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A novel therapeutic regime to eradicate established solid tumors with an effective induction of tumor-specific immunity

Short title: Sequential use of two oncolytic viruses for cancer treatment

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Abstract

Purpose: The efficacy of oncolytic viruses (OVs) 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 regime 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. 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 demonstrate 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.

Key words: oncolytic virus, pancreatic cancer, adenovirus, vaccinia virus, Syrian hamster.

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. OV's 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 OV's as single agent therapy has been limited. Exploiting our knowledge of cancer cell biology, immunology and virology, we have developed a novel therapeutic regime by sequential combination of oncolytic adenovirus and vaccinia virus. Not only does this regime eradicate established tumors in immuno-competent 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 antitumour therapeutic modality that can be directly translated into cancer patients.

Introduction

Oncolytic viruses (OVs) 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 OVs 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 anti-cancer potency of these agents. Most studies have focused on improving the direct anti-tumor 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 OV 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 two or more antigenically distinct viruses so that the specific immunity that arises subsequent to the first virus does not inhibit the therapeutic effects of the second therapeutic virus. However, the host immune response may be critical to the efficacy of oncolytic virotherapy. This may be mediated via innate immune effectors, adaptive antiviral immune responses eliminating infected cells or adaptive anti-tumor immune responses (2). Most interestingly, it has been shown that administration of OV such as measles virus (4), vaccinia virus (5, 6) and adenovirus (7) can not only infect and lyse tumor cells, but also result in tumor-specific immunity. Oncolytic virotherapy may therefore be considered as a method to achieve vaccination *in situ*, enabling the adaptive immune response to clear residual disease and provide long-term surveillance against relapse. Furthermore, the addition of a second OV may provide a boost if the anti-tumor immune response to the tumor cells infected by the second virus can be potentiated or primed by the host immune response to the first virus.

In the context of vaccination, a heterologous prime-boost immunization regime using recombinant adenovirus prior to vaccinia virus has been reported as an efficient approach to enhance CD8+ T cell immunogenicity with protective efficacy against malaria in a mouse model (8, 9). Therefore, it seemed possible that the combination of two different OV for cancer treatment may induce a stronger tumor-specific immunity. We have, for the first time, explored the potential of combining oncolytic adenovirus and vaccinia virus as a new approach for treatment of established tumors *in vivo* on the basis of their different mechanisms of action in an attempt to harness the host immune response to the infected tumors using a prime-boost strategy.

The lack of immune-competent models for investigating OV 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 OV, 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 OV in terms of the host immune response, since 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 (14) 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, since it is in part mediated by

natural killer cells, a feature not seen in mice (26). The T_h1-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 T_h1 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, VA, USA). 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 M3E3/C3 (fetal lung epithelial cell line maintained in RPMI with 10%) 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 epithelial cell line transformed with Ad5 DNA, was obtained from the Cancer Research UK Central Cell Services and maintained in DMEM with 10% FCS.

Viruses: The highly attenuated Lister vaccine strain of vaccinia virus (VVL_{ister}) was provided by Istvan Fodor (Loma Linda University, USA). Wild-type adenovirus (Ad5) was described previously (29).

Cytotoxicity assay: The assay was performed at least three 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 PFU/cell of Ad5 or VVLister in media with 2% FCS 16-18 hours later. Samples were harvested in triplicate at 24-hour intervals up to 96 hours after infection, freeze-thawed three times and titrated on JH293 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: 2×10^5 HPD-1NR or HaK cells were seeded in 60mm dishes and infected with 5 PFU/cell of Ad5 or VVLister in medium with 2% FCS after 16-18 hours. Cells were harvested at 3, 6, 12, 24, 48 and 72 hours following infection. 30 μ g of proteins were separated on precast NuPage[®] Novex 4-12% Bis-Tris gels (Invitrogen, CA, USA), electrotransferred onto nitrocellulose membranes and probed with a polyclonal rabbit antibody to the Lister vaccinia virus coat protein (MorphoSys UK Ltd, Bath, UK), a monoclonal murine antibody known to cross-react with Ad5 E1A (Calbiochem, Nottingham, UK) or a polyclonal rabbit antibody to Ad5 hexon (Autogen bioclear, Wiltshire, UK). Immunocomplexes were detected by incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (Autogen Bioclear, Wiltshire, UK) and visualized by enhanced chemiluminescence (ECL reagent; Amersham). α -tubulin was used as a loading control.

Real-time quantitative PCR: DNA was extracted from hamster cells collected as above using the QIAamp DNA blood mini kit (QIAGEN Ltd, Crawley, UK) according to the manufacturer's instructions. TaqMan[®] system primers and probes (Supplementary Table 1) were designed using Primer Express[®] v3.0 software (Applied Biosystems, New Jersey, USA) and constructed by Sigma-Aldrich and Applied Biosystems respectively. Samples, controls and standards (5 to 5×10^8 viral genome copies) were tested in triplicate in each plate by quantitative polymerase chain reaction (qPCR) using 7500 Real-time PCR System with samples at 48°C for 30min, 95°C for 10min followed by 40 cycles of 95°C for 15s and 60°C for 1min.

