

Efficient delivery of oncolytic enterovirus by carrier cell line NK-92

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Many members of the enterovirus family are considered as promising oncolytic agents; however, their systemic administration is largely inefficient due to the rapid neutralization of the virus in the circulation and the barrier functions of the endothelium. We aimed to evaluate natural killer cells as carriers for the delivery of oncolytic enteroviruses, which would combine the effects of cell immunotherapy with virotherapy. We tested four strains of nonpathogenic enteroviruses against the glioblastoma cell line panel and evaluated the produced infectious titers. Next, we explored whether these virus strains could be delivered to the tumor by natural killer cell line NK-92, which is being actively evaluated as a clinically acceptable therapeutic. Several strains of enteroviruses demonstrated oncolytic properties, but only coxsackievirus A7 (CVA7) could replicate in NK-92 cells efficiently. We compared the delivery efficiency of CVA7 *in vivo*, using NK-92 cells and direct intravenous administration, and found significant advantages of cell delivery even after a single injection. This suggests that the NK-92 cell line can be utilized as a vehicle for the delivery of the oncolytic strain of CVA7, which would improve the clinical potential of this viral oncolytic for the treatment of glioblastoma multiforme and other forms of cancer.

INTRODUCTION

Small viruses with a single-stranded RNA genome, such as enteroviruses, have several advantages as oncolytic agents. They display a robust lytic activity and fast and highly productive replication, which occurs in the cytoplasm without affecting the genetic processes of the cell.^{1,2} They do not interact with the host-cell genome and do not pose a risk of malignant transformation. However, as with other viral oncolytics, one of the severe problems for the clinical application of oncolytic enteroviruses is the low efficiency of their delivery to the tumor.³ When administered systemically, viruses are rapidly inactivated in the bloodstream due to absorption by the reticuloendothelial system, neutralizing antibodies, and nonspecific factors of the innate immune system.⁴⁻⁶ The problem can be avoided by injecting the virus directly into the tumor if it is accessible. However, if cancer has spread in the form of distant metastases, systemic administration is required. Currently, several approaches are being considered to protect the virus from inactivation in the bloodstream and increase the efficiency of systemic delivery to the tumor.⁷ Perhaps the most promising is the

use of virus-sensitive cells as carriers, which can be infected *in vitro* and then introduced into the bloodstream.⁸ Various cells have been tested so far, including endothelial cells, mesenchymal and neural stem cells, modified tumor cells, and different immune cells, such as patient-derived dendritic cells, tumor-infiltrating lymphocytes (TILs), antigen-specific T lymphocytes, engineered CAR-T cells, monocytes, and macrophages.⁹ For this purpose, immune cells that can actively move to the location of tumors driven by the gradient of chemokines are particularly promising. Carrier cells of lymphoid origin could be directed to the tumor site either by priming with cancer-antigen-loaded dendritic cells or by ectopic expression of the chimeric antigen receptor.¹⁰ In this case, carrier cells would concentrate within the tumor site before the virus release, minimizing the systemic effect of viremia and delaying the antiviral response.

Although autologous immune cells are entirely suitable as carriers of oncolytic viruses, immune-cell-based permanent cell lines have certain advantages. Cells of continuous cell lines can be artificially modified to replicate the oncolytic virus, thoroughly tested on an extended panel of virus strains, targeted at most common cancer antigens with chimeric antigen receptors, and produced in bulk quantities.

There are several established cell lines from natural killer (NK) lymphomas, of which only the NK-92 cell line has been considered as a potential immunotherapeutic agent in a few clinical trials.¹¹ So far, there are no data on the ability of these cells to become infected and support the replication of oncolytic enteroviruses or at least passively carry them at their cell surface. However, it can be assumed that at least some viral strains can replicate in NK-92, or this property can be artificially imparted to them. In this case, such cells can potentially serve as a means for delivering oncolytic viruses to the tumor.

Glioblastoma multiforme is characterized by rapid progressive growth, active invasion, and an inferior prognosis.¹² Due to the active

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Table 1. Infectious titers of virus strains CVA7, CVB5, PV2, and EV12 measured as TCID₅₀ on respective cell lines

	CVB5	EV12	PV2	CVA7
T89G	1.00E+05	1.00E+07	4.64E+06	1.47E+07*
U118MG	1.00E+06	2.15E+07	3.16E+07	3.16E+07*
DBTRG	3.16E+07	4.64E+07	3.16E+07	2.15E+07*
LN229*	1.00E+05*	1.00E+08*	3.16E+07*	3.16E+07*
U87MG	3.16E+05	1.00E+06	1.00E+07	3.16E+07*
RD (control)	3.16E+03	3.16E+08	3.16E+08	3.16E+08*
C33A (control)	3.16E+08	3.16E+08	3.16E+08	3.16E+08*
Normal astrocytes	<2E+03	<2E+03	<2E+03	<2E+03*

*Selected virus strain and glioma cell lines.

secretion of immunomodulating cytokines and chemokines by the tumor cells, the tumor is usually infiltrated by immune cells, which, together with the tumor cells, form an immunosuppressive microenvironment. This microenvironment requires immunosuppression-resistant cellular immunotherapeutic agents, like CAR-T or CAR-NK cells, to be used for treatment. Relative success has been achieved with an antitumor vaccine based on NK-92 cells targeted to EGFRvIII and HER2 antigens, which are often overexpressed on the surface of glioma cells.¹³

Along with cellular immunotherapy, several families of oncolytic viruses have been evaluated for the treatment of glioblastoma.¹⁴ In particular, the modified PVSRIPPO poliovirus has recently shown impressive results in the first phase of clinical trials.¹⁵ Current routes of virus delivery for glioblastoma treatment are mostly limited to intratumoral administration or resection bed inoculation,¹⁶ with limited reports on systemic intravenous or local intra-arterial administration.¹⁷ The use of *ex vivo* infected cell carriers can overcome the disadvantages of existing methods and increase the efficiency of the delivery of oncolytic viruses to brain tumors. Delivery specificity can be enhanced if lymphoid host cells simultaneously express chimeric T cell receptors targeting tumor antigens.

