INTERFERON TYPE I-EXPRESSING RECOMBINANT VACCINIA VIRUS AS A PLATFORM FOR SELECTIVE IMMUNOTHERAPY OF GLIOBLASTOMA AND MELANOMA

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Immunotherapy with oncolytic viruses (OVs) becomes a full-fledged neoadjuvant therapy method in the paradigm of evidence-based medicine for the growing number of cancers. The use of OVs for immunologically "cold" tumors causing minimal immune response and having the clearly immunosuppressive tumor microenvironment is especially relevant. Recombinant OVs carrying the sequences of proteins activating the immune system can be used to stimulate antitumor response. The study aimed to assess oncoselectivity and antitumor activity of the recombinant OV designed based on the LIVP vaccinia virus strain showing expression of human and murine interpheron alpha sequences (hIFNα and mIFNα, respectively). The *in vitro* experiments showed that the recombinant OVs designed showed oncoselectivity in relation to tumor cell lines of appropriate species. The ability to effectively infect human adenocarcinoma and glioblastoma cell lines was reported for LIVP-hIFNα. LIVP-mIFNα showed selectivity in relation to glioma Gl261 and melanoma B16 *in vitro*. The *in vivo* experiment involving the C57Bl/6 mice with subcutaneous melanoma B16 showed the ability of the intravenously administered LIVP-mIFNα to reduce the size of the subcutaneous tumor allograft and increase tumor infiltration with the $CD8^+$ and NK cells. The recombinant virus designed can be a potential platform for the development of oncolytic virotherapy of human melanoma and glioblastoma.

Keywords: oncolytic viruses, interferon, viral oncolysis, recombinant vaccinia virus strains

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Author contribution: Naberezhnaya ER — implementation of in vitro and in vivo experiments, manuscript writing; Soboleva AV — flow cytometry data acquisition, determining the cell line sensitivity to viruses; Vorobyev PO — designing recombinant viruses, production of preparative amounts of strains; Vadekhina VV — conducting *in vivo* experiments; Yusubalieva GM — interpretation of *in vivo* experimental data, manuscript writing; Isaeva IV — histologic and immunohistochemical assessment; Baklaushev VP — microscopy, describing histology and immunohistochemistry data, preparing a drawing, manuscript writing; Chumakov PM — manuiscript editing; Lipatova AV — study concept, general project management.

Compliance with ethical standards: the *in vivo* study was approved by the Ethics Committee of the Federal Scientific and Clinical Center of FMBA of Russia (protocol No. 7 dated 06 September 2022) and conducted in accordance with the the Eurasian Economic Commission Board's guidelines No. 33 dated 14 November 2023 "On the Guidelines for handling laboratory (experimental) animals when conducting preclinical (non-clinical) studies". The number of animals per group was minimized; the subcutaneous tumor size in the groups did not exceed 2000 mm^3. *In vitro* experiments involved the commercially available animal and human cell lines.

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РЕКОМБИНАНТНЫЙ ВИРУС ОСПОВАКЦИНЫ, ЭКСПРЕССИРУЮЩИЙ ИНТЕРФЕРОН ТИПА 1, КАК ПЛАТФОРМА ДЛЯ СЕЛЕКТИВНОЙ ИММУНОТЕРАПИИ ГЛИОБЛАСТОМЫ И МЕЛАНОМЫ

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Иммунотерапия онколитическими вирусами (ОВ) становится полноценным методом неоадъювантной терапии в парадигме доказательной медицины для все большего числа онкологических заболеваний. Особенно актуально применение ОВ для иммунологически «холодных» опухолей, вызывающих минимальный иммунный ответ и обладающих выраженным иммуносупрессивным опухолевым микроокружением. Для стимуляции противоопухолевого ответа применимы рекомбинантные ОВ, несущие последовательности иммуноактивирующих белков. Целью работы было исследовать онкоселективность и противоопухолевую активность рекомбинантного ОВ, созданного на базе штамма LIVP вируса осповакцины, экспрессирующего последовательности интерферона-альфа человека и мыши (hIFNα и mIFNα соответственно). В экспериментах с помощью метода Рида и Менча было показано, что созданные рекомбинантные ОВ проявляют онкоселективность в отношении опухолевых линий соответствующего вида. Для LIVP-hIFNα показана способность эффективно заражать линии аденокарциномы и глиобластомы человека. Для LIVP-mIFNα *in vitro* продемонстрирована селективность в отношении глиомы Gl261 и меланомы B16. В эксперименте *in vivo* на мышах линии C57Bl/6 с подкожной меланомой B16 показана способность LIVP-mIFNα после внутривенного введения уменьшать объем подкожного аллографта опухоли и увеличивать инфильтрацию опухоли $CD8^+$- и NK-клетками. Созданный рекомбинантный вирус может быть потенциальной платформой для разработки онколитической виротерапии меланомы и глиобластомы человека.

Ключевые слова: онколитические вирусы, интерферон, вирусный онколиз, рекомбинантные штаммы вируса осповакцины

Финансирование: создание рекомбинантных штаммов вируса осповакцины было выполнено при поддержке гранта Российского научного фонда № 23-14-00370, изучение их свойств на моделях *in vitro* и *in vivo* проводилось при подержке гранта Российского научного фонда № 22-64-00057, гистологические и иммуногистохимические исследования опухолевой ткани проводили при поддержке ФМБА России.

