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: 8.5×10^3 PFU, ΔSCR-EEV: 6×10^4 PFU, B5R-IMV: 4.9×10^4 PFU, ΔSCR-IMV: 2.3×10^4 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 (ND_{50}) of serum

The ND_{50} of immunized monkey serum was determined with the plaque reduction assay as previously described.[1] LC16mO virus (10^4 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 ND_{50} 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 *Nhe*I and *Apa*I 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 *Nhe*I and *Apa*I 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

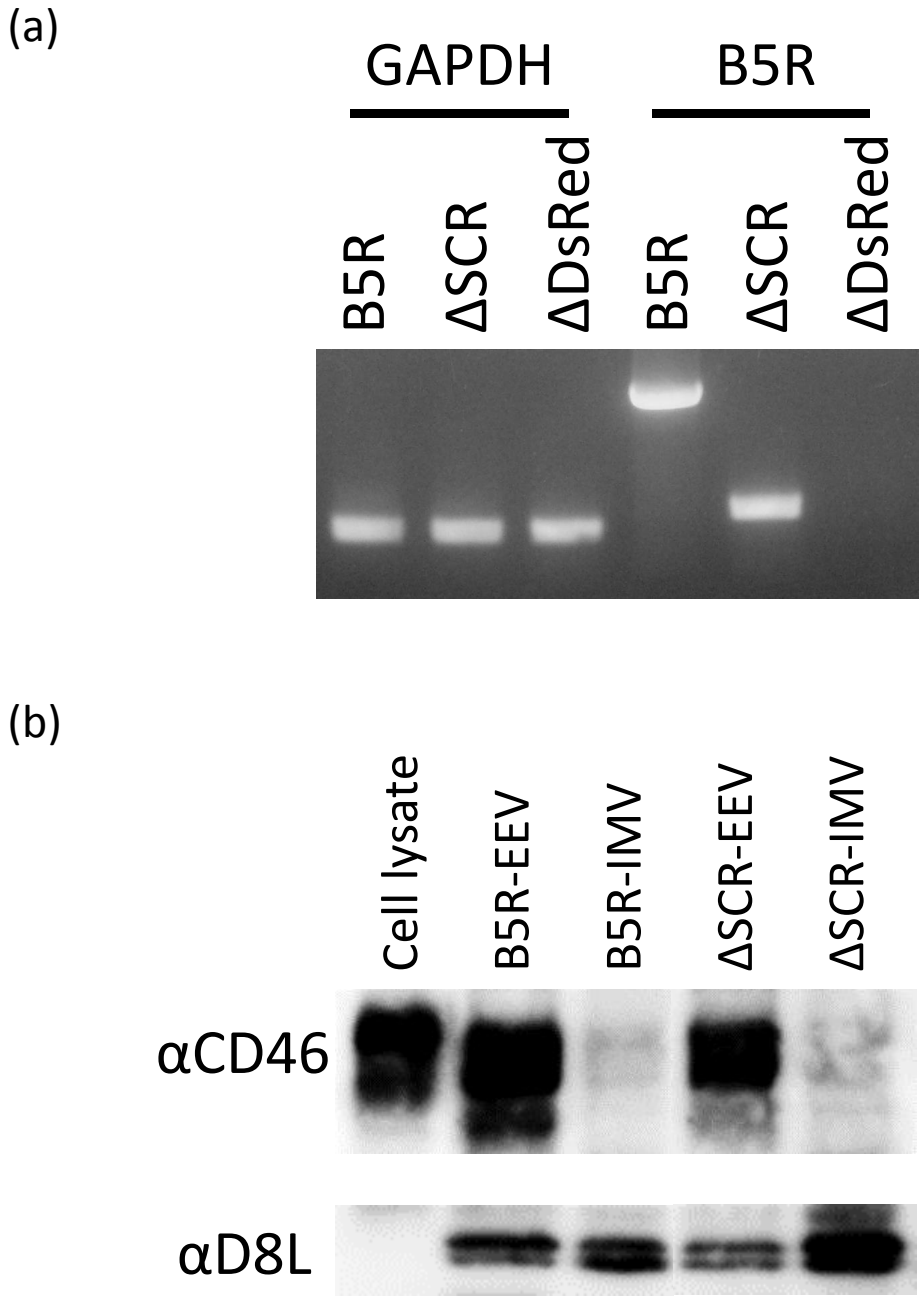

Figure S1. Detection of B5R expression and EEV phenotype

(a) cDNA were synthesized from total RNA of A549 cells infected with B5R, ϕSCR, or ϕ-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 ϕSCR-infected A2780 were resolved by SDS-PAGE and probed with anti-CD46 or anti-D8L antibodies.