We aimed to examine the susceptibility of glioma cell lines to several strains of oncolytic enteroviruses and to explore the ability of NK-92 cells to serve as carriers for these virus strains by assessing the efficiency of infection, virus replication, and binding the viruses to the surface of NK-92. To evaluate the advantages of this delivery strategy compared to the systemic intravenous administration of the virus, we examined the effect of the administration methods on the growth dynamics of mouse tumor xenografts.

RESULTS

Glioblastoma cell lines are sensitive to treatment with coxsackievirus A7

To assess the sensitivity of glioma cell cultures to various oncolytic enteroviruses, we compared the minimum number of infectious units that cause cytolysis on the glioblastoma cell line panel. All tested virus

strains were used in equal infectious titers. In parallel, infectious titers were evaluated on the control cell lines RD and C33a, which were utilized for virus production and 50% tissue culture infectious dose assay (TCID₅₀) determination, and normal human astrocytes to confirm cancer-specific oncolytic action (Table 1).

We found that EV12, PV2, and CVA7 can effectively infect every glioma cell line that we tested. The CVB5 showed the least ability to infect cultures; only in the glioblastoma cell line (DBTRG-05MG) was less than 100 IU required for infection. The CVA7 showed approximately the same minimum infectious dose in all cell lines, with less than 50 IU required for the cytolysis of each type of cell. This virus strain was selected for further animal experiments.

NK-92 cells efficiently replicate CVA7, but not EV12, PV2, or CVB5

To test the replication efficiency of each virus strain in NK-92 cells, we compared virus titers after infection of the cell culture with serial dilutions of virus stocks. For CVA7, on day 3 after infection, cells inoculated with minimal multiplicity of infection (MOI) produced roughly the same amount of infectious virus as cells with maximum MOI = 50 and demonstrated maximum daily titer growth, indicating that NK-92 cells are easily infected with CVA7 and can effectively replicate the virus. For EV12, PV2, and CVB5, cells inoculated with low MOI did not produce an infectious virus on day 3, and inoculation with a higher MOI of up to 50 led to the appearance of a virus, which, however, decreased from day 1 to day 3, indicating that these virus strains could not effectively replicate in NK-92 cells. EV12 demonstrated the worst replication efficiency among the strains tested (Figure 1).

The incubation time of NK-92 cells with the virus at MOI = 5 does not affect the oncolysis

Next, we checked whether prolonged incubation of NK-92 cells with the virus could increase their infection efficiency and their ability to transfer the virus to tumor cells. We determined the minimum ratio of NK-92 to tumor cells (NK/TC) required for complete lysis of glioma cells and examined how the increase of the incubation time with the virus affects the extent of NK-92-mediated oncolysis.

NK-92 cells infected with CVA7 for 1 h or 12 h were co-cultured with LN-229 cells at varying NK/TC values. We did not observe significant differences in oncolysis induction between samples that differ in the incubation time. Also, the co-culturing of cells at each NK/TC ratio, except for the minimum, 1/100, led to pronounced and complete lysis of cancer cells, while cells co-cultured in a ratio of 1/100 showed only occasional lysis in some replicates on day 4. When control noninfected NK-92 cells were used at any NK/TC ratio, no decrease in tumor cell culture growth was observed (Figure 2A).

A virus that is passively adsorbed on the surface of NK-92 cells has a minimal effect on the lysis of cancer cells

Besides infecting and replicating in the carrier cells, viruses could passively attach to the cell surface and then infect sensitive cancer cells

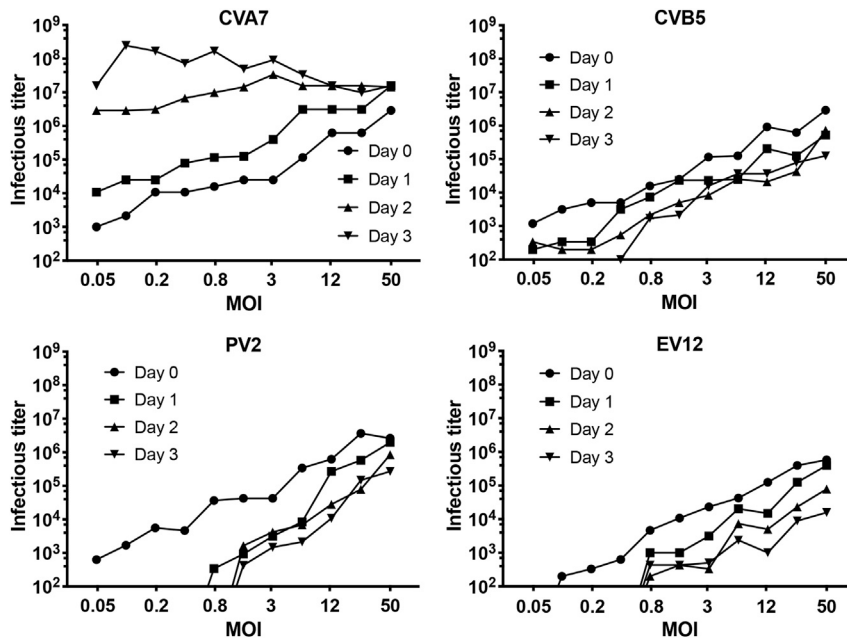


Figure 1. Virus replication in NK-92

TCID₅₀ of NK-92 cell cultures infected with different MOI of virus strains CVA7, CVB5, PV2, and EV12 at 3 h (day 0), day 1, day 2, and day 3 after infection, as measured on RD cells.

