IJC
International Journal of Cancer

Newcastle disease virotherapy induces long-term survival and tumor-specific immune memory in orthotopic glioma through the induction of immunogenic cell death

Carolien A. Koks[1], Abhishek D. Garg[2], Michael Ehrhardt[3], Matteo Riva[1], Lien Vandenberk[1], Louis Boon[4], Steven De Vleeschouwer[5,6], Patrizia Agostinis[2], Norbert Graf[3] and Stefaan W. Van Gool[1,7]

[1] Pediatric Immunology, Department of Microbiology and Immunology, KU Leuven, Herestraat 49, Leuven, Belgium
[2] Department of Cellular and Molecular Medicine, KU Leuven, Herestraat 49, Leuven, Belgium
[3] Department for Pediatric Oncology, University of Saarland Medical School, Kirrberger Straβe, Homburg, Germany
[4] Bioceros BV, Yalelaan 46, 3584 CM Utrecht, The Netherlands
[5] Department of Neurosciences, KU Leuven, Herestraat 49, Leuven, Belgium
[6] Department of Neurosurgery, University Hospitals Leuven, Herestraat 49, Leuven, Belgium
[7] Department of Pediatric Neuro-oncology, University Hospitals Leuven, Herestraat 49, Leuven, Belgium

The oncolytic features of several naturally oncolytic viruses have been shown on Glioblastoma Multiforme cell lines and in xenotransplant models. However, orthotopic glioma studies in immunocompetent animals are lacking. Here we investigated Newcastle disease virus (NDV) in the orthotopic, syngeneic murine GL261 model. Seven days after tumor induction, mice received NDV intratumorally. Treatment significantly prolonged median survival and 50% of animals showed long-term survival. We demonstrated immunogenic cell death (ICD) induction in GL261 cells after NDV infection, comprising calreticulin surface exposure, release of HMGB1 and increased PMEL17 cancer antigen expression. Uniquely, we found absence of secreted ATP. NDV-induced ICD occurred independently of caspase signaling and was blocked by Necrostatin-1, suggesting the contribution of necroptosis. Autophagy induction following NDV infection of GL261 cells was demonstrated as well. *In vivo*, elevated infiltration of IFN-γ^+ T cells was observed in NDV-treated tumors, along with reduced accumulation of myeloid derived suppressor cells. The importance of a functional adaptive immune system in this paradigm was demonstrated in immunodeficient Rag2$^{-/-}$ mice and in CD8$^+$ T cell depleted animals, where NDV slightly prolonged survival, but failed to induce long-term cure. Secondary tumor induction with GL261 cells or LLC cells in mice surviving long-term after NDV treatment, demonstrated the induction of a long-term, tumor-specific immunological memory response by ND virotherapy. For the first time, we describe the therapeutic activity of NDV against GL261 tumors, evidenced in an orthotopic mouse model. The therapeutic effect relies on the induction of ICD in the tumor cells, which primes adaptive antitumor immunity.

Tumor Immunology

Glioblastoma Multiforme (GBM) is a WHO grade IV neoplasm and the most frequent primary brain tumor in adults.[1] Current multimodal treatment consists of maximal surgical resection of the tumor, radiochemotherapy and maintenance chemotherapy. Despite great advances in these fields, the prognosis of GBM patients remains dismal and relapse

Key words: glioma, Newcastle disease virus, immunogenic cell death, antitumor immunity, necroptosis

Abbreviations: 7AAD: 7-aminoactinomycin D; AMPK: AMP-activated protein kinase; BSA: bovine serum albumin; CNS: central nervous system; CPE: cytopathic effect; CQ: chloroquine; DCm: mature dendritic cells; Ecto-CRT: surface-exposed Calreticulin; FBS: fetal bovine serum; FCS: fetal calf serum; GBM: glioblastoma multiforme; HMGB1: high mobility group box 1; HSP: heat-shock protein; ICD: immunogenic cell death; LLC: Lewis lung carcinoma; MDSC: myeloid derived suppressor cell; MHC: major histocompatibility complex; MOI: multiplicity of infection; NDV: Newcastle disease virus; NK: natural killer; OD: optical density; OV: oncolytic virus; OVT: oncolytic virotherapy: pAMPK: phosphorylated AMPK; PBS: phosphate buffered saline; PI: propidium iodide; PMEL17: premelanosome protein 17; PS: phosphatidylserine; Rep: replication initiator (proteins); RIPK 1: receptor-interacting protein kinase 1; RT: room temperature; TRP-2: tyrosinase-related protein 2; zVAD: Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone

Grant sponsor: Fund for Scientific Research; **Grant numbers:** G.0661.09, G.0728.10, G.0584.12N; **Grant sponsor:** Interuniversity Attraction Poles Program; **Grant number:** IAP7/32; **Grant sponsor:** KU Leuven; **Grant number:** GOA/11/009; **Grant sponsors:** Olivia Hendrickx Research Fund; the James E. Kearney Memorial Fund; Leuvenā s ARCH, Belgian Brain Tumor Support
DOI: 10.1002/ijc.29202
History: Received 21 Feb 2014; Accepted 27 Aug 2014; Online 10 Sep 2014
Correspondence to: Carolien Koks, Laboratory of Pediatric Immunology, Herestraat 49, bus 811, 3000 Leuven, Belgium, Tel.: +32-16-34-61-65, Fax: +32-16-34-60-35, E-mail: Carolien.Koks@med.kuleuven.be

What's new?

Glioblastoma multiforme has a particularly grim prognosis. One potential treatment is "oncolytic virotherapy" (OVT), which relies on viruses that specifically infect and lyse tumor cells. In this study, the authors found that mice whose gliomas were injected with Newcastle-disease virus (NDV) survived significantly longer than controls. Many researchers have assumed that, in order for OVT to work, the immune system must be suppressed. Here, however, NDV actually stimulated "immunogenic cell death" (ICD) and "necroptosis" (rather than apoptosis). OVT may thus be a promising therapeutic approach in combination with therapies that stimulate the immune response.

universal.[2,3] Therefore, much of the contemporary (pre)clinical research is focused on developing novel long-term and tumor-targeted treatments. One such emerging strategy is oncolytic virotherapy (OVT).[4] This approach employs viruses, which infect and lyse tumor cells, while leaving non-transformed cells unharmed, thereby helping in tumor eradication.

Within the broad group of oncolytic viruses (OVs), naturally occurring oncolytic strains, such as Newcastle disease virus (NDV), are particularly interesting as they show antitumor activity against a broad spectrum of malignancies and possible side toxicities are relatively easily predicted and addressed. Contrary to genetically engineered strains, these viruses are not human pathogens and safety databases concerning their use in humans are available.[5] Furthermore, NDV represents one of very few OVs that have completed the first phase I/II clinical trials in glioma patients to date, demonstrating appreciable evidence of antiglioma activity.[6] Preclinically, most *in vivo* studies performed to date have focused on the direct tumor cell killing capacity of NDV in (subcutaneous) xenotransplant models.[7] However, utilizing OVT solely for tumor eradication, as in this setting, does not validate the recent "oncolytic paradigm," which entails that OVT-mediated tumor cell death should be accompanied by elicitation of antitumor immunity that improves the efficacy of OVT.[4]

When considering the host immune system, earlier research in the glioma field mostly speculated it to be a strong hurdle to successful OVT and aimed to improve therapeutic efficacy with immunosuppressive co-treatments.[8,9] Recently, however, OVT efficacy was also shown in fully immunocompetent animals.[10,11] Evidence from other cancer models has further led to the suggestion that OVs might trigger antitumor immune reactivity relatively independent of viral replication. In this concept, the immune system is critical to mediating the efficacy of OVT and immunosuppression would actually reduce tumor therapy. In orthotopic glioma research, the data presented here is the first investigation into this hypothesis.

An OV may fulfill the "oncolytic paradigm" by inducing an emerging form of cancer cell death called immunogenic cell death (ICD).[12,13] While most anticancer therapies induce non-immunogenic cell death, which induces tolerance toward tumor cells, certain therapies can induce a cell death mechanism that is actively immunostimulatory because it is associated with the exposure/emission of potent danger signals, thereby leading to activation of antitumor immunity.[13] The *in vitro* molecular determinants of ICD include danger signals like surface-exposed calreticulin (ecto-CRT), secretion of ATP and passive release of heat shock proteins (HSPs)−70/90 or high mobility group box 1 (HMGB1) protein.[12,13] The *in vivo* immunological signatures include increased infiltration of IFN-γ^+ $CD4^+$/$CD8^+$ T cells into the treated tumor microenvironment and the ability to induce tumor-rejecting immunity.[13] Moreover, it has been recently proposed that an ideal ICD-inducing therapy should also principally lead to decreased sustenance of pro-tumorigenic immune cells like myeloid derived suppressor cells (MDSCs).[12]

Until now, as a standalone therapy, two naturally occurring OVs (*i.e.*, measles and coxsackie B3 virus) have been shown to elicit molecular signatures *in vitro* that may suggest induction of ICD (*e.g.*, ecto-CRT or passively released HMGB1) and to cause stimulation of immune cells.[14,15] However, neither of these were shown to induce concomitant tumor-rejecting anticancer immunity in an immunocompetent syngeneic animal model—an absolute prerequisite of *bona fide* ICD induction.[13]

Material and Methods

Animals, cell lines and viruses

Female 10-weeks-old C57BL/6J mice were purchased from Harlan (The Netherlands). Male 20-weeks-old $Rag2^{-/-}$ mice were kindly provided by Dr. Guy Boeckxstaens (KU Leuven). All animal experiments were approved by the bioethics committee of the KU Leuven.

