Targeting Poxvirus Decapping Enzymes and mRNA Decay to Generate an Effective Oncolytic Virus

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INTRODUCTION

A key challenge in developing viral platforms for oncolytic virus (OV) immunotherapy lies in identifying an attenuated variant that retains sufficient replicative capacity in cancer cells.1–3 By manipulating the genetic backbone of what was to become the first clinically approved oncolytic HSV1 (oHSV1), a powerful solution to this problem was exposed and effectively validated.4–8 The oHSV1 is attenuated because it lacks a critical viral virulence gene required to antagonize innate immune defenses.9–11 Most notably, the viral E3L and K3L genes prevent PKR activation and limit inactivation of its substrates, the cellular 5′-3′ RNA helicase (RIG-I) and 2′-5′ RNA oligonucleotide (DSG2).12–14 However, viruses deficient for E3L are profoundly attenuated and replicate poorly, likely the result of their inability to counter host dsRNA-induced defenses.14 In contrast, K3L-deficient viruses remain insufficiently attenuated for consideration as an OV candidate.14,15 Instead, much of the work on oncolytic VACV development has focused on strains deficient for the viral thymidine kinase (tk) gene, many of which are further attenuated by inactivation of additional virus genes, including the VACV growth factor VGF.16–18 Indeed, several of these mutant VACV derivatives have entered into clinical trials,19–21 although one failed to meet its primary end point,22 illustrating the need to investigate alternatives to multi-mutated tk-deficient VACV strains.

Unexpectedly, VACV mRNA decapping enzymes encoded by the D9 and D10 genes were recently found to limit dsRNA accumulation in infected cells.23–25 By removing the m7GTP cap on the mRNA 5′ end, D9 and D10 accelerate mRNA decay by generating substrates for the cellular 5′-3′ mRNA exoribonuclease Xrn1.26,27 Relative to WT VACV, D9- and D10-deficient strains accumulate higher levels of dsRNA and more effectively activate dsRNA-responsive host innate immune sensing pathways, including activation of PKR and RNase L.28–30 Moreover, D9- and D10-deficient viruses were both attenuated in mice with D10-deficient recombinants displaying the greatest attenuation following intranasal administration.31–34 Because D9 and D10 represent newly identified VACV-encoded virulence determinants controlling host dsRNA-activated defenses, the potential...
Here, we establish that D9- and D10-deficient VACV replicates to near wild-type (WT) levels in several established murine cancer cell lines and have OV activity in syngeneic murine cancer in vivo models. D9- and D10-deficient VACV also reduced growth of an established human hepatocellular carcinoma (HCC) xenograft in athymic mice. Moreover, greater levels of VACV antigen accumulated in HCC tumors. This shows that decapping-deficient VACV has anti-tumor activity against several murine syngeneic tumors and a human HCC model. Because D9- and D10-deficient VACV hyperactivates dsRNA innate immune defenses in non-tumorigenic cells, it further suggests a mechanism for its preferential replication in HCC tumors.

RESULTS

Productive Replication of Decapping-Deficient VACV in Established Murine Cancer Cell Lines

To evaluate the capacity of the decapping-deficient VACV mutants to replicate in murine tumor cell lines, their ability to direct viral protein production was first tested. MBT2 murine bladder carcinoma and 4T1 murine breast carcinoma cells were infected with either WT VACV, D9-deficient (ΔD9) VACV, or D10-deficient (ΔD10) VACV. After 18 hr, cultures were metabolically radiolabeled with 35S amino acids. Total protein was subsequently harvested, fractionated by SDS-PAGE, and analyzed by autoradiography (Figure 1A) or immunoblotting (Figure 1B). Compared to control primary human fibroblasts (NHDFs), less virus-induced suppression of ongoing host cell protein synthesis (host cell shutoff) was observed in murine cancer cell lines infected with WT, D9-deficient, or D10-deficient VACV (Figure 1A). Despite the apparent absence of host cell shut-off, VACV proteins accumulated to similar levels in 4T1 or MBT2 cells infected with either WT, D9-deficient, or D10-deficient VACV (Figure 1B). Thus, viral proteins accumulate similarly in murine cancer cell lines infected with decapping-deficient VACVs lacking either the D9 or D10 genes compared to WT VACV.

To compare the capacity of decapping-deficient VACV to productively replicate and spread in murine cancer cell lines, MBT2 (bladder carcinoma, H-2K) or 4T1 cells (breast carcinoma, H-2D) were infected with either WT, D9-deficient, or D10-deficient virus at low MOI (Figures 2A and 2B). Quantifying infectious virus production after 48 hr revealed decapping-deficient VACV mutants grow to similar levels as WT VACV, with only a minor reduction in yield (no more than 4-fold) detected in cells infected with either D9- or D10-deficient viruses. In addition, replication of decapping-deficient VACV mutants in MCA38 cells (colon adenocarcinoma, H-2B) was also comparable to WT virus (no more than 8-fold less) (Figure 2C). Thus, decapping-deficient VACV productively replicated and spread to near WT levels in representative murine cancer cell lines derived from different mouse genetic backgrounds.

Anti-tumor Activity of VACV Decapping-Deficient Mutants in Syngeneic Murine Cancer Models

To determine if decapping-deficient VACV lacking D10 could induce therapeutic anti-tumor responses, subcutaneous 4T1 tumors were established in syngeneic mice and once tumors reached approximately 50 mm³, they were directly injected with a virus-free control preparation from uninfected cells (mock) or D10-deficient VACV. Additional intra-tumoral injections were performed 3 and 6 days after the first treatment and tumor growth was measured over time. Between 6 and 9 days post-treatment, a statistically significant difference...
between mock and ΔD10-treated tumors was readily observed (Figure 3A). The volume of mock-treated tumors increased to a greater extent and more rapidly than ΔD9- or ΔD10-treated tumors. This difference increased and persisted throughout the entire course of the experiment (Figure 3A). This establishes that ΔD9- or ΔD10-deficient VACV treatment has anti-tumor activity against 4T1 tumors in syngeneic C57BL/6 mice and demonstrates that mutant VACVs lacking either ΔD9 or ΔD10 decapping enzymes are effective oncolytic viruses. In addition, this activity is not limited to a particular murine genetic background. The MCA38 tumors were particularly aggressive, progressing more rapidly than 4T1, as evidenced by the death of 3 mice treated with the virus-free control preparation. The rapid growth of the tumors necessitated that the animals be euthanized on day 12, effectively ending the experiment. Although one mouse died in the ΔD9-treated group and two mice died in the ΔD10-treated group, the fatalities in each experimental group were less than the control group treated with a virus-free preparation.