Вклад авторов: Е. Р. Набережная — реализация экспериментов *in vitro* и *in vivo*, написание рукописи; А. В. Соболева — получение данных проточной цитометрии, определение чувствительности линий к вирусам; П. О. Воробьёв — создание рекомбинантных вирусов, наработка препаративных количеств штаммов; В. В. Вадехина — проведение экспериментов *in vivo*; Г. М. Юсубалиева — интерпретация данных эксперимента *in vivo*, написание статьи; И. В. Исаева — проведение гистологического и иммуногистохимического исследования; В. П. Баклаушев — проведение микроскопии, описание гистологических и иммуногистохимических данных, подготовка рисунка, написание статьи; П. М. Чумаков, редактирование рукописи; А. В. Липатова — концепция исследования, общее руководство проектом.

Соблюдение этических стандартов: исследование *in vivo* одобрено этическим комитетом ФГБУ ФНКЦ ФМБА России (протокол № 7 от 06 сентября 2022 г.), проведено в соответствии с рекомендациями Коллегии ЕЭК от 14.11.2023 № 33 «О Руководстве по работе с лабораторными (экспериментальными) животными при проведении доклинических (неклинических) исследований». Число животных в группах было минимизировано, размеры подкожных опухолей в группах не превышали 2000 $мм^3$. Эксперименты *in vitro* проведены на коммерчески доступных линиях клеток животных и человека.

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Though many therapeutic approaches are now available including modern surgery with intraoperative navigation, radiotherapy, neoadjuvant therapy with targeted drugs, immune checkpoint inhibitors, the survival rates for patients with some cancers such as glioblastoma, metastatic melanoma and a number of carcinomas with primary metastasis remain extremely low. Development of new therapeutic approaches aimed at elimination or prevention of distant metastases is therefore of high significance.

An important requirement for developing new antitumor agents is a highly selective oncolytic action, the ability to detect and destroy malignant/metastatic tumor cells only. Non-pathogenic or attenuated viruses are ideal candidates for the development of tumor-selective agents. Tumor selective therapeutic strains are capable of destroying tumor cells by replicating efficiently in the cells without damaging normal tissues [1].

Currently, using Poxviridae viruses as prototypes of oncolytic viruses for the treatment of metastatic carcinomas and melanoma is of particular attention. A promising oncolytic virus belonging to the *poxvirus* family is vaccinia virus (VV) [2], in particular, the Russian variant of the Lister VV strain (LIVP VV). The strain was widely used in the Smallpox Eradication Campaign (SEC); it is highly tumor selective [3, 4], in particular, the thymidine kinase (TK) knockout leads to selective replication of the virus in tumor cells where TK is usually overexpressed [5].

To improve the antitumor properties of attenuated VV strains, recombinant VV strains expressing different transgenes are costructed [6]. To date, many transgenes are known to be effectively expressed in VV including genes encoding cytokines and their receptors [7], immunostimulators [8–10], oncotoxic proteins [11], and angiogenesis inhibitors [12].

In this study, we constructed highly tumor selective poxvirus strains expressing human or mouse IFNα and tagRFP in a bicistronic cassette. The expression of IFNα by a recombinant oncolytic strain can enhance indirect immune-mediated oncolytic action through inducing the expression of the major histocompatibility complex class I molecules, increased activity of cytotoxic T lymphocytes, activation of T helper cells, macrophages, and NK cells [13].

In normal cells, interferon secretion stops protein translation and cell cycle processes and slows down cell metabolism [14]; protecting cells from viruses interferon mechanisms are therefore interfere with tumor cell proliferation. Microevolution results in accumulating errors in the signaling pathways of interferon induction and interferon response in tumor cells and as a consequence, cells may lose their antiviral defense abilities [15, 16]. The loss of protective interferon response mechanisms is one of the factors responsible for the uncontrolled proliferation of tumor cells [17].

Another thing making the oncolytic virotherapy so promising is the ability of OVs to induce a long-lasting cancer-specific immune activation [18]. Viruses kill tumor cells releasing various molecules such as tumor-associated antigens, pathogen-associated molecular patterns, damage-associated molecular patterns and cytokines [19]. The molecules promote activation of immune antigen-presenting cells and T cells developing an effective adaptive immune response against the tumor [20, 21].

Type I interferons inhibit virus replication in normal cells however, in tumor cells the inhibition is less effective [8, 17]. Type I interferons therefore have antitumor effect and are able to induce tumor specific cytotoxic T-lymphocytes and activate antiangiogenic factors [22]. Furthermore, vaccinia virus inhibits the interferon response system through the expression of certain genes [22]. The aim of the study was to evaluate the significance of interferon expression by vaccinia virus for the oncolytic activity of the virus.

METHODS

Vaccinia virus strains

LIVP-hIFNα, vaccinia virus expressing human IFNα and tagRFP; LIVP-mIFNα, vaccinia virus expressing mouse IFNα and tagRFP; LIVP-RFP, vaccinia virus expressing tagRFP.

Cell cultures

BHK-21 — baby hamster kidney cell line; HEK293T — transformed human embryonic kidney cell line containing SV40-antigen; U-87 MG, DBTRG-05MG, U251-MG, PrGlioma 3821, PrGlioma 6067, PrGlioma 6138 — human glioblastoma cell lines; HeLa — human cervical carcinoma cell line; HEF — human embryonic fibroblast cell line; Embr.astro — human embryonic astrocyte cell line; B16 — murine melanoma cell line; 4T1 — murine breast cancer cell line.

Cell lines were cultured on DMEM medium (PanEco, Russia) with addition of 10% fetal bovine serum (FBS) (HyClone, USA), penicillin-streptomycin (PanEco, Russia) and 2 mM L-glutamine. The cells were cultured at 37 °C and 5% CO_2, and the medium changed every 3–5 days.