Methylcholanthrene-induced murine C57BL/6J syngeneic GL261 glioma cells were obtained from Dr. Ilker Eyupoglu (University of Erlangen, Germany). GL261 and Lewis Lung Carcinoma (LLC) cells were maintained in DMEM supplemented with 10% heat-inactivated FCS, 2 mM l-glutamine, 100 U ml^{-1} penicillin, 100 mg ml^{-1} streptomycin, and 100 mg ml^{-1} gentamycin sulfate (Lonza, Belgium). Primary murine astrocyte cultures were kindly provided by Dr. Matthew Holt (KU Leuven) and were maintained in DMEM/F12 (Lonza) supplemented with FCS, penicillin and streptomycin as described above. All cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C.

The lentogenic Hitchner B1 strain of NDV was kindly provided by Dr. Christian Grund (Friedrich-Loeffler Institut, Riems, Germany). It was propagated in embryonated eggs

from specific-pathogen free chicken and purified by sucrose gradient centrifugation as described.[16] Quantitative PCR for titer determination was performed on a Light Cycler 480 II (Roche, Germany). Primer sequences: B1S 5ÇGAG AAT TCA GAA TCG TCC CGT TAC3Ç (sense) and B1A 5ÇTCT TGA TGT CGC AGA AGA TAG GTG AT3Ç (antisense). Conditions: 15 min reverse transcription at 50°C, initial denaturation at 95°C, 40 denaturation cycles at 95°C for 8 sec and annealing and extension at 60°C for 45 sec.

In vitro cell death

GL261 cells were infected with NDV and harvested 24, 72 and 96 hr *post*-infection.

To investigate NDV-induced cytotoxicity, cells were infected with multiplicities of infections (MOIs) ranging from 4 to 1,000 and viability was analyzed *via* MTT assays, as described.[17]

To investigate the cell death pathways involved, GL261 cells were infected with NDV at MOI 125. Pan-caspase inhibitor Z-Val-Ala-DL-Asp(OMe)-fluoromethylketone (zVAD) (Bachem, Switzerland) and receptor-interacting protein kinase (RIPK) 1 inhibitor Necrostatin-1 (Sigma-Aldrich) were added 45 min prior to virus addition in some conditions and cell viability was measured using MTS, rather than MTT. This allows omitting cell lysis with DMSO and the resulting one-step protocol decreases intraexperimental variability.

Alternatively, cells were harvested, washed and stained for FITC-coupled Annexin V (Becton Dickinson (BD), Erembodegem, Belgium) and Propidium Iodide (PI) (Sigma–Aldrich) for 15 min at RT in the dark. Acquisition and analysis was performed using the Cellquest software on a FACSort flow cytometer (BD).

Also, whole cell lysates were prepared, followed by immunoblotting with anti-caspase-3 and anti-PARP antibodies (Cell Signaling Technology, USA), as described previously.[18]

Imaging analysis of cell death morphology was carried out in phase-contrast mode using the Leitz© Fluovert Inverted Microscope (fitted with the Leica DC200 camera) utilizing the NPL Fluotar L25/0.35 PHACO1 objective with 25× magnification.

Orthotopic glioma model and Newcastle disease virotherapy

Mice were injected intracranially with 5×10^5 GL261 tumor cells as previously described.[17] Some animals received $CD8^+$ T cell depleting antibody intraperitoneally 1 day before (200 μg) and 1 day after (100 μg) intracranial tumor induction.

For intratumoral virotherapy 5×10^6 U of NDV were injected into the preexisting burr hole using the same injection procedure as described above. After treatment mice were weighed three times per week. Animals were considered long-term survivors after 80 days (*i.e.*, approximately threefold the median survival of control animals). Long-term surviving animals received a second intracranial tumor cell injection with 5×10^5 GL261 cells 100 days after the initial tumor induction and at this time naïve mice of the same age were used as age-matched controls. To demonstrate the specificity for GL261 of the NDV-induced antitumor immune memory response, other long-term survivors and age-matched controls were (re)challenged subcutaneously with 5×10^5 LLC cells at this time.

Magnetic resonance imaging

MR images were recorded on the 9.4 T Biospec small animal MR system (Bruker Biospin, Germany) using a 7 cm linearly polarized resonator for transmission and an actively-decoupled dedicated mouse brain surface coil for receiving (Rapid Biomedical, Germany). For localization purposes, two-dimensional axial T2-weighted images (spin echo, TEeff = 48 ms, TR = 3,500 ms) and two-dimensional coronal T2-weighted images were obtained. Image processing and analysis were performed with Paravision 5.0 (Bruker BioSpin).

Immunogenic cell death

GL261 cells were treated with NDV at MOI 125 and recovered at several time-points *post*-infection. For ecto-CRT detection cells were incubated for 1 hr with primary anti-CRT rabbit antibody (Abcam, UK), diluted in 1% BSA, 2% FBS, at 4°C. This was followed by washing and 1 hr incubation with Alexa Fluor 488-conjugated anti-rabbit antibody (Invitrogen, Belgium). Thereafter the cells were washed and incubated with the viability detection/exclusion dye 7-aminoactinomycin D (7AAD) (Sigma–Aldrich). The samples were analyzed by Attune Acoustic Focusing Cytometer (Life Technologies, France). The fluorescent intensity of CRT positive cells was gated on 7AAD negative cells (thus detecting ecto-CRT rather than total CRT).

Extracellular ATP was measured in the conditioned media while intracellular ATP was determined after saponin-based lysis *via* an ATP assay mix (Sigma–Aldrich) based on luciferin-luciferase conversion, as described.[18] Bioluminescence was assessed by optical top reading *via* a FlexStation 3 microplate reader (Molecular Devices, USA).

For detection of released HMGB1 and HSP70/90 the conditioned culture media (5–8 ml) were collected and concentrated to 200–500 μl *via* centrifugation (2,000*g* for 5 min) using Pierce Concentrator 7 ml/9K filters (Pierce), according to the manufacturer's instructions. Simultaneously the treated cells were collected and whole cell lysates were prepared as described.[18] Lysates and media were analyzed by immunoblotting with antibodies against HMGB1, HSP90 (Cell Signaling Technology) and HSP70 (Santa Cruz Biotechnology, USA).

For analysis of GL261-associated antigen levels immunoblotting was performed on whole cell lysates with antibodies against premelanosome protein 17 (PMEL17) (Novus Biologicals, USA) and tyrosinase-related protein 2 (TRP-2) (Abcam).