Anti-tumor Activity of Decapping-Deficient VACV in a Human HCC Xenograft Cancer Model

To address the anti-tumor capacity of decapping-deficient VACV against human tumors, HepG2 HCC xenografts were established in athymic mice. When tumors reached approximately 50 mm³, they were injected with ΔD10-deficient (ΔD10) VACV, ΔD9-deficient (ΔD9) VACV, or an equivalent virus-free control preparation (mock) from uninfected cells and tumor volume was monitored. A statistically significant difference between tumors treated with a virus-free, mock preparation and ΔD9- or ΔD10-treated tumors was readily observed by day 14 (Figure 4). The volume of mock-treated tumors increased to a greater extent and more rapidly than tumors treated with VACV ΔD9 or ΔD10 (Figure 4). This difference increased and persisted throughout the entire 20-day course of the experiment, establishing that ΔD9- or ΔD10-deficient VACV treatment has anti-tumor activity against human HepG2 tumors in athymic, nude mice. Compared to the very large tumors in mice treated with a control, virus-free preparation, mice treated with either ΔD9- or ΔD10-deficient viruses had at most small tumors remaining and one animal had no palpable mass. All mice treated with a control, virus-free preparation survived until being euthanized on day 20, but 40% of the treated mice died (between day 17 and 18 for ΔD10 treated and between day 6 and 17 for ΔD9 treated) before the remaining animals were euthanized (day 20 for ΔD10 and mock treated and day 17 for ΔD9 treated). Thus, in athymic nude mice, the absence of the capacity to mount an acquired immune response likely accounts for the virulence of VACV deficient in only ΔD9 or ΔD10. Other VACV mutants that are attenuated in immunocompetent mouse models are likewise more virulent in athymic,
To analyze the anti-tumor response to ΔD9 and ΔD10 OV treatment, HCC tumors were fixed in formalin and embedded in paraffin. Sequential sections from representative tumors were stained with H&E and examined by light microscopy. All sections of the three mock-treated mice showed 45%–50% subcutaneous, viable HCC (Table 1). These tumors have a large main tumor and some smaller satellite tumor nodules, which represent tumor expansion outside the main tumor bed through infiltration or vascular invasion (Figure 5A). The viable HCC has a trabecular and solid growth pattern and is composed of cells with a scant to moderate amount of amphiphilic cytoplasm and moderately pleomorphic nuclei with prominent nucleoli. Mitotic figures were easily identified, and approximately 50%–65% tumor necrosis was noted. A thin pseudo-capsule composed of reactive spindle/stromal cell fibroblasts with patchy, mild, polymorphonuclear cell infiltrates was seen around the tumor nodules (Figure 5D). Compared to mock-treated tumors, ΔD9 and ΔD10 OV-treated HCC xenografts showed very small foci of residual viable tumor and satellite tumor nodules were not observed (Figure 5, compare B and C to A). Significantly, one ΔD9 OV-treated mouse (#67) showed no residual viable tumor in three different sections through the tumor and a second ΔD9-treated mouse (#69) had only three small foci representing about 4% of the xenograft (Figure 5B; Table 1). Both ΔD10-treated mice examined (#51 and #52) showed small foci of residual tumor in two out of three examined sections, representing about 3% of the xenograft (Figure 5C; Table 1). Collectively, the pathological analysis summarized in Figure 5 and Table 1 clearly demonstrate that ΔD9 and ΔD10 OV treatment resulted in substantial tumor necrosis and reduction of viable HepG2 human HCC xenografts that is compatible with their potent anti-tumor effect.

Although residual HCC in the OV-treated mice was similar in morphologic appearance to the control, mock-treated mice, the pseudo-capsule in the OV-treated mice focally shows a thick fibroblastic reaction, with abundant polymorphonuclear cell inflammatory infiltrates predominantly composed of neutrophils (Figures 5B, 5C, 5E, and 5F). To characterize the nature of these cellular infiltrates, immunohistochemistry was performed. Compared to mock-treated tumors, treatment with either ΔD9 or ΔD10 OVs resulted in an influx of slightly more F4/80+ myeloid cells (Figure 6) and a greater number of Ly6C+ cells (Figure 6), which are likely natural killer (NK) cells. These cell types reflect enhanced inflammation resultant from OV infection and are potentially related to the reduced rate of tumor growth in OV-treated tumors. The robust inflammatory infiltrate also appears activated by virtue of increased staining for Granzyme B (Figure 6), a marker of lytic function characteristic of NK cells.

To examine sites of VACV replication, sections from mock, ΔD9-treated, or ΔD10-treated tumor tissue sections were stained with antisera raised against VACV. In addition, human HCC cells were distinguished from normal mouse tissue by co-staining with anti-sera specific for a human nuclear mitotic apparatus component (NuMa). The HCC tumor is clearly demarcated from normal surrounding
mouse tissue by NuMA staining in a representative mock-treated tumor and tumors from four independent ΔD9- or ΔD10-treated mice (Figure 7). At the greatest magnification shown, NuMA antigen accumulation is evident within nuclei. In ΔD9- and ΔD10-treated tumors isolated from multiple animals, VACV antigen is enriched and appears contained within NuMA-positive cells over a range of magnifications (Figure 7). VACV antigen is observed to be confined to the cytoplasm within cells with NuMA-staining nuclei, providing evidence that NuMA-positive cells are infected with VACV (Figure 7). Moreover, cytoplasmic VACV antigen was not detected in the neighboring normal, NuMA-negative mouse tissue. This establishes that NuMA-positive cells are infected with VACV (Figure 7).

