B5R antibodies will certainly be faced, especially in patients with pre-existing antibodies. This antibody targets two major epitopes of B5R at the SCR1-SCR2 border and stalk.²⁶ Δ SCR harbors a deletion in the former region, which confers increased resistance against EEV-neutralizing antibody. The stalk, which is present in Δ SCR, is important not only for EEV entry³⁸ but also for the interaction with other EEV membrane proteins, such as A33R and A34R,^{61,62} which are required for B5R incorporation into the EEV outer membrane. Deletion of both the SCR and stalk regions leads to a dramatic reduction in plaque size, comparable to that observed in whole-B5R deletion.⁶³ Thus, VV with partial SCR deletion (Δ SCR) shows a good balance between oncolytic activity and immune resistance. This SCR-deleted phenotype was generated in other VV strains, including western reserve (WR) and IHD-J;¹⁸ therefore, it is adaptable for several oncolytic VVs.

In summary, our SCR-deleted virus produced highly neutralization-evasive enveloped virions that retained therapeutic efficacy irrespective of antiviral antibody. VV-specific antibodies are commonly low in patients. However, they are easily elicited after virus inoculation and strongly neutralize the virus, including its EEV form. Δ SCR-derived EEV has potential as a second candidate for treating the patients who have received one or more VV injection, due to its enhanced immune resistance against pre-existing host antibodies. Δ SCR can also serve as a vector for the delivery of therapeutic agents without interference by the host, and it can potently express loaded agents through stable viral replication. Thus, our SCR-deleted VV is a next-generation platform for oncolytic virotherapy.

MATERIALS AND METHODS

Plasmid Construction

All plasmids used for B5R recombination were generated from the pTN-B5R backbone vector and harbored the *B4R*, *B5R*, and *B6R* gene loci of LC16mO VV.¹ For whole-*B5R* deletion, the *B5R* gene in pTN-B5R was replaced with the DsRed gene derived from

pDsRed-Express-N1 (Takara Bio, Otsu, Japan). The B4R fragment was amplified from pTN-B5R using the primers 5'-CAG TCA CGA CGT TGT AAA-3' and 5'-CAT GCG CAC CTT GAA GCG CAT GAA CTC CTT GAT GAC GTC CTC GGA GGA GGC CAT TTT TAT TTA TGA GCG TTA A-3', and it was digested with *NotI* and *FspI*. The DsRed fragment was amplified from pDsRed-Express-N1 with the primers 5'-GAG TTC ATG CGC TTC AAG GT-3' and 5'-CTC AAT TGA TTC TAG CTA TAA GTC TTT AAT CTT TTG ATA CTT GTT CGT TAT TAA TTA TTA ATT ATT TTA ACG GAT TTA TAT CTA CAG GAA CAG GTG GTG-3', and it was digested with *FspI* and *MfeI*. The digested PCR fragments were subcloned into the *MfeI* and *NotI* sites of pTN-B5R by three-part ligation, yielding pTN-DsRed.

For SCR1–4 deletion, pTN-B5R was digested with *NotI* and *NspI* (B4R-B5R signal peptide) or *NspI* and *SacI* (B5R stalk-B6R), and the fragments were subcloned into the *NotI* and *SacI* sites of pTN-B5R by three-part ligation, yielding pTN- Δ SCR. The plasmids used for VGF and O1 deletion (pUC19-VGF-ST-lucGFP and pUC19-O1L-ST-BFP) were generated as previously described.³⁴

Cell Culture

Human carcinoma cell lines, including pancreatic AsPC1, BxPC-3, PANC-1, and SW1990 (cultured in RPMI-1640 medium); ovarian CaOV3 (DMEM) and SKOV3 (RPMI-1640); colon Caco2 (Eagle's minimal essential medium [EMEM]), HT-29 (RPMI-1640), LoVo (Ham's F12K), and SW480 (Leibovitz's L-15); lung A549 (Ham's F12K); breast MCF-7 (EMEM) and SK-BR-3 (EMEM); hepatocellular HepG2 (EMEM); neuroblastoma SK-N-AS (DMEM) and SK-N-BE (RPMI-1640); and epithelial A431 (EMEM) and Hep2 (EMEM) cells, as well as rabbit kidney-derived RK13 cells (EMEM), were purchased from the American Type Culture Collection (Manassas, VA, USA). The A2780⁶⁴ (DMEM) and RMG-1⁶⁵ (RPMI-1640) human ovarian carcinoma cell lines were provided by Osaka University (Suita, Japan) and Saitama Medical University (Hidaka, Japan), respectively. The cells were grown in the appropriate medium (Wako, Osaka, Japan) with 10% fetal bovine serum (FBS; Corning, Oneonta, NY, USA)—except for Caco2 (20% FBS) and RK13 (5% FBS) cells—at 37°C in a humidified atmosphere of 5% CO₂.

