A Novel Therapeutic Regimen to Eradicate Established Solid Tumors with an Effective Induction of Tumor-Specific Immunity

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Abstract

Purpose: The efficacy of oncolytic viruses depends on multiple actions including direct tumor lysis, modulation of tumor perfusion, and stimulation of tumor-directed immune responses. In this study, we investigated whether a sequential combination of immunologically distinct viruses might enhance antitumor efficacy through the induction of tumor-specific immunity and circumvention or mitigation of antiviral immune responses.

Experimental Design: The Syrian hamster as an immune-competent model that supports replication of both adenovirus and vaccinia virus was evaluated in vitro and in vivo. The antitumor efficacy of either virus alone or sequential combination of the two viruses was examined in pancreatic and kidney cancer models. The functional mechanism of the regimen developed here was investigated by histopathology, immunohistochemistry staining, CTL assay, and T-cell depletion.

Results: The Syrian hamster is a suitable model for assessment of oncolytic adenovirus and vaccinia virus. Three low doses of adenovirus followed by three low doses of vaccinia virus resulted in a superior antitumor efficacy to the reverse combination, or six doses of either virus alone, against pancreatic and kidney tumors in Syrian hamsters. A total of 62.5% of animals bearing either tumor type treated with the sequential combination became tumor-free, accompanied by the induction of effective tumor-specific immunity. This enhanced efficacy was ablated by CD3+ T-cell depletion but was not associated with humoral immunity against the viruses.

Conclusion: These findings show that sequential treatment of tumors with oncolytic adenovirus and vaccinia virus is a promising approach for cancer therapy and that T-cell responses play a critical role. Clin Cancer Res; 18(24); 6679–89. ©2012 AACR.

Introduction

Oncolytic viruses have been developing as an attractive class of therapeutics for treatment of cancers that are resistant to conventional therapies (1). Although the safety data of oncolytic viruses are encouraging, the therapeutic outcomes of clinical trials of replication-selective oncolytic viruses used alone are disappointing. Therefore, it is imperative to develop new strategies to improve the anticancer potency of these agents. Most studies have focused on improving the direct antitumor properties of these viruses, although there is now an increasing body of evidence that the host immune response is critical to the efficacy of oncolytic virotherapy (2).

For oncolytic virus–based therapeutics, the host immune response is a double-edged sword. On the one hand, a vigorous host immune response to the oncolytic virus can result in rapid viral clearance before the virus is able to exert a therapeutic effect or even result in tumor progression due to immunosuppression. The efficacy of multiple injections of the same virus may be further limited by a neutralizing antibody response (3). One approach that may overcome this obstacle is to sequentially apply 2 or more antigenically distinct viruses so that the specific immunity that arises subsequent to the first virus does not inhibit the therapeutic
Translational Relevance

Despite advances in minimally invasive surgery, hyperfractionated radiotherapy, and new combinations of chemotherapeutic agents, the survival rates for patients with many solid tumor types have remained unchanged. Oncolytic viruses have been developed as a new approach for the treatment of cancers that are resistant to standard therapies. Although the clinical safety profiles are encouraging, the efficacy of oncolytic viruses as single-agent therapy has been limited. Exploiting our knowledge of cancer cell biology, immunology, and virology, we have developed a novel therapeutic regimen by sequential combination of oncolytic adenovirus and vaccinia virus. Not only does this regimen eradicate established tumors in immunocompetent animal models, but strikingly it also results in long-lasting tumor-specific immunity. These findings provide proof-of-concept support for sequential use of oncolytic adenovirus and vaccinia virus as a powerful antitumor therapeutic modality that can be directly translated into patients with cancer.

The lack of immune-competent models for investigating oncolytic virus therapy, required us to first establish and validate an immune-competent animal model in which tumor cells could support the replication of both viruses. Like many oncolytic viruses, adenovirus replication is species-specific and this virus only replicates well in human tumors, with poor replication seen in most murine tumor cell lines (10, 11). Most published efficacy data have, therefore, come from human tumor xenografts in nude mice (12). These models do not accurately reflect the action of oncolytic virus in terms of the host immune response, as these mice are athymic and lack functional T cells (13).

