Supporting Information

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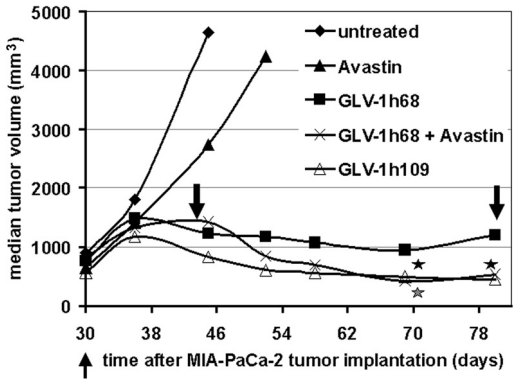

Fig. S1. Combination therapy of s.c. MIA PaCa-2 tumors with VACV GLV-1h68 and Avastin. Tumorous mice ($n = 7$ per group) were treated with the virus alone (5×10^6 pfu/mouse), with Avastin alone (5 mg/kg i.p. twice weekly for 5 weeks), with the virus initially (5×10^6 pfu/mouse) followed by Avastin treatment (5 mg/kg twice weekly for 5 weeks) 13 days later, with GLV-1h109, or with no treatment. The up-pointing arrow indicates the time of virus injection. The down-pointing arrows indicate the beginning and end of Avastin treatment. Statistical analysis was performed using one-way ANOVA (**$P < 0.01$, *$P < 0.05$). Stars indicate comparison of the GLV-1h68 group with the Avastin + GLV-1h68 group (gray) or with the GLV-1h109 group (black). Treatment with Avastin alone led to a delay in tumor growth compared with that in untreated mice. If given in combination with GLV-1h68, however, tumor regression was more pronounced than that seen in tumors treated only with GLV-1h68. The uniquely engineered VACV GLV-1h109 showed a similar effect as the combination treatment (GLV-1h68 and Avastin), which led to significantly smaller tumor volumes at later time points after infection.

