



A Dendritic Cell-Targeted Adenoviral Vector Facilitates Adaptive Immune Response Against Human Glioma Antigen (CMV-IE) and Prolongs Survival in a Human Glioma Tumor Model

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Abstract

Antitumor immunotherapeutic strategies represent an especially promising set of approaches with rapid translational potential considering the dismal clinical context of high-grade gliomas. Dendritic cells (DCs) are the body's most professional antigen-presenting cells, able to recruit and activate T cells to stimulate an adaptive immune response. In this regard, specific loading of tumor-specific antigen onto dendritic cells potentially represents one of the most advanced strategies to achieve effective antitumor immunization. In this study, we developed a DC-specific adenoviral (Ad) vector, named Ad5scFvDEC205FF, targeting the DC surface receptor, DEC205. *In vitro* analysis shows that 60% of DCs was infected by this vector while the infectivity of other control adenoviral vectors was less than 10%, demonstrating superior infectivity on DCs. Moreover, an average of 14% of DCs were infected by Ad5scFvDEC205FF-GFP, while less than 3% of non-DCs were infected following *in vivo* administration, demonstrating highly selective *in vivo* DC infection. Importantly, vaccination with this vehicle expressing human glioma-specific antigen, Ad5scFvDEC205FF-CMV-IE, shows a prolonged survival benefit in GL261^{CMV-IE}-implanted murine glioma models ($p < 0.0007$). Furthermore, when rechallenged, cancerous cells were completely rejected. In conclusion, our novel, viral-mediated, DC-based immunization approach has the significant therapeutic potential for patients with high-grade gliomas.

Key Words glioblastoma · adenovirus · dendritic cells · cytomegalovirus · vaccine

Introduction

Glioblastoma (GBM) is a highly aggressive, malignant primary brain tumor that carries a dismal prognosis even when treated by the standard care of surgery, chemotherapy, and radiation in most patients [1]. Given this poor outcome for patients and the inadequate therapeutic efficacy of current

treatments, the field of neuro-oncology demands new and advanced treatment options to extend patient survival. In this study, we explored the potential of a novel dendritic cell (DC)-based immunotherapy—a strategy that takes advantage of the body's own immune system to generate a specific adaptive immune response [2] against the tumor—by way of adenoviral tropism redirection via genetic manipulation to specifically target DCs and deliver a tumor antigen to elicit an anti-tumor immune response.

DCs, the body's professional antigen-presenting cells, are ideal for creating tumor-specific adaptive immune process [2]. In addition to antigen presentation, mature dendritic cells (mDCs) release cytokines and chemokines to induce the activation of tumor-specific T cells [2, 3]. Immature dendritic cells (iDCs), on the other hand, are incapable of inducing an antigen-specific immune response and may partially induce T-cell differentiation to immunosuppressive regulatory T cells,

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which negates the therapeutic benefit of a DC-based cancer vaccination. Therefore, it is imperative for any dendritic cell vaccine or immunization approach to selectively deliver the tumor antigen (TA) to mDCs or otherwise induce DC maturation upon TA delivery [2–6].

One potential GBM-specific TA that may be ideal for DC presentation is the cytomegalovirus immediate early gene product (CMV-IE) [7–10]. This CMV-IE antigen is reportedly seen in more than 90% of GBM patient samples evaluated, making CMV's presence in GBM a potential target for DC immunotherapy [7–10]. Our own analysis of CMV-IE in patient tissue corroborated these findings and an experiment in a glioma murine model cell line confirmed the validity of this TA target.

To create an adenoviral vector-mediated DC vaccination, we genetically engineered the human adenovirus serotype 5-based vector (Ad5). We selected the Ag receptor dendritic and epithelial cell receptor with a molecular weight of 205 kDa (DEC205), which is known to be nearly exclusively expressed by DCs and its expression is significantly upregulated in mature DCs [11–14], for the purpose of redirecting the adenovirus to DCs. To accomplish this, we incorporated a single-chain antibody (scFv) specific for DEC205 onto the adenoviral fiber domain, which dictates the adenoviral tropism (this new adenoviral vector is thus named Ad5scFvDEC205FF) [15–18]. Furthermore, previous work has suggested that the infection of adenovirus, itself, can induce DC maturation upon cell entry, an attractive trait for the *in vivo* translation of this viral-based DC immunotherapy [19, 20].

To further optimize this novel adenoviral vehicle, we overcame the generic hurdles facing systemic adenovirus administration. For example, most people have neutralizing antibodies (NAb) against Ad5, especially the fiber (i.e., the most structurally protruding viral domain) and the hexon (most structurally abundant domain) [21, 22]. In addition to this immune-mediated vector clearance, it is known that systemic delivery of adenovirus can cause liver sequestration and toxicity due to the interaction between coagulation factor X (FX) and the adenoviral hexon protein [23–26]. To overcome the first obstacle of NAb against the fiber domain, we modified the structurally exposed fiber domain with the scFvDEC205 and incorporated this into a chimeric fiber fibrin structure, which originates from bacteriophage (Sup Fig 1). Secondly, to minimize liver sequestration and the recognition of NAb against the viral hexon, we replaced the hexon domain of Ad5 with that of human Ad serotype 3 (Ad3), since it has been shown the hexon of Ad3 has relatively low affinity to FX, and this serotype 3 is less prevalent in humans, making pre-existing NAb formation less likely [23–26].

Hence, in this study, we generated a highly specific DC-targeting adenoviral vector suitable for the systemic

administration that carries a tumor antigen payload through a series of advanced genetic modifications. Next, we showed the capability of this modified virus to efficiently infect DCs both *in vitro* and *in vivo*, while simultaneously inducing DC maturation following viral infection. Moreover, we demonstrated that following viral infection, the infected DCs induce an antigen-specific T-cell activation, and these T cells can kill tumor cells expressing the TA. Finally, this adenoviral-mediated vaccination of DCs significantly prolonged the survival of mice bearing murine gliomas that expressed human glioma-specific antigen (CMV-IE) in both pre- and posttreatment models. Furthermore, the immunological antitumor memory was long-lasting, successfully preventing mortality in a tumor rechallenging experiment. Collectively, our data support the further exploration of this adenoviral vaccination strategy as a potential treatment strategy for humans with malignant gliomas.

Material and Methods

Cell Lines

Human embryonic kidney (HEK) 293, HEK 293-F28 stably expressing Ad5 wild-type fibers, Chinese hamster ovary (CHO) cells, CHO-hCAR cells stably expressing human CAR, murine glioma cell, and GL261 and GL261^{CMV-IE} cells stably expressing CMV-IE gene products were cultured in the media that contained 10% fetal bovine serum (FBS; Hyclone; Logan, UT), 100 unit/ml penicillin, and 100 mg/ml streptomycin (Mediatech, Inc., Herndon, VA) and incubated at 37 °C in 5% CO₂ in humidified conditions. To generate murine DCs, bone marrow from C57BL/6 mice was harvested from tibias and femurs. Red blood cells (RBCs) were lysed with RBC lysing (ACK) buffer (Lonza, Basel, Switzerland).