Sublethal irradiation does not prevent NK-92 from replicating CVA7

Clinical protocols for immunotherapy with NK-92 require the cells to be sublethally irradiated with 10 Gy before introducing them into the bloodstream. We tested how different doses of gamma irradiation affect the ability of NK-92 to replicate CVA7. Cells were irradiated by a cesium source, and a fraction of each sample was saved for culture; the rest were infected with CVA7 at MOI = 5 for 1 h, and the virus was removed. On day 3, culture supernatants were collected, and infectious viral titers were measured as TCID₅₀ along with an assessment of proliferation potential of uninfected irradiated NK-92 (Figures 2B and 2C). Application of doses

that appear nearby in the tumor. When viral particles are located on the cell's surface, they remain readily available for immune surveillance, which may reduce the main benefits of delivering the virus with carrier cells. To test whether passive sorption has a noticeable effect on CVA7 transfer by NK-92 cells, we raised a rabbit neutralizing antiserum against CVA7 and examined the ability of the antiserum-treated samples of infected NK-92 cells to transmit the virus.

The NK-92 cells were mixed with CVA7 for 20 h or 1 h and then treated with serial dilutions of the virus-neutralizing antiserum. For some of the samples, NK-92 cells were washed from antiserum before mixing with cancer cells (Table 2). The results show that if the virus-neutralizing antibody is washed off, neither concentration of the antiserum could prevent or weaken the lytic effect of the virus on target cancer cells. We also observed that even without the wash-off, at lower doses of the serum, the virus-neutralizing effect ceases, suggesting the limits of antibody titers.

We tried to estimate the total amount of infectious virus taken up by NK-92 cells during the infection to calculate the actual dose of the virus when it is introduced into the body with cell carriers. For this, we incubated NK-92 cells with CVA7 at MOI = 5 for 1 h, when the virus has not yet had time to replicate. Then, the cells were removed by centrifugation, and the infectious titers (as TCID₅₀) were compared in the supernatant and the control sample containing the input amount of the virus. Virus titers in both samples were identical (result not shown), indicating that the proportion of the virus bound to NK-92 cells was negligible. Thus, when the virus is introduced into the body using carrier cells, the viral load can be significantly reduced compared to the direct introduction of a viral preparation.

over 10 Gy substantially reduced the ability of NK-92 cells to proliferate, with doses over 25 Gy resulting in almost complete death of cells by day 3. Doses under 15 Gy did not prevent NK-92 from replicating CVA7: the registered TCID₅₀ dropped less than threefold compared to the nonirradiated sample. Doses of 25 and 50 Gy reduced the viral output by 32-fold and 125-fold, respectively.

LN-229 xenotransplants show the most uniform growth pattern

To select a suitable cell line for testing oncolytic effects of the virus *in vivo*, we compared the tumorigenicity of four glioblastoma cell lines in small cohorts of severe combined immunodeficiency (SCID) beige mice by xenograft transplantation. The cells of each line were transplanted subcutaneously into the right lower limb of two or four animals in the amount of 2×10^6 cells. Of the five cell lines tested, four revealed detectable tumors within 45 days after injection (Figure 3A). The T89G cell line did not form tumors in any mouse in this experiment.

The cell line LN229 showed the most uniform and synchronous growth pattern in all mice of the corresponding group. We selected this cell line for further *in vivo* experiments.

Two consecutive administrations of CVA7 or CVA7-infected NK-92 cells significantly reduced tumor growth

The therapeutic effect of the virus delivered systemically or using the cell-based carrier was compared *in vivo* in tumor-bearing mice. The virus was delivered in two consecutive rounds at days 49 and 69 after tumor transplantation. Groups were treated with CVA7 (10^6 IU), or with NK-92 (10^6) cells, or with CVA7-infected NK-92 (NK-92-CVA7) (10^6) cells infected at MOI = 5. By day 78, significant suppression of tumor growth was observed in the group of mice where the virus was delivered using NK-92 carrier cells (Figures 3B and 3C).