Figure S2

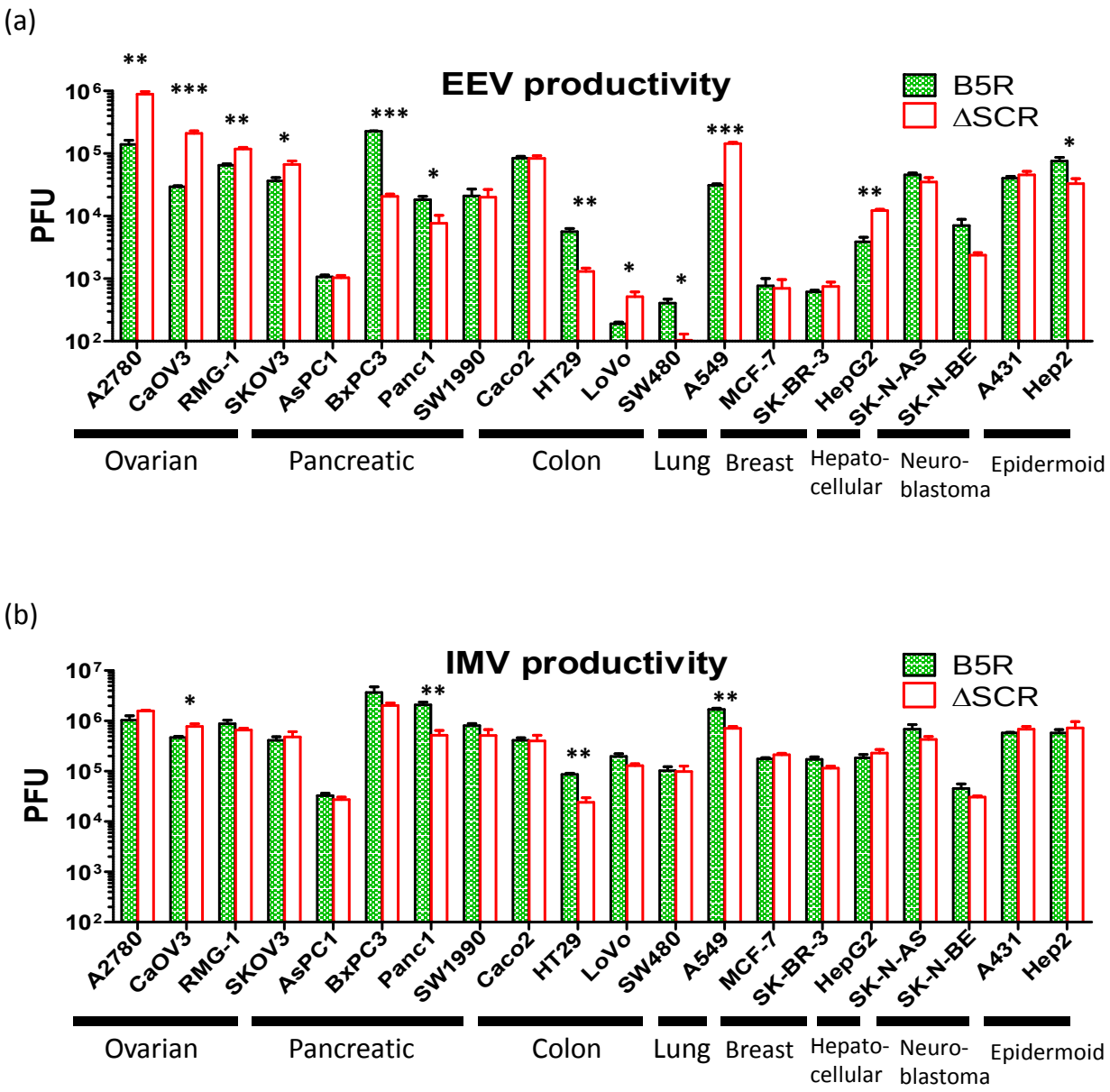

Figure S2. Comparison of EEV or IMV production in various tumor cell lines.

(a, b) EEV (a) and IMV (b) titers from various human cancer cell lines associated with Table 1. Titers are presented as mean ± SD (n = 3). *p <0.05, **p<0.01, ***p<0.001 (unpaired t test).