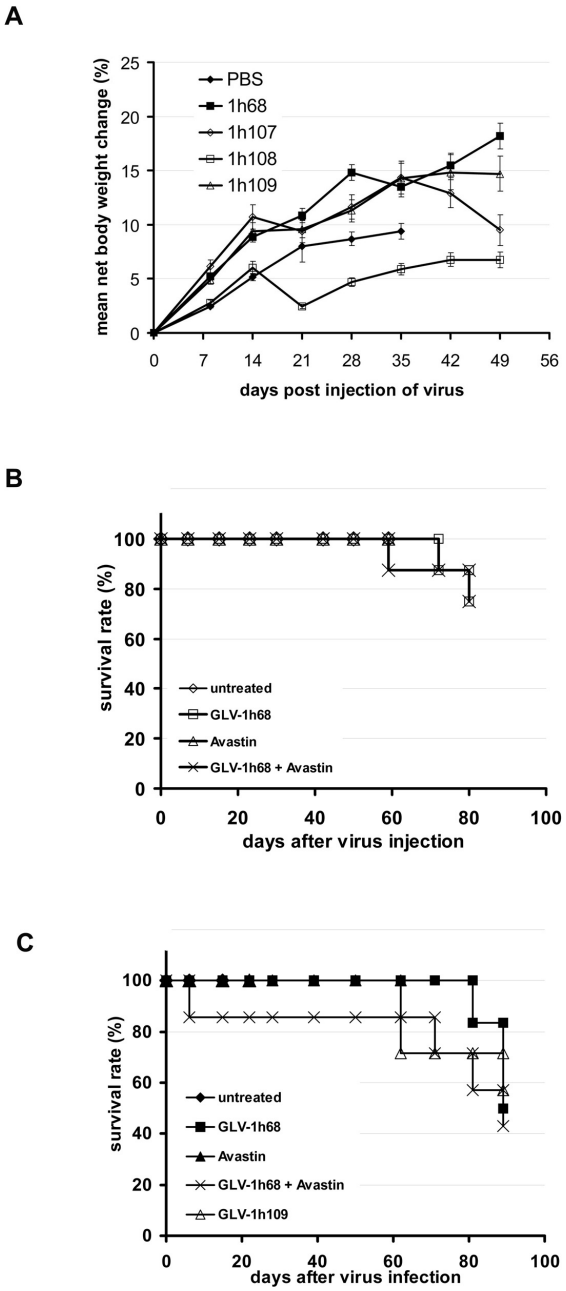

Fig. S2. (*A*) VACV strains expressing GLAF-1 showed safety and toxicity similar to GLV-1h68. To determine the safety and toxicity of the injected VACV strains in nude mice, the change in net body weight of A549 tumor-bearing animals was monitored over time. Injection of the GLV-1h107 VACV resulted in tolerance of the treatment for 35 days after virus injection. Body weight data were similar to those of mice treated with GLV-1h68. After 35 days, however, there was a significant drop in the net body weight of mice in the GLV-1h107 treatment group up to 2 weeks, which was not observed in the GLV-1h68 group. The reason for the late body weight loss is not known. Based on net body weight change data, GLV-1h109 showed comparable toxicity profiles to GLV-1h68 throughout the study. GLV-1h108 showed a significantly increased toxicity in comparison to GLV-1h68. The experimental groups contained 8 to 10 animals. (*B* and *C*) Survival rates of animals bearing different tumor xenografts after VACV treatment. Survival of animals after systemic application of VACVs GLV-1h68 and GLV-1h109 or GLV-1h68 and Avastin combination treatment is shown for PANC-1 (*B*) and MIA PaCa-2 (*C*). Untreated PANC-1 and MIA PaCa-2 tumor-bearing mice were killed because of extensive tumor burden at days 60 and 15 after virus injection, respectively. Avastin-treated PANC-1 and MIA PaCa-2 tumor-bearing mice were killed because of extensive tumor burden at days 60 and 22 after virus injection, respectively. The VACV-treated groups showed a survival rate of 75% in the PANC-1 experiment. However, the combination treatment was less well tolerated, because mice started to die earlier in the course of the experiment. Also in the MIA PaCa-2 animal model, an initial drop in survival was seen in the combination group. GLV-1h109–treated mice seemed to tolerate the VACV treatment slightly less well compared with GLV-1h68–treated mice earlier in the course of the experiment, even though the survival rate at the end of the experiment was lower in the GLV-1h68–treated group than in the GLV-1h109–treated group.