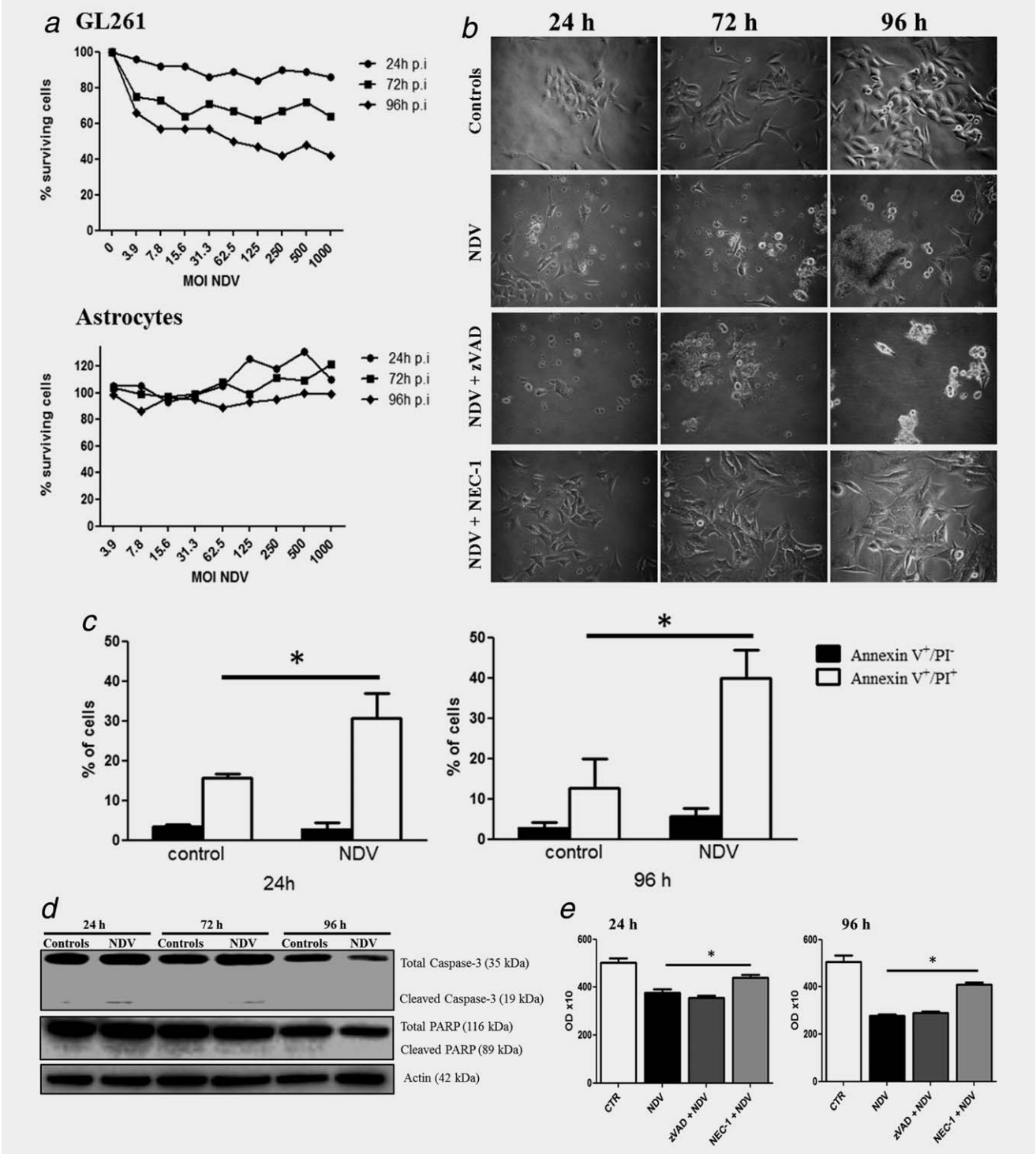


Figure 1. NDV-induced cell death in GL261 cells occurs through necroptosis. (*a*) Cell viability of GL261 cells and freshly isolated murine astrocytes 24 (•), 72 (■) and 96 (◆) hr *post*-NDV infection. OD of uninfected control conditions was set to 100%. Data is pooled from three independent experiments. (*b*) Phase-contrast microscopy images depicting GL261 monolayers 24, 72 and 96 hr *post*-NDV infection. Magnification: ×25. (i) Annexin V / PI staining on GL261 cells, analyzed by flow cytometry, 24 and 96 hr *post*-NDV infection. Data is pooled from three independent experiments. (*d*) Western blot analysis demonstrating absence of cleaved caspase-3 and PARP in GL261 cells 24, 72 and 96 hr *post*-NDV infection, as in uninfected control cultures. Blots were cropped to improve clarity. One representative of two independent experiments is shown. E: Cell viability of GL261 cells untreated, NDV-treated, NDV-treated with zVAD pre-treatment or NDV-treated with Necrostatin-1 pre-treatment. One representative of three independent experiments is shown. These conditions are visualized in (*b*).

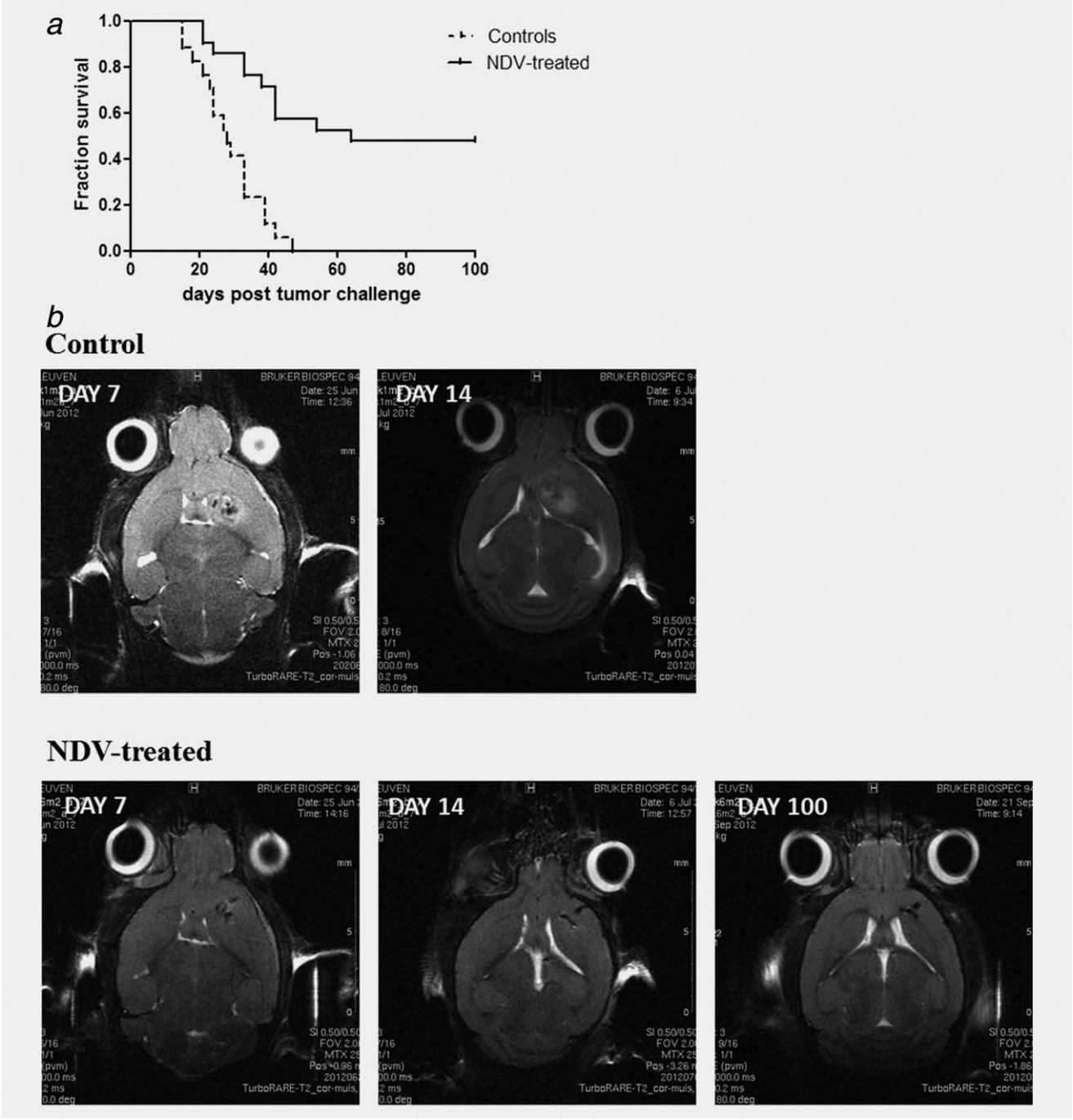


Figure 2. NDV therapy improves survival of glioma-bearing mice. (*a*) Kaplan–Meier curve depicting survival of glioma-bearing mice treated intratumorally with NDV ($n = 21$, black line) or PBS ($n = 17$, dotted line). Median survival after ND virotherapy was 64 days, as compared to 28 days for the controls ($p < 0.0001$). (*b*) Representative MR images from a control and a NDV-treated glioma-bearing mouse, obtained on day 7, 14 and 100 after tumor induction.

Autophagy analysis

GL261 cells were treated with NDV at MOI 125 and whole cell lysates were prepared 24 and 96 hr *post*-infection. Immunoblotting was performed using antibodies against LC3-I/LC3-II, p62, AMP-activated protein kinase (AMPK) and phosphorylated AMPK (pAMPK) (Cell Signaling Technology), as described.[19] To confirm active autophagic flux, autophagosome-lysosome fusion inhibitor Chloroquine (CQ) (5 μM) was added in some conditions.

Ex vivo immunomonitoring

Brain mononuclear cells were isolated as described.[17] Digestion medium consisted of preheated culture medium supplemented with 2.5 mg ml^{-1} collagenase D and 5 U ml^{-1}

DNase (Roche). Subsequently, surface staining was performed for 30 min at 4°C using specific antibodies to CD45, CD3, CD8, CD4, CD11b, Ly6C and Ly6G (BD). Intracellular IFN-γ staining was performed after 4 hr *in vitro* restimulation of the cells with 100 ng ml^{-1} phorbol 12-myristate 13-acetate, 1 μg ml^{-1} Ionomycin and 0.7 μg ml^{-1} Monensin (Sigma–Aldrich). After restimulation, surface staining was performed and cells were washed with a 0.5% Saponin, 0.5% BSA (Sigma–Aldrich) containing permeabilisation buffer. The intracellular staining was then performed for 30 min at 4°C using an IFN-γ specific antibody (BD). Acquisition and analysis was performed using the Cellquest software on a FACSort flow cytometer (BD).

Spleens were isolated and single cell suspensions were prepared by passage through a cell strainer (BD). Next, nylon wool fiber (Polysciences, Germany) was used to enrich the T cell fraction within the splenocyte population.[20] These responder cells were co-cultured with mature dendritic cells that were either unloaded (DCm) or loaded with GL261 lysate; 200 μg/10^6 DC (DCm-GL261), synthetic PMEL17 and TRP2 peptides; 10 μg ml^{-1} (DCm-pep) or LLC cell lysate; 200 μg/10^6 DC (DCm-LLC), at a 5:1 ratio, for 5 days. DCm were generated and loaded as described.[21] PMEL17 and TRP2 peptides were ordered from LifeTein (South Plainfield, USA). The supernatant was then harvested and analyzed for secreted IFN-γ levels using a CBA kit (BD) according to the manufacturer's instructions.