Virus Construction

Recombinant viruses were constructed as previously described.¹ Briefly, RK13 cells were infected with LC16mO at an MOI of 0.04, then transfected with pTN-DsRed. Infected cells were harvested 2–5 days later, and recombinant LC16mO Δ -DsRed virus was selected based on DsRed expression and plaque size reduction, and it was isolated over several cycles of plaque purification. Using LC16mO Δ -DsRed as the parent virus, recombination was induced in cells transfected with pTN- Δ SCR to obtain LC16mO Δ SCR, which was selected based on the loss of DsRed fluorescence and plaque expansion.

VGF- and O1-deleted viruses (LC16mO VGF⁻/O1⁻) were generated by insertion of a gene cassette expressing luciferase-fused EGFP and

BFP into the VGF and O1 gene loci, respectively. For VGF deletion, RK13 cells were infected with LC16mO and transfected with pUC19-VGF-ST-lucGFP, and LC16mO VGF⁻ was purified as described above and used to infect RK13 cells, which were simultaneously transfected with pUC19-O1L-ST-BFP. LC16mO Δ SCR VGF⁻/O1⁻ was generated by two-step recombination of the B5R gene locus with LC16mO VGF⁻/O1⁻ as the parent virus, using pTN-DsRed and pTN- Δ SCR as described above. All viruses were propagated in A549 cells and titrated in RK13 cells. Viral plaque phenotype was examined and photographed under a phase-contrast or fluorescence microscope (BZ-X700; Keyence, Osaka, Japan) during titration.

EEV and IMV Production

Progeny virus productivity was evaluated by titration. Tumor cell lines were infected with LC16mO, LC16mO Δ -DsRed, LC16mO Δ SCR, LC16mO VGF⁻/O1⁻ (B5R), or LC16mO Δ SCR VGF⁻/O1⁻ (Δ SCR) at an MOI of 0.1; 48 h later, the culture medium was collected and centrifuged at 700 × g for 5 min, and the supernatant was used as the EEV. Infected cells were harvested in 1 mL Opti-MEM (Thermo Fisher Scientific, Waltham, MA, USA), and the cell-associated virions—namely, IMV—were extracted from cell lysates by freeze-thawing, sonication, and centrifugation. Both virions were titrated in RK13 cells, and viral plaques were counted 3–4 days after infection; total PFU was calculated as plaque number relative to fluid volume.

Cytotoxicity Assay

Viral cytotoxicity was determined by measuring cell viability with the (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt)/phenazine methosulfate assay. The oncolytic activity of EEV was evaluated based on the viability of cells treated with the culture medium of infected cells. Ovarian carcinoma cell lines were infected with LC16mO VGF⁻/O1⁻ (B5R) or LC16mO Δ SCR VGF⁻/O1⁻ (Δ SCR) at an MOI of 0, 0.001, 0.01, 0.1, or 1 at 37°C for 1 h, and virus diluents were removed and replaced with the appropriate culture medium. After 48 h, 50 μ L medium (including EEV) was recovered from each culture and pipetted onto the newly seeded ovarian cancer cells, which were the same as the EEV-producing cells; 120 h later, cells were photographed under a fluorescence microscope, and viability was assessed with the CellTiter 96 Aqueous Nonradioactive Cell Proliferation Assay (Promega, Madison, WI, USA). Whole viral cytotoxicity was evaluated in cells infected with B5R or Δ SCR at the same MOI, which were also photographed and assessed for viability 120 h after infection.

Preparation of Serum Sample

Human serum samples were prepared from ovarian cancer patients at Saitama Medical University International Medical Center (Hidaka, Japan). Approval for this project was obtained from the Ethics Committee of Saitama Medical University International Medical Center (12-096) and Tottori University (2543). Immunized monkey serum was prepared for the viral toxicity test at Tsukuba Primate Research Center, National Institutes of Biomedical Innovation, Health, and

Nutrition (Tsukuba, Japan). Male *Macaca fascicularis* (2–4 years old) were intravenously injected with LC16mO VGF⁻/O1⁻ VV at 1×10^8 PFU (001, 002, and 003) or 1×10^7 PFU (005, 006, and 008), or they were mock infected (004, 007, and 009). The monkeys were sacrificed 30 days later, and serum was collected for analysis. The experiment was approved by the Animal Experiment Committee of National Institutes of Biomedical Innovation, Health, and Nutrition.