The Syrian hamster has been established as a suitable immune-competent model for the assessment of adenovirus as the level of replication observed is significantly higher than that in murine and cotton rat tumors (10, 15, 16) and adenovirus serotype 5 (Ad5), the serotype most commonly used in gene therapy, replicates in the lungs in humans and the Syrian hamster (15) but not in mice (17) and rats (18).

Oncolytic vaccinia virus does not suffer from the same paucity of immunocompetent animal models due to its wide tropism for mammalian cells (19). Gene therapy delivered by vaccinia virus has been evaluated in murine (20–22), rat (23) and guinea pig (24) models in vivo. Vaccinia virus has been found to replicate in Syrian hamster cell lines in vitro (25). The immune response of the Syrian hamster to vaccinia virus infection has also been shown to mimic that of humans, as it is in part mediated by natural killer cells, a feature not seen in mice (26). The Th1-dominant cytokine response of Syrian hamsters to the parasite that causes visceral leishmaniasis was similar to humans, whereas that observed in mice was strikingly different (27). The Th1 response is also known to be critical for the host immune response to vaccinia virus (28). The Syrian hamster was, therefore, chosen for investigation as a candidate likely to support the replication of both oncolytic vaccinia virus and adenovirus.

Materials and Methods

Cell lines

The Syrian hamster cell lines RPMI-1846 [melanotic melanoma maintained in McCoy’s 5A with 10% fetal calf serum (FCS)], DDT1-MF-2, and HaK [leiomyosarcoma and kidney tumors, respectively maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 10% FCS] were obtained from the American Type Culture Collection (ATCC). The Syrian hamster cell lines HAP-T1 (pancreatic carcinoma maintained in DMEM with 10% FCS), HPD-1NR and HPD-2NR (pancreatic carcinomas maintained in RPMI with 10% FCS), HKT-1097 (kidney carcinoma maintained in DMEM with 10% FCS), and M3ES/C3 (fetal lung epithelial cell line maintained in RPMI with 10% FCS) were obtained from the German Collection of Microorganisms and cell cultures. CV1, the African Green Monkey normal kidney cell line was obtained from ATCC and cultured in DMEM supplemented with 10% FCS. JH293, the human kidney cell line was obtained from ATCC and cultured in DMEM supplemented with 10% FCS.
kidney epithelial cell line transformed with Ad5 DNA, was obtained from the Cancer Research UK Central Cell Services (London, United Kingdom) and maintained in DMEM with 10% FCS.

Viruses

The highly attenuated Lister vaccine strain of vaccinia virus (VVLister) was provided by Istvan Fodor (Loma Linda University, Loma Linda, CA). Wild-type adenovirus (Ad5) was described previously (29).

Cytotoxicity assay

The assay was conducted at least 3 times and the EC₅₀ value (viral dose killing 50% of tumor cells) calculated as previously described (29).

Viral replication assay

Cells were infected with 5 plaque-forming unit (PFU)/cell of Ad5 or VVLister in media with 2% FCS 16 to 18 hours later. Samples were harvested in triplicate at 24-hour intervals up to 96 hours after infection, freeze-thawed 3 times, and titrated on H293 cells for Ad5 or CV1 cells for VVLister to determine the 50% tissue culture infective dose (TCID₅₀) as previously described (29).

Western blotting for detection of oncolytic viral protein expression

A total of 2 × 10⁸ HPD-1NR or HaK cells were seeded in 60-mm dishes and infected with 5 PFU/cell of Ad5 or VVLister in medium with 2% FCS after 16 to 18 hours. Cells were harvested at 3, 6, 12, 24, 48, and 72 hours following infection. Thirty microgram of proteins were separated on precast NuPage Novex 4% to 12% Bis–Tris gels (Invitrogen), electrotransferred onto nitrocellulose membranes, and probed with a polyclonal rabbit antibody to the Lister vaccinia virus coat protein (MorphoSys UK Ltd.), a monoclonal murine antibody known to cross-react with Ad5 E1A (Calbiochem) or a polyclonal rabbit antibody to Ad5 hexon (Autogen Bioclear). Immunocomplexes were detected by incubation with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies (Autogen Bioclear Ltd.), a monoclonal murine antibody known to cross-react to Ad5 E1A (Calbiochem) or a polyclonal rabbit antibody to Ad5 hexon (Autogen Bioclear). Immunocomplexes were detected by incubation with appropriate horseradish peroxidase (HRP)–conjugated secondary antibodies (Autogen Bioclear) and visualized by enhanced chemiluminescence (ECL reagent; Amersham). a-Tubulin was used as a loading control.