Cells were plated in 6 well plates at 1–3 × 10⁵ cells/ml in 2 ml of DC culture media containing RPMI 1640 (Mediatech) with 10% fetal bovine serum (Hyclone), 100 unit/ml penicillin, 100 mg/ml streptomycin (Mediatech), and murine GM-CSF (20 ng/ml, Miltenyi, Auburn, CA). On day 3, half of the media was removed without any agitation and 2 ml of the DC culture media was supplemented. Cells were cultured for 6 days to obtain dendritic cells.

Genetic Modifications and Virus Production

To generate a fiber shuttle vector, pKan-566 containing the gene of fiber fibrin was used [27]. The plasmids containing single-chain antibody against DEC205 were kindly offered by Dr. Miguel A. Gómez-Lima. The nucleotide sequence of the single-chain antibody against DEC 205 was inserted at the C-terminal of fiber fibrin by using a standard molecular technique, resulting in pKan566-scFvDEC205. Recombinant

HAd5 backbones containing an enhanced green fluorescence protein (eGFP) or ovalbumin or CMV-IE under the control of the CMV promoter in the E1 deleted region was used to perform homologous recombination with pKan566-scFvDEC205, generating Ad5scFvDEC205FF-GFP, Ad5scFvDEC205-OVA, and Ad5scFvDEC205-CMV-IE, respectively. Hexon modification was done in the same manner as previously described [26]. The recombinant viruses were rescued and propagated in HEK293-F28 until the last propagation step, and the last propagation in 20×175 ml flasks was done in HEK293 cells. Viruses were purified by two rounds of CsCl gradient ultracentrifugation and their titer was determined at 260 nm [28]. The virus titer of Ad5scFvDEC205 was highly equivalent to that of Ad5WT (Sup. Fig. 2).

PCR Analysis and Western Blot

Purified adenoviral DNA contained in Ad5GFP and Ad5scFvDEC205-GFP was used as templates for PCR amplification of fiber genes using a HAdV-5-specific primer set: (fwd) 5'-CAGCTCCATCTCCTAACTGT-3' and (rev) 5'-TTCTTGGGCAATGTATGAAA-3', and a scFvDEC205-specific primer set: (fwd) 5'-ATGGGATGGTCTTG CATCATT-3' and (rev) 5'-GAGCTCGTCGACTCTCTTAA GC-3'. For Western blot analysis, purified adenoviral particles (1×10^{10}) were diluted in Laemmli buffer, incubated either at room temperature (unboiled samples) or at 95 °C (boiled samples) for 10 min and loaded to a 4–10% SDS-polyacrylamide gel (Bio-Rad, Hercules, CA). After electrophoretic separation, samples were transferred onto a PVDF membrane and detected with a 4D2 monoclonal antifiber tail antibody (1:3000) (Thermo Scientific, IL, USA) followed by HRP tagged anti-mouse IgG secondary antibody (1:5000) (Santa Cruz, CA, USA).

Virus Infectivity Analysis

For *in vitro* analysis, 3×10^5 cells were plated in 24 well plates and incubated overnight. Each virus sample was diluted to a multiplicity of infection of in-text-described viral particles (vp)/cells in 500 μ l of infection media containing 2% FBS in DMEM. The cells were infected with each virus for 2 h at 37 °C. Virus-containing media was replaced with the fresh media containing 2% FBS and cells were maintained at 37 °C in atmospheric humidification containing 5% CO₂ for 3 days until flow cytometry analysis. In virus infectivity analysis of DCs, percentage of GFP-positive cells in CD45⁺/CD11⁺ DC population were utilized. For *in vivo* analysis, 10^9 viral particles were subcutaneously administrated. After 5 days, spleens and draining lymph nodes were harvested, followed by FACS analysis. For DC maturation analysis, bone marrow-derived DCs were prepared and the viral infections were done as described above. After confirmation of bone

marrow-derived DCs, DCs were analyzed against the expression level of maturation markers.

FACS Analysis

Spleen or draining lymph nodes from mice administrated with Ad5scFvDEC205-GFP (10^9 vp, s.c.) were harvested into complete RPMI (Mediatech) 3 days after the injection. Single-cell suspension was prepared by processing organs through a 70- μ m nylon cell strainer (Thermo Fisher, Waltham, MA). Red blood cells were lysed using 1 ml ACK buffer (Lonza) followed by inactivation, centrifugation, and resuspension. All antibody staining was performed in 2% PBS/FBS. Cells were stained for 15 min at 4 °C with fluorescently labeled antibodies for the following surface markers (and their dilutions): anti-CD45 APC-Cy7 1:200, anti-CD11b Pacific Blue 1:400, and anti-CD11c APC 1:200, anti-CD8 α BV605 1:200, anti-CD80 FITC 1:200, and anti-CD40 PE 1:200 were all purchased from Biolegend. Samples were acquired using a LSR-Fortessa flow cytometer (Becton Dickinson, Franklin Lakes, NJ). All flow cytometry gating and data analysis were performed using FLOWJO Software (Flowjo LLC, OR, USA).

Activated T Cell-Mediated Cytotoxicity Analysis

For LDH assay, DC culture and virus infection were performed in the same way as described above. After 3 days, isolated CD8⁺ T cells (EasySep Mouse CD8⁺ T Cell Isolation Kit, Stemcell, MA, USA) were incubated with the post Ad5scFvDEC205FF-CMV-OVA-infected DCs. Activated CD8⁺ T cells were transferred to GL261 or GL261-OVA in different ratios as indicated in the text, followed by LDH cytotoxicity analysis according to the company instruction (Thermo Scientific). For MTT assay, virus infection was done in the same way as described above except for which MOI of 100 vp/cells was used instead. MTT assay was performed using cell proliferation kit I according to the manufacture's instruction (Roche, Mannheim, Germany). For chromium⁵¹ (Cr⁵¹) release assay, standard chromium (Cr⁵¹) release assays were performed as previously described.

Briefly, 1×10^5 target cells were labeled with 0.1 mCi (3.7 MBq) Cr⁵¹ and mix with activated CD8⁺ T cells by virus-infected DCs. The ratio of the virus to DCs and DCs to CD8⁺ T cells is as described in the text. Spontaneous and maximum Cr⁵¹ release controls were generated incubating the target cells (GL261 or GL261-OVA) in complete medium alone or in 1% Triton X-100, respectively. After incubating in each condition for 4 h, the radioactivity from the supernatants was measured in a gamma counter (Cobra Quantum; PerkinElmer, Wellesley, MA). The cytotoxicity was calculated according to the following a formula (%): (test release –

spontaneous release) / (maximal release – spontaneous release) × 100.