Altered Activation of Host dsRNA Responses by D9 or D10 Decapping-Deficient VACV in Human Tumor Cells Compared to Non-tumorigenic Cells

To determine if replication of D9 or D10 decapping-deficient VACVs might be preferentially restricted in non-tumorigenic human cells compared to tumor cells, the activation state of the interferon-induced, dsRNA-dependent protein kinase PKR was investigated. PKR is an interferon-induced host gene that is activated by dsRNA, a pathogen associated molecular pattern (PAMP) that accumulates in virus-infected cells and is a signature of virus infection. Upon activation, PKR phosphorylates the host translation initiation factor eIF2 on its alpha subunit, inactivating this critical translation initiation factor and restricting virus protein synthesis and replication (reviewed by Mohr et al.11). PKR activation is routinely measured by immunoblotting for the autophosphorylated form (on residue T446) using a phospho-specific anti-PKR antibody.36 Following mock-infection or infection (ΔD9, ΔD10, or WT VACV) of several tumorigenic human HCC cell lines (HepG2, Hep3B, and Huh7), the overall abundance of total PKR and phosphorylated (activated) PKR was assessed by immunoblotting. In HepG2 and Hep3B cells, PKR was similarly activated after infection with WT, ΔD9, or ΔD10 VACV beyond levels detected in mock-infected cells as evidenced by phosphorylated PKR abundance (Figure 8). Although the background level of activated PKR was detected in mock-infected Huh7 cells, activated PKR abundance was reduced similarly upon infection with WT, ΔD9, or ΔD10 VACV (Figure 8). Thus, in tumorigenic human cell lines, PKR was not detectably hyperactivated following infection with ΔD9 or ΔD10 VACV compared to WT VACV.

To compare PKR activation upon infection of non-tumorigenic cells with WT, ΔD9, or ΔD10 VACV, NHDFs and cBAL111 cells were either mock-infected or infected. Although NHDFs are primary cells, the cBAL111 cell line was derived from human fetal liver cells immortalized by overexpressing the telomerase reverse transcriptase.43 These cells display hepatic differentiated functionality similar to the parental cells prior to immortalization and express immature hepatocyte markers.43 Significantly, cBAL111 cells do not grow in soft agar and are not tumorigenic in nude mice.43 Remarkably, although phosphorylated PKR was undetectable in mock-infected or WT VACV-infected cBAL111 cells, activated, phosphorylated PKR was readily detected in cells infected by either ΔD9 or ΔD10 VACV (Figure 8). This demonstrates that PKR is selectively hyperactivated upon infection with ΔD9 or ΔD10 decapping-deficient
VACV compared to WT VACV in non-tumorigenic cBAL111 cells and normal, primary human cells. Moreover, it establishes that D9 or D10 decapping-deficient VACV hyperactivates cell intrinsic antiviral responses selectively in non-tumorigenic cells compared to tumorigenic cells.

**DISCUSSION**

Nearly all oncolytic VACV strains have been attenuated by removing viral tk or ribonucleotide reductase genes.\(^{25,28,41,44,45}\) Although presumed to restrict virus growth to dividing cells, these mutations over-attenuate the virus, restricting its capacity to directly destroy cancer cells and induce systemic, anti-tumor immune responses. Indeed, although largely safe, one first-generation VACV OV did not meet expectations in a phase 2b clinical trial.\(^{34,35}\) In lieu of removing genes required for metabolism like tk that enable robust virus replication, a different attenuation strategy involves deleting genes required for pathogenesis. In this regard, VacV encodes two mRNA decapping enzymes (D9 and D10) that hydrolyze the m\(^7\)GTP mRNA cap from 5’ termini that is critical for mRNA translation and stability.\(^{38,39}\) Besides controlling virus and host gene expression, D9 and D10 antagonize host anti-viral responses by limiting dsRNA accumulation.\(^{36,37}\) Although decapping enzymes are not essential for VACV productive growth, viruses deficient for D9 or D10 are attenuated.\(^{37,40}\) Here, we demonstrate that decapping-deficient VACV as a new, tk-positive platform for OV therapy. It further suggests that replication of decapping-deficient VACV in normal cells is restricted by cell intrinsic, anti-viral host defenses.

![Histopathology of HCC Tumors Treated with Decapping-Deficient VACV OV](image)

(A–F) On day 20 (A, C, D, and F) or day 17 (B and E) post-treatment, animals treated as in Figure 4 were sacrificed and explanted tumors were fixed in formalin. Paraffin-embedded sections were prepared and stained with H&E and evaluated by light microscopy. At low magnification (20X; A–C), the amount of residual viable tumor (HCC) in mock-treated mice (A) was substantially greater than that observed in ΔD9- (B) or ΔD10-treated mice (C). Viable HCC is highlighted between the blue lines; the red line shows a satellite tumor nodule present only in mock-treated mice (A). At high magnification (400X; D–F), viable HCC appears surrounded by a capsule composed of fibroblasts and few inflammatory cells in mock-treated mice (D). In contrast, ΔD9- (E) and ΔD10-treated (F) mice show a marked fibro-inflammatory response to the viable and necrotic HCC (left of the blue line: viable HCC; right of the blue line: fibro-inflammatory response). Tumor sections from individual mice are identified by the number at the top of the panels.
defenses like PKR, which are often disabled in tumors, thereby enhancing the tumor specificity of VACV OVs.