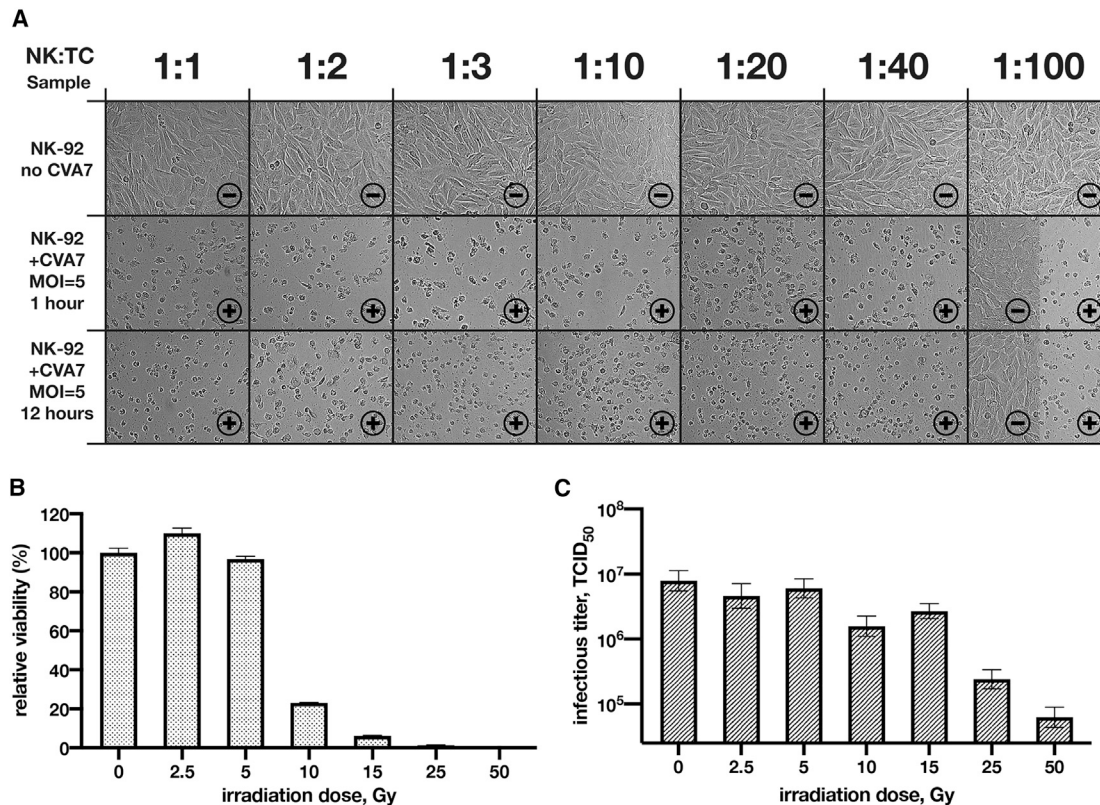


Figure 2. Virus transmission by co-culturing and virus replication in irradiated NK-92

(A) Cytolytic effect of co-culturing of LN-229 cells with CVA7-infected NK-92 at different NK:TC ratios. (B) Proliferation of NK-92 cells measured 3 days post-irradiation (mean ± SD). (C) TCID₅₀ of irradiated NK-92 cell cultures infected with CVA7 at MOI = 5, as measured on C33A cells (mean ± SD).

By day 84, both groups of mice treated with the virus showed significant tumor suppression compared to the control group of mice treated with mock-infected NK-92 cells (Figures 3B and 3D). Treatment based on both virus-infected carrier cells and direct injection of the virus was well tolerated, and by the 84th day, the administration was not accompanied by significant weight loss. Control treatment with noninfected NK-92 cells did not lead to any therapeutic effect.

A single injection of CVA7-infected NK-92 cells significantly delays tumor growth

We examined whether a single injection is sufficient for marked tumor suppression. To better visualize tumors, we created the LN-229-Luc cell line, which constitutively expresses the firefly luciferase delivered by a lentiviral vector. Before the experiment, we tested LN-229-Luc cells for tumorigenicity in a small group of animals (n = 4) and found that they form tumors with the same frequency as the parental cell line, although the tumor growth rate was reduced (Figure S1).

The mice were divided into four groups and treated with the same doses of virus or virus-carrying cells as in the previous experiment.

On day 104, we found a statistically significant decrease in tumor volumes in the group that received the virus-carrying cells compared to the control group. This difference was further confirmed by bioluminescence imaging (Figure 4).

DISCUSSION

Four tested strains of nonpathogenic enteroviruses use different cell receptors for the cell entry: CXADR for CVB5,¹⁸ CD155/PVR for PV2,¹⁹ CD55 for EV12,²⁰ and SCARB2 for CVA7.²¹ All glioblastoma cell lines demonstrated a high sensitivity to PV2, EV12, and CVA7, which suggests that the corresponding virus entry receptors are present on their surface. The sensitivity of glioblastoma cells to CVB5 varies significantly, which suggests the deficiency in some of the cell lines of components required for effective replication of this viral strain, for example, a low expression level of the CXADR receptor. Testing glioblastoma cell lines reveals their high sensitivity to enteroviruses, as a relatively low infectious dose is sufficient for productive replication of the virus. This suggests that the intrinsic mechanisms of antiviral defense in glioblastoma cells are frequently compromised, making this type of tumor a suitable target for oncolytic virotherapy with many strains of enteroviruses.

Table 2. Effect of treatment with dilutions of antiserum raised against CVA7 on virus transmission by CVA7-infected NK-92

Antiserum dilution	1/4	1/8	1/16	1/40	1/80
NK-92 + CVA7, MOI = 5, 1 h treatment	–	–	–	+	+
NK-92 + CVA7, MOI = 5, 1 h treatment, unbound antibodies removed	+	+	+	+	+
NK-92 + CVA7, MOI = 5, 20 h treatment	–	–	+	+	+
NK-92 + CVA7, MOI = 5, 20 h treatment, unbound antibodies removed	+	+	+	+	+

Carrier cells were added to cancer cells at ratio of 1:40.