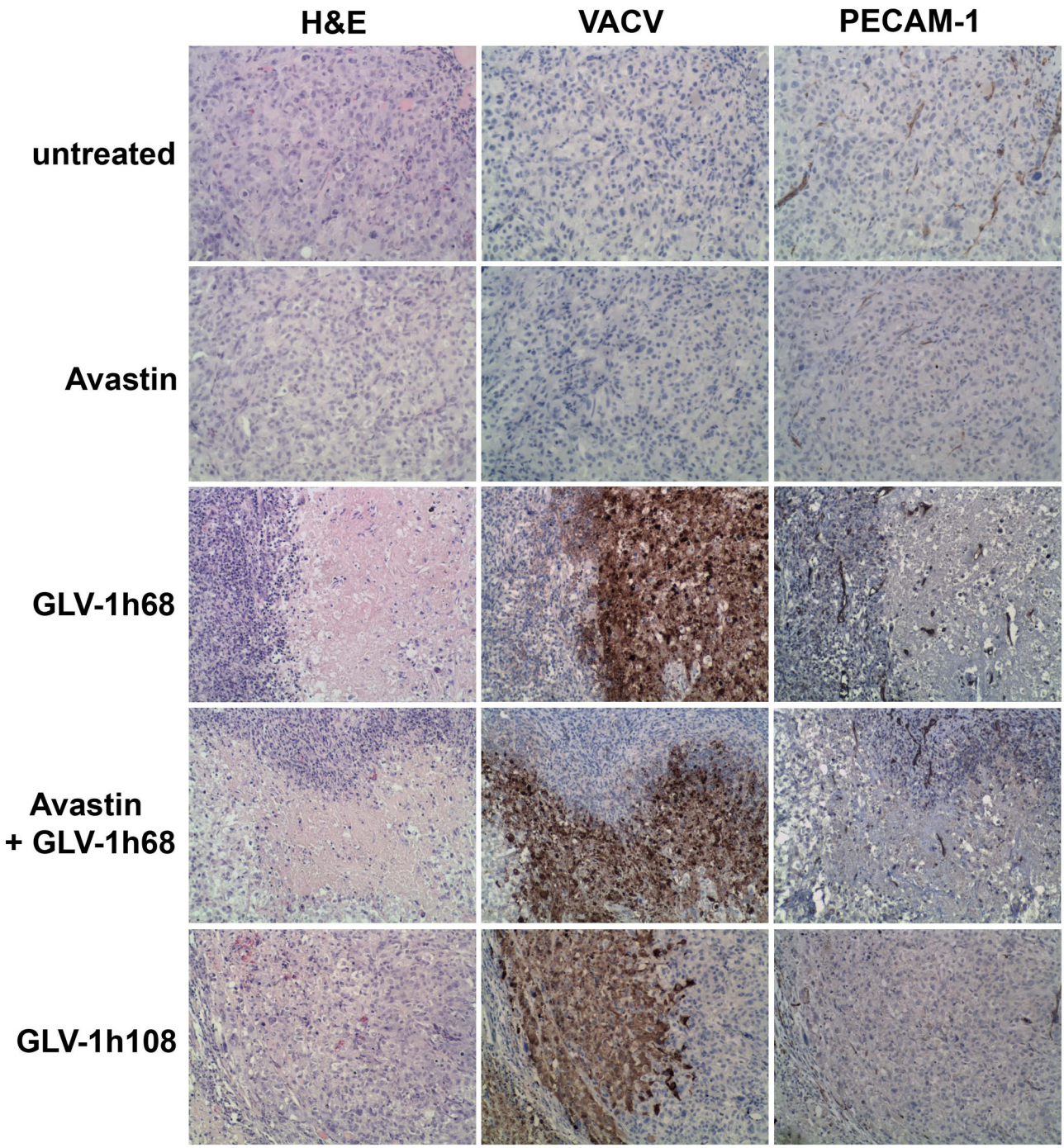

Fig. S3. IHC of DU-145 tumor xenografts after VACV infection. Tumorous mice ($n = 3$ per group) were treated with the virus alone (5×10^6 pfu/mouse), with Avastin alone (5 mg/kg i.p. twice weekly for 5 weeks), with the virus initially (5×10^6 pfu/mouse) followed by Avastin treatment (5 mg/kg twice weekly for 5 weeks) 14 days later, with GLV-1h108, or with no treatment. Tumors were excised at day 21 after VACV infection. Formalin-fixed and paraffin-embedded tumor tissue was cut in 5-μm sections, and H&E staining was performed. Adjacent slides were stained for PECAM-1 and VACV, respectively. Antigen retrieval was performed with citrate buffer. For the PECAM-1 staining, tissue was blocked with normal rabbit serum, peroxidase treated, incubated with anti-PECAM-1 (Santa Cruz Biotechnology), and then incubated with a rabbit anti-goat IgG as a secondary antibody (Vector Laboratories). For the VACV staining, tissue was blocked with normal goat serum, peroxidase treated, and incubated with an anti-A27L antibody custom-made against a VACV synthetic peptide (GenScript Corporation). A goat anti-rabbit IgG was used as a secondary antibody (Vector Laboratories). Detection was performed with Vectorstain Elite ABC reagent and Vector ImmPact DAB Peroxidase substrate (Vector Laboratories). Sections were counterstained with hematoxylin. Shown are representative areas of tumor tissue obtained at $\times$100 magnification. Untreated tissue showed a regular distribution of blood vessels. After Avastin treatment alone, BVD was reduced. The combination treatment of GLV-1h68 and Avastin led to reduced BVD compared with GLV-1h68 alone. After treatment with GLV-1h108, in areas of VACV infection, a very low BVD was observed. In GLV-1h108–infected areas, more advanced necrosis was present than that observed in GLV-1h68–treated tumors.