Selective activation of dsRNA-dependent host defenses in normal, non-tumor tissue likely helps restrict virus replication and spread to cancer cells, a desirable feature for an OV. Replication of D9- and D10-deficient VacV D9 in normal cells and their virulence in animals is impaired because these viruses, although attenuated, potently activate cellular intrinsic anti-viral defenses. In the absence of VACV decapping enzymes or the host endoribonuclease Xrn1, which degrades decapped miRNA, dsRNA accumulates in infected cells and activates host antiviral defense molecules, including the interferon-inducible, dsRNA-dependent eIF2α kinase PKR and RNase L. Stimulation of PKR and RNase L arrests infected cell protein synthesis and virus replication. The restriction of decapping-deficient VACV by host defenses, including PKR and RNase L, coupled with their attenuation, strongly resembled the phenotype of an HSV1 OV approved by US and European regulatory agencies. Previously, we and others have established that HSV-1 ICP34.5-deficient derivatives, which are defective in controlling PKR and RNase L, are effective OVs. Although HSV1 ICP34.5-deficient viruses and derivative strains are strongly attenuated, they remain capable of destroying cancer cells because tumor cells have deficiencies in many cell intrinsic host defenses. Even though there are no homologs of ICP34.5 in VACV or any other poxvirus, it is remarkable that the PKR-RNase L host defense axis can be harnessed to attenuate VACV by deleting D9 or D10 to create effective OVs. This not only highlights the importance of dsRNA-responsive host defenses in controlling virulence, but hints at a powerful general strategy for OV design that may well have utility across virus families.

Having established decapping-deficient, attenuated VACV mutants as effective OVs with anti-tumor activity in murine and human models, how this platform might be developed and modified going forward can be rationally considered. Our data show that both innate and adaptive anti-tumor immune responses are elicited by VACV OV treatment. Inclusion of additional transgene armaments, such as GM-CSF or perhaps even agents that target the transporter associated with antigen processing (TAP) within tumor cells, could further increase anti-tumor immune responses. Similarly, therapeutic synergy with an immune checkpoint blockade can be investigated. As decapping-deficient VACV retains a functional tk gene, its more robust replication within cancer cells may engender more effective antitumor responses compared to tk-negative viruses. Should further attenuation be indicated once formal safety and toxicology studies are performed, for example, in treating immune compromised patients, the F4L gene encoding a ribonucleotide reductase subunit or the VACV growth factor gene (VGF), which does not significantly impact virus reproduction in a primate cell line, might also be removed. Alternatively, a doubly deficient variant lacking both D9 and D10 might be evaluated for OV therapy, although it might be overly compromised for productive replication. Finally, the VACV tk gene could be replaced with the HSV1 tk gene. Prior studies have shown that VACV infection cells expressing HSV tk is sensitive to acyclovir, an effective antiviral agent. This could...
contribute greatly to the safety of VACV OVs in the clinic by providing access to a well-tested, widely used antiviral drug should any adverse clinical events occur.

MATERIALS AND METHODS

Cells and Viruses

The D9- and D10-deficient recombinant VACVs in the western reserve strain genetic background were kindly provided by Dr. B. Moss (NIAID). 4T1 and MCA38 cells were kindly provided by Dr. S. Demaria (Weil Cornell). NHDFs were purchased from Lonza (Walkersville, MD). Huh-7 cells, originally obtained from JCRB Genebank (Shinjuku, Japan), HepG2 and Hep3B cells, originally obtained from ATCC (ATCC HB-8064, CRL-2522), and cBAL111 cells were generously provided by Dr. A. Epstein (Lyon, France).

Mouse In Vivo Tumor Models

All animal procedures were performed in accordance with protocols approved by the institutional animal care & use committee at New York University (NYU) School of Medicine. 4T1 cells (1 × 10⁴) in DMEM without additives were injected subcutaneously (s.c.) into the right flank of 8-week-old, female BALB/c mice anesthetized by intraperitoneal (i.p.) injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). Tumor growth was monitored every day using an electronic digital caliper, and tumor volume was calculated as described. When tumors reached approximately 50 mm³ (8 to 9 days after 4T1 inoculation), they were directly injected on days 0, 3, and 6 with 5.4 × 10⁶ PFU of D10-deficient VACV (N = 10 mice) or an equivalent volume of virus-free control preparation from uninfected cells (N = 10 mice). Tumor size was monitored over time, and animals were euthanized when control-treated tumors reached approximately 1,200 mm³.