Statistical methods

All data was analyzed using GraphPad Prism 5 (USA). Survival analysis was performed using the Log-rank test and groups were compared using the student *t* test or one-way ANOVA.

Results

NDV-treated glioma cells undergo necroptotic cell death

To characterize the *in vitro* cytopathic effect (CPE) of NDV we inoculated monolayers of GL261 cells with NDV at MOI ranging from 4 to 1,000 and quantified cell viability 24, 72 and 96 hr *post*-infection. GL261 cells were sensitive to cytotoxic killing by NDV in a time- and dose-dependent manner (Fig. 1*a*). The LD_{50} dose was found to be 62.5 MOI, reached following 96 hr of NDV treatment. In contrast, NDV did not decrease healthy astrocyte viability, even at the high MOI of 1,000 (Fig. 1*a*).

Next, we decided to probe into the cell death mechanics behind this NDV-induced CPE. Phase-contrast microscopy analysis showed that NDV treatment induced GL261 cell killing, predominantly with necrosis-like characteristics such as extensive membrane and cell disintegration, cellular swelling, extensive karyolysis and the presence of large amounts of cellular debris (Fig. 1*b*). Thus, NDV-mediated GL261 cell death was not associated with morphological traits of apoptosis. To confirm the lack of apoptosis on the molecular level we analyzed the presence of *bona fide* apoptosis markers after NDV treatment *i.e.* externalization of phosphatidylserine (PS; an early marker) and activation of executioner caspase-3 (a late marker). For PS externalization we analyzed the staining for Annexin-V (a PS-binding protein) and propidium iodide (PI; an agent that stains permeabilized cells) *via* flow cytometry. We found absence of Annexin-V staining in PI negative cells (early apoptotic cells) but a strong staining pattern for Annexin-V staining in PI positive cells (necrotic cells) at both early (24 hr) as well as late (96 hr) time-points after NDV infection (Fig. 1*c*). Presence of Annexin-V^+PI^+ cells without appreciable amounts of Annexin-V^+PI^- cells at different time-points is a typical characteristic of necrosis. This point was further substantiated by analysis of caspase-3 activation. Following NDV treatment, we found no major evidence for caspase-3 or PARP cleavage (PARP being the downstream target of activated caspase-3) (Fig. 1*d*). To confirm the dispensability of caspase activity for the pro-death effects of NDV we pretreated the cells with the pan-caspase chemical inhibitor zVAD. As expected, pretreatment with zVAD did not strongly affect the NDV-induced CPE (Figs. 1*b* and 1*e*).

Altogether, these results clearly show that NDV treatment induces necrotic cell death in GL261 cells evidently devoid of typical apoptotic characteristics. However, the NDV-induced cell death did not develop in an unordered, accidental manner, as primary necrosis typically does. Rather, we saw a progressive decrease in survival from 24 to 96 hr *post*-treatment (Fig. 1*a*), which is a possible property of programmed cell death routines like apoptosis or necroptosis. Indeed, pretreatment with Necrostatin-1, a well-established *in vitro* chemical inhibitor of RIPK1-mediated necroptosis,[22] significantly rescued the NDV-induced CPE, both at 24 and at 96 hr *post*-NDV treatment, suggesting that NDV causes glioma cell demise through necroptosis in this model (Figs. 1*b* and 1*e*).

Newcastle disease virotherapy significantly prolongs survival of glioma-bearing mice

To analyze the efficacy of OVT *in vivo*, we challenged C57BL/6J mice intracranially with GL261 cells. After 7 days, groups of glioma-bearing animals received a single intratumoral injection of NDV. Control animals received an injection consisting of PBS only. In the group of NDV-treated animals 50% reached long-term survival *versus* none in the placebo-treated control group (Fig. 2*a*). Median survival was also significantly improved from 28 to 64 days. Surviving mice were followed for 100 days and showed no external signs of tumor recurrence or virus-related toxicity.

To explore signs of toxicity after NDV injection, we subjected healthy mice to a single intracranial dose of 5×10^6 copies NDV. On T2-weighted MR images of the brain taken 1 and 2 weeks after the injection, no signs of edema were present (data not shown). Brain water content measurements, often used as a measure for edema formation in the brain,[23] also showed no differences between treated and naïve mice (data not shown). We further analyzed infiltration of immune cell into the brain after intracranial virus injection but found no influx of macrophages or $CD4^+$ and $CD8^+$ T cells after NDV injection (data not shown).

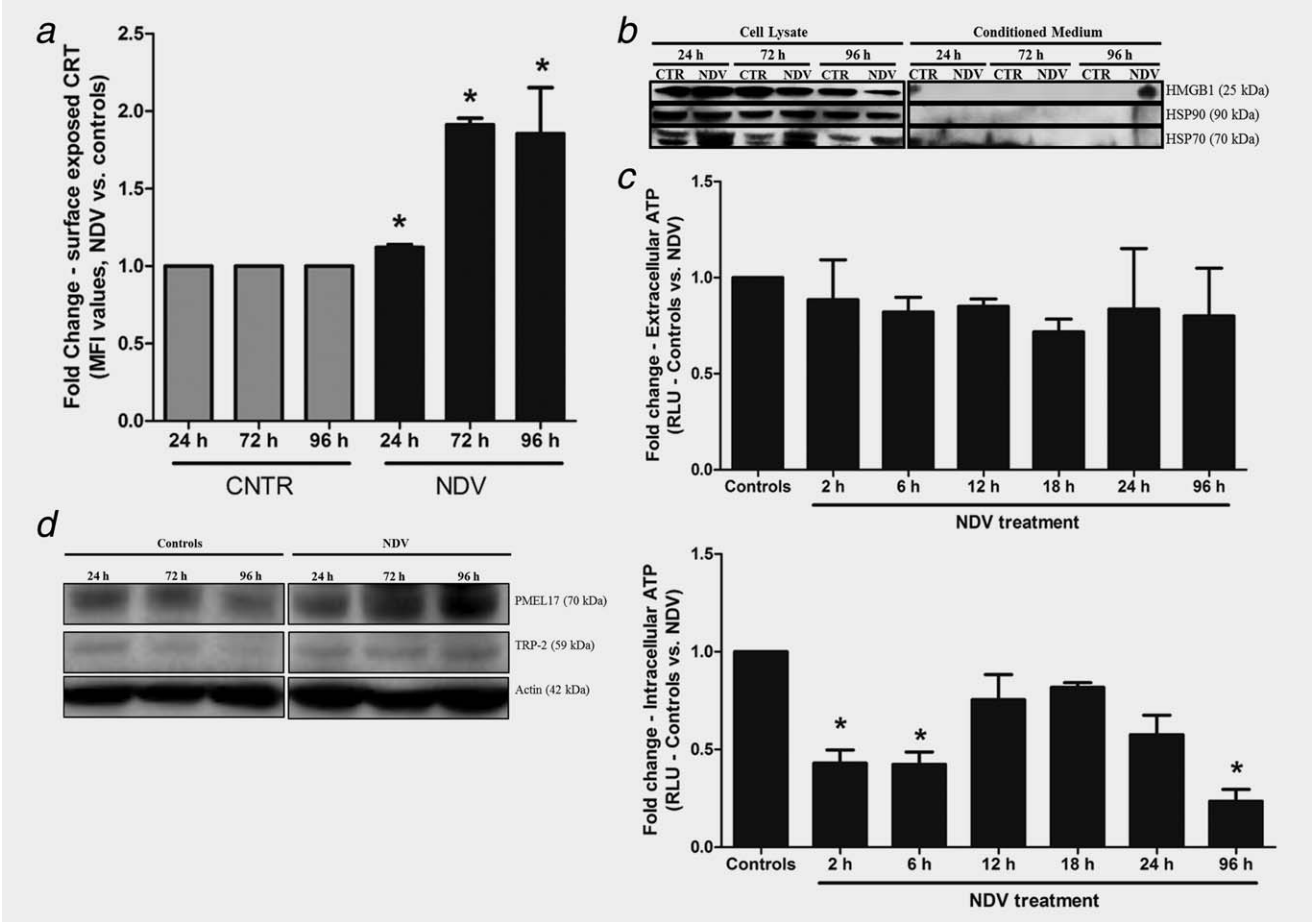


Figure 3. NDV induces immunogenic cell death in GL261 cells. (*a*) Flow cytometric analysis of ecto-CRT staining on GL261 cells 24, 72 and 96 hr *post*-NDV infection. Relative mean fluorescence intensities are shown, with values for uninfected control conditions set to 1. Data is pooled from two independent experiments. (*b*) Western blot analysis detecting HMGB1 and HSP70/90 expression in GL261 cell lysates and conditioned media 24, 72 and 96 hr *post*-NDV infection. Blots were cropped to increase clarity. One representative of two independent experiments is shown. (*c*) Relative amounts of extracellular and intracellular ATP levels, measured in GL261 conditioned media and cell lysates, respectively. Bioluminescence intensities for uninfected control conditions were set to 1. Data is pooled from two independent experiments (*d*) Western blot detection of PMEL17 and TRP-2 expression on the surface of GL261 cells 24, 72 and 96 hr *post*-NDV infection. Blots were cropped to improve clarity. One representative of two independent experiments is shown.