Construction of recombinant vaccinia virus strains expressing mouse and human IFNα

Preparation of plasmid constructs for recombination

To obtain mouse IFNα cDNA fragment, Balb/c female was infected with Sendai virus (Moscow strain), $1x10^9$ infectious particles. After 24 h, the mouse was euthanized. mRNA was extracted from spleen (Total RNA Isolation Kit, Evrogen, Russia) according to manufacturer's recommendations. cDNA was prepared by reverse transcription using Superscript III cDNA synthesis kit according to the manufacturer's instructions. Fragments were amplified by PCR using specific primers:

Forward: 5'-ATGGGGCTAGGCTCTGTGTG-3'

Reverse: 5'-TTTCTCTCTTCTCTCTCTCAGTCTTCCCA-3'

Human IFNa cDNA was obtained by reverse transcription of total RNA from peripheral blood mononuclear cells of a healthy donor. Primer sequences used for amplification:

Direct 5'-ATGGCCCCTCGCCCTTTGC-3'

Reverse: 5'-TTCCTTCCTCCTCCTCCTTAATCTTTCTTTCT TGCAAG-3'

After obtaining the amplicon, one more PCR was performed with primers containing restriction sites. The fragments were cloned using sticky ends into a shuttle vector developed previously in the Cell Proliferation Laboratory. The constructs were sequenced using Sanger method.

The constructs were transfected into the HEK293T cells using PEI [23] followed by infection with LIVP variant of the Lister strain of vaccinia virus. Using the plaque method, recombinant strains were selected on the BHK-21 line [7] and amplified as described previously [24].

Assessment of sensitivity of tumor cell lines to vaccinia virus strains

The sensitivity of tumor cell lines to vaccinia virus strains was assessed using resazurin staining. For this purpose, cells were spread on 96-well plates and infected with 10-fold serial

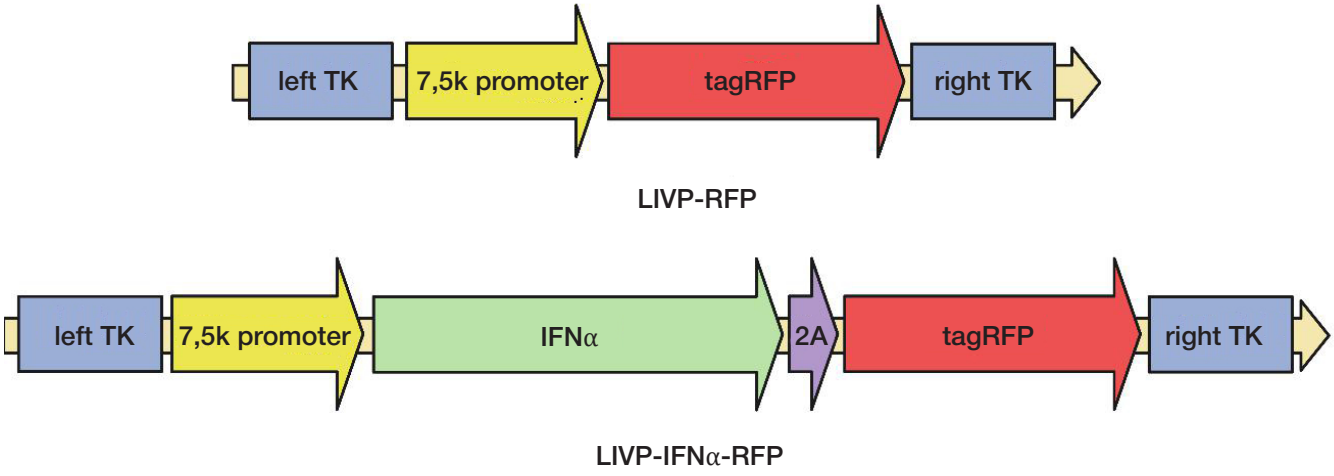

Fig. 1. Schematic illustration of the plasmid design

dilutions of the virus. Virus-free medium was used as a control. The cells were incubated at 37 °C, 5% CO_2. Cytotoxicity was assessed 72 h after infection. The test is based on the ability of viable cells to convert resazurin to resorufin by redox reactions. Cells were incubated with the dye for 4 h. The fluorescence level was measured at 590 nm using an excitation wavelength of 560 nm on a CLARIOstar microplate reader (BMG Labtech, Germany). Based on obtained data, the number of live cells was counted as a percentage relative to control, with $TCID_{50}$ values calculated.

Assessment of viral kinetics by flow cytometry

RFP expression in infected cells correlated with virus replication. Cell lines were seeded into 24-well plates (1x 10^5 /well). Cells were infected with LIVP-hIFNα (MOI of 1, 0.1). Cells were collected 24, 48, 72, 96 h after infection for analysis by flow cytometry. Samples were analyzed by red fluorescence detection in PE channel using a BD LSR Fortessa cytofluorimeter (Beckman Dickinson, Franklin Lakes, NJ, USA) with 10,000 events per sample.