MCA38 murine colon adenocarcinoma cells in media were injected (1 × 10⁵) s.c. into the flank of 4- to 6-week-old, female C57/B16 mice. When tumors reached approximately 50 mm³ (approximately 7 days after MCA38 inoculation), they were directly injected on days 0, 3, and 6 with 1.0 × 10⁶ PFU of D10-deficient (ΔD10) VACV (N = 10 mice), 1.0 × 10⁶ PFU of D9-deficient (ΔD9) VACV (N = 10 mice), or an equivalent volume of virus-free control preparation from uninfected cells (N = 10 mice). Tumor size was monitored over time and animals were euthanized when control-treated tumors reached approximately 1,200 mm³.
were incubated for 1 hr at 60
stored at room temperature. Immediately prior to staining, slides
tumors were
in media were injected (1/C2
8-week-old, female, athymic (nude) mice. HepG2 human HCC cells
thick) cut from formalin-
embedded HepG2 xenograft tumors were stained with H&E, exam-
Sequentially cut sections (4 m
Histopathology and Immunohistochemistry
Human HCC (HepG2, Hep3B, and Huh7) or untransformed, non-tumorigenic
cBAL111 human liver cells were either mock-infected or infected (MOI = 5) with WT
VACV, D9-deficient VACV (ΔD9), or D10-deficient VACV (ΔD10). After 18.5 hr, total
protein was isolated and analyzed by immunoblotting with either total PKR or a PKR
phospho-specific antibody as described. Anti-NuMa antibody was optimized and validated on tissue microar-
oids composed of both human and murine tissues. Anti-VACV was
optimized on HepG2 HCC xenografts infected with VACV. Sequential
chromogenic immunohistochemistry was performed on a Ventana Medical Systems Discovery XT plat-
form, with online deparaffinization, using Ventana’s reagents and
detection kits unless otherwise noted. Slides stained for Ly6G/Ly6c were
deparaffinized online and treated with protease-3 (Ventana Medical Systems) for 8 min. Samples for staining with F480 were
heat retrieved using Cell Conditioner 2 (citrates pH 6.0) for 20 min,
and samples for staining with Granyme-B were heat retrieved using Cell Conditioner 1 (Tris-Borate-EDTA, pH 8.5) for 36 min. Endoge-
rous peroxidase activity was blocked for all samples. Ly6G/Ly6c, F480, and Granyme-B antibodies were diluted in Tris-buffered saline
(Thermo Scientific) to 1:800, 1:100, and 1:100, respectively. Ly6G/Ly6c and Granyme-B antibodies were incubated for 12 hr and
F480 was incubated for 6 hr, each at room temperature. Ly6G/Ly6c and F480 antibodies were detected with goat anti-rat horseradish
peroxidase-conjugated multimer incubated for 16 min, and anti-
Granyme-B was detected with goat anti-rabbit horseradish-peroxi-
dase-conjugated multimer incubated for 8 min. The complex was
visualized with 3,3 diaminobenzidene and enhanced with copper sul-
fate. Slides were washed in distilled water, counterstained with hema-
toxyl, dehydrated, and mounted with permanent media.

HepG2 human HCC xenografts were established in the flanks of
8-week-old, female, athymic (nude) mice. HepG2 human HCC cells
in media were injected (1 × 10⁶ s.c. and when tumors reached
approximately 50 mm³ (approximately 7 days after HepG2 inocula-
tion), they were directly injected on days 0, 3, 6, and 9 with
1.0 × 10⁶ PFU of D10-deficient (ΔD10) VACV (N = 10 mice),
1.0 × 10⁶ PFU of D9-deficient (ΔD9) VAC (N = 10 mice), or
an equivalent virus-free control preparation from uninfected cells
(N = 10 mice).

Histopathology and Immunohistochemistry
Sequentially cut sections (4 μm thick) from formalin-fixed, paraffin-
embedded HepG2 xenograft tumors were stained with H&E, exam-
ined on an Olympus BX53 light microscope, and photographed.
The first, fourth, and seventh sections were evaluated from each
representative tumor. For immunohistochemistry, sections (4 μm
thick) cut from formalin-fixed, paraffin-embedded HepG2 xenograft
tumors were floated onto Plus slides (Fisher Scientific), air dried, and
stored at room temperature. Immediately prior to staining, slides
were incubated for 1 hr at 60°C in a convection oven. The following
antibodies were used for chromogenic immunohistochemistry: rabbit
anti-mouse Ly6G/Ly6c, clone NIMP-R14 IgG2b (Abcam catalog #
ab2557, Lot GR135037-1, AB_303154), rat anti-mouse F480, clone
BM8 IgG2a (Biologicals catalog # 14-4801, Lot # E016242, RRID: AB_2314387), and polyclonal rabbit anti-mouse Granyme-B
(Abcam catalog # ab4059, Lot # GR276728-4, RRID: AB_304251),
 polyclonal rabbit anti-Vaccinia virus (VAC, ViroStat catalog #
8101), and unconjugated, polyclonal rabbit anti-Human Nuclear
Mitotic Apparatus Protein (NuMA, Abcam catalog # ab97585, Lot GR268490-12, RRID: AB_10680001). Immunohistochemistry was
performed on a Ventana Medical Systems Discovery XT plat-
form, with online deparaffinization, using Ventana’s reagents and
detection kits unless otherwise noted. Slides stained for Ly6G/Ly6c were
deparaffinized online and treated with protease-3 (Ventana Medical Systems) for 8 min. Samples for staining with F480 were
heat retrieved using Cell Conditioner 2 (citrates pH 6.0) for 20 min,
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rous peroxidase activity was blocked for all samples. Ly6G/Ly6c, F480, and Granyme-B antibodies were diluted in Tris-buffered saline
(Thermo Scientific) to 1:800, 1:100, and 1:100, respectively. Ly6G/Ly6c and Granyme-B antibodies were incubated for 12 hr and
F480 was incubated for 6 hr, each at room temperature. Ly6G/Ly6c and F480 antibodies were detected with goat anti-rat horseradish
peroxidase-conjugated multimer incubated for 16 min, and anti-
Granyme-B was detected with goat anti-rabbit horseradish-peroxi-
dase-conjugated multimer incubated for 8 min. The complex was
visualized with 3,3 diaminobenzidene and enhanced with copper sul-
fate. Slides were washed in distilled water, counterstained with hema-
toxyl, dehydrated, and mounted with permanent media.