Cells used as carriers for delivering the virus to the tumor must satisfy several requirements. They must efficiently replicate the virus and penetrate the tumor sites, while the duration of the infection cycle of the virus inside the carrier cell must be no less than the time required for cells to reach the tumor site. Our results indicate that NK-92 cells can produce substantial amounts of the virus even after infection with low MOI, while passive sorption of viral particles on the cell surface is negligible. The cells were efficiently infected after 1-h incubation with the virus, and therefore taking the known enterovirus replication cycle for 6–7 h,²² the cell carrier has enough time to reach the location of the tumor. During the delivery of the virus to the tumor, NK-92 cells provide reliable protection of the virus against neutralization by specific antibodies, nonspecific blood factors, and the uptake of viruses by the reticuloendothelial system. The release of the newly formed virus from the carrier cells occurs directly in the tumor microenvironment, thereby contributing to the effective infection of tumor cells.

According to *in vitro* experiments, the number of carrier cells necessary to mediate viral oncolysis is at least an order of magnitude lower than the number of NK cells sufficient for the NK-mediated cytotoxicity. Non-virus-loaded NK-92 cells introduced at NK:TC ratios of up to 1:1 did not cause any inhibition of tumor cell growth in cell culture, while the virus-bearing cells consistently initiated oncolysis at a NK:TC ratio of 1:40 and higher. This has also been confirmed *in vivo*. In both experiments, in the groups of mice injected with uninfected NK-92 cells, the tumor growth rates were similar to those in the control groups. A decrease in the effective dose of the introduced carrier cells is undoubtedly preferable, as it will reduce the incidence of side effects of cell therapy.

Groups of mice treated with two consecutive administrations of either the virus or virus-loaded carrier cells demonstrated a consistent suppression of tumor growth, unlike the groups treated with only NK-92 cells or the mock-infected control. However, we did not notice a significant difference in the growth rate of tumors in the groups of mice treated with either the virus or the virus-loaded NK-92 cells. By analyzing the tumor growth curves for each group, we suggested that even a single administration of CVA7 or CVA7-infected NK-92 cells could lead to significant suppression of tumor

growth. The following experiment with the planned single treatment administration was to reveal the possible advantages of one of the delivery methods over another. It was found that a single treatment administration is not sufficient for stable suppression of tumor growth, although, on day 104 after xenotransplantation, we were able to detect noticeable differences in the mean tumor volume in the animal groups treated with CVA7-loaded NK-92 cells and mock control.

The above results provide indirect evidence for more efficient delivery of CVA7 carried by NK-92 cells than the direct intravenous administration of the virus. However, some additional factors may cause the observed variations in the responses to the first administration and explain the results of two experiments on the treatment of tumor xenografts. In the second *in vivo* experiment, the luciferase-expressing LN229 cells were used, displaying a slower growth rate, leading to the formation of tumors with different vascularization profiles and less accessible for infiltration with NK-92 cells and subsequent viral infection. Another possible explanation is that the first administration of the virus partially disrupts tumor vascular structure and makes it more susceptible to the virus administered in the second round of therapy.²³

In both *in vivo* experiments, NK-92 cells were not specifically targeted to tumor cells. Thus, the observed differences in the treatment efficacy can be explained either by a decrease in a nonspecific virus clearance from the circulation or by a more prolonged viremia provided by infected carrier cells.

Most cell-carrier-based virus delivery strategies developed to date are based on cell populations obtained directly from patients. These are myeloid or lymphoid cells or their precursors. The preparation of a therapeutic involves the isolation of certain subfractions of immune cells, their expansion *ex vivo*, and subsequent processing, including transfection of DNA constructs for expression of transgenes, such as chimeric antigen receptors. Concerning the proposed method of viral therapy, this approach will include a loading of carrier cells with viruses. Such an approach is costly compared to the direct administration of the virus. When using the NK-92 cell line as a virus carrier, all preparatory procedures are significantly simplified, which would allow extending this method as a standard and relatively cheap process for administering viral therapeutics to patients with various types of cancer. As was demonstrated in the experiment with gamma-irradiated NK-92 cells, sublethal irradiation with 10 Gy does not prevent the cells from replicating CVA7, making this delivery strategy compatible with current clinical protocols for immunotherapy with NK-92. The use as a virus carrier of NK-92 cells, which retain the functions of killer cells by recognizing and destroying cancer cells based on some distinguishing features, allows them to rely on an additional immunotherapeutic effect. The NK-92 cells and other similar permanent cell lines of NK origin are suitable for CAR therapy after the introduction of constructs expressing the chimeric T cell receptor.^{11,24} Using CAR-expressing NK-92 cells as virus carriers may increase the specificity of targeting tumor cells, allowing for even