As a long-term follow up for tumor recurrence, MR images were obtained immediately prior to NDV treatment, 1 week after treatment and 100 days after tumor induction (Fig. 2*b*). Images taken 1 week after tumor cell injection showed a developing tumor mass. In control animals the tumor continued to develop, by day 14 after tumor challenge starting to show distortion of the ventricles and shifting of the midline in some cases. In a subset of NDV-treated animals however, the tumor mass was reduced at day 14. On the long-term follow up MRI of these animals the needle tract was still visible in an otherwise normal, healthy murine brain.

Newcastle disease virus-treated glioma cells undergo immunogenic cell death

On the basis of the strong antiglioma effects induced by NDV as described above, we found it important to assess whether antitumor immune activities might be involved in the therapeutic effect of ND virotherapy, next to the direct tumor cell killing capacities we demonstrated *in vitro*. To see if NDV could validate this "oncolytic paradigm" we investigated the induction of molecular ICD determinants in GL261 cells after NDV infection.

First, we checked ecto-CRT, the most important determinant of ICD.[12] GL261 glioma cells treated with NDV exposed ecto-CRT actively (*i.e.*, in absence of plasma membrane permeabilization, tested by 7-AAD exclusion) in a time-dependent fashion (Fig. 3*a*). NDV-induced ecto-CRT emerged as early as 24 hr *post*-treatment, albeit in a small but significant amount. Thereafter ecto-CRT increased considerably reaching a saturation level at 72 hr and remaining at similar levels until 96 hr *post*-treatment.

Second, we checked for released or secreted danger signals like HMGB1 and HSP70/90. GL261 cells treated with NDV released HMGB1 passively in the conditioned media (*i.e.*, in presence of plasma membrane permeabilization) at 96 hr *post*-treatment (Fig. 3*b*). This was accompanied by a considerable

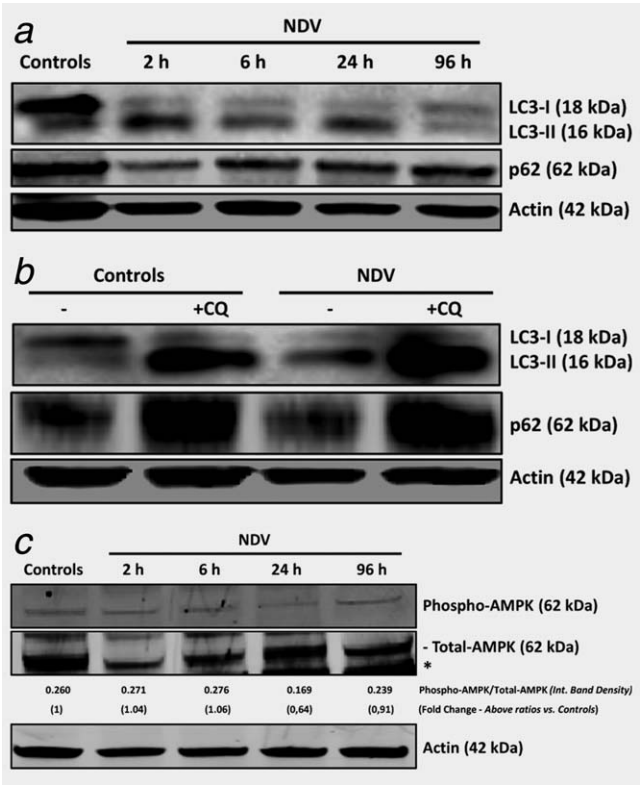


Figure 4. NDV induces autophagy in GL261 cells. Western blot analysis detecting (*a*,*b*) LC3-I/II, p62 and (*c*) AMPK, pAMPK protein levels in GL261 cell lysates 2, 6, 24 and 96 hr *post*-NDV infection. In (*b*) CQ was added to some conditions. Blots were cropped to improve clarity. One representative of two independent experiments is shown.

intracellular drop in HMGB1 levels (analyzed in the cell lysate)—a surrogate marker of ICD.[12] Moreover, we found that NDV therapy was not associated with passive release of chaperones like HSP70/90—in line with previous instances advocating that depending on the anticancer therapy, the release of HMGB1 and HSP70/90 is interchangeable.[18,24,25]

Next, we looked at extracellularly secreted/released ATP after NDV therapy. We did not find any palpable presence of extracellulary secreted/released ATP—both at early time-points (2–18 hr) as well as late time-points (24 and 96 hr) *post*-NDV treatment of GL261 cells (Fig. 3*c*). This lack of extracellular ATP secretion from stressed/dying GL261 cells after NDV treatment was accompanied by a significant drop in intracellular ATP levels in the cells relatively early after NDV infection (as early as 2 hr *post*-treatment). This was followed by a brief period of recovery of intracellular ATP (between 12 and 24 hr *post*-treatment); finally culminating in a strong drop in intracellular ATP levels at 96 hr *post*-treatment possibly owing to significant cell death in these conditions.

Last but not least, we also checked the effects of NDV treatment on the antigenicity of GL261 cancer cells by analyzing the effect of this therapy on levels of GL261-associated cancer antigens *i.e.* PMEL17 and TRP-2.[27] We found that NDV treatment increased the levels of PMEL17 protein in a time-dependent fashion (Fig. 3*d*). However NDV treatment did not strongly affect the levels of TRP-2. Thus, NDV treatment, apart from inducing various ICD determinants in the glioma cells, also led to (at least partial) overexpression of tumor-associated antigens.

Newcastle disease virus treatment induces autophagy in GL261 cells

The decreased intracellular ATP levels, observed following NDV treatment of GL261 cells, implied that autophagy might be triggered as a stress-response in this set-up.[27] On the other hand, the demonstrated lack of ATP secretion during NDV-induced ICD might suggest that NDV blocks autophagy induction, as autophagy is known to be crucial for ATP secretion during ICD.[28]

Immunoblotting analysis demonstrated the induction of active autophagy in GL261 cells as early as 2 hr *post*-infection (Fig. 4*a*). This was evident from an increased emergence of the autophagosome-associated, lipidated form of LC3, *i.e.* LC3-II (a well-established marker of autophagy induction) and a decrease in the p62 protein levels (a marker of active autophagic degradation). Autophagy induction was also present at 24 hr *post*-infection, but diminished towards the 96 hr time-point, as NDV-induced necroptosis reached a peak. Co-treatment with the autophagosome-lysosome fusion inhibitor CQ confirmed that NDV treatment induced an active autophagic flux (Fig. 4*b*).

Drastic drop in intracellular ATP levels can induce AMPK activation/phosphorylation, which can in turn induce autophagy.[29] However, immunoblotting analysis to detect the phosphorylation status of AMPK after NDV treatment did not reveal significant changes (Fig. 4*c*), suggesting the drop in intracellular ATP levels caused by NDV treatment is probably not sufficient to activated AMPK in our set-up.

Newcastle disease virotherapy primes adaptive antitumor immunity

As the *in vitro* hallmarks of ICD were clearly present in GL261 cells after NDV infection, we further analyzed the presence of the *in vivo* ICD characteristics, which include increased infiltration of IFN-γ^+ $CD4^+$/$CD8^+$ T cells into the tumor microenvironment, along with a decreased sustenance of pro-tumorigenic MDSCs and the ability to induce tumor-rejecting immunity.[12] To investigate the presence of these features we isolated the mononuclear cells from the brain of glioma-bearing animals treated intratumorally with NDV and from control animals, 3 weeks after tumor induction. The percentage of IFN-γ^+ cells within the $CD8^+$ and $CD4^+$ T cell populations was significantly increased in the brains of NDV-treated animals as compared to placebo-treated controls (Fig. 5*a*). Furthermore, the tumor-infiltrating lymphocyte population contained a significantly higher percentage of $CD4^+$ T cells and significantly lower percentages of $CD45^+CD11b^+Ly6G^+$ granulocytic and $CD45^+CD11b^+Ly6C^+$ monocytic MDSC subsets in NDV-treated animals, as compared to controls (Fig. 5*b*). We saw a