Anti-NuMa antibody was optimized and validated on tissue microar-rays composed of both human and murine tissues. Anti-VACV was
optimized on HepG2 HCC xenografts infected with VACV. Sequential
chromogenic immunohistochemistry was performed on a Ventana Medical Systems Discovery XT platform as described in the
preceding paragraph. Sections were deparaffinized online and
endogenous peroxidase activity was blocked. Anti-VACV was diluted
1:1,500 in Tris, 0.15 M NaCl, and 1% BSA, incubated for 1 hr at 37°C, and detected with goat anti-rabbit horseradish
peroxidase-conjugated multimer for 8 min. The complex was
visualized with 3,3 diaminobenzidene and enhanced with copper sul-
fate. Slides were washed in distilled water, and epitope retrieval was
performed in a 1,200-W microwave oven at 100% power in 10 mM
sodium citrate buffer, pH 6.0, for 10 min. Slides were allowed to
cool for 30 min, rinsed in distilled water, and reloaded onto the instru-
ment. Anti-NUMA was diluted 1:5,000 in Tris-BSA (25 mM Tris, 0.15 M NaCl, and 1% BSA), incubated for 1 hr at 37°C, and detected with goat anti-rabbit horseradish-peroxidase-conjugated multimer followed by alpha-naphthol
pyronin (8 min each). Slides were counterstained with hematoxylin,
dehydrated, and mounted with permanent media. Double-negative
controls substituted PBS for primary antibody in the sequential stain-
ing. Because both primary antibodies are made in a rabbit, we
included an additional control test to eliminate cross-over detection.
The test slide consisted of application of all reagents for anti-VACV
antibody detection, except for the application of goat anti-rabbit
secondary. The test slide was epitope retrieved (as described above)
to strip off previous reagents and then re-probed with goat anti-rabbit
horseradish-peroxidase-conjugated multimer as described above.

Figure 8. Hyperactivation of PKR in Non-tumorigenic Human Cells
Following Infection with D9- or D10-Deficient VACV
Double-negative and cross-over controls did not have demonstrable labeling.

**Antibodies**

The following antibodies for immunoblotting were purchased from the indicated commercial sources: total PKR (Cell Signaling Technology # 12297) and phospho(T446)-PKR (Abcam # 32036).

**AUTHOR CONTRIBUTIONS**

I.M. conceived the project, H.M.B., A.P., A.B.F., and I.M. designed the experiments. H.M.B., A.P., C.H.H., and L.C. performed the experiments. H.M.B., A.B.F., C.H.H., L.C., A.P., and I.M. wrote and edited the manuscript.

**CONFLICTS OF INTEREST**

The authors declare no conflict of interest.