Animal Study

Intracranial glioma xenograft implantation: GL261 or GL261CMV-IE (1×10^5 cells) murine glioma cells were implanted via cranial guide screws as described previously [29]. The 10^9 viral particles of Ad5scFvDEC205-CMV-IE (prevaccination) and Ad5scFvDEC205H3-CMV-IE (postvaccination) were subcutaneously administered. Detailed vaccination strategies are in each figure. For survival analysis, animals losing $\geq 30\%$ of their body weight or having trouble ambulating, feeding, or grooming were euthanized by CO₂ administration followed by cervical dislocation. For rechallenging analysis, the same number of glioma cells was implanted on the opposite site of the initial injection site. After 75 days, animals were sacrificed. Brains were harvested, sectioned, and H&E-stained.

Statistical Analysis

All statistical analyses were performed using GraphPad Prism 4 (GraphPad Software Inc., San Diego, CA). The sample size for each group was ≥ 3 and numerical data was reported as mean \pm S.E. Student's *t* test was used for comparisons between two groups, and ANOVA with Tukey's post hoc test was used for comparisons between more than two groups. Kaplan–Meier survival curve was generated and log rank test was applied to compare survival distributions. All reported *p* values were two-sided and were considered to be statistically significant at **p* < 0.05, ***p* < 0.01, ****p* < 0.001.

Results

Generation and Confirmation of DC-Targeting Adenoviral Vector

The natural tropism of Ad5 is dictated by the fiber knob domain which can be genetically modified to redirect Ad5 to a specific cell/tissue other than those that express the *coxsaekie* and *adenovirus receptor* (CAR), which is the primary receptor for determining Ad5 tropism [15, 18, 30]. DCs are deficient in CAR expression, which makes targeting DCs with wild-type Ad5 unachievable [31–33]. To address this critical barrier, a novel Ad5 that can infect DCs was developed by incorporating a single-chain fragment variable (scFv) antibody against a cell surface DC marker, DEC205, into the knob domain with an additional fiber-stabilizing modification of the fiber shaft domain, called fiber fibrin (namely, Ad5scFvDEC205FF, Sup. Fig 1) [17]. Generation of this modified virus DC targeting Ad5scFvDEC205FF was confirmed at the DNA

level (PCR analysis, Fig. 1A) and at the protein level (Western blot analysis Fig. 1B). Purified viral DNA of wild-type (WT) Ad5 and that of Ad5scFvDEC205 (DEC205) were used for PCR analysis with a fiber-specific primer set and scFvDEC205-specific primer set. For Western blot analysis, purified virus particles (10^9) were boiled (B) to dissociate fiber trimer (a monomeric form of the fiber) and unboiled (U) to keep the trimeric fiber intact. The fiber proteins were detected by an antifiber tail antibody, recognizing trimeric and monomeric form of recombinant fiber. Thus, PCR analysis revealed the insertion of the scFvDEC205 gene on the fiber gene of the purified viral gene (Fig. 1A) and Western blot analysis shows that scFvDEC205 incorporated fibers are stably trimerized and incorporated into Ad5 (Fig. 1B) as the bands of scFvDEC205 incorporated fibers from the trimers and monomers were comparable to those of wild-type Ad5 with an increased molecular weight. Since the knob and shaft domains of Ad5scFvDEC205FF were replaced with scFvDEC205FF (Sup Fig 1), it should not be able to infect a cell in a CAR-dependent manner. To verify CAR-independent infectivity of Ad5scFvDEC205FF-GFP, we performed a viral infection (300 virus particles(vp)/cell) followed by a flow cytometric analysis in a human CAR-negative cell line, Chinese hamster ovary (CHO) cell line, and a CHO-derived cell line, CHO-hCAR (Fig. 1C), which stably expresses human CAR. While the infection rate of Ad5GFP is dramatically increased in CHO-hCAR cells, as expected, there was no difference in the infection rate of Ad5scFvDEC205FF-GFP from both cell lines (Fig. 1D). Therefore, we confirmed the generation of Ad5scFvDEC205FF and demonstrated that Ad5scFvDEC205FF infects cells in a CAR-independent manner.

Ad5scFvDEC205FF Can Efficiently and Selectively Infect DC *In Vitro* and *In Vivo*

Next, to determine whether scFvDEC205 in the fiber of Ad5scFvDEC205FF dictates the viral infection to DCs, we isolated the murine splenic cells for the viral infection. The infectivity of DCs by Ad5scFvDEC205FF was compared against three other adenoviral vectors: Ad5PK7-GFP (infectivity enhanced adenoviral vector via addition of poly-lysine on the fiber which binds to anionic surface proteins) [34–36], Ad5/3-GFP (DC infectivity enhanced adenoviral vector by replacing the Ad5 knob with the Ad3 knob, which binds to CD46, CD80, CD86, and desmoglein 2) [32, 34, 37], and wild-type Ad5-GFP. After viral infection, cells were gated on CD45⁺/CD11c⁺ (general DC surface markers [3, 5, 38–41]) and GFP-positive populations were analyzed via flow cytometric analysis. As shown in Fig. 2A, the infectivity of Ad5scFvDEC205FF on DCs was superior to that of all other viruses and the infectivity kinetics was observed as highly stable (Sup. Fig 2). To further examine the *in vivo* DC selectivity of Ad5scFvDEC205FF-GFP, we subcutaneously