Analysis of Antibody Response by ELISA

Whole-virus antigen- and B5R-specific antibody titers in immunized serum were determined by ELISA as previously described.^{19,66} Briefly, Immulon 2HB plates (Thermo Fisher Scientific) were coated with 5×10^6 PFU/well of whole-virus antigen, 660 ng/well of B5R, or PBS (mock). The wells were blocked at 37°C for 2 h with PBS containing 10% FBS, then washed with PBS containing 10% FBS and 0.5% Tween-20. The plates were incubated for 2 h with 4-fold serial dilutions (32×, 128×, and 512× or 4×, 16×, 64×, 256×, 1,024×, and 4,096×) of serum from human patients (221, 183, 195, 175, and 214) or monkeys immunized with high (001, 002, and 003) or low (005, 006, and 008) doses of VV or from mock-immunized monkeys (004, 007, and 009). Rabbit anti-VV serum (IgG fraction; Capricorn Scientific, Ebsdorfergrund, Germany) was used as a positive control in human serum analysis.

After washing, the wells were incubated at 37°C for 1 h with horseradish peroxidase-conjugated rabbit anti-human IgG H&L (diluted 1:4,000; SouthernBiotech, Birmingham, AL, USA), goat anti-monkey IgG H&L (diluted 1:5,000; Abcam, Cambridge, UK), or goat anti-rabbit IgG H&L (diluted 1:4,000; SouthernBiotech). A 100 μL volume of SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Fisher Scientific) was added to the washed wells, and optical density at 405 nm (OD₄₀₅) was recorded with an ARVO MX (PerkinElmer, Waltham, MA, USA). Antibody endpoint titers were defined as the reciprocal of dilutions corresponding to twice the mean OD₄₀₅ value of PBS-coated wells.

EEV and IMV Neutralization Assays

EEV and IMV were neutralized by mixing with anti-virus IgG and complement or immunized monkey serum. EEV and IMV were recovered from the supernatant or lysate of SKOV3 cells infected with LC16mO VGF⁻/O1⁻ (B5R) or LC16mO ΔSCR VGF⁻/O1⁻ (ΔSCR) at an MOI of 0.1, as described for EEV or IMV production. In the case of rabbit IgG and complement, about 1,500–2,000 PFU EEV or 5,000–7,000 PFU IMV was mixed with 0%, 0.2%, 0.5%, or 1% rabbit anti-VV immunized serum (IgG fraction; Capricorn Scientific) and 0%, 1%, 3%, 10%, or 25% rabbit complement (Cedarlane Labs, Burlington, ON, Canada), followed by incubation at 37°C for 30 min. The virus-serum-complement mixture was used to infect newly seeded SKOV3 cells, and, after incubation at 37°C for 2 h, the mixture was removed and replaced with the appropriate culture medium. After 96 h, the cells were photographed under a fluorescence microscope, and fluorescence intensity was quantified using the Hybrid Cell Count software (Keyence), according to the manufacturer's protocol.

When RMG-1 cells were used to measure cell viability, about 1,500–5,000 PFU EEV or 6,000–7,000 PFU IMV derived from RMG-1 cells infected with B5R or ΔSCR was mixed with 0%, 0.2%, or 0.5% rabbit anti-vaccinia IgG and 0%, 1%, 3%, 10%, or 25% complement or with 0%, 0.5%, 1%, 3%, or 5% immunized monkey serum. The mixtures were incubated and used to infect newly seeded RMG-1 cells as described above. After 120 h, cells were photographed, and fluorescence and viability were quantified.

In Vivo Antibody-Treated Mouse Model

To establish an antibody-treated tumor-bearing mouse model, 6-week-old female athymic nude mice (Charles River Laboratories, Yokohama, Japan) were intraperitoneally injected with A2780 cells stably expressing Rluc (5×10^6 cells in 100 μL PBS [pH 7.4]), and they were treated 11 days later (1 day before virus injection) with 100 μL PBS or rabbit anti-VV serum (500 μg/mouse, IgG fraction; Capricorn Scientific) by intraperitoneal injection. Meanwhile, cultured A2780 cells were infected with B5R or ΔSCR at an MOI 0.05, and, 24 h later, each EEV was recovered from the culture supernatant as described above.