Evaluation of the functional activity of interferon expressed by viruses

Vesicular stomatitis virus (Indiana strain) is a strain sensitive to the antiviral state of cells having no cytopathic effect (CPE) on cells with a functional interferon response system after treatment with interferon. IFNα-containing supernatant was obtained from virus-containing medium taken from BHK-21 cells infected with LIVP-hIFNα strain or HEK293T line in case of the LIVP-mIFNα strain. Virus-containing medium was collected after 48 h, centrifugated at 4 °C 4000 rpm for 20 min, filtered through 0.22 μm filter, and a supernatant containing IFNα was obtained. Only trace amounts of vaccinia virus remained in the filtered supernatant. The tumor cell line was treated in three repeats with recombinant human IFNα2β (Pharmaclone, Russia) or mouse recombinant interferon alpha (752802, Biolegend, USA) at different concentrations, and the supernatant with IFNα interferons at different concentrations. Supernatant from cells infected with LIVP-RFP and cells without interferon treatment were used as control. One day after interferon treatment, the absence of viral infection was checked using fluorescence

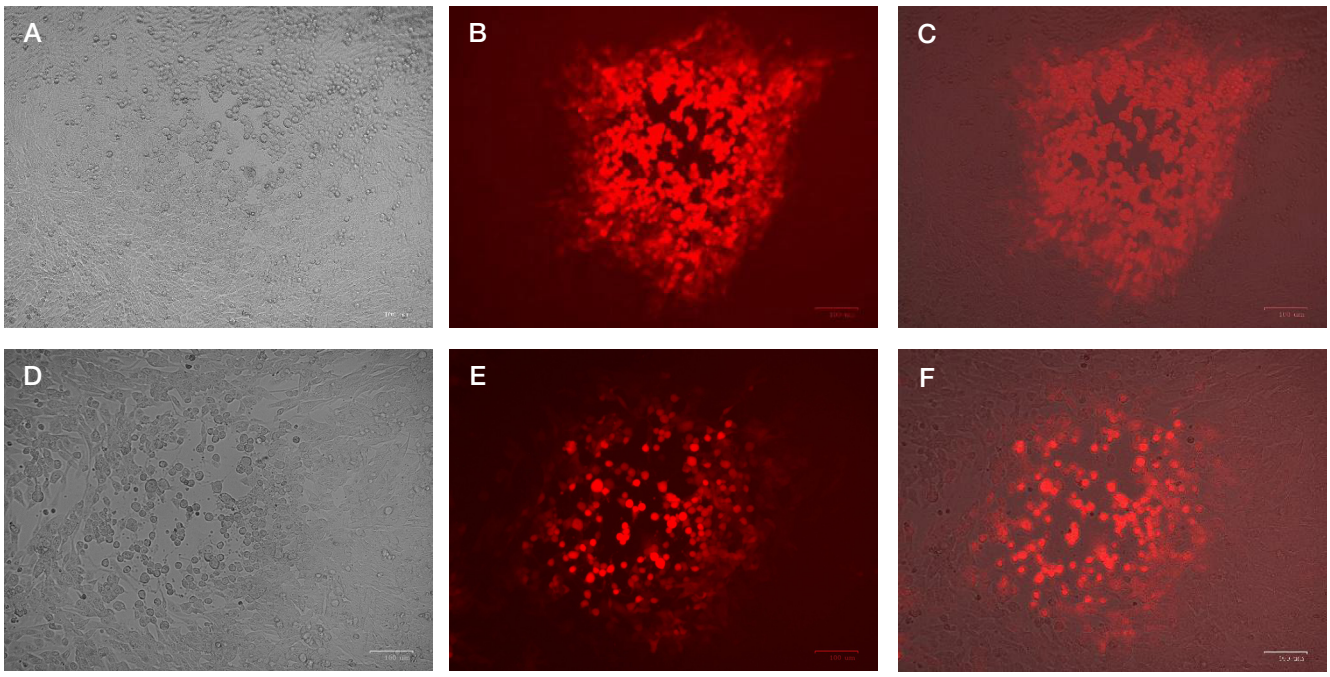

Fig. 2. Photographs of BHK-21 cells 24 h after infection with LIVP-mIFNα (**A–C**) and LIVP-hIFNα (**D–F**) (**A** and **D** — light field; **B** and **E** — red fluorescent channel; **C** and **F** — combined image). 200x magnification; scale bar = 100 μm