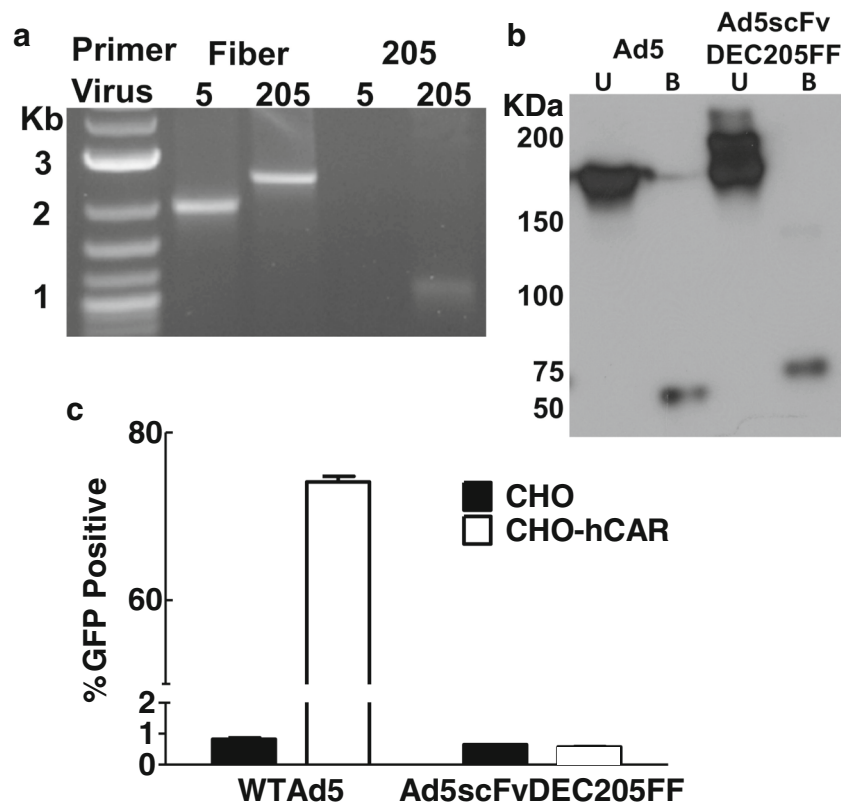


Fig. 1 Generation and confirmation of the novel virus. (A) PCR analysis of fiber genes of the adenoviral DNA from purified viral particles as PCR templates. Purified viral DNA from Ad5GFP (lanes 2 and 4) and Ad5scFvDEC205FFGFP (lanes 3 and 5) were used as DNA templates to amplify each fiber gene with a fiber-specific primer set (lanes 2 and 3) or with scFvDEC205-specific primers (lanes 3 and 5). Lane 1 shows DNA size standards (Kb). (B) Detection of fiber trimerization and incorporation in the viral particles by Western blot analysis with an HAdV-5 tail antibody (4D2). Purified viral particles (1×10^{10} vp) of Ad5GFP (lanes 1 and 2) or those of Ad5scFvDEC205GFP (lanes 3 and 4) were analyzed. Samples were identified as U (unboiled, incubation at room temperature for 10 min) in lanes 1 and 3 and as B (boiled,

incubation at 95 °C for 10 min) in lanes 2 and 4 show the nondenatured trimeric fiber composition and the denatured monomeric fiber composition, respectively. Makers indicate kiloDaltons (kDa). (C) Flow cytometric analysis of CAR-independent infection of Ad5scFvDEC205GFP. Cells were infected with Ad5CMV-GFP or Ad5scFvDEC205GFP at an MOI of 300 vp/cell for 2 h on CAR-negative CHO and CAR-positive CHO-hCAR cell line, and the virus containing media was removed and replaced with fresh media containing 2% FBS. 72 h after infection, flow cytometric analysis was performed. Each column is the average of three independent replicates. Data presented as mean \pm S.E.

(s.c.) administered 10^9 vp of Ad5scFvDEC205FF-GFP and Ad5-GFP in mice. Three days after the viral infection, spleens and lymph nodes were isolated and analyzed with flow cytometric analysis. After gating with CD45⁺ (nucleated hematopoietic cells), the DC population (CD11c⁺) was analyzed for the presence of an infected DC population (GFP positive). Of note, we did not detect GFP-positive cells from wild-type Ad5-GFP-administrated mice since it is well known that wild-type Ad5-GFP gets aggregated in the liver and cleared out fast (data not shown). An average of 14% of DCs were infected by Ad5scFvDEC205FF-GFP, while less than 3% of non-DCs were infected following *in vivo* Ad5scFvDEC205FF-GFP administration (Fig. 2B). It has been suggested that the expression of DEC205 on DCs is highly correlated with CD8 α^+ DC subset which is known to cross-present antigens more efficiently and activate robust cytotoxic CD8⁺ T cell responses [13, 14, 42, 43]. For this reason, we further analyzed the Ad5scFvDEC205FF-GFP-infected DC population. After gating with CD45⁺

(nucleated hematopoietic cells), the DC population (CD11c⁺) was analyzed for the presence of a CD8 α^+ DC population. We observed ~19% of CD8 α^+ DCs from CD45⁺CD11c (Fig. 2C). An average of 91% of CD45⁺CD11c⁺ CD8 α^+ DCs were infected by Ad5scFvDEC205FF-GFP (Fig. 2D). Therefore, Ad5scFvDEC205FF possess enhanced and selective DC infectivity (highly preferential for CD8 α^+ DC subset) not only *in vitro* but also *in vivo*. On this basis, the tropism-modified Ad5scFvDEC205FF represents a promising agent for active GBM immunotherapy.

Infection of Ad5scFvDEC205FF Itself Induces DC Maturation

It is imperative to deliver TA to mature DCs or induce DC maturation upon TA delivery [5, 38–41, 44, 45]. The targeting motif, DEC205, is known to be highly upregulated when DCs are mature [11, 13, 14]. Furthermore, it is perceived that the

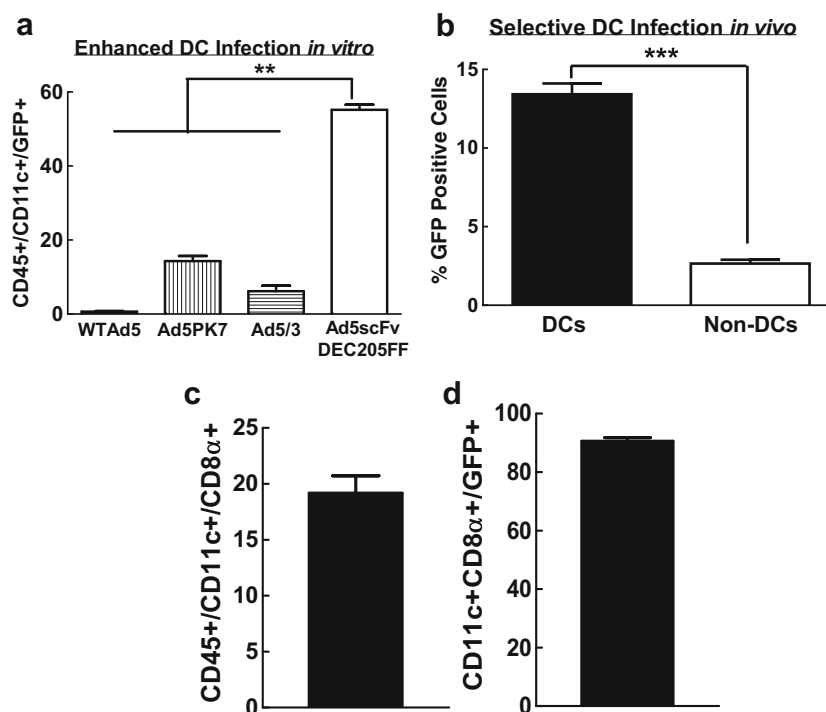


Fig. 2 Enhanced and selective DC infection of Ad5scFvDEC205. **(A)** *In vitro* infectivity enhancement analysis. Spleen-derived DCs were infected with wild-type Ad5-GFP, Ad5PK7-GFP, Ad5/3-GFP, and Ad5scFvDEC205FF-GFP at an MOI of 300 vp/cell for 2 h on DCs and the virus-containing media was removed and replaced with fresh media containing 2% FBS. After 72 h, cells were stained with DC markers (CD45 and CD11c) followed by flow cytometric analysis. Infected DCs were identified with GFP expression. **(B)** *In vivo* DC flow analysis. After gating with CD45⁺, the DC population (CD11c⁺) was analyzed to identify

GFP-positive DC population. **(B)** *In vivo* selective DC infectivity analysis. 10⁹ vp of Ad5scFvDEC205FF-GFP were administrated subcutaneously. Spleens and lymph nodes were isolated 5 days after the viral administration, followed by flow cytometric analysis. GFP-positive DCs (CD45⁺/CD11c⁺/GFP⁺) were first analyzed. Among DCs, percentage of CD8 α ⁺ DC subset **(C)** and that of GFP positive in CD8 α ⁺ DC subset **(D)** are shown. Each data point is the average of three independent replicates. Data presented as mean \pm S.E.