Cycle thresholds (CT) were determined using 7500 System SBS software and used to create standard curves in Prism® (GraphPad Software, CA, USA). 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 (TM) microplates (Corning Incorporated, USA) were coated with 1×10^7 PFU/ml Ad5 or 1×10^6 PFU/ml of vaccinia virus in 50 μ L Carbonate Buffer (pH 9.6) per well at 4°C overnight. Wells were blocked with 200 μ L PBS (pH 7.4) containing 10% FCS for 1 h at room temperature, and then incubated for 2 h with 50 μ L 100-fold diluted serum samples in PBS (pH 7.4) containing 10% FCS. After washing four times with PBS (pH 7.4) containing 0.05% Tween 20, the wells were incubated for 1 h with 50 μ L HRP-conjugated Anti-Hamster (Syrian) IgG (H+L) (Alpha Diagnostic International, China, 1:5000 dilution). After washing, 50 μ L 1-Step™ Ultra TMB-ELISA solution (34028, Thermo Scientific) were added as substrate and the color reaction was terminated by addition of 50 μ L 2N 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 CPE at six days post-infection. Neutralizing antibody (NAb) 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 three days. Non-adherent cells were harvested as effector cells by Lymphocyte Separation Media (LTS1077, TBD Corp, Tianjing, China). 3×10^4 target cells (HPD-1NR cells) or control target cells (HaK cells) were incubated with effector cells for 6 h at 37°C in U-bottom 96 well plates in a volume of 150µl per well at different ratio of 1:20, 1:10, 1:5, 1:2.5 (in triplicate wells). Supernatant from each well was harvested, and the percentage of specific lysis was determined using CytoTox 96 (Promega, Beijing, China) according to the manufacturer's instructions.

***In vivo* animal studies:** 1×10^6 HPD-1NR or 5×10^6 HaK cells were implanted subcutaneously into the right flank of female, 4-5 week-old Syrian hamsters. When tumors reached 6-7mm in diameter, hamsters were stratified by tumor size into groups of 7 or 8 to receive 100µl intratumoral (IT) injections of VVLister or Ad5, either alone or in combination, or PBS on days 0, 2, 4, 6, 8 and 10. Tumor volumes were estimated ($\text{Volume} = (\text{length} \times \text{width}^2 \times \pi)/6$) twice weekly until tumors reached 1.55cm in diameter or tumor ulceration occurred, whichever came first. For biological time-point experiments to investigate functional mechanisms, when tumors reached 6-7mm in diameter, hamsters were stratified into different groups and treated with the same regimens used in the efficacy experiment. On day 5, day 11 and day 20, tumors, sera and spleens were harvested from three animals in each group to investigate histopathological 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.

Histopathological examination and immunohistochemistry (IHC) for viral proteins and Caspase-3 staining: The tissues harvested at different time points were processed and stained with haematoxylin and eosin (H&E) as well as 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, Hitchin, Hertfordshire, UK).

CD3 depletion *in vivo*: At day 1, 1×10^6 HPD-1NR cells (or 5×10^6 HAK cells) were implanted subcutaneously into 4-5 week-old Syrian hamsters. When tumors reached 6-7mm in diameter, hamsters were distributed between the treatment and the control groups by matched tumor size to receive 100 μ l intratumoral (IT) injections of with 5×10^8 Pfu Ad5 and 5×10^7 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 3-4 needles tracks were made radially from the center while virus was injected as the needle was withdrawn. Depletion mAb against Syrian hamster CD3 (clone 4F11) or control Ig (mouse anti-KLH mAbs) were administered intraperitoneally at doses of 500mg/g every 4th 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 above.

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. 1) and produced infectious virions of both viruses *in vitro* (Fig. 1C). The level of viral replication for both viruses in the three cell lines are comparable to 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 demonstrated 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 six doses of oncolytic adenovirus (5×10^8 PFU, much lower than 1×10^{10} PFU, the most commonly reported dose in the literature (15)) or vaccinia virus (5×10^7 PFU, much lower than 1×10^9 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 three doses of adenovirus (5×10^8 PFU) followed by three doses of vaccinia virus (5×10^7 PFU) resulted in a superior anti-tumor 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 regime survived significantly longer than the other groups (Fig. 2B), and 75% of animals were still alive at the end of the study (four months). A similar therapeutic effect was also shown in the established kidney cancer HaK model as the sequential Ad-VV regime displayed superior efficacy to the reverse combination or six 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 regime 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, while there were only a few TIL and apoptotic cells observed in other groups (Fig. 3A, B, C and D). To test whether sequential application of two 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 regimes. The total antibody titres against adenovirus and vaccinia virus were not significantly different between groups ($p>0.05$; Fig. 4 A and B). In fact, Ad-VV treatment induced a slightly higher level of antibody against adenovirus than the Ad treatment alone. The titres of neutralizing antibody against adenovirus and vaccinia virus were also detected (Fig. 4C and D). There was no significant difference between one virus alone and the combination of the two 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 compared to 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 three Syrian hamsters bearing pancreatic tumors that had been treated with different viral regimes was detected at different

time points. The cytotoxic T lymphocyte activity against tumor cells was highest in the animals treated with Ad5 x 3 then VVLister x 3, followed by those treated with VV x 3 then Ad x 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 re-challenged with HPD-1NR, whereas tumors grew rapidly in 3 of 4 animals challenged with HaK (Fig. 5C). One animal re-challenged 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

In order to investigate further the role of T cell responses in combination OV therapy, an antibody against Syrian Hamster CD3 (mAB4F11) was first developed and characterised (Supplementary method and supplementary Fig.2 and supplementary Fig. 3). Interestingly, when injected into hamsters intraperitoneally, the mAb 4F11 was found to deplete T cell subsets in both lymphoid and non-lymphoid tissues compared to animals receiving relevant mouse isotype control antibody (Supplementary Fig. 3). The efficacy of depletion was >98% and lasted for more than a week in the lymph nodes as well as the spleen and 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 one day prior to three intratumoral administration of adenovirus then vaccinia virus or the reverse. The superior efficacy of the sequential OV regime 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. 4). These results demonstrate that T-cell responses play a critical role in combination OV therapy.