Real-time quantitative PCR

DNA was extracted from hamster cells collected as earlier using the QIAamp DNA Blood Mini Kit (QIAGEN Ltd.) according to the manufacturer’s instructions. TaqMan system primers and probes (Supplementary Table S1) were designed using Primer Express v3.0 software (Applied Biosystems) and constructed by Sigma-Aldrich and Applied Biosystems, respectively. Samples, controls, and standards (5–5 × 10⁷ viral genome copies) were tested in triplicate in each plate by quantitative PCR (qPCR) using 7500 Real-time PCR System with samples at 48°C for 30 minutes, 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds, and 60°C for 1 minute. Cycle thresholds (Cₜ) were determined using 7500 System SBS software and used to create standard curves in Prism (GraphPad Software). Results were expressed as genome copy number/cell based on the mean number of cells per sample at viral infection.

Detection of the total and neutralizing antibodies against Ad5 and vaccinia virus

In brief, for detection of the total antibodies against Ad5 and vaccinia virus in serum, Stripwell microplates (Corning Incorporated) were coated with 1 × 10⁷ PFU/mL Ad5 or 1 × 10⁶ PFU/mL of vaccinia virus in 50 μL carbonate buffer (pH 9.6) per well at 4°C overnight. Wells were blocked with 200 μL PBS (pH 7.4) containing 10% FCS for 1 hour at room temperature, and then incubated for 2 hours with 50 μL 100-fold–diluted serum samples in PBS (pH 7.4) containing 10% FCS. After washing 4 times with PBS (pH 7.4) containing 0.05% Tween 20, the wells were incubated for 1 hour with 50 μL HRP-conjugated anti-hamster (Syrian) immunoglobulin G (IgG; H+L; Alpha Diagnostic International, 1:3,000 dilution). After washing, 50 μL 1-Step Ultra TMB-ELISA solution (34028, Thermo Scientific) were added as substrate and the color reaction was terminated by addition of 50 μL 2 N sulfuric acid. Color intensity was determined using a photometer at a wavelength of 450 nm. A standard curve was drawn for each plate using a reference serum. The relative levels of total antibodies in sera were calculated using the level of the reference serum as 100. Virus-neutralizing antibody was detected as previously described (30) by serially diluting the sera (1:2), incubating with Ad5 or VVLister (100 PFU/well) for 1 hour at 37°C before incubation with the indicator cells A549. Wells were individually scored (positive or negative) for cytopathic effect (CPE) at 6 days postinfection. Neutralizing antibody titers were determined by the highest dilution of serum that resulted in at least 50% inhibition of CPE (<2 of 4 wells positive for CPE).

CTL assay

Spleen cells harvested from Syrian hamsters bearing tumors that had been treated with different viral regimens were stimulated with mitomycin C (MMC)-treated HPD-1NR cells at a ratio of 10:1 in RPMI-1640 supplemented with 0.1% 2-mercaptoethanol for 3 days. Nonadherent cells were harvested as effector cells by Lymphocyte Separation Media (LTS1077, TBD Corp.). A total of 3 × 10⁴ target cells (HPD-1NR cells) or control target cells (HaK cells) were incubated with effector cells for 6 hours at 37°C in U-bottom 96-well plates in a volume of 150 μL per well at different ratio of 1:20, 1:10, 1:5, and 1:2.5 (in triplicate wells). Supernatant from each well was harvested, and the percentage of specific lysis was determined using CytoTox 96 (Promega) according to the manufacturer’s instructions.