At 12 days after tumor transplantation, the mice were intraperitoneally injected with 500 μL PBS (mock), B5R EEV, or ΔSCR EEV (6.3×10^4 PFU or 3.5×10^5 PFU, which was determined by titration). Both antibody and EEVs were treated once. For non-invasive monitoring of tumor growth or viral replication, mice were intraperitoneally injected with 150 μL ViviRen *In Vivo* Renilla Luciferase Substrate (18.5 μg/mouse; Promega) on days 3, 8, 11, and 15 and with 200 μL VivoGlo Luciferin, *In Vivo* Grade (3 mg/mouse; Promega) on days 1, 2, 3, 5, 7, and 10 after virus injection. The mice were anesthetized with isoflurane during bioimaging; the bioluminescence of tumor Rluc or viral Rluc was detected by the injection of ViviRen and VivoGlo (Promega), respectively, and it was visualized using NightSHADE LB985 (Berthold Technologies, Bad Wildbad, Germany) and quantified according to the manufacturer's protocol. The experiment was approved by the Animal Experiment Committee of Tottori University.

Statistical Analysis

Differences in progeny virus titers and cell viability among groups were evaluated with the two-tailed unpaired t test, and *in vivo* bioluminescence was analyzed by two-way ANOVA followed by the Bonferroni test when the ANOVA showed an overall significance. Survival curves were generated with the Kaplan-Meier method and were analyzed with the log rank test. p values <0.05 were considered statistically significant. All statistical analyses were performed using Prism version (v.)5 (GraphPad, La Jolla, CA, USA).

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.omto.2019.05.003>.

AUTHOR CONTRIBUTIONS

T.N. supervised the study. T.N., M.N., and H. Kurosaki designed the study and interpreted the results. M.N. performed most of the

experiments. M.N. and N.K. constructed recombinant vaccinia viruses. K. Horita, M.I., and H. Kono maintained the tumor cell lines. K. Hasegawa prepared human serum. T.O. and Y.Y. prepared vaccinia-immunized monkey serum. T.N. and M.N. wrote the manuscript.

CONFLICTS OF INTEREST

The authors declare no competing interests.

ACKNOWLEDGMENTS

We thank Dr. Masahiko Nishiyama (Saitama Medical University, Hidaka, Japan) for providing RMG-1 cells and Dr. Seiji Mabuchi (Osaka University, Suita, Japan) for providing A2780 cells. This work was supported by Japan Society for the Promotion of Science KAKENHI grants (26640103 and 15H04310 to T.N.).

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OMTO, Volume 14

Supplemental Information

Partial Deletion of Glycoprotein B5R Enhances

Vaccinia Virus Neutralization Escape

while Preserving Oncolytic Function

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Supplementary Materials and Methods

B5R detection by RT-PCR

A549 cells were infected with LC16mO VGF⁻/O1⁻ (B5R), LC16mO ΔSCR VGF⁻/O1⁻ (ΔSCR), or LC16mO Δ-DsRed VGF⁻/O1⁻ (Δ-DsRed [B5R-]) at an MOI of 0.1; after 48 h, the total RNA was extracted using NucleoSpin RNA (Takara Bio, Otsu, Japan), according to the manufacture's protocol. cDNA was synthesized using TaqMan Reverse Transcription Reagents (Invitrogen, CA, USA) at the following conditions: 25°C for 10 min, 37°C for 2 h, and 85°C for 5 min. B5R was amplified using the following primers: 5'-ATG AAA ACG ATT TCC GTT G-3' and 5'-TTA CGG TAG CAA TTT ATG G-3'. The control, GAPDH, was amplified with the following primers: 5'-GAA GGT GAA GGT CGG AGT C-3' and 5'-GAA GAT GGT GAT GGG ATT TC-3'.