Discussion

The efficacy of OVs 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 anti-tumor efficacy of OVs is dependent on the interaction of virus, tumor cells and the host immune response to the virus as well as to tumor cells (35). OV replication in tumor cells typically leads to direct destruction of tumor cells, releasing tumor antigens and other danger signals into the extracellular environment, while 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.

In order to prove the hypothesis, we first validated the immunocompetent Syrian hamster as an ideal model for combination OV as the Syrian hamster tumors can support replication of human adenovirus and vaccinia virus (Fig. 1) and both OV can induce lysis of tumor cells and induce a host immune response *in vivo* (Fig. 1D and Fig. 3). We, for the first time, demonstrated 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 (Fig. 2 and 5), to which the T cell response is critical (Fig. 3, Fig. 6 and Supplementary Fig. 4) and humoral immunity to the viruses is unchanged (Fig. 4). Of note, combination of two 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 regime. The functional mechanisms that underlie the efficacy of treatment with adenovirus prior to vaccinia virus are not fully understood, but it is possible to speculate. Adenovirus may be superior when 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 pre-primed 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 OV. An alternative mechanism may be the expression of adenovirus E3 14.7kDa protein, which has been shown to enhance the virulence of vaccinia virus through attenuation of the effect of Tumor Necrosis Factor 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 regime 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 IL-7, Fl3tL and IL-15 (41) because arming oncolytic viruses with cytokines, such as vaccinia virus armed with GM-CSF (JX963), has been demonstrated 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 two OV 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 anti-tumor efficacy through a combination of oncolytic activity and the induction of cellular immunity through T-cells. These findings demonstrate 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 viro-immunotherapy and vaccines.

Lastly, 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, since 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

The authors have no conflicting financial interests. The funders had no role in the study design, data collection, analysis, interpretation of results, nor the preparation of the manuscript.

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Figure Legends

Figure 1 Potency, replication and expression of VVLister and Ad5 early and late proteins in Syrian hamster tumor cell lines. **A**, cell death as a percentage of uninfected cells by MTS assay 144 hours post-infection. Mean EC_{50} values \pm SEM are shown; **B**, viral protein expression in Syrian hamster cancer cells. Cells were infected with 5 PFU/cell of VVLister or Ad5, the viral protein was detected by Western Blot; **C**, production of infectious virions of adenovirus and vaccinia virus in Syrian hamster tumor cell lines. Syrian hamster tumor cells were infected with 5 PFU/cell VVLister or Ad5, cell lysates harvested at 24 hour intervals up to 96 or 120 hours. Mean viral replication \pm SEM was determined by TCID₅₀ assay. **D**, Viral protein expression in established HPD-1NR tumors *in vivo* after treatment with PBS, adenovirus or vaccinia virus. The viral proteins against E1A of adenovirus (indicating viral replication) and vaccinia virus were detected by IHC as described in Materials and Methods. The positive cells were arrowed. Original magnification x200.

Figure 2 Efficacy of oncolytic viruses in combination or alone against HPD-1NR and HaK Syrian hamster tumor models *in vivo*.

1×10^6 HPD-1NR or 5×10^6 HAK cells were seeded by subcutaneous injection into the right flank of Syrian hamsters. When tumors reached 6-7mm in diameter, eight hamsters were each injected IT with 5×10^8 PFU Ad5 on days 0, 2 and 4, followed by 5×10^7 VVLister on days 6, 8 and 10; the reverse combination; six doses of either virus alone, or PBS. Tumors were measured twice weekly. Mean tumor size \pm SEM are displayed until the death of the first hamster in each group and compared by one-way ANOVA with *post-hoc* Bonferroni testing. **A**, tumor growth curve of HPD-1NR; **B**, Kaplan-Meier survival analysis of Syrian hamsters bearing HPD-1NR tumors after different treatments; **C**, tumor growth curve of HaK; **D**, Kaplan-Meier survival analysis of Syrian hamsters bearing HaK tumors after different treatments. *** $p < 0.001$.