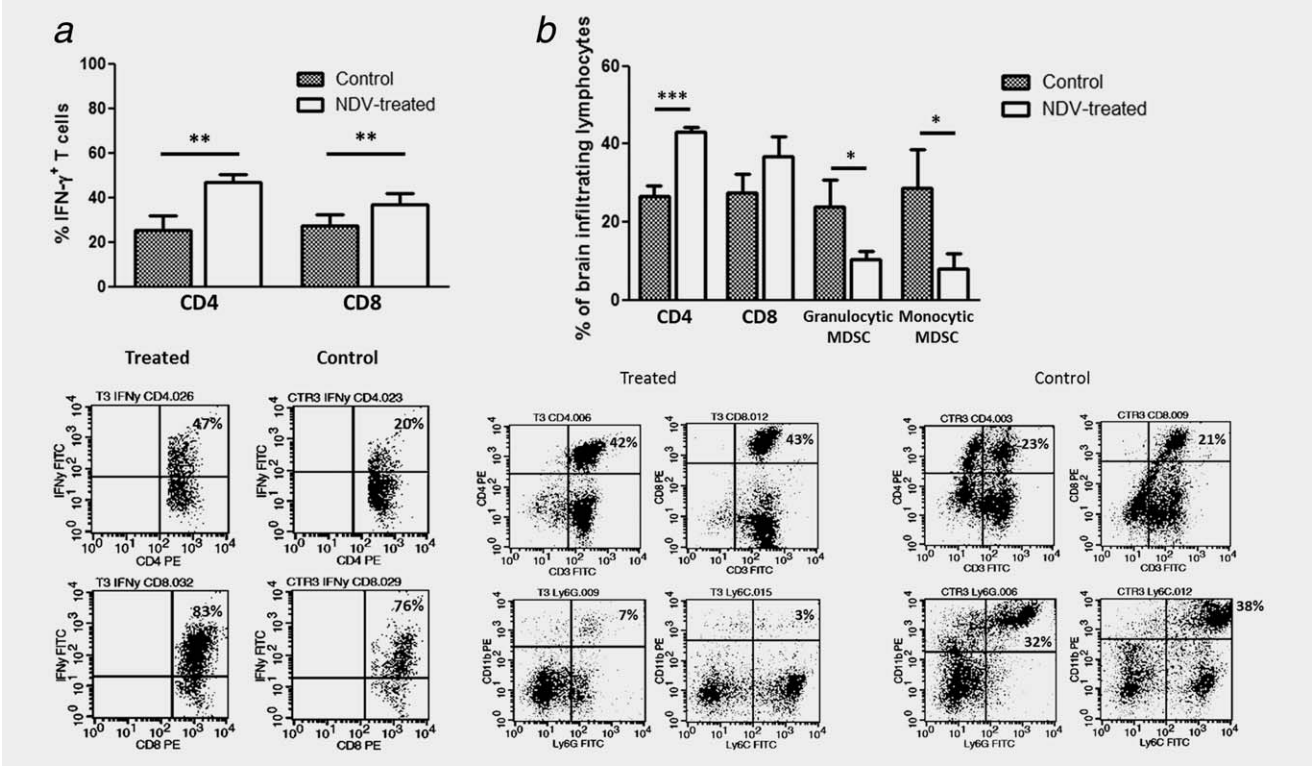


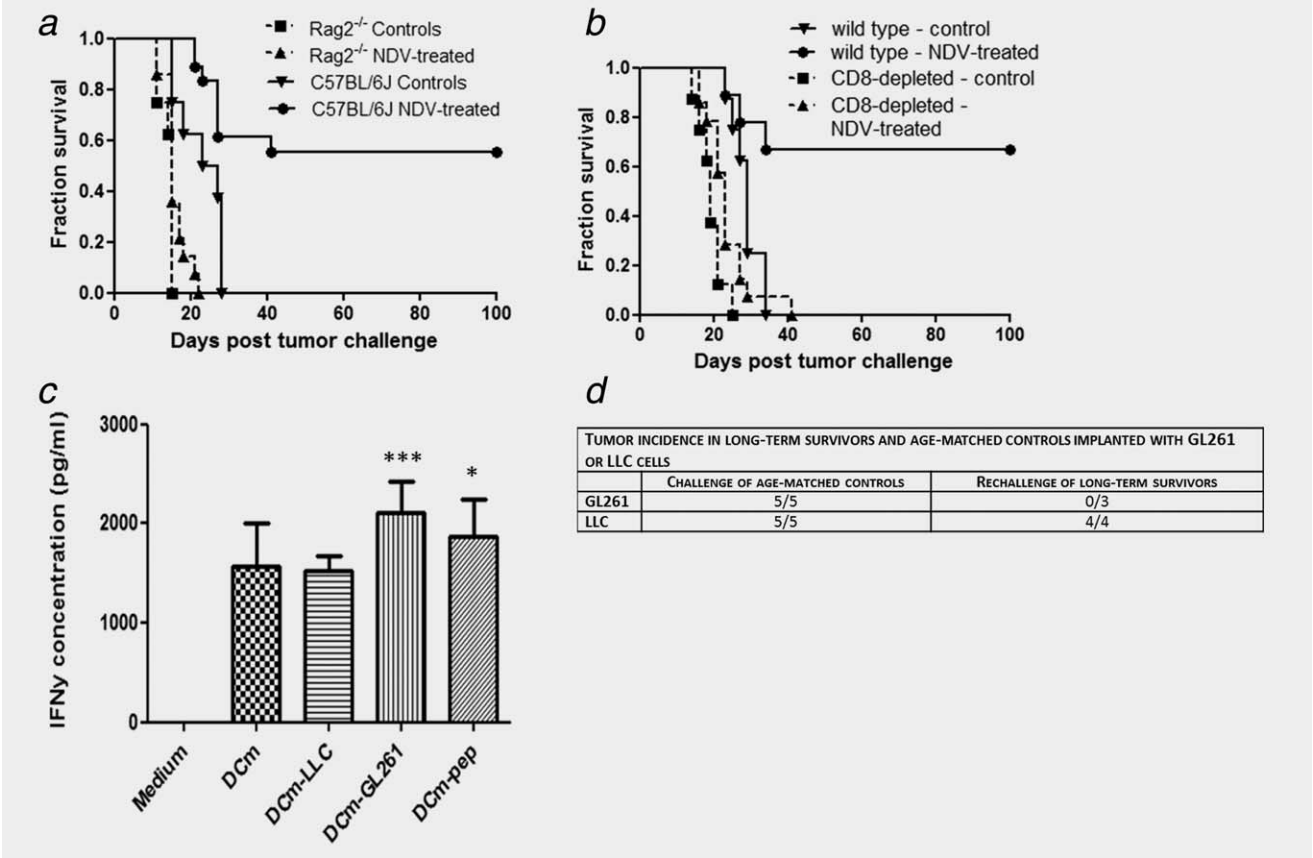
Figure 5. NDV therapy modulates the immunological tumor microenvironment. Flow cytometry on the mononuclear brain-infiltrating lymphocytes from NDV- and PBS-treated glioma-bearing mice ($n = 3$ per group). (*a*) Percentage of IFN-γ^+ cells in the $CD4^+$ and $CD8^+$ T cell fractions in the brain of treated ($n = 3$, white bar) and control ($n = 3$, black bar) animals, analyzed by intracellular IFN-γ staining. Summary data as well as representative dot plots are shown. (*b*) Percentages of $CD4^+$ T cells, $CD8^+$ T cells, granulocytic macrophages and monocytic macrophages within the population of brain-infiltrating lymphocytes from treated ($n = 3$, white bars) and control ($n = 3$, black bars) animals, analyzed by cell surface staining for CD45, CD4, CD8, CD11b, Ly6C and Ly6G. summary data as well as representative dot plots are shown.

clear trend toward a higher percentage of $CD8^+$ T cells as well, although this was not significant. This shift towards more immune activation and less immune suppression within the local tumor microenvironment after ND virotherapy confirms presence of the first *in vivo* hallmark of ICD.

To investigate the importance of these activated T cell populations in the therapeutic effect of ND virotherapy, we first analyzed NDV-mediated tumor suppression after intratumoral injection in immunocompetent C57BL/6J mice and in immunodeficient $Rag2^{-/-}$ mice lacking functional B and T cells (Fig. 6*a*). Without treatment, immunocompetent animals survived significantly longer than their immunodeficient counterparts, demonstrating that the GL261 tumor is immunogenic and capable of eliciting a limited endogenous antitumor immune response, which prolongs survival of glioma-bearing mice but is insufficient to induce cure. This confirms data previously published by our group.[21] NDV treatment cured 50% of immunocompetent animals of established glioma. On the contrary, in immunodeficient animals no long-term survival was induced, although NDV treatment could prolong median survival slightly. This demonstrates that although NDV can slow tumor growth by direct killing of tumor cells, a functional adaptive immune system is essential to allow cure by ND virotherapy. The effects seen in $Rag2^{-/-}$ animals could also be induced in immunocompetent C57BL/6J mice by $CD8^+$ T cell depletion, identifying the $CD8^+$ T cell fraction as the main player in the observed adaptive antitumor immunity following ND virotherapy (Fig. 6*b*).

The antitumor immune reactivity induced by Newcastle disease virotherapy is tumor-specific

Having identified the T cell fraction of the immune system as the main mediator of the NDV-induced antitumor immunity, we next investigated the presence of an activated T cell population *in vivo* that specifically recognizes GL261 cells following ND virotherapy. Thereto, we challenged mice with GL261 tumor cells and treated them with NDV after 7 days. On day 21 *post*-tumor challenge T cell-enriched splenocytes were restimulated with either DCm, DCm-GL261, DCm-pep (a mixture of PMEL17 and TRP2 immunodominant peptides) or DCm-LLC. T cell-enriched splenocytes from NDV-treated animals produced significantly more IFN-γ upon restimulation with DCm-GL261 or DCm-pep, as compared to restimulation with DCm or DCm-LLC (Fig. 6*c*). IFN-γ production did not differ following restimulation with DCm or DCm-LLC. This indicates the presence of a T cell population after ND virotherapy, which specifically recognizes GL261 tumor material and not presented tumor antigens



Tumor incidence in long-term survivors and age-matched controls implanted with GL261 or LLC cells		
	Challenge of age-matched controls	Rechallenge of long-term survivors
GL261	5/5	0/3
LLC	5/5	4/4

Figure 6. Efficient ND virotherapy requires functional adaptive immunity and induces tumor-specific immune memory. (*a*,*b*) Kaplan–Meier curves depicting survival of immunocompetent (black lines) mice—untreated ($n = 8$, ▼) or treated with NDV ($n = 18/9$, •)—and immunodeficient (dotted lines) mice—untreated ($n = 8$, ■) or treated with NDV ($n = 14$, ▲). Immunodeficient mice are either $Rag2^{-/-}$ (*a*) or CD8-depleted (*b*). C: Concentration of IFN-γ, measured in the supernatant of co-cultures consisting of T cell-enriched splenocytes from NDV-treated animals ($n = 10$) and DCm, DCm-LLC, DCm-GL261 or DCm-pep. D: Tumor incidence in long-term survivors following ND virotherapy and age-matched controls implanted with GL261 or LLC cells.

from an irrelevant tumor source (*i.e.*, LLC). IFN-γ production did not differ significantly after restimulation with DCm-GL261 or DCm-pep, indeed indicating PMEL17/TRP2 as the immunodominant epitopes of the GL261 tumor. This GL261-specific population was not present in control animals (data not shown).