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**REFERENCES**

1. Russell, S.J., and Peng, K.W. (2017). Oncolytic virotherapy: a contest between apples and oranges. Mol. Ther. 25, 1107–1116.
2. Saha, D., Wakimoto, H., and Rabkin, S.D. (2016). Oncolytic herpes simplex virus interactions with the host immune system. Curr. Opin. Virol. 21, 26–34.
3. Lichty, B.D., Breitbach, C.J., Stojdl, D.F., and Bell, J.C. (2014). Going viral with cancer immunotherapy. Nat. Rev. Cancer 14, 559–567.
4. Mohr, I., and Gluzman, Y. (1996). A herpesvirus genetic element which affects translation in the absence of the viral GADD34 function. EMBO J. 15, 4759–4766.
5. Mohr, I., Sternberg, D., Ward, S., Leib, D., Mulvey, M., and Gluzman, Y. (2001). A herpes simplex virus type 1 gamma34.5 second-site suppressor mutant that exhibits enhanced growth in cultured glioblastoma cells is severely attenuated in animals. J. Virol. 75, 5189–5196.
6. Taneja, S., MacGregor, J., Markus, S., Ha, S., and Mohr, I. (2001). Enhanced antitumor efficacy of a herpes simplex virus mutant isolated by genetic selection in cancer cells. Proc. Natl. Acad. Sci. USA 98, 8804–8808.
7. Todd, T., Martuza, R.L., Rabkin, S.D., and Johnson, P.A. (2001). Oncolytic herpes simplex virus vector with enhanced MHC class I presentation and tumor cell killing. Proc. Natl. Acad. Sci. USA 98, 6396–6401.
8. Liu, B.L., Robinson, M., Han, Z.Q., Branston, R.H., English, C., Reay, P., McGrath, Y., Thomas, S.K., Thornton, M., Bullock, P., et al. (2003). ICP34.5 deleted herpes simplex virus with enhanced oncolytic, immune stimulating, and anti-tumour properties. Gene Ther. 10, 292–303.
9. Chou, J., Kern, E.R., Whiteley, R.J., and Roizman, B. (1990). Mapping of herpes simplex virus-1 neurorovirulence to gamma 134.5, a gene nonessential for growth in culture. Science 250, 1262–1266.
10. Sadler, A.J., and Williams, B.R. (2008). Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8, 559–568.
11. Mohr, I., and Sonenberg, N. (2012). Host translation at the nexus of infection and immunity. Cell Host Microbe 12, 470–483.
12. Mulvey, M., Poppers, J., Ladd, A., and Mohr, I. (1999). A herpesvirus ribosome-associated, RNA-binding protein confers a growth advantage upon mutants deficient in a GADD34-related function. J. Virol. 73, 3375–3385.
13. Sanchez, R., and Mohr, I. (2007). Suppression of PACT-induced type I interferon production by herpes simplex virus 1 Us11 protein. J. Virol. 81, 3455–3464.
14. Mulvey, M., Camarena, V., and Mohr, I. (2004). Full resistance of herpes simplex virus type 1 infected primary human cells to alpha interferon requires both the Us11 and gamma134.5 gene products. J. Virol. 78, 10193–10196.
15. Kew, C., Lui, P.Y., Chan, C.P., Liu, X., Xu, S.W., Mohr, I., Jin, D.Y., and Kok, K.H. (2013). Suppression of PACT-induced type I interferon production by herpes simplex virus 1 Us11 protein. J. Virol. 87, 13141–13149.
16. Herdy, B., Jaramillo, M., Svitkin, Y.V., Rosenfeld, A.B., Kobayashi, M., Walsh, D., Alain, T., Sean, P., Robichaud, N., Topisirovic, I., et al. (2012). Translational control of the activation of transcription factor NF-kB and production of type I interferon by phosphorylation of the translation factor elf4E. Nat. Immunol. 13, 543–550.
17. Mulvey, M., Poppers, J., Sternberg, D., and Mohr, I. (2003). Regulation of elf2alpha phosphorylation by different functions that act during discrete phases in the herpes simplex virus type 1 life cycle. J. Virol. 77, 10917–10928.
18. Critchley-Thorne, R.J., Simons, D.L., Yan, N., Miyahira, A.K., Dirbas, F.M., Johnson, D.L., Swetter, S.M., Carlson, R.W., Fisher, G.A., Koong, A., et al. (2009). Impaired interferon signaling is a common immune defect in human cancer. Proc. Natl. Acad. Sci. USA 106, 9010–9015.
19. Mohr, I. (2005). To replicate or not to replicate: achieving selective oncolytic virus replication in cancer cells through translational control. Oncogene 24, 7697–7709.
20. Pourcher, A., Fuhrmann, S.R., Päonès, K.A., Demaria, S., Frey, A.B., Mulvey, M., and Mohr, I. (2016). CD9(+)-T cell immune evasion enables oncolytic virus immunotherapy. ElBioMedicine 5, 59–67.
21. Jan, E., Mohr, I., and Walsh, D. (2016). A cap-to-tail guide to mRNA translation strategies in virus-infected cells. Annu. Rev. Virol. 3, 283–307.
22. Langland, J.O., and Jacobs, R.L. (2002). The role of the PKR-inhibitory genes, E3L and GADD34, in determining vaccinia virus host range. Virology 299, 133–141.
23. Rice, A.D., Turner, P.C., Embury, J.E., Moldawer, L.L., Baker, H.V., and Moyer, R.W. (2011). Roles of vaccinia virus genes E3L and K3L and host genes PKR and RNase L during intratracheal infection of C57BL/6 mice. J. Virol. 85, 550–567.
24. Brandt, T.A., and Jacobs, R.L. (2001). Both carboxy- and amino-terminal domains of the vaccinia virus interferon resistance gene, E3L, are required for pathogenesis in a mouse model. J. Virol. 75, 850–856.
25. McCart, J.A., Ward, J.M., Lee, J., Hu, Y., Alexander, H.R., Libutti, S.K., Moss, B., and Bartlett, D.L. (2001). Systemic cancer therapy with a tumor-selective vaccinia virus mutant lacking thymidine kinase and vaccinia growth factor genes. Cancer Res. 61, 8751–8757.
26. Kim, J.H., Oh, J.Y., Park, B.H., Lee, D.E., Kim, J.S., Park, H.E., Roh, M.S., Je, J.E., Yoon, J.H., Thorne, S.H., et al. (2006). Systemic armed oncolytic and immunologic therapy for cancer with JX-594, a targeted poxvirus expressing GM-CSF. Mol. Ther. 14, 361–370.
27. Thorne, S.H., Hwang, T.H., O’Gorman, W.E., Bartlett, D.L., Sei, S., Kanji, F., Brown, C., Werier, J., Cho, J.H., Lee, D.E., et al. (2007). Rational strain selection and engineering creates a broad-spectrum, systemically effective oncolytic poxvirus, JX-963. J. Clin. Invest. 117, 3350–3358.
28. Breitbach, C.J., Parato, K., Burke, J., Hwang, T.H., Bell, J.C., and Kirn, D.H. (2015). Pexa-Vec double agent engineered vaccinia: oncolytic and active immunotherapeutic. Curr. Opin. Virol. 13, 49–54.
29. Downs-Canner, S., Guo, Z.S., Ravindranathan, R., Breitbach, C.J., O’Malley, M.E., Jones, H.L., Moon, A., McCart, J.A., Shuai, Y., Zeh, H.J., et al. (2016). Phase I study of intravenous oncolytic poxvirus (vvDD) in patients with advanced solid cancers. Mol. Ther. 24, 1492–1501.
30. Breitbach, C.J., Burke, J., Jonker, D., Stephenson, J., Haas, A.R., Chow, L.Q., Nieva, J., Hwang, T.H., Moon, A., Patt, R., et al. (2011). Intravenous delivery of a multi-mechanistic cancer-targeted oncolytic poxvirus in humans. Nature 477, 99–102.
31. Heo, J., Reid, T., Ruo, L., Breitbach, C.J., Rose, S., Bloomston, M., Cho, M., Lim, H.Y., Chung, H.C., Kim, C.W., et al. (2013). Randomized dose-finding clinical trial of oncolytic immunotherapeutic vaccinia virus JX-594 in liver cancer. Nat. Med. 19, 329–336.

32. Park, S.H., Breitbach, C.J., Lee, J., Park, J.O., Lim, H.Y., Kang, W.K., Moon, A., Mun, J.H., Sommermann, E.M., Maruni Avidal, L., et al. (2015). Phase 1b trial of biweekly intravenous Pexa-Vec (JX-594), an oncolytic and immunotherapeutic vaccinia virus in colorectal cancer. Mol. Ther. 23, 1532–1540.

33. Zeh, H.J., Downs-Canner, S., McCart, J.A., Bartlett, D.L., and Moss, B. (2013). Vaccinia virus expression vector for vaccination. European Patent 2051881A1.

34. Carroll, J. (2013). Korean CRO grabs failed cancer vaccine in $150m-max Jennerex buyout. https://www.fiercebiotech.com/financials/korean-cro-grabs-failed-cancer-vaccine-150m-max-jennerex-buyout.

35. ClinicalTrials.gov (2016). A study of recombinant vaccinia virus prior to sorafenib to treat unresectable primary hepatocellular carcinoma. https://clinicaltrials.gov/ct2/show/study/NCT01171651.

36. Burgess, H.M., and Mohr, I. (2015). Cellular 5′-3′ mRNA exonuclease Xrn1 controls double-stranded RNA accumulation and anti-viral responses. Cell Host Microbe 17, 332–344.

37. Liu, S.W., Katsafanas, G.C., Liu, R., Wyatt, L.S., and Moss, B. (2015). Poxvirus decapping enzymes enhance virulence by preventing the accumulation of dsRNA and the induction of innate antiviral responses. Cell Host Microbe 17, 320–331.