Figure 3 Sequential combination of oncolytic adenovirus and vaccinia virus induces higher levels of TIL infiltration and apoptotic tumor cells. On day 10 after the last viral treatment, Syrian hamsters were killed and tumors harvested and processed for histopathology and IHC. **A**, H&E staining of sections of tumors derived from five different groups of Syrian hamsters, original magnification x200; **B**, immunoreactivity for cleaved Caspase-3 for detection of apoptotic cells, original magnification x 200; **C**, quantitative score of lymphocyte infiltration within tumors. Inflammatory cell infiltration was assessed on day 10 after treatments were finished. Lymphocytes were counted in five high-power fields randomly selected from each tumor section (HPF, x200). The scoring was performed within the tumor and stroma; necrotic areas were avoided. The extent of lymphocyte infiltration was categorized into the following four grades: 1, <25 cells/HPF; 2, 25-49 cells/HPF; 3, 50-75 cells/HPF; 4, >75 cells/HPF; **D**, quantitative score of apoptotic cancer cells. Cleaved Caspase-3-positive cancer cells were assessed on day 10 after treatments were finished. Caspase-3-positive cells were counted in 10 higher-power fields randomly selected from each tumor section (HPF, x200) and the mean number of caspase-3-positive cells per HPF from three animals presented. **p<0.01, ***p<0.001.

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

Figure 5 Induction of tumor-specific immunity *in vivo* following treatment with the combination of oncolytic adenovirus and vaccinia virus or one 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 regimes; **B**, cytotoxic activity of splenocytes against HaK cells. Splenocytes were harvested from Syrian hamsters bearing HPD-1NR tumors after treatment with different regimes. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; Tumor-free Syrian hamsters (**C**) or Naïve Syrian hamsters (**D**) were kept for more than 30 days and then rechallenged with 1×10^6 HPD-1NR cells or HaK cells into the left flank. Tumor volumes were measured twice weekly. Mean tumor size \pm SEM are displayed until the death of the first hamster in each group.

Figure 6 Superior antitumor efficacy by sequential use of oncolytic adenovirus and vaccinia virus is mediated by CD3+ T cells in the immunocompetent Syrian hamster.

4-5 week-old Syrian hamsters were inoculated s.c. with 1×10^6 HPD-1NR cells. The established tumors (about 6-7mm in diameter) were injected directly with 5×10^8 PFU of Ad5 and 5×10^7 PFU of VVlister in combination, or PBS (n=5/group) on day0, 2, 4, 6, 8 and 10. Mouse anti-hamster CD3e MAbs (4F11) or control Ig were injected intraperitoneally at doses of 500mg/g every 4th day from the day before the viral therapy to the end of the experiment. Tumor sizes of individual mice were monitored twice weekly. **A**, tumor growth curve of HPD-1NR; **B**, Kaplan-Meier survival analysis of Syrian hamsters bearing HPD-1NR tumors after different treatments. * $p < 0.05$, *** $p < 0.001$.