Western blot analysis for EEV membrane detection

A2780 cells were infected with B5R or ΔSCR at an MOI of 0.1; after 48 h, the EEV was recovered from the supernatant of culture media by centrifugation at 700 × g for 5 min. IMV was extracted from infected cell lysates by freeze-thawing, sonication, and centrifugation. Control uninfected A2780 lysate was harvested using M-PER Mammalian Protein Extraction Reagent (Thermo Fisher Scientific, Waltham, MA, USA), according to the manufacture's protocol. Viruses and cell lysate were mixed with Laemmli sample buffer (Bio-Rad, CA, USA) and heated for 5 min at 95°C. After electrophoresis on SDS polyacrylamide gels, proteins were transferred to PVDF membranes (sample loading; cell lysate: 3μg, B5R-EEV: 8.5 × 10³ PFU, ΔSCR-EEV: 6 × 10⁴ PFU, B5R-IMV: 4.9 × 10⁴ PFU, ΔSCR-IMV: 2.3 × 10⁴ PFU, determined by titration). After blocking for 2 h at 25°C in western BLoT blocking buffer (Takara Bio), the membrane was incubated with anti-Vaccinia Virus Cell Surface Binding Protein (D8L) Ab (1:2000; Abcam, Cambridge, UK) or rabbit anti-human CD46 (1:2000; Abcam) in blocking buffer at 4°C overnight. Following washes in Tris-buffered saline containing 0.1% Tween-20, the membrane was incubated with a secondary antibody, anti-rabbit IgG-HRP conjugated (1:2500; Promega WI, USA), in blocking buffer for 1h at 25°C. Immune complexes were detected using western BLoT chemiluminescence HRP substrate (Takara Bio) and photographed using Lumino Graph I (ATTO, Tokyo, Japan).

50% Neutralization dose (ND₅₀) of serum

The ND₅₀ of immunized monkey serum was determined with the plaque reduction assay as previously described.¹ LC16mO virus (10⁴ PFU/ml) was mixed with serial dilutions (0×, 32×, 64×, 128×, 256×, and 512×) of heat-inactivated monkey serum (60°C for 20 min), followed by incubation for 1 h at 37°C. After further

incubation for 16 h at 4°C, 100 µl of virus/serum mixture (approximately 100 PFU of input infectivity) were titrated onto RK13 cells, and ND₅₀ was calculated from calibration curves of plaque reduction ratio vs. serum dilution.

Antigen preparation

Whole virus antigen was prepared from the lysate of infected cells. RK13 cells were infected with LC16mO at an MOI of 0.05 and recovered 48 h later. Infected cells were freeze-thawed to release intracellular viruses, and after centrifugation at 700 × *g* for 10 min to remove cellular debris, the lysate supernatant was used as an antigen reagent.

To prepare B5R antigen, a DNA fragment harboring *NheI* and *ApaI* sites at the 5' signal peptide and 3' stalk terminal of the *B5R* gene was amplified with the primers 5'-GAG CTA GCC ACC ATG AAA ACG ATT TCC GTT GTT AC-3' and 5'-TGG GCC CTT CTA ACG ATT CTA TTT CTT GTT CAT ATT G-3' using LC16mO genomic DNA as a template. The PCR product was subcloned into the *NheI* and *ApaI* restriction sites of the pSecTag2/Hygro A vector (Thermo Fisher Scientific) to generate B5R-producing plasmid (pSecTag2-B5R), which was transfected into 293T cells. Hexahistidine (6× His)-tagged B5R protein was purified from the culture supernatant using the His tagged Protein Purification kit (MBL, Nagoya, Japan).

Supplementary references

1. Law, M., and Smith, G.L. (2001). Antibody neutralization of the extracellular enveloped form of vaccinia virus. *Virology* 280, 132–142.