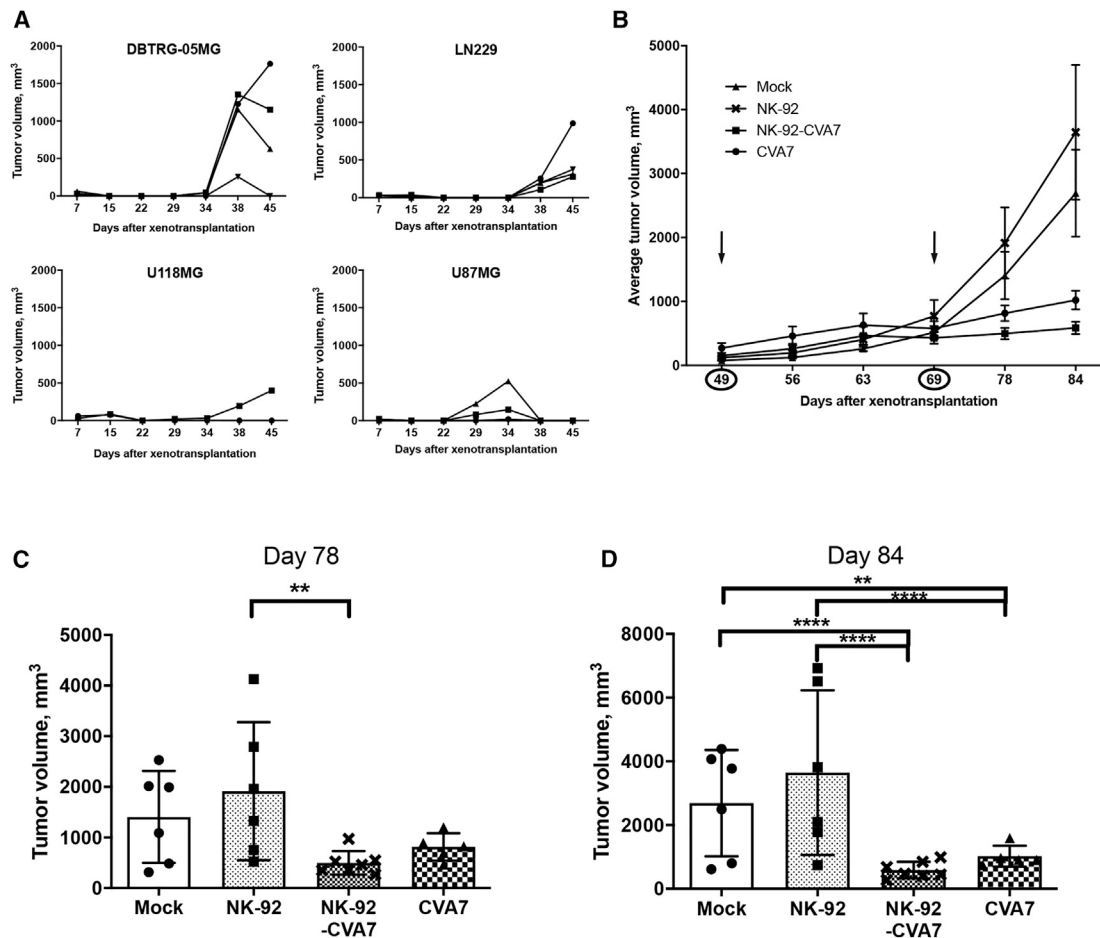


Figure 3. Treatment with two-dose administration of CVA7-infected NK-92

(A) Growth dynamics of tumor xenotransplants of cell lines DBTRG-05MG, LN229, U118MG, and U87MG. (B) Tumor progression dynamics after first treatment administration. (C) Comparison of tumor volumes measured on day 78 (mean \pm SD), ** $p = 0.0056$. (D) Comparison of tumor volumes measured on day 84 (mean \pm SD), ** $p = 0.0021$, **** $p < 0.0001$.

lower therapeutic doses to be efficient. Furthermore, the immunotherapeutic action of CAR-NK-92 therapy could be combined with virus delivery by knocking out specific virus entry receptors in a fraction of the NK-92 cells, thus making them resistant to infection. The resulting mixture of virus-bearing and virus-resistant NK-92 cells could serve as a ready-made double-acting therapeutic agent.

MATERIALS AND METHODS

Cells and viruses

Enteroviruses coxsackievirus B5, strain LEV14 (CVB5); coxsackievirus A7, strain LEV8 (CVA7); echovirus 12, strain LEV7 (EV12); and poliovirus type 2, Sabin vaccine strain (PV2) were provided from the Prof. M.K. Voroshilova collection. Stocks of viruses were prepared by infection of C33A cervical carcinoma at MOI = 1. After 1 h of adsorption, the infected cultures were incubated for 48 h in complete Dulbecco's modified Eagle's medium

(DMEM) supplemented with 2% fetal bovine serum (FBS). After complete degradation of the monolayers, the cultures were freeze-thawed three times to release the virus from cell debris, and the virus-containing fluid was clarified by low-speed centrifugation. For virus concentration, the supernatants were pulled down by at 45,000 rpm in a Beckman TL100 ultracentrifuge (TLA-100.3 rotor) at 4°C for 2 h. The virus pellet was resuspended in 55 μ L of PBS for every 1.5 mL of virus-containing liquid. Virus titers were quantified using a standard 50% tissue culture infectious dose assay (TCID₅₀) in RD and C33A cells, and stocks were stored at -20°C in single-use aliquots.

Human RD (rhabdomyosarcoma), C33A (cervical carcinoma), U-118MG (glioblastoma), LN-229 (glioblastoma), DBTRG-05MG (glioblastoma), U87MG (glioblastoma), T89G (glioblastoma), NK-92 (NK-lymphoma), and HEK293T cell lines were obtained