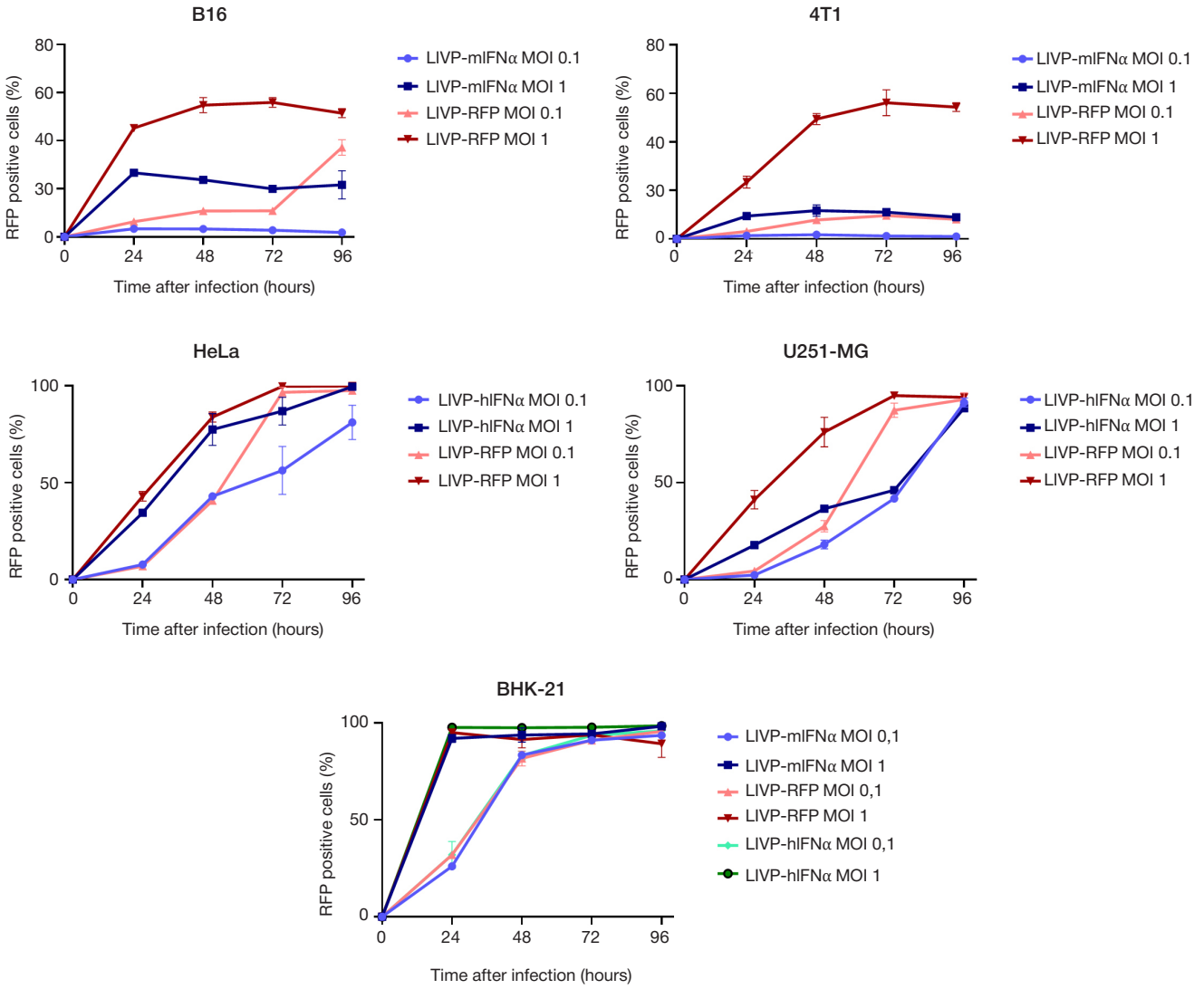

Fig. 3. Evaluation of virus replication kinetics 24, 48, 72 and 96 h after infection with recombinant VV strain on different cell lines

microscope. 24h after supernatant/interferon treatment, cells were infected with VSV in different multiplicities (100, 10, 1, 0.1, 0.01, 0.001). After 24 h, the cytopathic effect was assessed relative to the uninfected VSV control. And $TCID_{50}$ was calculated using the Reed–Muench method.

Modeling of subcutaneous melanoma in C57Bl/6 mice, virus administration

Female (n = 12) and male (n = 12) C57Bl/6 mice at 6-8 weeks of age were injected subcutaneously, above the posterior thigh, with 1x106 B16 cells to model the melanoma. Each day, tumor growth and development were assessed by visual inspection and measurement of tumor nodule size using a caliper. On the seventh day, mice with confirmed growth of subcutaneous tumor allografts were randomly assigned into three groups, LIVP-mIFNα (n = 8), LIVP-RFP (n = 8), and control (n = 8), with 4 females and 4 males in each group. LIVP-mIFNα and LIVP-RFP virus-containing solutions of 5×10^6 BOU/mL were dissolved in 100 µL PBS and injected intravenously into mice of the respective groups on day 7 and day 10 after tumor implantation. Animals in the control group were injected with saline at the same time points. The animals were observed during 24 days, and tumor size was measured to assess the dynamics of tumor growth or regression before treatment and every 2 days thereafter. Tumor volume was calculated using the formula:

$$V = \frac{a \times b^2}{2}, \quad (3)$$

where a is the smaller of the two orthogonal tumor measurements, b is the second orthogonal measurement. The average tumor volume (Vcp) was calculated in each group.

Histologic and immunohistochemical analysis

Histologic analysis was performed using three additional groups of C57Bl/6 mice that were injected with LIVP-mIFNα (n = 3), LIVP-RFP (n = 3), and saline (n = 3) in the same manner. On the 24th day after tumor implantation (14^{th} day after the last virus injection), the animals were deeply anesthetized by intraperitoneal injection of a prohibitive dose of propofol; after apnea occurred, the mice were euthanized by dislocation of the cervical vertebrae; subcutaneous tumor allografts were carefully isolated together with the surrounding tissue to avoid damage to cystic cavities and placed in a tenfold volume of 10% neutral buffered formalin for 72 hours. Fixed tissues were dehydrated in ethanol solutions of ascending concentration (70%, 80%, 96%), then in isopropanol and O-xylene, and then embedded in paraffin. Paraffin sections 3–5 µm thick were