In vivo animal studies

A total of 1 × 10⁸ HPD-1NR or 5 × 10⁸ HaK cells were implanted subcutaneously into the right flank of female, 4- to 5-week-old Syrian hamsters. When tumors reached 6 to 7 mm in diameter, hamsters were stratified by tumor size into
groups of 7 or 8 to receive 100 μL intratumoral (IT) injections of VVLister or Ad5, either alone or in combination, or PBS on day 0, 2, 4, 6, 8, and 10. Tumor volumes were estimated (volume = length × width² × π)/6) twice weekly until tumors reached 1.55 cm in diameter or tumor ulceration occurred, whichever came first. For biologic time-point experiments to investigate functional mechanisms, when tumors reached 6 to 7 mm in diameter, hamsters were stratified into different groups and treated with the same regimens used in the efficacy experiment. On day 5, 11, and 20, tumors, sera, and spleens were harvested from 3 animals in each group to investigate histopathologic changes, immunohistochemical staining for cleaved caspase-3–positive cells, total and neutralizing antibodies against adenovirus and vaccinia virus, and tumor-specific CTL assays. All animal experiments were approved by the Animal Welfare and Research Ethics Committee of the Institute of Biophysics, Chinese Academy of Sciences (Beijing, China) and were conducted in accordance with institutional regulations.

**Histopathologic examination and immunohistochemistry for viral proteins and caspase-3 staining**

The tissues harvested at different time points were processed and stained with hematoxylin and eosin (H&E) as well as immunohistochemistry (IHC) for viral proteins and cleaved caspase-3 (ASP175) as previously described (29, 31). The antibody against cleaved caspase-3 (ASP175) was purchased from Cell Signaling (New England Biolabs [UK] Ltd.).

**CD3 depletion in vivo**

At day 1, 1 × 10⁶ HPD-1NR cells (or 5 × 10⁶ HaK cells) were implanted subcutaneously into 4- to 5-week-old Syrian hamsters. When tumors reached 6 to 7 mm in diameter, hamsters were distributed between the treatment and the control groups by matched tumor size to receive 100 μL IT injections of with 5 × 10⁹ PFU Ad5 and 5 × 10⁷ PFU VVLister in combination, or PBS on day 0, 2, 4, 6, 8, and 10. The injections were introduced through a single central tumor puncture site, and 3 to 4 needles tracks were made radially from the center while virus was injected as the needle was withdrawn. Depletion monoclonal antibody (mAb) against Syrian hamster CD3 (clone 4F11) or control Ig (mouse anti-KLH mAbs) were administered intraperitoneally at doses of 500 mg/g every fourth day from the day before the viral therapy to the end of the experiment. Tumor dimensions were measured twice a week using digital calipers, and tumor volumes were calculated as described earlier.

**Results**

**The Syrian hamster is a suitable immunocompetent model to evaluate the efficacy of combination therapy with oncolytic adenovirus and vaccinia virus**

Eight Syrian hamster tumor cell lines were screened for sensitivity to both oncolytic adenovirus and vaccinia virus (Fig. 1A). Three of these (HPD-1NR, HPD-2NR, and HaK) supported viral gene expression (Fig. 1B), DNA amplification (Supplementary Fig. S1), and produced infectious virions of both viruses in vitro (Fig. 1C). The level of viral replication for both viruses in the 3 cell lines are comparable with some human cancer cell lines, although they are still lower than those in the majority of human cancer cell lines that we previously screened (31, 32). After intratumoral administration of adenovirus and vaccinia virus into the established tumor model of HPD-1NR in vivo, proteins of both viruses were expressed in tumor cells (Fig. 1D), with induction of tumor cell death and infiltration of inflammatory cells.

**Sequential use of oncolytic adenovirus and vaccinia virus eradicates established tumors in the immunocompetent Syrian hamster**