Newcastle disease virotherapy primes tumor-specific immune memory

To investigate whether the NDV-induced antitumor immune reactivity also led to the establishment of antitumor immune memory long-term survivors, cured of their primary glioma through NDV treatment, received a second orthotopic tumor cell injection 100 days after the initial tumor induction. Whereas all age-matched controls developed rapidly growing gliomas, NDV-cured animals resisted secondary glioma development without additional treatment (Fig. 6*d*). Intracranial tumor growth, or lack thereof, was confirmed on MRI (data not shown). To investigate the specificity of the NDV-induced immune response for GL261, we challenged other groups of long-term survivors subcutaneously with LLC cells. Contrary to GL261 rechallenge, challenge with LLC cells resulted in the establishment of rapidly growing tumors in all mice (Fig. 6*d*).

Discussion

Here, we show the antiglioma activity of NDV in the murine orthotopic immunocompetent GL261 glioma model. NDV exerts a direct cytotoxic effect on the murine glioma cells. However, the curative effect of NDV depends mainly on the elicited cellular tumor-specific immune response. NDV treatment ameliorates the endogenous antitumor immune response resulting in immunological antitumor memory. The capability to trigger antitumor immunity may be supported by the induction of ICD by viral infection in the GL261 tumor cells.

A relatively small number of studies exist that have tried to analyze the cell death pathways responsible for cancer cell demise following NDV treatment. Uncovering the mechanisms behind NDV-induced tumor cell death and its immune components is important, however, as NDV is presently under clinical consideration for cancer therapy.[6,30] While the use of replication-competent OVs in recurrent GBM patients was

demonstrated by initial clinical trials to be safe,[6,31–33] the moderate clinical efficacy has not yet matched the encouraging preclinical results. After NDV treatment, 1 patient (9%) showed complete remission, coinciding with a significant improvement in neurological status.[6] Trials using other OVs have shown similar response rates, along with lymphocytic cell infiltrates in the tumor and radiological and neurological improvements.[31,33] The moderate clinical success obtained thus far indicates a strong need to explore the reasons behind, and ways of improving, the limited efficacy of OVT in cancer patients. Most preclinical studies performed to date employ cell culture systems or xenotransplant models to evaluate the efficacy of OVT. These studies focused on (improving) the direct cytotoxic effect of viral strains but did not take into consideration the role of the host immune system in limiting or enhancing the efficacy of OVT. An increasing amount of evidence from other tumor fields now demonstrates the induction of antitumor immunity by OVT, which greatly contributes to the oncosuppressive effect of certain OVs. Therefore, we found it important to analyze the immunological aspects of ND virotherapy in a fully immunocompetent, orthotopic model.

In vitro we demonstrated NDV-induced cytotoxicity towards the GL261 cells in a dose- and time-dependent manner, though about 50% of cells remained viable at the highest MOI tested. The *in vitro* cytotoxicity of NDV towards human GBM cell lines and primary cell cultures has been established as well.[7]

In vivo we showed a significant increase in median survival of glioma-bearing animals treated with a single intratumoral injection of NDV, as compared to placebo-treated controls. Furthermore, a substantial percentage (50%) of glioma-bearing mice became long-term survivors after treatment, showing no signs of tumor recurrence for up to 100 days. Taken together this data demonstrated a highly beneficial safety-efficacy profile of NDV treatment in this model. These results compare favorably to those obtained with genetically engineered oncolytic strains in the intracranial GL261 model to date. Recombinant forms of vaccinia virus (JX-594m) and adenovirus (Ad24TYR) were previously shown to improve median survival and induce 20–25% long-term survival in this model, respectively.[11,34]

To uncover the role of the immune system in the therapeutic activity of NDV we investigated the ability of NDV to induce ICD in glioma cells. It is noteworthy that ICD so far has been reported to be of apoptotic nature.[35] However, conventional cell death concepts have limited applicability in case of OVT because, following infection, OVs typically take over and control molecular cell death machinery allowing induction of multimodal cell death sub-routes only after available cellular resources (like ATP) have been maximally exploited for production of new virions.[4] Accordingly, depending on the context and cancer type, NDV has been shown to induce both caspase-dependent as well as -independent cell death routes.[36–38] There is no information available on NDV-induced cell death pathways in glioma cells. Here NDV infection induced necroptosis in GL261 glioma cells. Furthermore, NDV-infection induced the *in vitro* characteristics of ICD in GL261 cells, including ecto-CRT and release of HMGB1, and led to accentuation of their antigenicity. Remarkably, we could not detect any secreted ATP after NDV infection in the cells.

ATP secretion has been reported to be mediated by two main mechanisms; *via* secretory pathway or *via* autophagy.[18,28] As evident by active exposure of ecto-CRT (which is established to mandatorily require secretory pathway[18]) following NDV infection, NDV does not seem to compromise this mechanism. Interestingly, we also observed the induction of autophagy following NDV treatment. Together, this indicates that the induction of autophagy during ICD may not guarantee ATP secretion in all conditions/contexts. Furthermore, though neither of the two mechanisms which can mediate ATP secretion is compromised, we fail to observe ATP secretion following NDV infection (though intracellular levels decrease in a cyclic pattern *post*-treatment). We speculate this to be due to increased ATP utilization in the cell during viral replication. Indeed, several papers describe declining ATP levels due to increased consumption in cells undergoing active viral infection[39,40] and the existence of ATP-dependent replication initiator (Rep) proteins has been described for several viruses.[41,42] This suggests that OVs, as ICD-inducing therapeutics, might bend the presently existing definition of ICD due to their interactions with the host cell for their own replication.

The *in vivo* hallmarks of ICD, being increased infiltration of IFN-γ^+CD4$^+$/CD8$^+$ T cells, decreased sustenance of protumorigenic immune cells and induction of tumor-rejecting immunity,[12] were clearly present in this model as well, further demonstrating the induction of ICD. To demonstrate the important antitumoral role of the activated T cells present within the brain after NDV therapy, we used Rag2$^{-/-}$ animals, specifically defected in their B- and T-cell responses. Here NDV was able to prolong median survival slightly, but failed to induce any long-term survival, suggesting the involvement of the T cell arm of the immune system in conjunction with NDV. The CD8$^+$ T cells were identified as prime players in this effect, as demonstrated in a CD8$^+$ T cell depletion experiment in immunocompetent C57BL/6J animals, where the effects seen in Rag2$^{-/-}$ animals were recapitulated. These results suggest that NDV-infected tumor cells might activate the immune system, which then takes over from the direct oncolytic effect to allow for full tumor regression in a situation where either component alone is not efficient.

Research within the field has also focused on the potential of natural killer (NK) cells to diminish OVT efficacy by initiating antiviral immunity. Conflicting results were however obtained. Although NK cells are rapidly recruited to sites of viral infection and mediate viral clearance,[43] they also possess strong tumor cell killing capacities, which can be increased by OV infection.[44,45] In our model the presence of functional NK cells in mice lacking B/T cell responses (*i.e.*, Rag2$^{-/-}$) was not sufficient to establish successful OVT. However, the

effect of NK cell depletion as a potential benefit to NDV therapy has not been studied in this model.

Long-term surviving animals after ND virotherapy were able resist glioma outgrowth after a second GL261 cell injection. This strongly indicates the induction of immunological antitumor memory by OVT. We have furthermore demonstrated—following both initial tumor challenge and tumor rechallenge—that the induced antitumor immune reactivity is directed specifically towards the GL261 tumor antigens.

To our knowledge this is the first description of an immune response as part of the antitumoral activity of OVs in an orthotopic glioma model. GBM is an immunogenic tumor and clinical evidence has been reached that tumor control can be induced by immunostimulatory approaches. ND virotherapy seems to represent a unique approach that combines tumor cell eradication with activation of adaptive antitumor immune responses and modulation of the immunosuppressive tumor microenvironment. Therefore, we hypothesize that OVT might work synergistically with other immunostimulatory approaches. In the future, this model will allow us to study ND virotherapy combination regimes. Furthermore, our results suggest studies are needed to investigate optimal schedules for combining OVT with immunosuppressive therapeutics such as corticosteroids, Temozolomide and radiation therapy, which form the present standard of care for GBM patients.

In conclusion, we report successful OVT using NDV in an orthotopic, immunocompetent GBM model. This is the first report demonstrating the induction of a long-term, tumor-specific immune memory response after OVT in this model. This immune response is likely promoted by ICD after viral infection of the tumor cells.