Figure 1

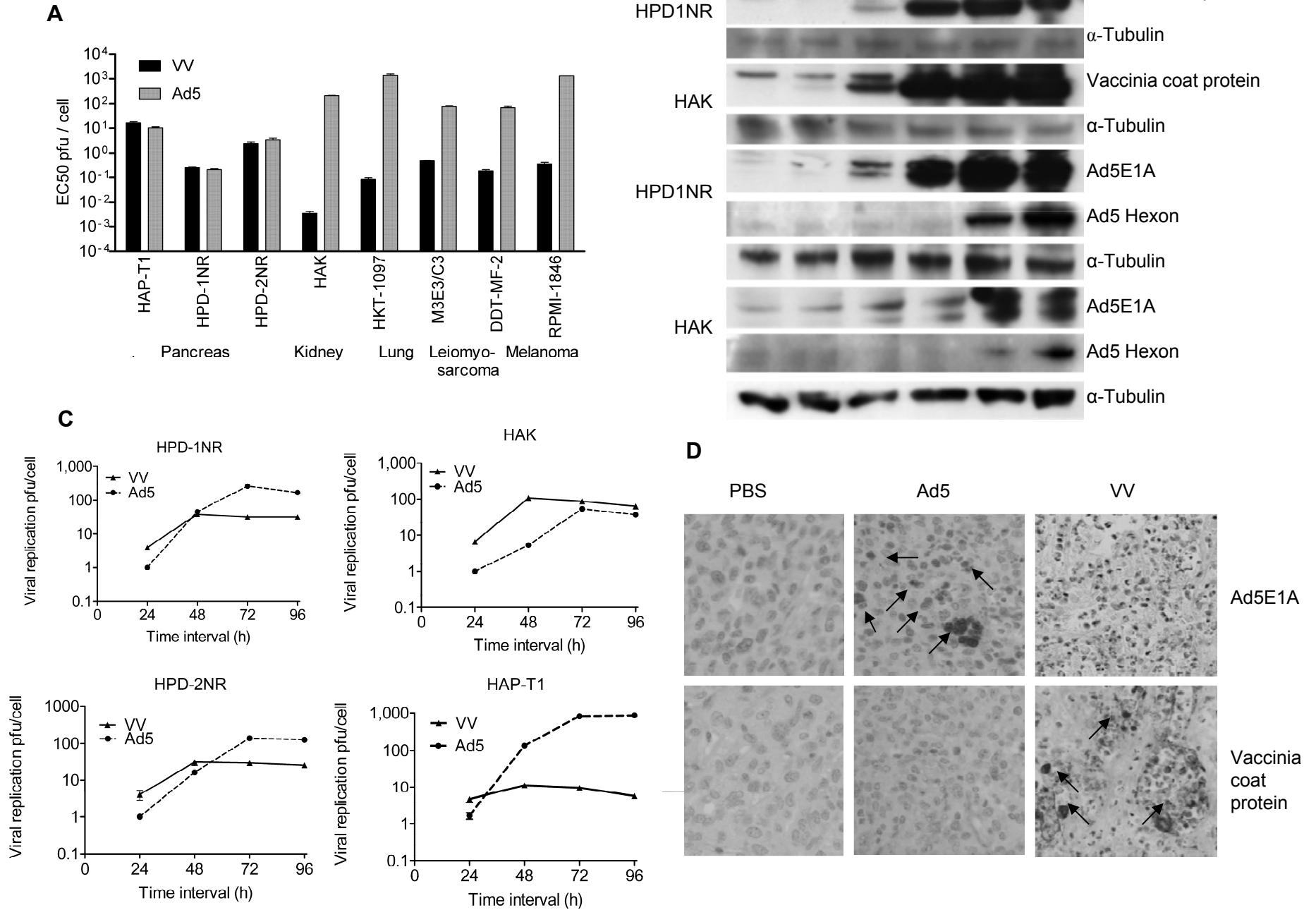


Figure 2

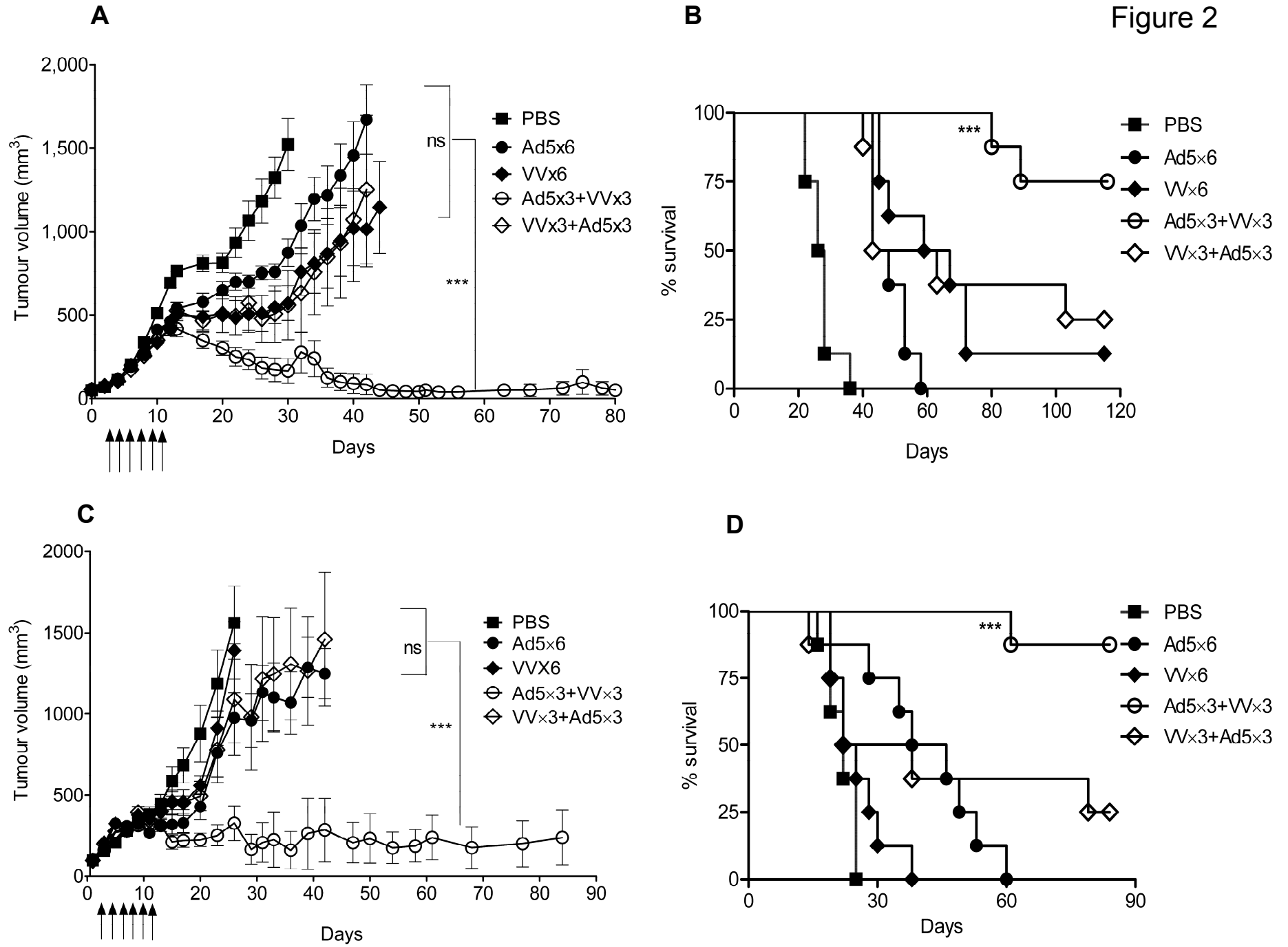


Figure 3