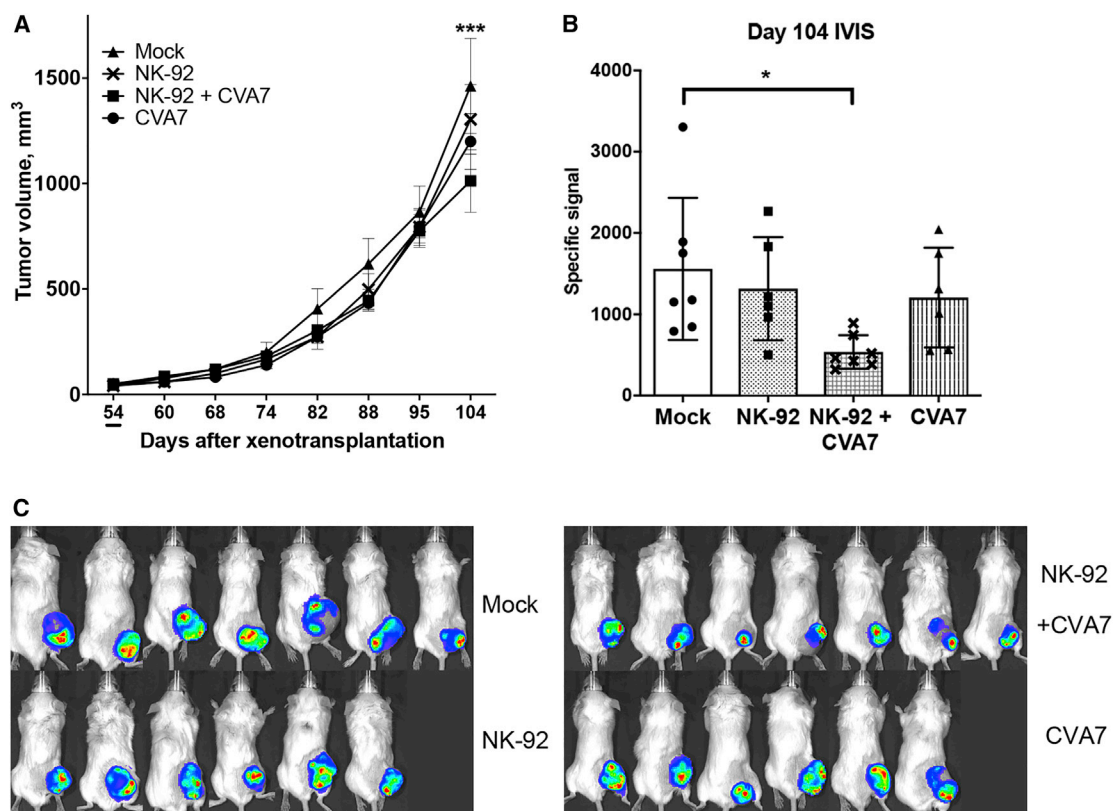


Figure 4. Treatment with a single administration of CVA7-infected NK-92

(A) Tumor progression dynamics after treatment administration. *** $p = 0.0009$, Mock versus NK-92+CVA7, two-way ANOVA with post hoc Sidak's test. (B) Comparison of specific bioluminescence signals obtained from tumors at day 104 after xenotransplantation (mean \pm SD) * $p = 0.0165$. (C) Bioluminescent scans of mice on day 104. The bioluminescence signal was recorded as the total light emission in the region of interest (photons/s). A specific signal was calculated as the ratio of the bioluminescent signal in the region of interest to that in the contour of a mouse with no tumors.

from the American Type Culture Collection (ATCC). NK-92 cells were grown in RPMI-1640 medium supplemented with 2 mM alanyl-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, 10% heat-inactivated fetal bovine serum and 10% heat-inactivated horse serum, 100 μ M β -mercaptoethanol, 20 μ M folic acid, 200 μ M inositol, 20 ng/mL IL-2, and 1 μ M hydrocortisone. Other cell lines were grown in DMEM or RPMI-1640 medium supplemented with 2 mM alanyl-glutamine, 100 U/mL penicillin, 100 mg/mL streptomycin, and 10% heat-inactivated fetal bovine serum. Cell lines were kept at 37°C in a 5% CO₂ humidified atmosphere.

Animals

1- to 8-month-old male and female SCID beige mice were from Charles River Laboratories, maintained in the individually ventilated cages at the vivarium of the Institute of Bioorganic Chemistry, Russian Academy of Sciences (IBCH RAS). All procedures were approved by IBCH RAS Institutional Animal Care and Use Committee. The use of animals of both sexes is associated with the lack of differences in survival and growth dynamics in

our experiments. All manipulations were performed in aseptic conditions.

Testing of the sensitivity of cell lines to viruses and TCID₅₀ measurements

For testing infectious titers of viral preparations (as TCID₅₀) in five glioblastoma cell lines and two control tumor cell lines (RD and C33A), cells were seeded at a density of 10⁴ cells per well of 96-well plates. The next day the plates were infected in triplicate with 10-fold serial dilutions of viruses in a volume of 0.1 mL, incubated for 1 h, and the medium was replaced with fresh DMEM with 2% fetal bovine serum. Counting was performed on the fourth day after infection. The TCID₅₀ values were calculated using the Reed-Muench method.²⁵ For precise measurements of TCID₅₀ values, C33A cells were infected in triplicate with 2-fold serial dilutions of virus samples in the same conditions as described above. In the experiment with gamma-irradiated NK-92, C33A cells were infected in six replicates with 5-fold serial dilutions. The TCID₅₀ values were calculated using the Spearman-Kärber method.²⁶

Construction of luciferase-expressing LN-229

For tumor bioimaging, the LN-229 cell line was transduced with a pLACMV-Luc-puro lentiviral construct expressing firefly luciferase in the form of lentiviral particles. Following the transduction, the cells were selected in the medium containing 1 $\mu\text{g}/\text{mL}$ of puromycin. To assess the transduction efficiency, LN-229 and LN-229-LucPuro cells were lysed with Luciferase Cell Lysis Buffer (NEB, Ipswich, MA, USA) and incubated for 20 min, followed by the addition of 100 μL luciferin solution (40 mg/mL) and luminescence measurement.

Loading of viruses onto NK-92 cells

Target NK-92 cells were counted and diluted in RPMI-1640 medium to a final concentration of 5×10^6 cells/mL. NK-92 cells were infected with CVB5, PV2, EV12, or CVA7 at MOI = 5 for 1.5 h, washed twice in PBS, centrifuged at $1,000 \times g$ for 5 min, and resuspended in the RPMI-1640 medium.