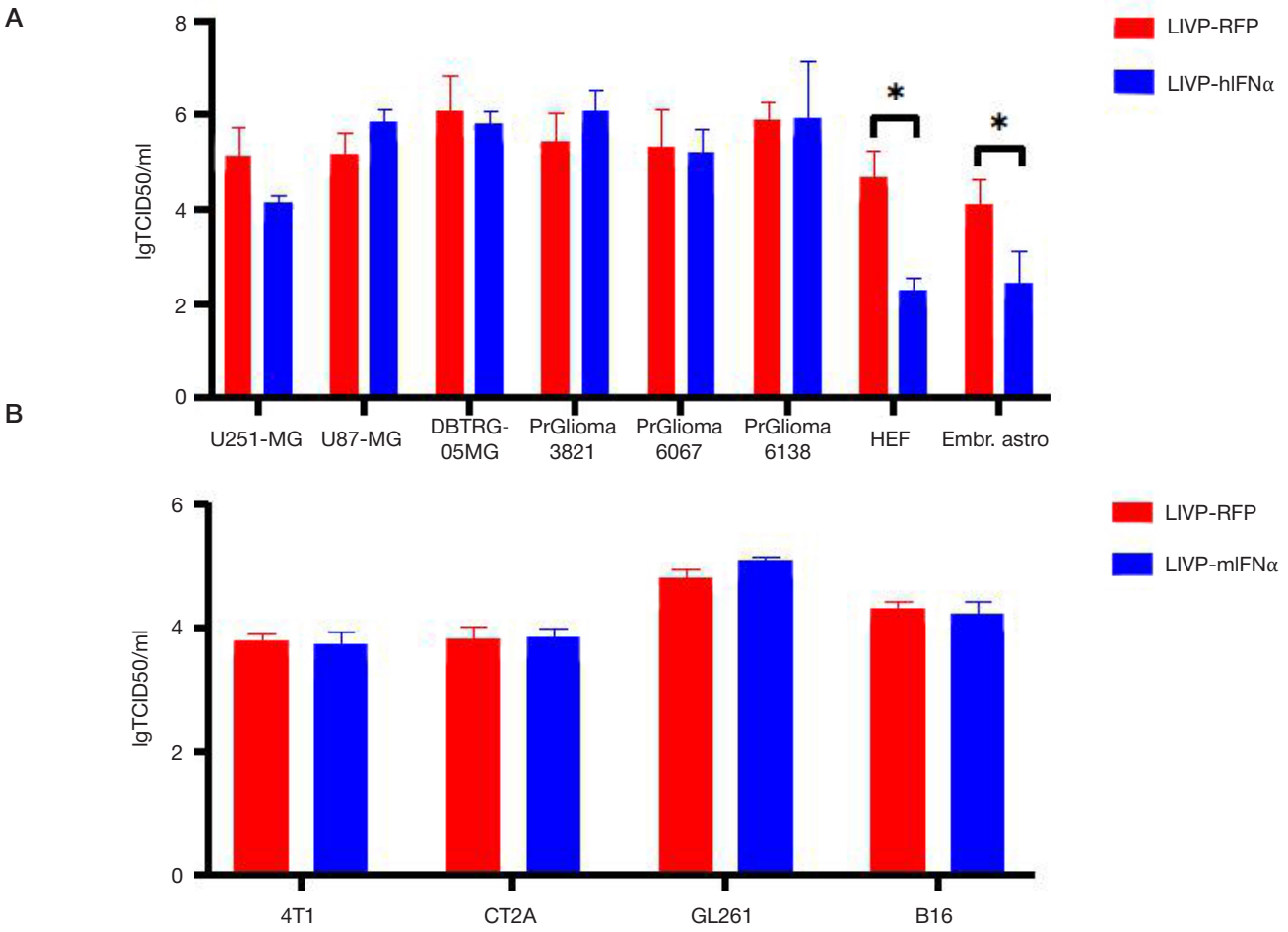

Fig. 4. Sensitivity of tumor and normal cell lines to vaccinia virus expressing tagRFP and a) human IFNα and b) mouse IFNα. X-axis: tumor cell lines, Y-axis: lgTCID$_{50}$/ml

prepared using a rotary microtome and mounted on slides. The sections were deparaffinized in O-xylene, isopropanol and ethanol before staining. For histologic examination, slices were stained with hematoxylin for 10 min, washed with distilled water, then with running tap water and stained with eosin for 30 s. Slices were rehydrated in 96% ethanol, isopropanol, and O-xylene and mounted using Vitrogel medium and 0.15 mm coverslips. Immunoperoxidase staining of paraffin sections for immune cell markers was performed using Benchmark Ultra Immunostainer (Ventana, USA) with primary antibodies to CD4, CD8, CD56 (Roche, USA) and OptiView DAB IHC Detection Kit (Roche, USA) according to the manufacturer's protocols. The stained and coverslipped preparations were scanned using Leica Aperio GT450 DX scanner (Leica Biosystems, USA) and processed at 20x magnification using Aperio ImageScope software.

RESULTS

Construction of recombinant strains

The IFNα gene is one of the promising transgenes that can enhance virus tumor selectivity due to its immunostimulatory properties and inhibition of viral replication in normal tissues. In our work, we constructed recombinant strains of the LIVP biovariety expressing under the control of the p7.5k VV IFNα promoter (human or mouse) as part of a bicistronic cassette with the red fluorescent protein RFP (Fig. 1). The use of this early promoter allowed to achieve a high level of interferon expression in tumor cells.

For further cultivation of the recombinant vaccinia virus strain, BHK-21 cell culture was used due to its high sensitivity to vaccinia virus. The recombinant variants expressed fluorescent protein (Fig. 2). The correctness of transgene expression was confirmed by Sanger sequencing of the amplicons of transcripts flanked by regions of the thymidine kinase gene of the vaccinia virus.

The efficiency of virus replication in different tumor lines was determined by flow cytometry 24, 48, 72, and 96 h after infection. Human HeLa adenocarcinoma and U251-MG glioblastoma, mouse B16 melanoma and 4T1 adenocarcinoma tumor cell lines were tested. BHK-21 cell line was used as a reference cell line. HeLa and U251-MG cell cultures were infected at MOI 1 and 0.1 with LIVP-hIFNα, LIVP-RFP strains. B16 and 4T1 cell cultures were infected in the same multiplicities with LIVP-mIFNα, LIVP-RFP strains. At 24, 48, 72, and 96 h after infection, the percentage of RFP-positive cells was determined.