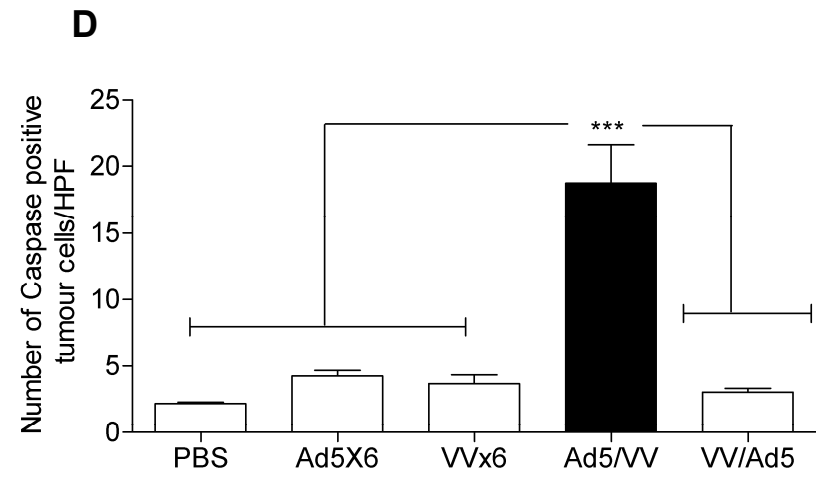
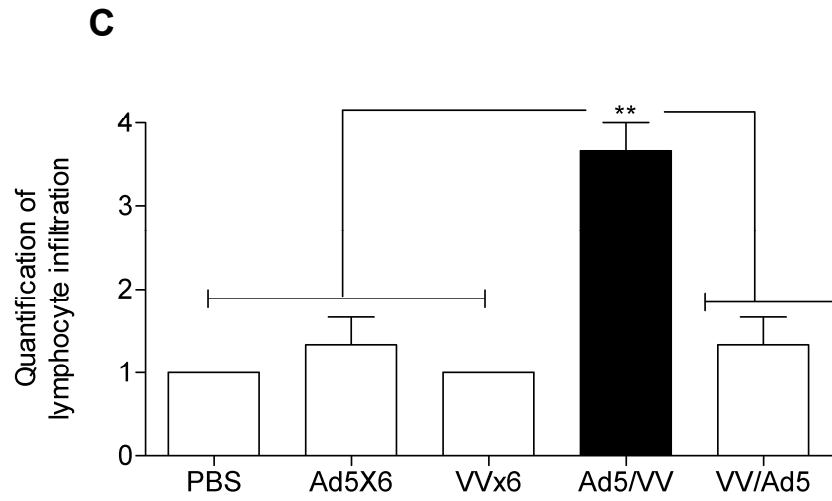
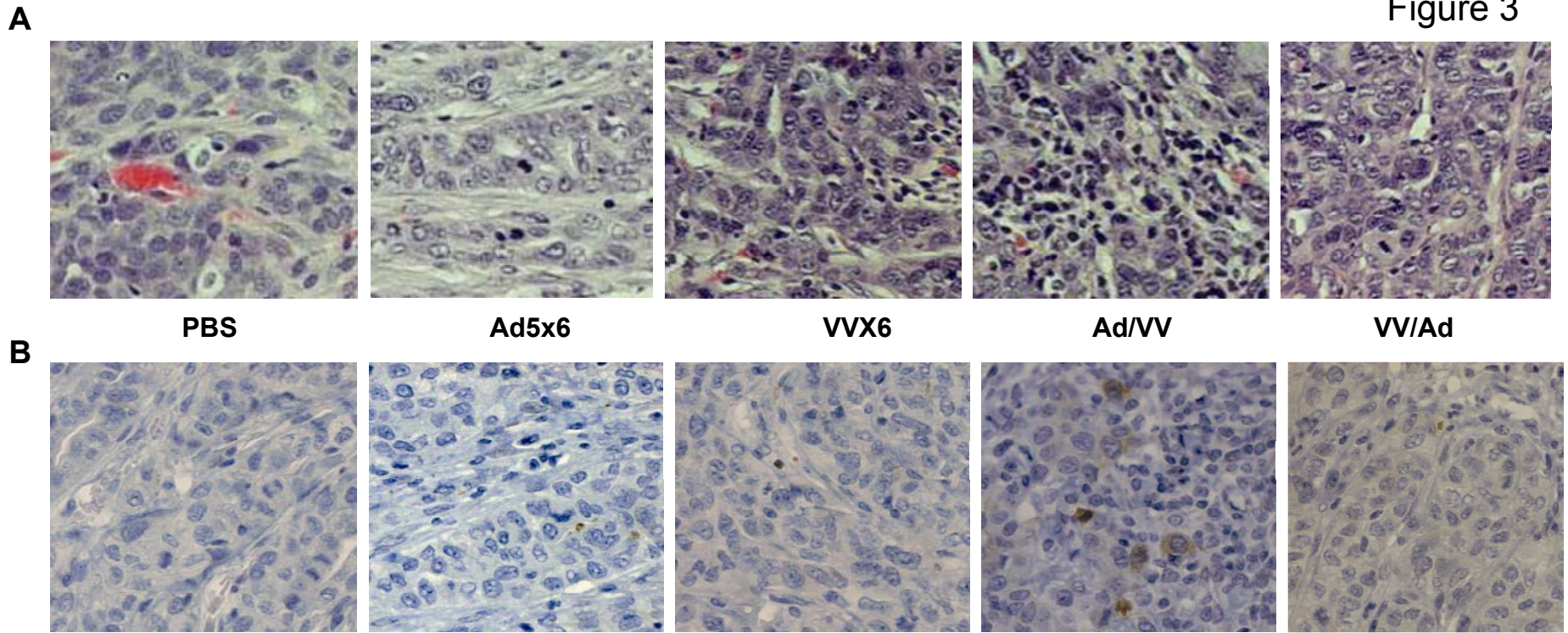