Virus replication in NK-92 cells

To determine the kinetics of viral replication in NK-92 cells, infectious viral titers were evaluated for 3 days. NK-92 cells were infected in 12-well plates, 5×10^5 cells/well, with MOI ranging from 0.024 to 50. Plates with infected NK-92 were frozen at -80°C at 3, 24, 48, and 72 h after infection. Infectious titers were evaluated by standard TCID₅₀ assay on RD and C33A cells using 96-well plates.

Evaluation of the effect of virus absorption on NK-92 cells on the ability of infected cells to transfer the virus to sensitive cells

LN-229 cells were seeded on 96-well plates, 12,000 cells per well. The next day, virus-loaded NK-92 cells 1 h (day 0) and 24 h (day 1) after infection were incubated with 2-, 4-, 8-, 20-, and 40-fold dilutions of rabbit antiserum to CVA7 for 1 h. In some samples, the antisera were removed by washing three times in PBS. NK-92 cells were added in 10,000, 5,000, 3,000, 1,000, 500, or 250 cells in serum-free RPMI-1640 medium to the wells with LN-229 cells. The viability of LN-229 cells was evaluated on day 4.

NK-92 irradiation and proliferation assessment

Irradiation of NK-92 cell cultures was performed with Gammacell 3000 Elan (Best Theratronics). Cells were suspended in a complete growth medium in the density of 10^6 cells/mL, in 1 mL aliquots in 1.5 mL microcentrifuge tubes. Following irradiation, half of the cells were infected with CVA7 at MOI = 5, and the other half was cultured for 3 days, after which the proliferation was assessed with Promega CellTiter Glo 2.0 homogeneous assay in 96-well plates in triplicate.

Assessment of tumorigenicity of glioblastoma cell lines and tumor growth dynamics

Tumor cells were injected subcutaneously (s.c.) in the amount of 1×10^6 to 2×10^6 cells at both sides in the hind region of 2 male SCID beige mice, except for the U-118MG cells, which were injected to a single site. The tumorigenicity of the LN-229 LucPuro cell line was evaluated in 4 mice. The number of injected cells depended on the tumor cell line: for the T89G, LN-229, and LN-229 LucPuro cell lines,

2×10^6 cells/site were used; for U118MG, 1.5×10^6 cells/site; and for DBTRG-05MG and U87MG, 1×10^6 cells/site.

Subcutaneous glioblastoma tumor model

Glioblastoma cells were injected at a dose of 2×10^6 cells s.c. into hind regions of SCID beige mice (day 0). Treatment was started after xenografts reached a size of approximately 50–100 mm³. At the beginning of the treatment, animals with similar xenograft growth dynamics were selected and divided into groups per the equal sex ratio and medial xenograft size between the groups. Sizes of the xenografts were evaluated every 6–7 days by measuring with a caliper. Xenograft tumor volumes were calculated according to the equation $V = L \times W^2/2$.²⁷

The appearance of statistically significant differences between the groups or the achievement of an individual xenograft size of 3,000 mm³ were considered valid experimental endpoints. Animals with signs of distress (ascites, sudden weight loss, etc.) were euthanized by decapitation.

Dual therapy with NK-92 cells

Mice aged 4 to 8 months with xenografts on both thighs were divided into groups and were injected intravenously (i.v.) into the tail vein with 0.1 mL of RPMI-1640 medium (control group, n = 6), CVA7 (10^6 TCID₅₀, n = 5), NK-92 cells (10^6 cells, n = 6), or NK-92 + CVA7, MOI = 5 (10^6 cells, n = 7) on 49 and 69 days after injection of tumor cells. All preparations were based on the RPMI-1640 medium. NK-92 cells were infected with the CVA7 with MOI = 5. The tumor volumes of each mouse were estimated as a sum of their volumes.

Single therapy with NK-92 cells

Mice aged 1 to 8 months with xenografts of tumors on the right thigh were divided into groups and injected i.v. into the tail vein in a volume of 0.1 mL of RPMI-1640 medium (control group, n = 7), CVA7 (10^6 TCID₅₀, n = 6), NK-92 cells (10^6 cells, n = 6), or NK-92 + CVA7, MOI = 5 (10^6 cells, n = 7) on day 54 after injection of tumor cells.

Tumor xenograft bioimaging

Tumor xenograft areas were shaved before imaging. 100 μL of luciferin at a dose of 150 mg/kg was administered by retroorbital injection. Thirty seconds after the injection, the bioluminescence of the tumor was recorded under isoflurane anesthesia as the IVIS spectra.

Statistical analyses

Statistical analysis was performed using GraphPad Prism 8. The Shapiro-Wilk normality test was used to verify the normality of the distribution of quantitative data. To verify the significance of differences between samples with a normal distribution, we used Student's t test for two samples, one-way ANOVA for more than two samples without repeating values, and two-way ANOVA with repeated measurements, followed by post hoc the Sidak's multiple comparisons test for samples with duplicate values. The significance level was $p < 0.05$.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omto.2021.03.013>.

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AUTHOR CONTRIBUTIONS

Conceptualization, E.S.P. and S.P.C.; methodology, S.P.C.; investigation, E.S.P., A.S.S., D.S.K., and S.P.C.; visualization, E.S.P. and A.S.S.; resources, E.I.F. and S.P.C.; writing – original draft, S.P.C.; funding, E.I.F.

DECLARATION OF INTERESTS

The authors declare that there is no conflict of interest.

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