Figure S1

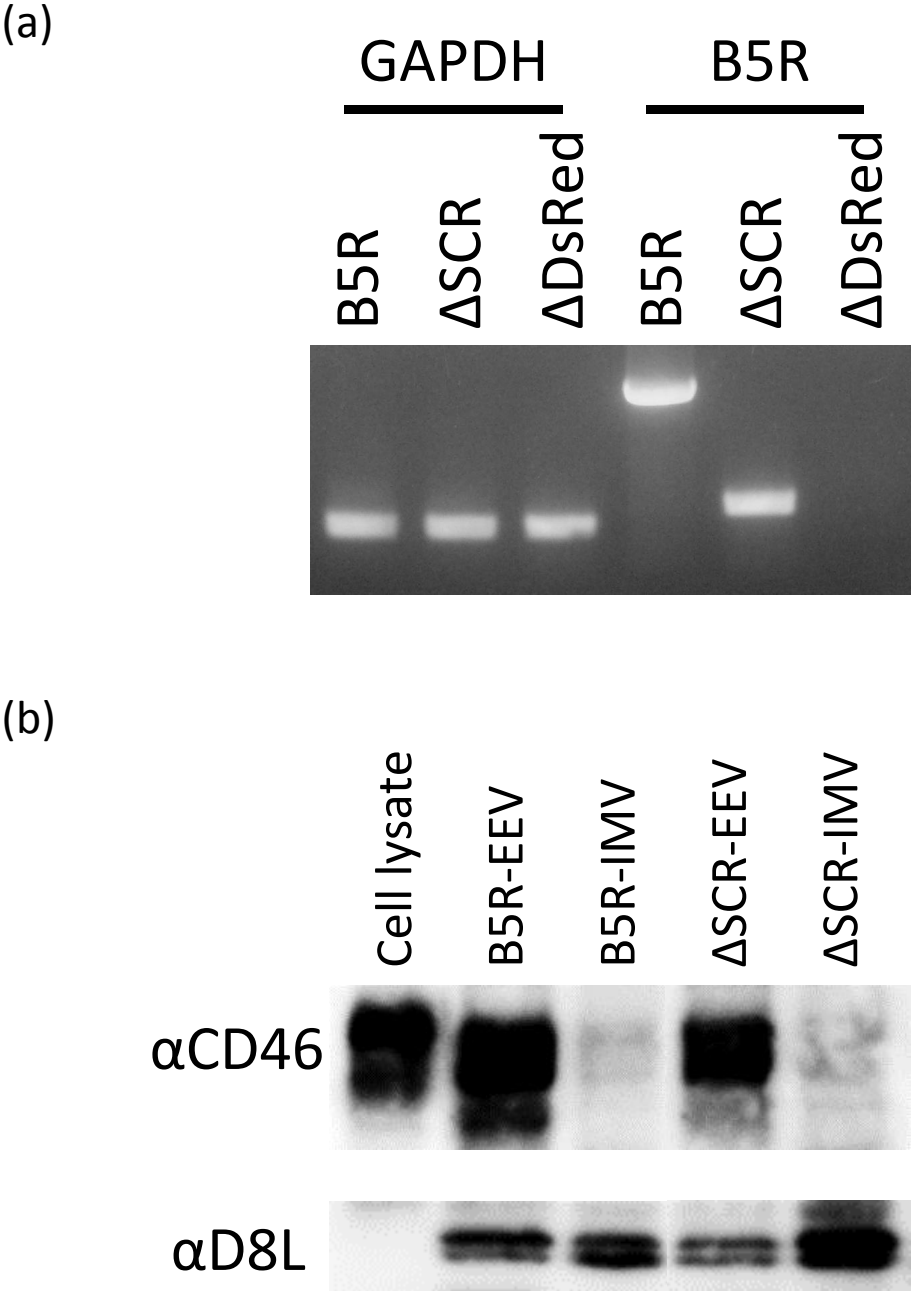


Figure S1. Detection of B5R expression and EEV phenotype

- (a) cDNA were synthesized from total RNA of A549 cells infected with B5R, SCR, or -DsRed and were amplified by PCR using the primers targeting GAPDH or B5R.
- (b) A2780 cell lysate and EEV or IMV samples derived from B5R- or SCR-infected A2780 were resolved by SDS-PAGE and probed with anti-CD46 or anti-D8L antibodies.