Figure 4

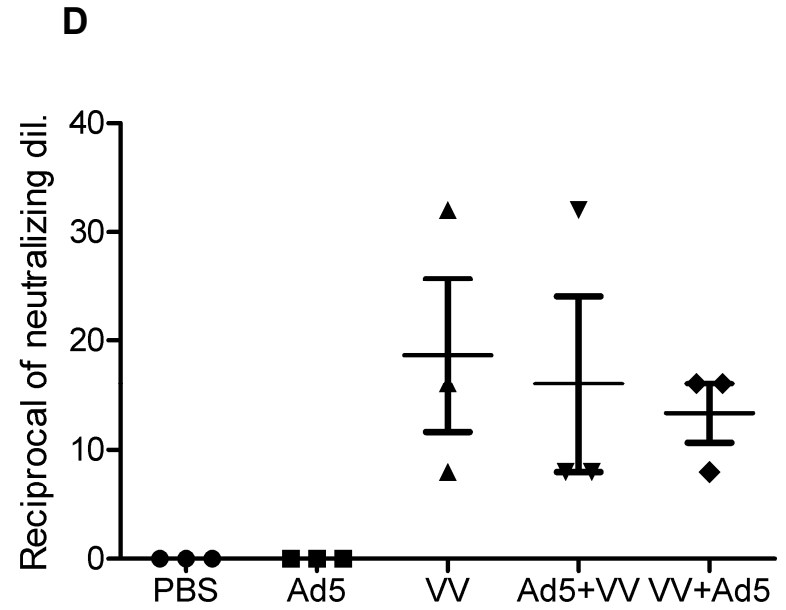
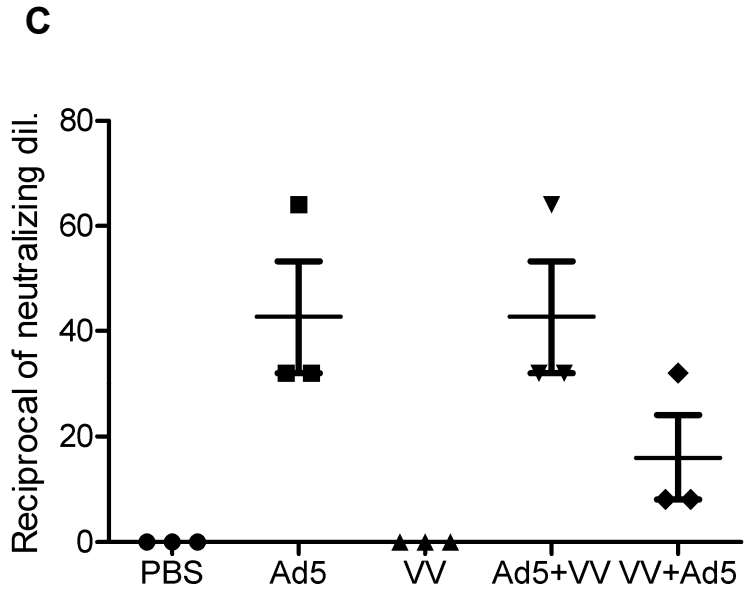
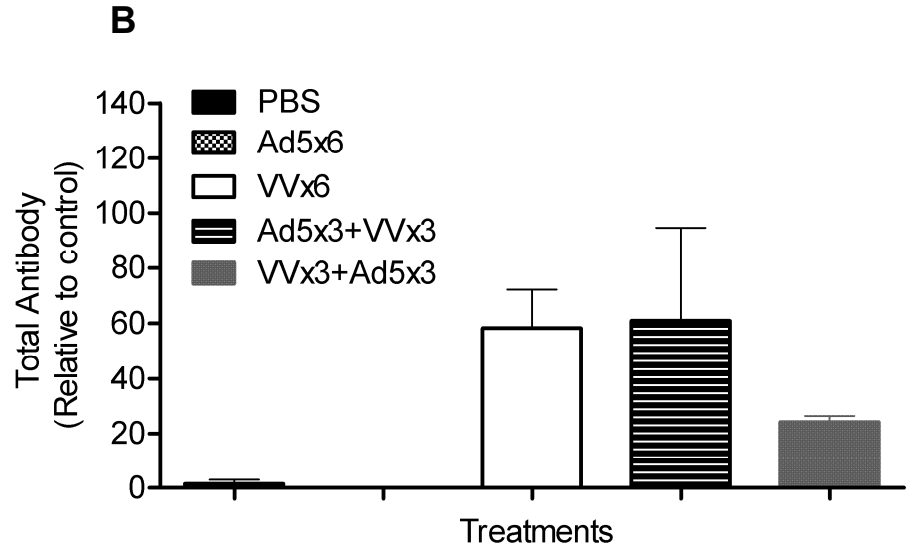
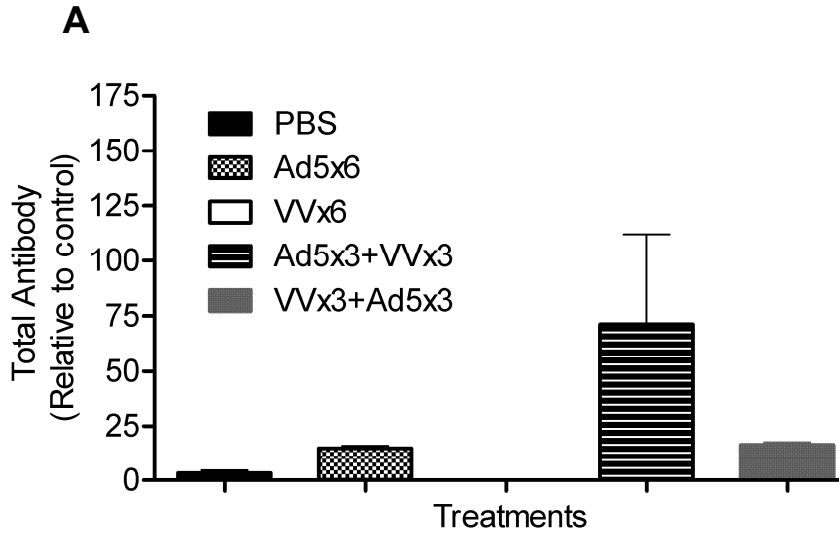