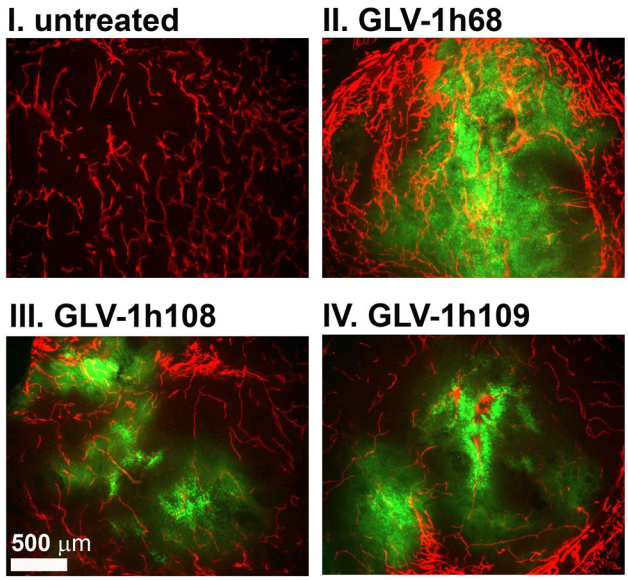

Fig. S4. Effect of VACV treatment on tumor vasculature in A549 tumor xenografts. A549 cells (5×10^6) were implanted s.c. into the lateral thigh of 4–5-week-old mice. Twelve days later, mice were injected i.v. with the different VACV strains at 5×10^6 pfu/mouse. Tumors were excised 7 days p.i. and snap-frozen in liquid N_2. Paraformaldehyde-fixed tumors were cut in 100-μm sections using a vibratome. Specimens were then labeled with anti-CD31 antibody (BD Pharmingen), followed by incubation with Cy3-conjugated donkey anti-rat antibody (Jackson ImmunoResearch). Examination of the tumor sections was conducted with an MZ16 FA fluorescence stereomicroscope (Leica) equipped with a digital CCD camera (Leica). GFP expression of virus-infected cells and CD31 expression (red) in tumor sections were monitored. Digital images were processed using Adobe Photoshop 7.0 software. All images are representative examples. (Scale bar: 500 μm.) Representative examples of CD31 and GFP expression in A549 tumors treated with the VACV strains GLV-1h68, GLV-1h108, and GLV-1h109 are shown. Untreated control tumors and tumors treated with GLV-1h68 (I and II) showed highly vascularized tumor tissues. In contrast, tumors obtained from mice infected with GLV-1h108 and GLV-1h109 revealed drastically less CD31-positive BVD in virus-infected areas (III and IV).

Table S1. Biodistribution of GLV-1h107, GLV-1h108, and GLV-1h109 VACV in A549 tumor-bearing nude mice at 1 week p.i.

	GLV-1h68				GLV-1h107				GLV-1h108				GLV-1h109			
Animal no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Bladder	0	0	0	0	0	48.1	0	0	0	0	0	0	0	0	0	0
Testes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen	0	78	0	0	0	0	40.3	0	0	0	0	0	0	40	0	0
Kidneys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	31.6	0	0	0	28.7	0	139	0	0	0
Heart	0	0	0	0	0	0	0	0	33.3	0	0	0	0	0	0	0
Brain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tumor	2.1×10^6	1.9×10^6	3.5×10^6	1.4×10^6	4.2×10^5	4.0×10^6	2.4×10^6	2.0×10^6	3.2×10^4	2.9×10^7	3.0×10^6	7.5×10^5	1.2×10^6	2.9×10^6	2.7×10^6	2.6×10^5
Tumor, median			2×10^6				2.2×10^6				1.88×10^6				1.95×10^6	