Having confirmed the infection and replication of both viruses in Syrian hamster tumor models, the efficacy of Ad5 and VVLister was first investigated in a subcutaneously established pancreatic cancer HPD-1NR model. Both oncolytic viruses showed dose-dependent efficacy in independent experiments (data not shown). This enabled the selection of doses with similar efficacy for use as combination oncolytic viral therapy. Treatment with 6 doses of oncolytic adenovirus [5 × 10⁹ PFU, much lower than 1 × 10¹⁰ PFU, the most commonly reported dose in the literature (15)] or vaccinia virus [5 × 10⁹ PFU, much lower than 1 × 10¹⁰ PFU, the most commonly reported dose in the literature (33, 34)] as single agents did not induce significant tumor regression (Fig. 2A) in the established pancreatic cancer tumor HPD-1NR. Strikingly, treatment with 3 doses of adenovirus (5 × 10⁸ PFU) followed by 3 doses of vaccinia virus (5 × 10⁷ PFU) resulted in a superior antitumor efficacy, in which 62.5% (5/8) of animals were tumor-free by day 40 after the first viral treatment. The reverse combination was not significantly different from the single viral therapies, although it resulted in 25% (2/8) of animals tumor-free at the same time point. Animals treated with the Ad-VV sequential regimen survived significantly longer than the other groups (Fig. 2B), and 75% of animals were still alive at the end of the study (4 months). A similar therapeutic effect was also shown in the established kidney cancer HaK model as the sequential Ad-VV regimen displayed superior efficacy to the reverse combination or 6 doses of either virus alone, leading to 62.5% (5/8) of animals tumor-free and 87% (7/8) of animals surviving 87 days after the viral treatment (Fig. 2C and D). This suggests that this therapeutic regimen is not tumor type–specific and might have a broad application.

**The sequential use of oncolytic adenovirus and vaccinia virus results in more tumor-infiltrating lymphocytes and does not affect humoral immunity to each virus**

To dissect the mechanisms underlying the combined therapy-mediated tumor regression, we first assessed tumors histologically. It was noted that many tumor-infiltrating lymphocytes (TIL) and apoptotic tumor cells were observed in the Ad-VV–treated tumors on day 10 after the last treatment, whereas there were only a few TIL and
apoptotic cells observed in other groups (Fig. 3A–D). To test whether sequential application of 2 different oncolytic viruses could reduce the host humoral immunity to each virus, the levels of all circulating antibodies and specific neutralizing antibodies against adenovirus and vaccinia virus were detected in the serum of animals after treatment with different regimens. The total antibody titers against adenovirus and vaccinia virus were not significantly different between groups \((P > 0.05; \text{Fig. 4A and B})\). In fact, Ad-VV treatment induced a slightly higher level of antibody against adenovirus than the Ad treatment alone. The titers of neutralizing antibody against adenovirus and vaccinia virus were also detected (Fig. 4C and D). There was no significant difference between 1 virus alone and the combination of the 2 viruses \((P > 0.05)\). These results suggest that the enhanced antitumor efficacy induced by sequential combination of adenovirus and vaccinia virus was not a result of reduction of humoral immunity to each virus. Of note, the neutralizing antibody against adenovirus is much lower in our experiments as compared with the previous study \((30)\). This is likely due to the lower doses of adenovirus that we used.

**Sequential combination of oncolytic adenovirus and vaccinia virus resulted in tumor-specific immunity**

Next, we investigated whether the sequential use of adenovirus and vaccinia virus induced a higher level of tumor-specific immunity as we hypothesized. The cytotoxic activity of splenocytes from groups of 3 Syrian hamsters bearing pancreatic tumors that had been treated with different viral regimens was detected at different time points. The CD8 activity against tumor cells was highest in the animals treated with Ad5 \(\times\) 3 then VVLister \(\times\) 3, followed by those treated with VV \(\times\) 3 then Ad \(\times\) 3 (Fig. 5A). If the
pancreatic tumor cells (HPD-1NR) were replaced by kidney cancer cells (HaK), none of the splenocytes harvested from animals in any group displayed cytotoxic activity (Fig. 5B).

One month after a complete response as a result of combination viral therapy had been observed in animals bearing HPD-1NR tumors, animals were rechallenged with either the original pancreatic cancer cells (HPD-1NR, \(n = 3\)) or kidney cancer cells (HaK, \(n = 4\)), respectively. There was no tumor growth 105 days after animals were rechallenged with HPD-1NR, whereas tumors grew rapidly in 3 of 4 animals challenged with HaK (Fig. 5C). One animal rechallenged with HaK, initially developed a tumor that regressed 63 days later, which might be due to some commonality of tumor-associated antigens between HaK and HPD-1NR. Tumors grew rapidly in control naïve Syrian hamsters injected with HPD-1NR or HaK (Fig. 5D). This confirmed that the sequential combination of adenovirus followed by vaccinia virus was the most effective approach to induce tumor-specific immunity in vivo.