infection of adenovirus, itself, induces DC maturation [46]. Since Ad5scFvDEC205FF selectively infects DCs both *in vitro* and *in vivo*, we confirmed in this study that the infection of Ad5scFvDEC205FF induces DC maturation of infected immature DCs. Bone marrow-derived DCs were infected with Ad5scFvDEC205FF-CMV-OVA. Within this novel DC-specific delivery vehicle, the glioma-specific antigen gene (i.e., CMV-IE) was inserted by replacing a viral replication essential early gene 1 (E1). This modification ensured that the viral delivery vehicle cannot replicate in or lyse the infected DCs but instead would transiently express the loaded tumor antigen within the DCs so that these DCs can present and/or cross-present this TA. Three days after infection, the levels of DC maturation markers have been analyzed via flow cytometric analysis. As shown in Fig. 3, the levels of CD40 and CD80 markers of DC maturation from infected DCs were highly elevated compared to those of non-infected DCs, confirming that Ad5scFvDEC205FF-CMV-OVA infection of DCs indeed induces the maturation of DCs. Therefore, the infection of Ad5scFvDEC205FF can facilitate the maturation of DCs if the infected DCs are immature.

Vaccinated DCs Induce TA-Specific T-Cell Activation, Leading to T Cell-Mediated Cytotoxicity of TA-Expressing Tumor Cells

Since Ad5scFvDEC205FF can infect DCs both efficiently and selectively *in vitro* and *in vivo*, and can further induce the maturation of DCs, we investigated the question as to whether Ad5scFvDEC205FF-CMV-OVA-mediated DC vaccination can induce TA-specific T-cell activation so as to kill the TA-expressing tumor cell line. To assess this, we first performed a LDH release assay. After DC infection with Ad5scFvDEC205FF-CMV-OVA at different MOIs, isolated CD8⁺ T cells were added to activate these T cells in a TA (OVA)-specific manner. Activated CD8⁺ T cells were transferred to TA-expressing target tumor cells (GL261-OVA). The cell death of TA-expressing glioma cells (GL261-OVA) by activated T cells was analyzed. As DCs were infected with a high titer of Ad5scFvDEC205FF-CMV-OVA, CD8⁺ T cells could be more efficiently activated in a TA-specific manner, rendering these activated T cells able to kill target tumor cells more effectively (Fig. 4A). In order to investigate the activated CD8⁺ T cell-

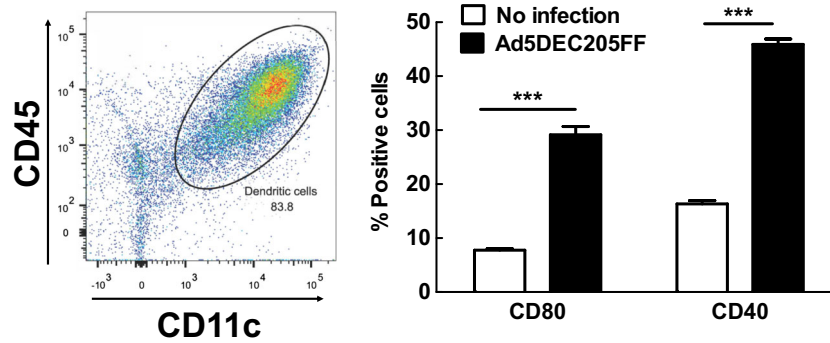


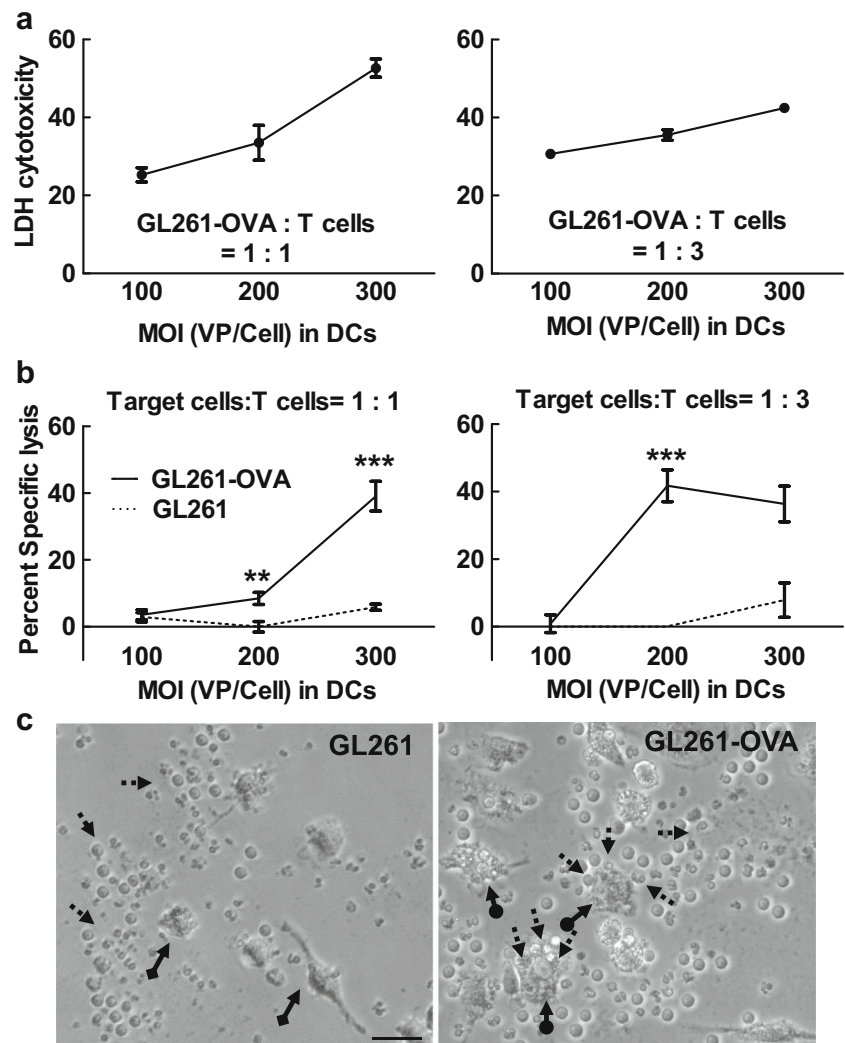
Fig. 3 Induction of DC maturation via Ad5scFvDEC205FF-GFP infections. The 3×10^5 bone marrow-derived DCs and an MOI of 300 vp/cell of Ad5scFvDEC205FF-GFP were used for the infection. After 72 h, DCs were stained with (A) DC markers (CD45 and CD11c)

and (B) DC maturation markers (CD80 and CD40) followed by cytometric analysis. Each data point is the average of three independent replicates. Data presented as mean \pm S.E.

mediated cytotoxicity at different time points, we also performed an MTT (Supp. Fig 3). DCs were infected with Ad5scFvDEC205FF-CMV-OVA at different MOIs, followed by CD8⁺ T-cell incubation for 72 h. Since the LDH assay showed the optimal ratio of CD8⁺ T cells to tumor target cells (GL261-