References

1. Louis DN, Ohgaki H, Wiestler OD, et al. The 2007 WHO classification of tumours of the central nervous system. *Acta Neuropathol* 2007;114: 97–109.
2. Stupp R, Hegi ME, Mason WP, et al. Effects of radiotherapy with concomitant and adjuvant temozolomide versus radiotherapy alone on survival in glioblastoma in a randomised phase III study: 5-year analysis of the EORTC-NCIC trial. *Lancet Oncol* 2009;10:459–66.
3. Stummer W, Pichlmeier U, Meinel T, et al. Fluorescence-guided surgery with 5-aminolevulinic acid for resection of malignant glioma: a randomised controlled multicentre phase III trial. *Lancet Oncol* 2006;7:392–401.
4. Russell SJ, Peng KW, Bell JC. Oncolytic virotherapy. *Nat Biotechnol* 2012;30:658–70.
5. Lam HY, Yeap SK, Rasoli M, et al. Safety and clinical usage of newcastle disease virus in cancer therapy. *J Biomed Biotechnol* 2011;2011: 718710.
6. Freeman AI, Zakay-Rones Z, Gomori JM, et al. Phase I/II trial of intravenous NDV-HUJ oncolytic virus in recurrent glioblastoma multiforme. *Mol Ther* 2006;13:221–8.
7. Alkassar M, Gartner B, Roemer K, et al. The combined effects of oncolytic reovirus plus Newcastle disease virus and reovirus plus parvovirus on U87 and U373 cells in vitro and in vivo. *J Neurooncol* 2011;104:715–27.
8. Friedman A, Tian JP, Fulci G, et al. Glioma virotherapy: effects of innate immune suppression and increased viral replication capacity. *Cancer Res* 2006;66:2314–9.
9. Lun X, Alain T, Zemp FJ, et al. Myxoma virus virotherapy for glioma in immunocompetent animal models: optimizing administration routes and synergy with rapamycin. *Cancer Res* 2010;70: 598–608.
10. Geletneky K, Kiprianova I, Ayache A, et al. Regression of advanced rat and human gliomas by local or systemic treatment with oncolytic parvovirus H-1 in rat models. *Neuro Oncol* 2010;12: 804–14.
11. Lun X, Chan J, Zhou H, et al. Efficacy and safety/toxicity study of recombinant vaccinia virus JX-594 in two immunocompetent animal models of glioma. *Mol Ther* 2010;18:1927–36.
12. Krysko DV, Garg AD, Kaczmarek A, et al. Immunogenic cell death and DAMPs in cancer therapy. *Nat Rev Cancer* 2012;12:860–75.
13. Kroemer G, Galluzzi L, Kepp O, Zitvogel L. Immunogenic cell death in cancer therapy. *Annu Rev Immunol* 2013;31:51–72.
14. Miyamoto S, Inoue H, Nakamura T, et al. Coxsackievirus B3 is an oncolytic virus with immunostimulatory properties that is active against lung adenocarcinoma. *Cancer Res* 2012;72:2609–21.
15. Donnelly OG, Errington-Mais F, Steele L, et al. Measles virus causes immunogenic cell death in human melanoma. *Gene Ther* 2013;20:7–15.
16. Chucholowius HW, Rott R. A new method for purification of myxoviruses by zonal centrifugation with two different sucrose density gradients. *Proc Soc Exp Biol Med* 1972;140:245–7.
17. Maes W, Rosas GG, Verbinnen B, et al. DC vaccination with anti-CD25 treatment leads to long-term immunity against experimental glioma. *Neuro Oncol* 2009;11:529–42.
18. Garg AD, Krysko DV, Verfaillie T, et al. A novel pathway combining calreticulin exposure and ATP secretion in immunogenic cancer cell death. *EMBO J* 2012;31:1062–79.
19. Maes H, Van ES, Krysko DV, et al. BNIP3 supports melanoma cell migration and vasculogenic mimicry by orchestrating the actin cytoskeleton. *Cell Death Dis* 2014;5:e1127.
20. Hathcock KS. T cell enrichment by nonadherence to nylon. *Curr Protoc Immunol* 2001;Chapter 3: Unit.
21. Verschuere T, Toelen J, Maes W, et al. Glioma-derived galectin-1 regulates innate and adaptive antitumor immunity. *Int J Cancer* 2013;134:873–84.
22. Vandenabeele P, Grootjans S, Callewaert N, et al. Necrostatin-1 blocks both RIPK1 and IDO: consequences for the study of cell death in experimental disease models. *Cell Death Differ* 2013;20: 185–7.
23. Whalen MJ, Carlos TM, Dixon CE, et al. Reduced brain edema after traumatic brain injury in mice deficient in P-selectin and intercellular adhesion molecule-1. *J Leukoc Biol* 2000;67:160–8.
24. Garg AD, Dudek AM, Ferreira GB, et al. ROS-induced autophagy in cancer cells assists in evasion from determinants of immunogenic cell death. *Autophagy* 2013;9:1292–307.
25. Apetoh L, Ghiringhelli F, Tesniere A, et al. Toll-like receptor 4-dependent contribution of the immune system to anticancer chemotherapy and radiotherapy. *Nat Med* 2007;13:1050–9.
26. Prins RM, Odesa SK, Liau LM. Immunotherapeutic targeting of shared melanoma-associated antigens in a murine glioma model. *Cancer Res* 2003; 63:8487–91.
27. Russell RC, Yuan HX, Guan KL. Autophagy regulation by nutrient signaling. *Cell Res* 2014;24:42–57.
28. Martins I, Wang Y, Michaud M, et al. Molecular mechanisms of ATP secretion during immunogenic cell death. *Cell Death Differ* 2014;21:79–91.
29. Roach PJ. AMPK -> ULK1 -> autophagy. *Mol Cell Biol* 2011;31:3082–4.
30. Laurie SA, Bell JC, Atkins HL, et al. A phase 1 clinical study of intravenous administration of PV701, an oncolytic virus, using two-step desensitization. *Clin Cancer Res* 2006;12:2555–62.
31. Chiocca EA, Abbed KM, Tatter S, et al. A phase I open-label, dose-escalation, multi-institutional trial of injection with an E1B-attenuated adenovirus, ONYX-015, into the peritumoral region of recurrent malignant gliomas, in the adjuvant setting. *Mol Ther* 2004;10:958–66.
32. Forsyth P, Roldan G, George D, et al. A phase I trial of intratumoral administration of reovirus in patients with histologically confirmed recurrent malignant gliomas. *Mol Ther* 2008;16:627–32.
33. Markert JM, Liechty PG, Wang W, et al. Phase Ib trial of mutant herpes simplex virus G207 inoculated pre-and post-tumor resection for recurrent GBM. *Mol Ther* 2009;17:199–207.
34. Ulasov IV, Rivera AA, Nettelbeck DM, et al. An oncolytic adenoviral vector carrying the tyrosinase promoter for glioma gene therapy. *Int J Oncol* 2007;31:1177–85.
35. Garg AD, Nowis D, Golab J, et al. Immunogenic cell death, DAMPs and anticancer therapeutics: an emerging amalgamation. *Biochim Biophys Acta* 2010;1805:53–71.
36. Ravindra PV, Tiwari AK, Sharma B, et al. Newcastle disease virus as an oncolytic agent. *Indian J Med Res* 2009;130:507–13.
37. Fournier P, Arnold A, Wilden H, et al. Newcastle disease virus induces pro-inflammatory conditions and type I interferon for counter-acting Treg activity. *Int J Oncol* 2012;40:840–50.

38. Elankumaran S, Rockemann D, Samal SK. Newcastle disease virus exerts oncolysis by both intrinsic and extrinsic caspase-dependent pathways of cell death. *J Virol* 2006;80:7522–34.
39. Bardeletti G. Respiration and ATP level in BHK21/13S cells during the earlist stages of rubella virus replication. *Intervirology* 1977;8:100–9.
40. Ando T, Imamura H, Suzuki R, et al. Visualization and measurement of ATP levels in living cells replicating hepatitis C virus genome RNA. *PLoS Pathog* 2012;8:e1002561.
41. Desbiez C, David C, Mettouchi A, et al. Rep protein of tomato yellow leaf curl geminivirus has an ATPase activity required for viral DNA replication. *Proc Natl Acad Sci USA* 1995;92:5640–4.
42. Dignam SS, Correia JJ, Nada SE, et al. Activation of the ATPase activity of adeno-associated virus Rep68 and Rep78. *Biochemistry* 2007;46:6364–74.
43. Alvarez-Breckenridge CA, Yu J, Price R, et al. NK cells impede glioblastoma virotherapy through NKp30 and NKp46 natural cytotoxicity receptors. *Nat Med* 2012;18:1827–34.
44. Ogbomo H, Zemp FJ, Lun X, et al. Myxoma virus infection promotes NK lysis of malignant gliomas in vitro and in vivo. *PLoS One* 2013;8:e66825.
45. Song DZ, Liang Y, Xiao Q, et al. TRAIL is involved in the tumoricidal activity of mouse natural killer cells stimulated by Newcastle disease virus in vitro. *Anat Rec (Hoboken)* 2013;296:1552–60.