Viral titer in tissue samples, pfu per organ or grams of tumor. To determine the clearance of the 3 new VACV strains, A549 tumor-bearing mice (250 mm^3) were injected i.v. with GLV-1h107, GLV-1h108, and GLV-1h109 and with GLV-1h68 as a control (5×10^6 pfu/mouse). Titers were recovered from different organs and tumors at 1, 2.5, 4, and 6 weeks p.i. Tissues were dissected and disrupted in homogenization tubes (Roche MagNA Lyser Green Beads; Roche). After 3 freeze/thaw cycles, tissues were sonicated 3 times for 60 seconds. Viral titers were determined after culture on CV-1 cells. Serial dilutions were applied in case of tumor tissue to determine viral titers. Virus was rapidly cleared from all organs except tumors less than 1 week p.i. $^{+++}$ signifies large amount of plaques in the brain of 1 mouse. Overall, the viral titers in tumors were found to be 10^4 to 10^7 logs higher than titers found in all other organs combined. The median viral titers in tumors increased gradually over the course of 4 weeks with all viruses tested, with the exception of GLV-1h109–infected tumors, in which the median viral titer reached the maximum 2.5 weeks p.i. After 6 weeks, median viral titers in all tumors decreased in comparison to the 4-week time points (C and D). Median control virus GLV-1h68 titers increased over the first 4-week period and were found to be generally higher than the titers in tumors colonized by the 3 novel VACV strains. These results also showed that all 3 new VACV strains, GLV-1h107, GLV-1h108, and GLV-1h109, exhibited similar tumor specificity.

Table S1 (continued). Biodistribution of GLV-1h107, GLV-1h108, and GLV-1h109 VACV in A549 tumor-bearing nude mice at 2.5 weeks p.i.

	GLV-1h68				GLV-1h107				GLV-1h108				GLV-1h109			
Animal no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Bladder	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidneys	0	0	25.5	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs	0	29.1	32.9	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tumor	0	1.6×10^7	6.6×10^6	1.9×10^7	3.9×10^6	9.6×10^6	1.0×10^7	1.6×10^6	1.0×10^7	4.9×10^6	2.1×10^7	2.2×10^5	1.1×10^6	3.7×10^6	6×10^6	2.5×10^6
Tumor, median			1.6×10^7				6.75×10^6				7.45×10^6				3.1×10^6	

Table S1 (continued). Biodistribution of GLV-1h107, GLV-1h108, and GLV-1h109 VACV in A549 tumor-bearing nude mice at 4 weeks p.i.

	GLV-1h68				GLV-1h107				GLV-1h108				GLV-1h109			
Animal no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
Bladder	0	0	0	0	45.5	1,180	0	0	0	0	0	0	0	0	0	0
Testes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen	0	0	0	0	37.3	0	0	0	0	0	0	0	0	0	0	0
Kidneys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Tumor	0	2.4×10^7	0	2.5×10^7	0	1.0×10^7	5.9×10^6	9.8×10^6	1.9×10^7	1.1×10^7	1.2×10^7	4.8×10^6	4.6×10^6	2.3×10^6	3.9×10^6	1.1×10^6
Tumor, median		2.45×10^7				9.8×10^6				1.15×10^7				3.1×10^6		

Table S1 (continued). Biodistribution of GLV-1h107, GLV-1h108, and GLV-1h109 VACV in A549 tumor-bearing nude mice at 6 weeks p.i.

	GLV-1h68				GLV-1h107				GLV-1h108				GLV-1h109		
Animal no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Bladder	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Testes	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Spleen	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Kidneys	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Liver	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Lungs	0	0	0	0	0	0	59.5	732	0	0	0	0	0	0	0
Heart	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Brain	0	0	0	0	0	0	0	+++	0	0	0	0	0	0	0
Tumor	1.64×10^6	4.89×10^6	3.81×10^6	5.07×10^6	3.49×10^6	4.39×10^6	5.21×10^6	1.98×10^7	1.17×10^6	2.60×10^6	0	5.90×10^5	7.81×10^6	1.73×10^6	1.37×10^6
Tumor, median			4.35×10^6				4.8×10^6				8.8×10^5			1.73×10^6	