The recombinant LIVP-mIFNα strain was found to replicate poorly in 4T1 adenocarcinoma however, more than 20% replication was observed in B16 melanoma at MOI 1 (Figure 3). LIVP-hIFNα showed efficient replication in human tumor cell cultures. After 24 h, the HeLa line infected with different multiplicities showed the maximum infectivity among the lines tested: 34.44 ± 1.38% for MOI 1 and 7.82 ± 0.96% for MOI 0.1. In human glioblastoma culture LIVP-hIFNα virus replication in case of MOI 1 was almost 100% 96 h after infection. To confirm the hypothesis of tumor selectivity of LIVP-hIFNα against glioblastoma, we tested its cytopathic effect on expanded panel of the tumor lines.

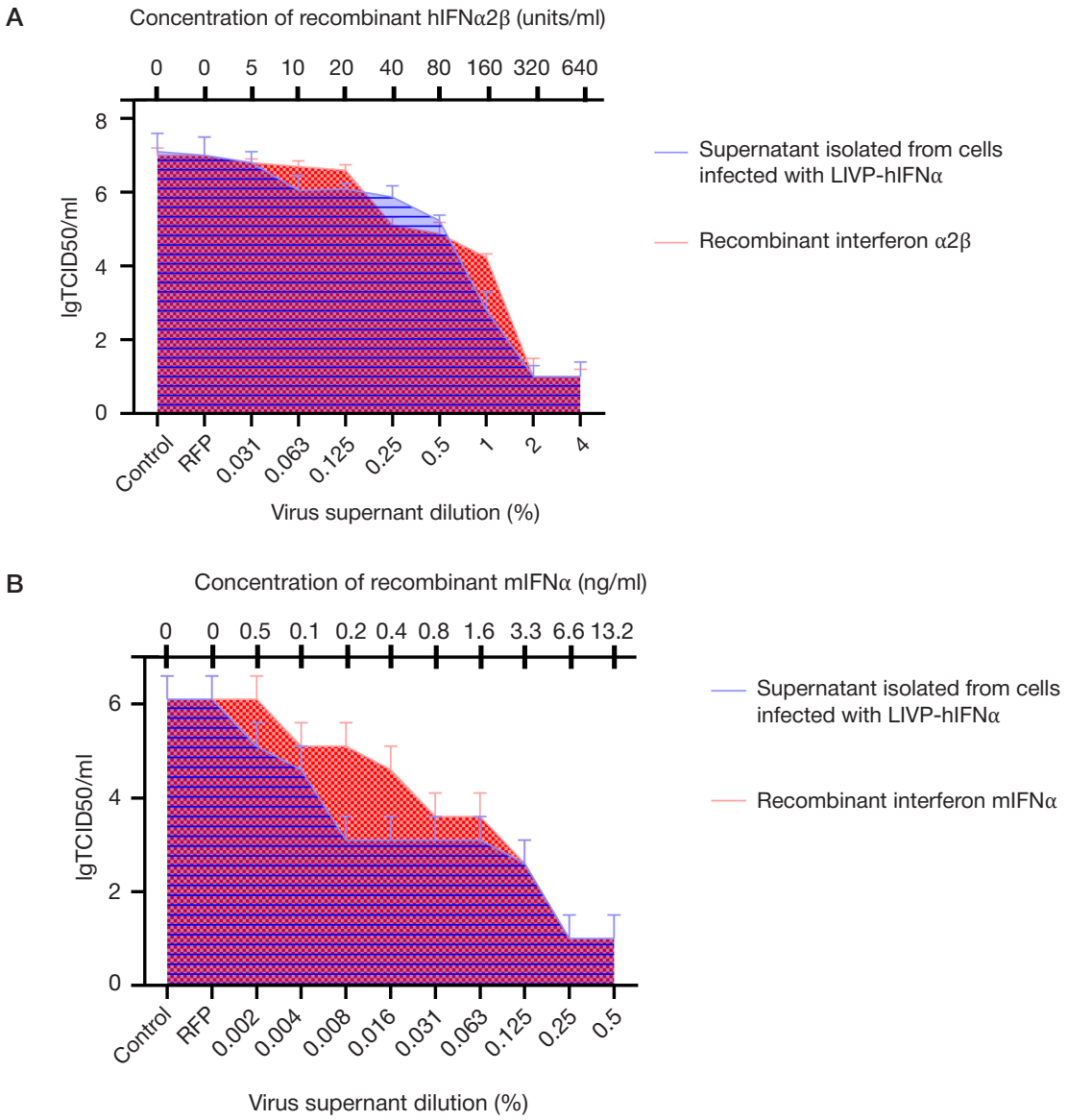

Fig. 5. Evaluation of functional activity of interferons expressed by LIVP-hIFNα strains on U87-MG cell line (**A**) and LIVP-mIFNα on the B16 cell line (**B**)

Evaluation of the cytopathogenic action of recombinant strains on a panel of tumor and normal cell lines

The oncolytic activity of vaccinia viruses expressing interferon alpha was examined in comparison with the tagRFP expressing strain on a panel of human and mouse tumor and normal cells. By titrating LIVP-hIFNα virus using Reed–Muench method and determining $TCID_{50}$ value, the sensitivity of glioblastoma cell lines U-87 MG, DBTRG-05MG, U251-MG, PrGlioma 3821, PrGlioma 6067, PrGlioma 6138 was evaluated (Fig. 4A) as well as of the two normal cell lines: human embryonic fibroblast line HEF, human embryonic astrocyte line Embr.astro. A strain expressing mouse interferon was tested on murine glioma cell lines CT2A and GL261 as well as on adenocarcinoma 4T1 and melanoma B16 (Fig. 4B).