Figure 5

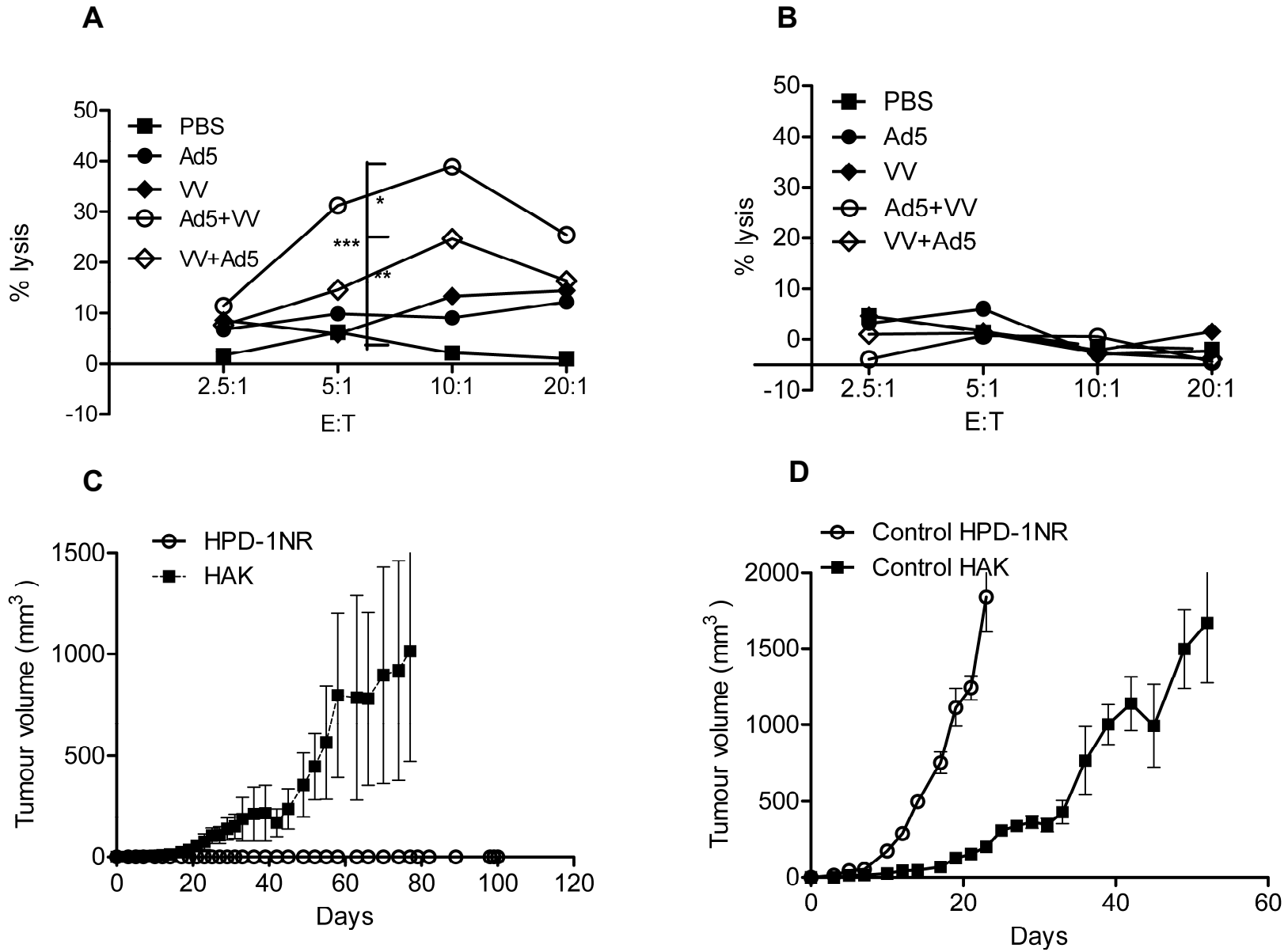


Figure 6