OVA) at 1:1 (Fig. 4A), we incubated these activated CD8⁺ T cells in GL261-OVA in 1:1 ratio and analyzed the cytotoxicity in a time-dependent manner (12, 24, and 48 h). As observed from the LDH assay, the cytotoxic activity of activated CD8⁺ T cells was the most efficient when DCs were infected with

Fig. 4 Cytotoxicity analysis of TA-specific CD8⁺ T cell activated by Ad5scFvDEC205FF-OVA-transduced DCs. 3×10^5 bone marrow-derived DCs were infected with Ad5scFvDEC205FF-OVA at an MOI of 100, 200, and 300 vp/cell for 2 h on DCs and the virus-containing media was removed and replaced with a fresh media containing 2% FBS. After 72 h, CD8⁺ T cells isolated from syngeneic mice were incubated with infected DCs in the ratio of 1:1. Activated CD8⁺ T cells were transferred to either GL261 or GL261-OVA followed by (A) LDH assay and (B) Cr⁵¹ release assay. The ratio of 1:3 = target cells: T cells from LDH analysis were used for (C) the microscopic observation. Arrows and dot arrows indicate glioma cells and CD8⁺ T cells, respectively. The bar indicates 100 μ m. Each data point is the average of three independent replicates. Data presented as mean \pm S.E.



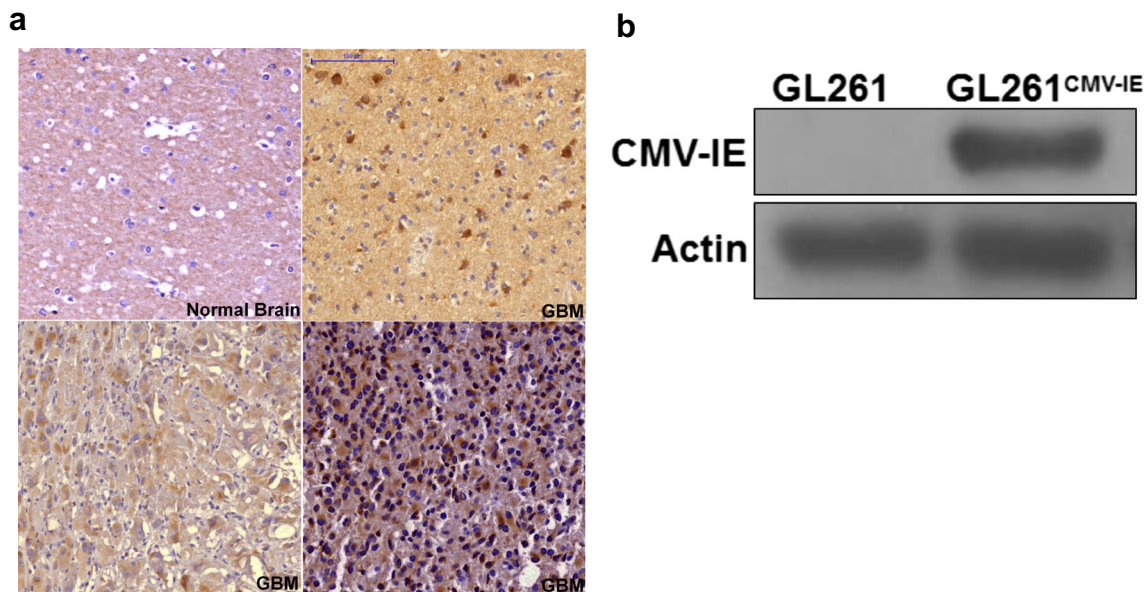


Fig. 5 Validation of human glioma-specific antigen (CMV-IE) and generation of the CMV-IE-expressing murine glioma model cell (GL261^{CMV-IE}). **(A)** Brain cancer and adjacent normal tissue array (GL803b, Biomax) were stained with anti-CMV-IE antibody. All the GBM tissue staining with anti-CMV-IE showed CMV-IE positive. The

representative tissue staining is shown including a normal brain tissue staining. **(B)** Murine glioma GL261 cells were lenti-virally transduced to generate a stably CMV-IE-expressing GL261^{CMV-IE} cell line. The expression of CMV-IE were confirmed by Western blot analysis

Ad5scFvDEC205FF-CMV-OVA at an MOI of 300 vp/cell at all time points (Fig. 4).

To further confirm this finding, we utilized another classic assay for accessing T cell-mediated cytotoxic function using Cr⁵¹. Since Cr⁵¹ is only loaded in target tumor cells, it is a classic and efficient way to measure cytotoxicity by T cells by analyzing the release of Cr⁵¹ from dying target cells. Infection of DCs and T-cell activation was done in the same way as described already. Next, to analyze if T cells were activated in a TA-specific manner (OVA-specific), activated T cells were added into the Cr⁵¹-loaded GL261 and GL261-OVA cell lines, followed by analyzing the release of Cr⁵¹ from dead target tumor cells. Since CD8⁺ T cells were activated specifically against an antigen (OVA), GL261 cells (no antigen-expressing cells) were not recognized by OVA-specifically activated T cells evidenced by no observable cytotoxicity (Fig. 4B). On the other hand, antigen-expressing GL261-OVA cells were efficiently recognized by OVA-specifically activated CD8⁺ T cells, thereby causing high cytotoxicity in GL261-OVA cells (Fig. 4B). Interestingly, OVA-specifically activated T cells localized evenly away from GL261 cells while these T cells clustered around GL261-OVA cells, inducing cell death (Fig. 4C). Therefore, combining the data from the experiments described, strongly supports that 1) Ad5scFvDEC205FF-CMV-OVA-infected DCs can be matured and present OVA antigens, 2) this OVA antigen-presenting mature DCs can activate CD8⁺ T cells against OVA antigen, specifically, and 3) these T cells can recognize the specific antigen (OVA) on the tumor cells and kill the tumor cells in an antigen-specific manner (Figs. 3 and 4).