Figure S2

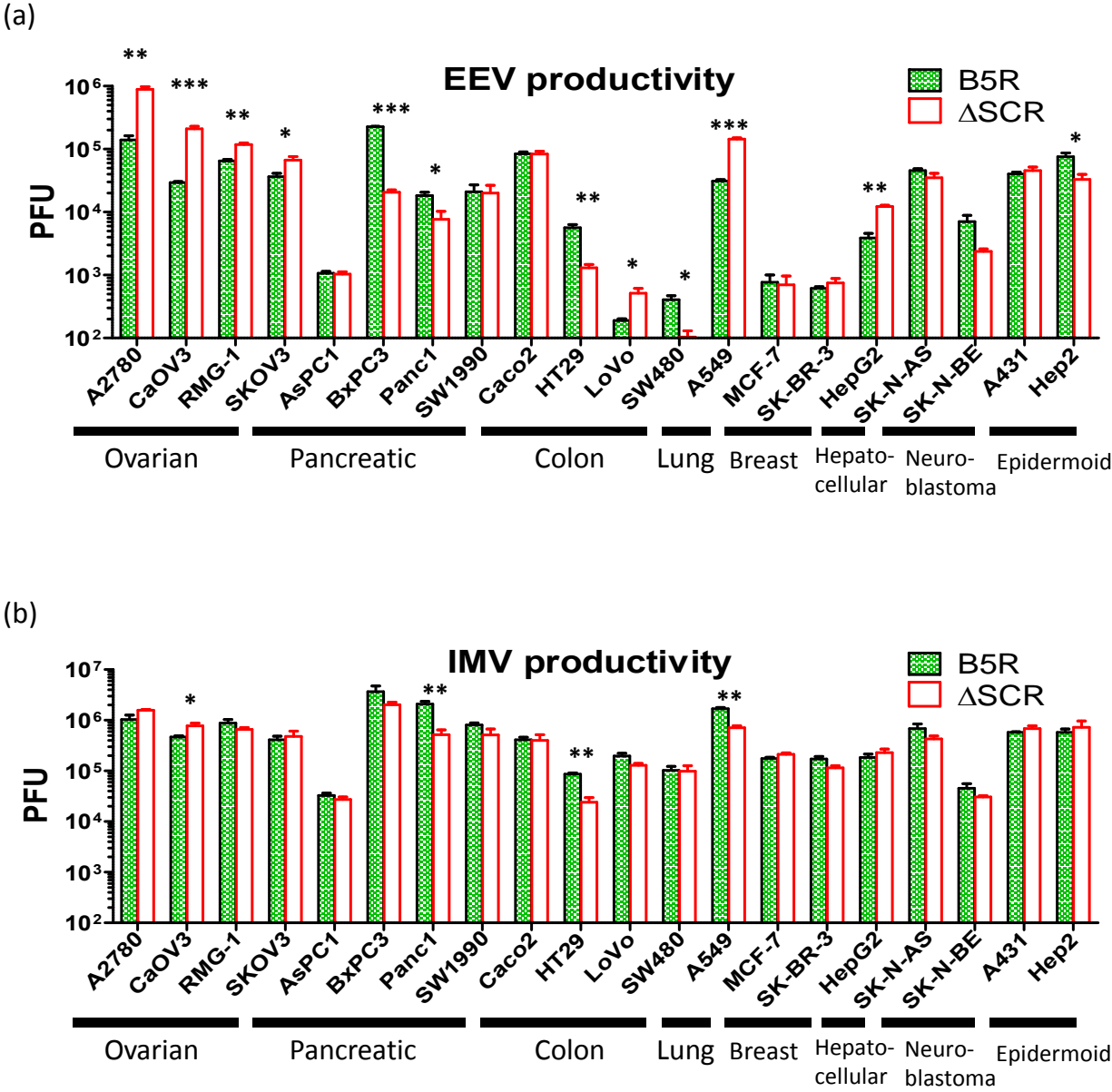


Figure S2. Comparison of EEV or IMV production in various tumor cell lines.
 (a, b) EEV (a) and IMV (b) titers from various human cancer cell lines associated with Table 1. Titers are presented as mean \pm SD (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001 (unpaired t test).