T cells play a critical role in the efficacy of sequential oncolytic adenovirus and vaccinia virus therapy

To investigate further the role of T-cell responses in combination oncolytic virus therapy, an antibody against Syrian hamster CD3 (mAb4F11) was first developed and characterized (Supplementary Method and Supplementary Figs. S2 and S3). Interestingly, when injected into hamsters intraperitoneally, the mAb 4F11 was found to deplete T-cell subsets in both lymphoid and nonlymphoid tissues compared with animals receiving relevant mouse isotype control antibody (Supplementary Fig. S3). The efficacy of depletion was more than 98% and lasted for more than a week in the lymph nodes as well as the spleen and peripheral blood mononuclear cell (PBMC; data not shown).

Syrian hamsters bearing subcutaneous HPD-1NR tumors were rendered CD3⁺ T-cell–deficient by injecting them intraperitoneally with the anti-hamster CD3e mAb (clone 4F11) just 1 day before 3 intratumoral administration of adenovirus, then vaccinia virus, or the reverse. The superior...
efficacy of the sequential oncolytic virus regimen seen in control antibody–treated hamsters was completely ablated in CD3+ T-cell–depleted animals (Fig. 6A and B). Depletion of CD3+ T cells also significantly inhibited the superior antitumor efficacy of sequential use of AdV and VV in the HaK model (Supplementary Fig. S4). These results show that T-cell responses play a critical role in combination oncolytic virus therapy.

Discussion

The efficacy of oncolytic viruses depends on multiple actions including direct tumor lysis, modulation of tumor perfusion, and stimulation of tumor-directed innate and adaptive immune responses. It has become apparent that the antitumor efficacy of oncolytic viruses is dependent on the interaction of virus, tumor cells, and the host immune response to the virus as well as to tumor cells (35). Oncolytic virus replication in tumor cells typically leads to direct destruction of tumor cells, releasing tumor antigens and other danger signals into the extracellular environment, whereas the ultimate clearance of these viruses from the tumors indicates that the localized immunosuppression induced by viral gene expression is eventually overcome. All these actions should, unsurprisingly, induce a long-term memory immune response targeting tumor-associated antigens (36). Therefore, in the present study, we hypothesized that sequential combination of immunologically distinct viruses might enhance antitumor efficacy through the induction of tumor-specific immunity and circumvention or mitigation of antiviral immune responses.

To prove the hypothesis, we first validated the immunocompetent Syrian hamster as an ideal model for combination oncolytic virus as the Syrian hamster tumors can support replication of human adenovirus and vaccinia virus (Fig. 1) and both oncolytic virus can induce lysis of tumor cells and induce a host immune response in vivo (Figs. 1D and 3). We, for the first time, have shown that sequential use...
of oncolytic adenovirus and vaccinia virus, even at low doses, resulted in a complete tumor response in vivo and induction of effective tumor-specific immunity (Figs. 2 and 3), to which the T-cell response is critical (Figs. 3 and Fig. 6; Supplementary Fig. S4) and humoral immunity to the viruses is unchanged (Fig. 4). Of note, combination of 2 genetically distinct viruses, vesicular stomatitis virus (VSV) and VV, has been reported to show a synergistic antitumor efficacy previously (37). However, the synergistically enhanced antitumor efficacy of VV and VSV is induced through a different mechanism of action from our combination of Ad and VV (37).

Currently, we are not able to dissect which subtype of T cells plays more important role due the lack of research tools such as hamster-specific antibodies and microarrays, but it is highly likely that tumor-specific CD8+ T cells play an important role in this regimen. The functional mechanisms that underlie the efficacy of treatment with adenovirus before vaccinia virus are not fully understood, but it is possible to speculate. Adenovirus may be superior when

Figure 4. The humoral immune response to adenovirus and vaccinia virus in HPD-1NR bearing Syrian hamster animals after treatment with different regimens. A, total antibody against adenovirus in sera of Syrian hamster bearing HPD-1NR tumors on day 10 after treatment with different regimens. B, total antibody against vaccinia virus in sera of Syrian hamster bearing HPD-1NR tumors on day 10 after treatment with different regimens. C, neutralizing antibody against adenovirus in sera of Syrian hamsters bearing HPD-1NR tumors on day 10 after treatment with different regimens. D, neutralizing antibody against vaccinia virus in sera of Syrian hamsters bearing HPD-1NR tumors on day 10 after treatment with different regimens.