Confirmation of GBM-Specific Tumor Antigen (CMV-IE) Expression and Generation of a Murine Glioma Cell Line Expressing the Antigen, GL261-CMV-IE

Although several GBM-specific targets for immunotherapy have been identified, such as EGFRvIII and IL13R α 2, these

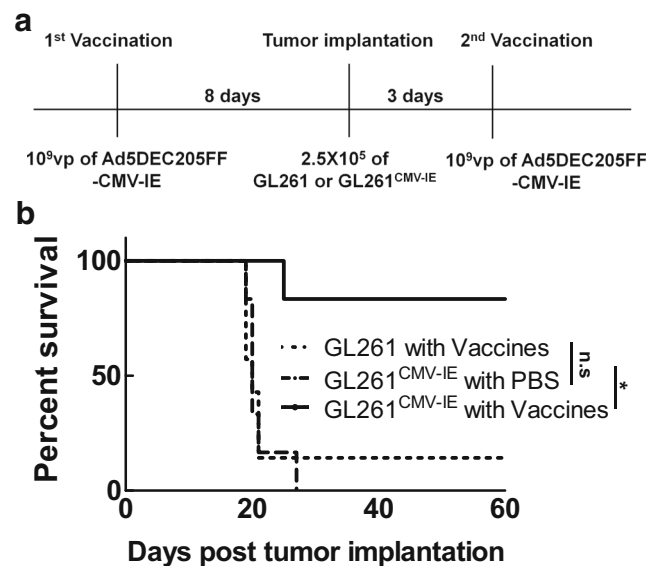


Fig. 6 Survival analysis of the prevaccinated (Ad5scFvDEC205-CMV-IE) murine model. Tumor implantation and vaccination procedures were done as described in (A). The Kaplan–Meier survival curves of treated mice were analyzed (B). * $p < 0.05$

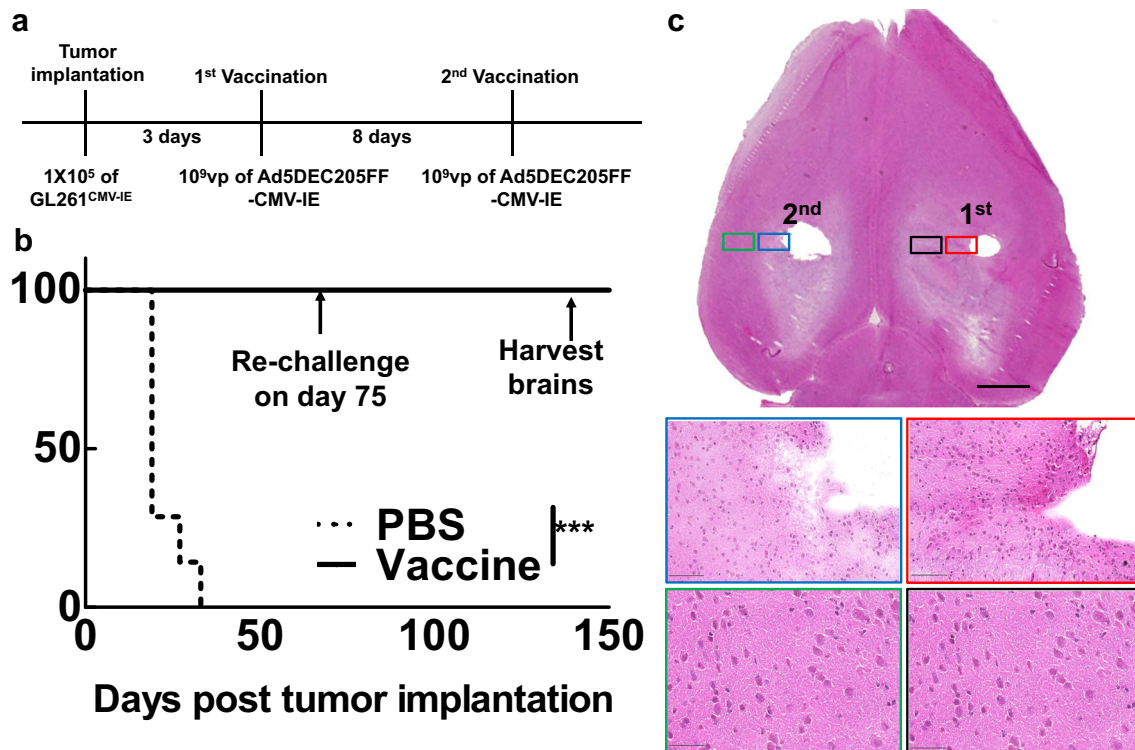


Fig. 7 The efficacy of the postvaccination (Ad5scFvDEC205-CMV-IE) on the primary and rechallenged glioma murine models. Tumor implantation and vaccination procedures were done as described in (A). After 75 days of prolonged survivals from the vaccinated mice, the same number of GL261CMV-IE cells was rechallenged on the opposite site of

molecules are, unfortunately, only targetable in a limited percentage (< 30%) of GBM patients [46]. Recently, there have been reports demonstrating the presence of cytomegalovirus (CMV) in more than 90% of GBM patients [47–49] examined and though some established relationship between CMV persistence [50–52] and glioma tumorigenesis has been elucidated, this intriguing correlation and its potential for targeted immunotherapy inspired us to further verify the presence of CMV immediate early gene product (CMV-IE) in patient-derived glioma tissues (Fig. 5A). Brain cancer and adjacent normal tissue array (GL803b, Biomax, Rockville, MD) were stained with anti-CMV-IE antibody. We could only observe CMV-IE-specific staining from glioma tissues but none from peripheral normal tissue (Fig. 5A). This patient tissue-based analysis suggests that CMV-IE could be a highly applicable molecular target for GBM immunotherapy. Hence, to investigate our GBM immunotherapeutic approach with this CMV-IE antigen, we generated a murine glioma model cell line, GL261^{CMV-IE}. The gene of *CMV-IE* was intrachromosomally incorporated into GL261 cell line with a Lentivirus encoding *CMV-IE*, generating GL261-expressing CMV-IE stably. The expression of CMV-IE from GL261^{CMV-IE} cell line was confirmed via Western blot analysis (Fig. 5B). Therefore, CMV-IE is a highly suitable target for human glioma vaccine therapy and our established murine cell line (GL261^{CMV-IE}) can serve

the initial tumor implantation (B). After 75 days of tumor rechallenge, brains were harvested and sectioned and H&E staining was done to check any residual tumor cells (C). The Kaplan–Meier survival curves of treated mice were analyzed (B). *** $p < 0.001$

as a highly valuable therapeutic experimental model cell line in this regard.

Prevaccination with Ad5scFvDEC205FF-CMV-IE Shows a Prolonged Survival Benefit in TA-Specific Manner

Our *in vitro* analysis showed that Ad5scFvDEC205FF efficiently infects, loads an antigen, and induces maturation of DCs (Figs. 2 and 3). These Ad5scFvDEC205FF-infected and matured DCs can activate CD8⁺ T cells in an antigen-specific manner that efficiently lyses the target tumor cells (Fig. 4). Therefore, we utilized the versatile Ad5scFvDEC205FF virus to selectively load the GBM-TA (CMV-IE, Fig. 5) in DCs in order to trigger a strong antitumor immune response via generating Ad5scFvDEC205FF-CMV-IE. As an initial approach, we first investigated the prevaccination-based tumor-specific therapeutic efficacy as depicted in Fig. 6A. In antigen-expressing tumor cell line (GL261^{CMV-IE})-implanted mice, there is no survival benefit from PBS subcutaneous-injected group (mean survival, 20 days) while Ad5scFvDEC205FF-CMV-IE showed a statistically significant prolong survival ($p < 0.02$). Moreover, when Ad5scFvDEC205FF-CMV-IE is subcutaneously injected in non-antigen-expressing glioma cell (GL261)-implanted mice, there is no survival benefit which indicates that Ad5scFvDEC205FF-CMV-IE specifically induces an immune

response against CMV-IE (Fig. 6B). Therefore, our *in vivo* prevaccinated survival analysis suggests that Ad5scFvDEC205FF-CMV-IE is highly efficient as a vaccination strategy against a human glioma antigen (CMV-IE) and extended survival periods in the human glioma-carrying murine model.