Figure S3

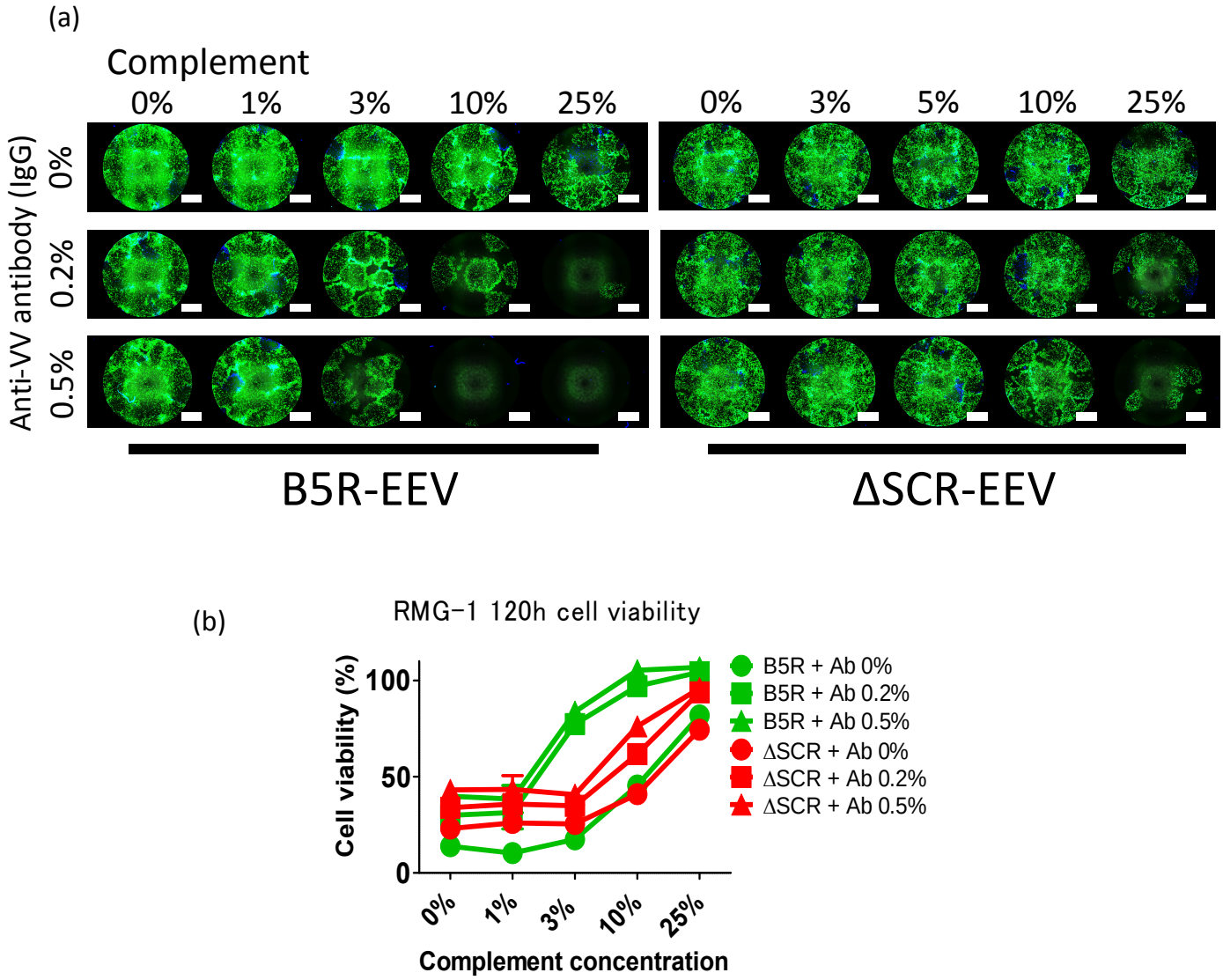

Figure S3. Immune resistance and cytolytic effect of EEV.

(a) EEV from the supernatant of RMG-1 cells infected with B5R or ΔSCR 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 μm.

(b) Viability of infected RMG-1 cells described in (a) expressed as percent survival of mock-infected cells. Data represent mean ± SD (n = 3).

Table S1. ND_{50} 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.	**ND_{50}**	**Sample No.**	**ND_{50}**
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. ND_{50} 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 agaisnt 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.