Figure S3

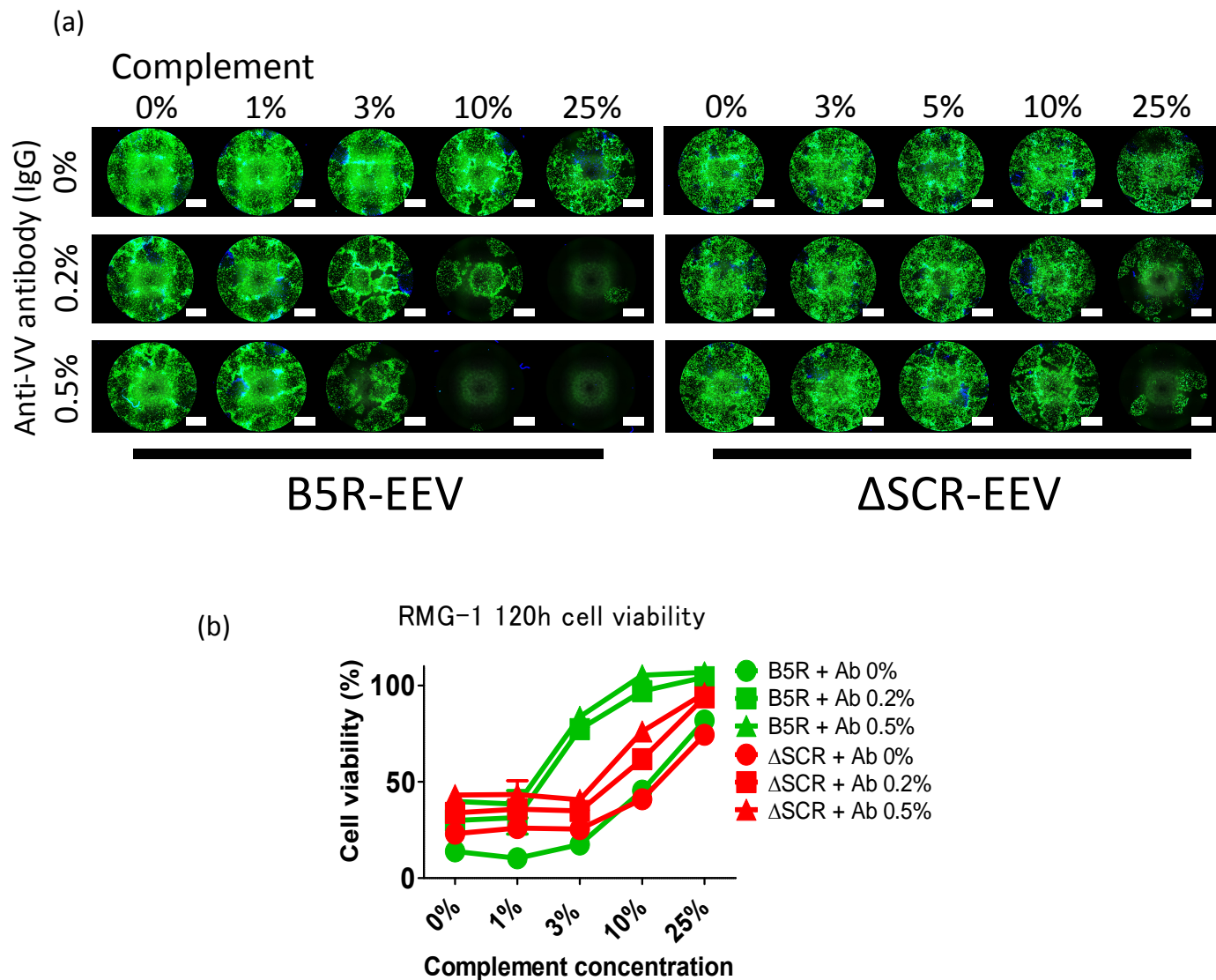


Figure S3. Immune resistance and cytolytic effect of EEV.

- (a) EEV from the supernatant of RMG-1 cells infected with B5R or Δ SCR was mixed with 0%, 0.2%, or 0.5% rabbit anti-VV-immunized IgG and/or 0%, 1%, 3%, 10% or 25% rabbit complement. The cells were imaged by fluorescence microscopy 120 h later. Scale bar, 1500 μ m.
- (b) Viability of infected RMG-1 cells described in (a) expressed as percent survival of mock-infected cells. Data represent mean \pm SD (n = 3).

Table S1. ND₅₀ against vaccinia virus in human serum.

50% neutralization dose of human serum samples was determined based on viral plaque-reduction activity.

(N.D.: Not detected)

Sample No.	ND₅₀	Sample No.	ND₅₀
139	N.D.	246	N.D.
159	N.D.	248	N.D.
161	N.D.	268	N.D.
175	53.55	269	N.D.
183	16.09	280	9.64
195	50.26	281	N.D.
214	114.767	295	N.D.
219	N.D.	308	N.D.
221	N.D.	311	N.D.
230	N.D.	316	N.D.

Table S2. ND₅₀ and specific antibody titers against whole VV antigen or B5R in immunized monkey serum.

50% neutralization dose of immunized monkey serum was determined based on viral plaque-reduction activity, and antibody end-point titers against whole VV antigen or B5R were determined from linear regression plots of the ELISA presented in Figure 3c and 3d. (N.D.: Not detected)

Monkey serum		Whole (ND ₅₀)	Whole (ELISA)	B5R (ELISA)
High immunized (1x10 ⁸ PFU)	#001	2250.5	1308.18	6230
	#002	1212.25	1135.68	4836.72
	#003	134.963	591.59	1518
Low immunized (1x10 ⁷ PFU)	#005	N.D.	1.17	742.8
	#006	200.75	170.46	1486.04
	#008	14.96	38.79	2246
Mock (0 PFU)	#004	N.D.	N.D.	N.D.
	#007	N.D.	N.D.	N.D.
	#009	N.D.	N.D.	N.D.