Figure 5. Induction of tumor-specific immunity in vivo following treatment with the combination of oncolytic adenovirus and vaccinia virus or 1 virus alone. A, cytotoxic activity of splenocytes against HPD-1NR cells. Splenocytes were harvested from Syrian hamsters bearing HPD-1NR tumors after treatment with different regimens. B, cytotoxic activity of splenocytes against HaK cells. Splenocytes were harvested from Syrian hamsters bearing HPD-1NR tumors after treatment with different regimens. C, HPD-1NR or HaK cells were kept for more than 30 days and then rechallenged with 1 x 10^6 HPD-1NR cells or HaK cells into the left flank. Tumor volumes were measured twice weekly. Mean tumor size ± SEM are displayed until the death of the first hamster in each group.
given first as it is more effective than vaccinia virus at activating Toll-like receptors, which are necessary for antigen presentation, so eliciting a better response to tumor-associated antigens than vaccinia virus. Vaccinia virus might create a more effective “boost” of preprimed CD8+ T cells in preference to vaccinia epitopes due to the expression of its own immunomodulatory proteins (38). Perhaps vaccinia virus is able to reduce the host immune response and improve replication of both oncolytic virus. An alternative mechanism may be the expression of adenovirus E3 14.7 kDa protein, which has been shown to enhance the virulence of vaccinia virus through attenuation of the effect of TNF in the local microenvironment (39, 40). Given that in this study the sequential use of wild-type adenovirus and vaccinia virus induced effective tumor-specific immunity (Fig. 6), further investigation is required to understand how to optimize this novel therapeutic regimen by using different mutants of engineered oncolytic viruses. We believe that antitumor efficacy can be improved further through engineering the viruses by deleting viral genes that inhibit the host immune response, such as adenovirus E3gp19k (29), and rationally expressing immunotherapeutic genes, such as interleukin (IL)-7, Fl3tL, and IL-15 (41) because arming oncolytic viruses with cytokines, such as vaccinia virus armed with granulocyte macrophage colony-stimulating factor (GM-CSF; JX963), has been shown to have improved antitumor efficacy and enhanced tumor-specific immunity (42).

Both oncolytic adenovirus and vaccinia virus have been safely used separately in clinical trials (43–45). Despite this, the use of this combination to treat patients with cancer may raise safety concerns. It is important to highlight the fact that the sequential combination of these 2 oncolytic virus did not induce any overt side-effects in the tumor-bearing Syrian hamsters. Our findings suggest that the sequential use of oncolytic adenovirus and vaccinia viruses achieve antitumor efficacy through a combination of oncolytic activity and the induction of cellular immunity through T cells. These findings show that sequential combination of oncolytic adenovirus and vaccinia virus, both of which have been used individually in clinical trials, could be a promising approach for curing cancer in humans. These results have significance for the design of new regimens for cancer viroimmunotherapy and vaccines.

Finally, this study strongly supports the development of the Syrian hamster as a model for the assessment of oncolytic viruses, although there are limitations to its use at present. Scientists are in general less experienced in the husbandry and use of Syrian hamsters for research. It is far easier to give mice intravenous injections, as they have tails with superficial tail veins. However, the femoral veins of Syrian hamsters are easily accessible, so this should not be a major issue (14). Fewer tumor cell lines and transgenic cancer models are available than for the mouse. The Syrian hamster genome has not yet been fully sequenced, there are
few antibodies and no gene microarrays are currently available. These important tools should now be developed to meet this demand. In addition, it is also very important to investigate whether adenovirus and vaccinia virus proteins, especially immune evasion proteins are active in Syrian hamster once research tools are available.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed. The funders have no role in the study design, data collection, analysis, interpretation of results, and the preparation of the article.

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Conception and design: J.R. Tysome, X. Li, S. Wang, N.R. Lemoine, Y. Wang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J.R. Tysome, X. Li, S. Wang, P. Du, D. Chen, Y. Wang
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doi:10.1158/1078-0432.CCR-12-0979

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