Hexon-Modified Adenoviral Vaccine Mediates Superior Vaccine Efficacy on a Postvaccination Glioma Model and Rejected Rechallenged Tumor Implantation

It is known that liver sequestration of human adenovirus serotype 5 (Ad5) limits therapeutic efficacy. Recent findings suggest that interactions of Ad5 capsid protein, hexon, with the blood coagulation factor X (FX) lead these complexes to attach to cell surface heparin sulfate proteoglycans (HSPGs) on hepatocytes [22, 24, 26]. The attachment of this complex causes sequestration of adenoviral therapeutic vehicles on liver [22, 24]. One strategy to overcome this challenge is to replace the Ad5 hexon with the hexon from Ad3, which has been shown to minimize the liver sequestration and toxicity since the hexon of human adenovirus serotype 3 has a relatively low affinity to FX [26]. Based on this rationale, we generated hexon-modified DC-targeting adenoviral vector, Ad5H3scFvDEC205FF-CMV-IE and investigated the vaccine efficacy after the tumor implantation (postvaccine approach). As shown in Fig. 7, while the median survival of PBS-treated group was 19 days, all the vaccinated group showed no signs of tumor and was healthy until day 75. To further verify this vaccination efficacy and possibility to induce memory cells against CMV-IE, we rechallenged GL261^{CMV-IE} on the other side of previously tumor-implanted site. Again, all the tumor-rechallenged mice were healthy without any sign of illness. After another 75 days of tumor rechallenging (150 days in total), we sacrificed mice and looked for any signs of tumor on the brain. As shown in Fig. 7B, there were no observable tumor cells around the injection site confirming that the vaccinated mice remained tumor free following tumor rechallenging.

Discussion

Antitumor vaccination (ATV) strategies represent an especially promising set of approaches with rapid translational potential, especially considering the dismal clinical context of high-grade gliomas. In this regard, the most recent strategies to achieve effective antitumor immunization have sought to capitalize on the active loading of tumor-specific antigen(s) to the most professional antigen-presenting cells: dendritic cells (DCs) [2–4, 44]. Therefore, efficient and selective *in vivo* DC delivery vehicles that can facilitate highly efficient multi-epitope TA loading for maximal antitumor immunity are needed to achieve efficacious GBM immunotherapy [3, 4, 41, 45].

Here, we engineered a novel adenovirus (named Ad5scFvDEC205FF) that specifically infects DCs by incorporating a single-chain fragment variable (scFv) region antibody against a DC surface marker, DEC205 [13, 14], with an additional fiber-stabilizing modification [17]. This novel viral vector was optimized with a series of genetic modifications to specifically infect dendritic cells and deliver a tumor-specific antigen for presentation to the adaptive immune system. Furthermore, these modifications have the additional benefit of reducing the risk of liver toxicity following systemic viral administration and limiting the effect of potential preformed neutralizing antibodies from previous adenovirus exposure [21, 22]. Also, to investigate our virus in clinically relevant experiments, we generated a murine glioma model cell line that stably expresses a human glioma antigen (CMV-IE) suitable for experimentation in immunocompetent mice.

We first evaluated whether scFvDEC205 in the fiber of Ad5scFvDEC205FF dictates efficient DC infection by comparing the infectivity of DCs by Ad5scFvDEC205FF with three other control adenoviral vectors: Ad5PK7-GFP, Ad5/3-GFP, and Ad5-GFP. By undertaking this study with the use of a DC infectivity assay, we were able to see that DCs had increased infection when subject to the experimental fiber as shown in Fig. 2A. Qualitatively, the infectivity kinetics for the DCs exhibited were highly stable (Sup Fig 3). Furthermore, subcutaneous administration of this vector, Ad5scFvDEC205FF-GFP, showed highly selective infectivity on DC population (14%) as compared to that on non-DC population (3%). As the expression of DC-targeting molecule, DEC205, is highly correlated with the CD8 α DC subset, which is known to cross-present an antigen and activate cytotoxic T cells more efficiently [42, 43], our data indicate that Ad5scFvDEC205FF-GFP infects preferentially to these DC subsets. These lines of data suggest the high potential for the therapeutic usages of our novel vector.

Another key aspect of DC targeting for T-cell immune responses is the DC maturation index. DCs must reach maturity in order to facilitate an effective T-cell immune response and it has shown that the DC maturation is affected by adenoviral infection [46]. In this study, we examined that the maturation of DCs as a function of adenoviral infection is a fundamental factor in the maturation of DCs—a factor that is instrumentally important in considering a DC vaccination strategy such as this one. In doing so, we learned that in addition to being a highly efficient and selective DC infector, this adenovirus-induced DC maturation, augmenting the efficacy of a T-cell immune response is mediated by the antigen presentation of DCs.

We then examined the premise of CD8⁺ T-cell activation via antigen-loaded and mature DCs. Three cytotoxicity analyses (LDH, MTT, and Cr⁵¹) were used to test the assumption that such CD8⁺ T cells are activated by TA-presenting and mature DCs in an antigen-specific manner. Additionally, we found that activated CD8⁺ T cells can execute a murine glioma cell antitumor response due to resultant cytotoxicity. Such

cytotoxic activity for DC infection was found to be dependent on viral particle dose and thus indicative of the antitumor effectiveness.

Finally, we tested this novel adenoviral vector in the GL261^{CMV-IE} murine glioma cell line to evaluate the success and effectiveness of DC vaccination strategy. We found that both pre- and postvaccination strategies resulted in enhanced murine survival to support marked therapeutic benefit in a TA-specific manner that was supported by additional pathological examination, both grossly and histologically. Impressively, surviving mice that were rechallenged with tumor cells still survived without any additional therapeutic injections, indicating long-term immunological memory against the tumor following virus administration.

Our approach differs radically from current antitumor immunotherapies, which typically passively deliver a tumor antigen to DCs following DC harvesting and *ex vivo* manipulation. Rather, we actively deliver the tumor antigen specifically to DCs *in vivo* using a viral vector that also stimulates DC maturation upon infection. This has the advantage of being easier to administer and being easier to redose with an additional virus if needed. Future projects can combine this viral-based dendritic cell vaccine with other immunomodulatory agents, such as checkpoint inhibition, to remove the immunosuppression present in human malignant gliomas.

In conclusion, using a series of viral modifications, we created a viral vector capable of being delivered systemically and specifically delivering a tumor antigen directly to dendritic cells that were able to activate effector T cells and lead to a robust and long-lasting immune response against the tumor. This novel approach is an exciting combination of virotherapy and immunotherapy and represents a creative dendritic cell-based immunotherapeutic approach for human tumor with persistence of protumorigenic viruses such as cytomegalovirus.

Required Author Forms [Disclosure forms](#) provided by the authors are available with the online version of this article.

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