38. Parrish, S., and Moss, B. (2007). Characterization of a second vaccinia virus mRNA-decapping enzyme conserved in poxviruses. J. Virol. 81, 12973–12978.

39. Parrish, S., Resch, W., and Moss, B. (2007). Vaccinia virus D10 protein has mRNA decapping activity, providing a mechanism for control of host and viral gene expression. Proc. Natl. Acad. Sci. USA 104, 2139–2144.

40. Liu, S.W., Wyatt, L.S., Orlandi, M.S., Minai, M., and Moss, B. (2014). The D10 decapping enzyme of vaccinia virus contributes to decay of cellular and viral mRNAs and to virulence in mice. J. Virol. 88, 202–211.

41. Buller, R.M., Smith, G.L., Cremer, K., Notkins, A.L., and Moss, B. (1985). Decreased virulence of recombinant vaccinia virus expression vectors is associated with a thymidine kinase-negative phenotype. Nature 317, 813–815.

42. McCart, J.A., Bartlett, D.L., and Moss, B. (2013). Vaccinia virus expression vector for selective replication in a tumor cell and induction of exogenous nucleotide sequence into a tumor cell. U.S. patent 8506947.

43. Deurholt, T., van Til, N.P., Chhatta, A.A., ten Bloemendaal, L., Schwartlander, R., Payne, C., Plevris, J.N., Sauer, I.M., Chamuleau, R.A., Elferink, R.P., et al. (2009). Novel immortalized human fetal liver cell line, cBAL111, has the potential to differentiate into functional hepatocytes. BMC Biotechnol. 9, 89.

44. Gammon, D.B., Gowrishankar, B., Duraffour, S., Andrei, G., Upton, C., and Evans, D.H. (2010). Vaccinia virus-encoded ribonucleotide reductase subunits are differentially required for replication and pathogenesis. PLoS Pathog. 6, e1000984.

45. Potts, K.G., Irwin, C.R., Favis, N.A., Pink, D.B., Vincent, K.M., Lewis, J.D., Moore, R.B., Hit, M.M., and Evans, D.H. (2017). Deletion of F4L (ribonucleotide reductase) in vaccinia virus produces a selective oncolytic virus and promotes anti-tumor immunity with superior safety in bladder cancer models. EMBO Mol. Med. 9, 638–654.

46. U.S. Food and Drug Administration (2015). IMLYGIC (talimogene laherparepvec). https://www.fda.gov/BiologicsBloodVaccines/CellularGeneTherapyProducts/ApprovedProducts/ucm469411.htm.

47. European Medicines Agency (2017). Imlygic. http://www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002771/human_med_001941.jsp&mid=WCIb01ac058001d124.

48. Liu, Z., Ravindranathan, R., Kalinski, P., Guo, Z.S., and Bartlett, D.L. (2017). Rational combination of oncolytic vaccinia virus and PD-L1 blockade works synergistically to enhance therapeutic efficacy. Nat. Commun. 8, 14754.

49. Rojas, J.J., Sampath, P., Hou, W., and Thorne, S.H. (2015). Defining effective combinations of immune checkpoint blockade and oncolytic virotherapy. Clin. Cancer Res. 21, 5543–5551.

50. Buller, R.M., Chalerbarbi, S., Cooper, J.A., Twardzik, D.R., and Moss, B. (1988). Deletions of the vaccinia virus growth factor gene reduce virus virulence. J. Virol. 62, 866–874.

51. Darby, G., Larder, B.A., Bastow, K.F., and Field, H.J. (1980). Sensitivity of viruses to phosphorylated 9-(2-hydroxymethylguanine revealed in TK-transformed cells. J. Gen. Virol. 48, 451–454.

52. Panicali, D., and Paolotti, E. (1982). Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA 79, 4927–4931.

53. Parrish, S., and Moss, B. (2006). Characterization of a vaccinia virus mutant with a deletion of the D10R gene encoding a putative negative regulator of gene expression. J. Virol. 80, 553–561.

54. Demaria, S., Kawashima, N., Yang, A.M., Devitt, M.L., Babb, J.S., Allison, J.P., and Formenti, S.C. (2005). Immune-mediated inhibition of metastases after treatment with local radiation and CTLA-4 blockade in a mouse model of breast cancer. Clin. Cancer Res. 11, 728–734.

55. Hiebert, P.R., Bovin, W.A., Zhao, H., McManus, B.M., and Granville, D.J. (2013). Perforin and granzyme B have separate and distinct roles during atherosclerotic plaque development in apolipoprotein E knockout mice. PLoS One 8, e78939.

56. Mackler, A.M., Green, L.M., McMillan, P.J., and Yellon, S.M. (2000). Distribution and activation of uterine mononuclear phagocytes in peripartum endometrium and myometrium of the mouse. Biol. Reprod. 62, 1193–1200.

57. Perez-Aso, M., Montesinos, M.C., Mediero, A., Wilder, T., Schafer, P.H., and Cronstein, B. (2015). Apremilast, a novel phosphodiesterase 4 (PDE4) inhibitor, regulates inflammation through multiple cAMP downstream effectors. Arthritis Res. Ther. 17, 249.

58. Puentes, F., van der Stok, R.J., Victor, M., Kipp, M., Beyer, C., Peiferen Baert, R., Ummenhum, K., Pyce, G., Gerritsen, W., Huizinga, R., et al. (2013). Characterization of immune response to neurofilament light in experimental autoimmune encephalomyelitis. J. Neuroinflammation 10, 118.

59. Walsh, D., Arias, C., Perez, C., Halladin, D., Escandon, M., Ueda, T., Watanabe-Fukunaga, R., Fukunaga, R., and Mohr, I. (2008). Eukaryotic translation initiation factor 4F architectural alterations accompany translation initiation factor redistribution in poxvirus-infected cells. Mol. Cell. Biol. 28, 2648–2658.