Human glioblastoma lines were shown to be highly sensitivite to LIVP-hIFNα comparable to sensitivity of glioblastoma cells to the control virus expressing tagRFP (Fig. 4). Normal fibroblasts and astrocytes were found to have a lower sensitivity to vaccinia virus expressing hIFNα than to virus expressing tagRFP. The data suggest that the recombinant strain is more tumor selective compared to the control strain LIVP-RFP. Similar sensitivity levels of the cell lines to IFNα-expressing and tagRFP-expressing viruses are in turn an evidence of the persisting direct cytopathic activity of the interferon-expressing virus against tumor cells.

Assessment of the functional activity of interferons expressed by vaccinia virus

The functional activity of interferons expressed by vaccinia virus was tested in the common way using vesicular stomatitis virus (VSV). Vesicular stomatitis virus is sensitive to the antiviral state of cells having no cytopathic effect (CPE) on cells with a functional interferon response system after treatment with interferon. Tumor cell lines U87-MG and B16 were shown to have a functional interferon response system [25]. Cell cultures were treated with filtered supernatant taken from cells infected with LIVP-hIFNα or LIVP-mIFNα as well as manufactured recombinant IFNαs (Fig. 5). Cells without interferon treatment and cells treated with filtered supernatant isolated from LIVP-RFP-infected cells were used as controls.

For the human interferon experiment, we used supernatant isolated from the BHK-21 cell line infected with LIVP-hIFNα. For the experiment with LIVP-mIFNα strain, we used supernatant from the HEK293T cell line, since mouse and hamster interferons are cross-reactive.

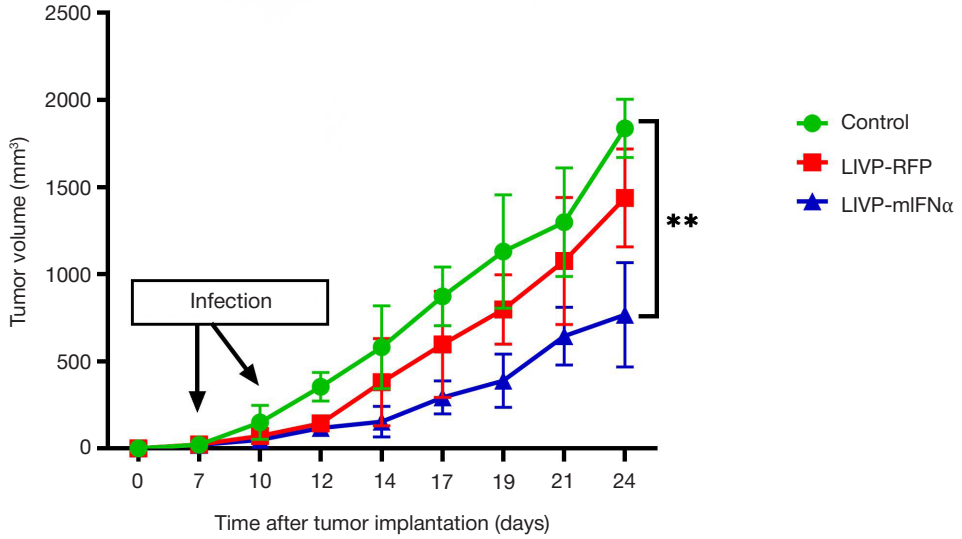

Fig. 6. Dynamics of tumor growth after LIVP-mIFNα injection

One day after interferon treatment, an absence of viral infection was checked using fluorescence microscope. 24 hafter interferon treatment, cells were infected with VSV in different multiplicities of infection.

Cytopathic effect (CPE) of vesicular stomatitis virus was not observed in cells treated with 320 units/ml recombinant interferon and for cells treated with 2% dilution of conditioned medium in the case of human interferon. For mouse interferon, CPE was not observed in cells treated with 6.6 ng/ml recombinant interferon or 0.25% dilution of conditioned medium.

Antitumor activity of LIVP-mIFNα *in vivo*

The oncolytic activity of LIVP-mIFNα vaccinia virus was investigated in C57Bl/6 mice with murine melanoma B16 (Fig. 6). Intravenous injections of virus were performed on day 7 and day 10 after tumor implantation. The control group of mice was injected with saline solution.

Tumor volumes were measured once every 2 days. Smaller tumor volumes were observed during treatment with vaccinia virus compared to the control group, especially when treated with virus expressing murine interferon alpha. The mean tumor volume was 1800 mm^3 in the control group, 1450 mm^3 in the group receiving LIVP-RFP, and 650 mm^3 in the group receiving virus with murine interferon alfa (p-value < 0.01). Tumor volume reduction was 64% as compared to the control group (Fig. 6).

Summarizing the results, we can conclude that the developed tumor-selective strain of vaccinia virus expressing functionally active murine interferon alpha has significant antitumor activity in the murine melanoma B16 model.

DISCUSSION

DNA-containing vaccinia virus is a promising platform for construction of selective recombinant oncolytic strains as up to 25 kb fragments can be inserted into the virus genome [6]. To date, recombinant VV strains containing sequences of cytokines, chemokines, oncotoxic proteins [11], bacterial immune attractants (e.g., the ligand of the innate immune system receptor TLR5 flagellin [10]), and other proteins

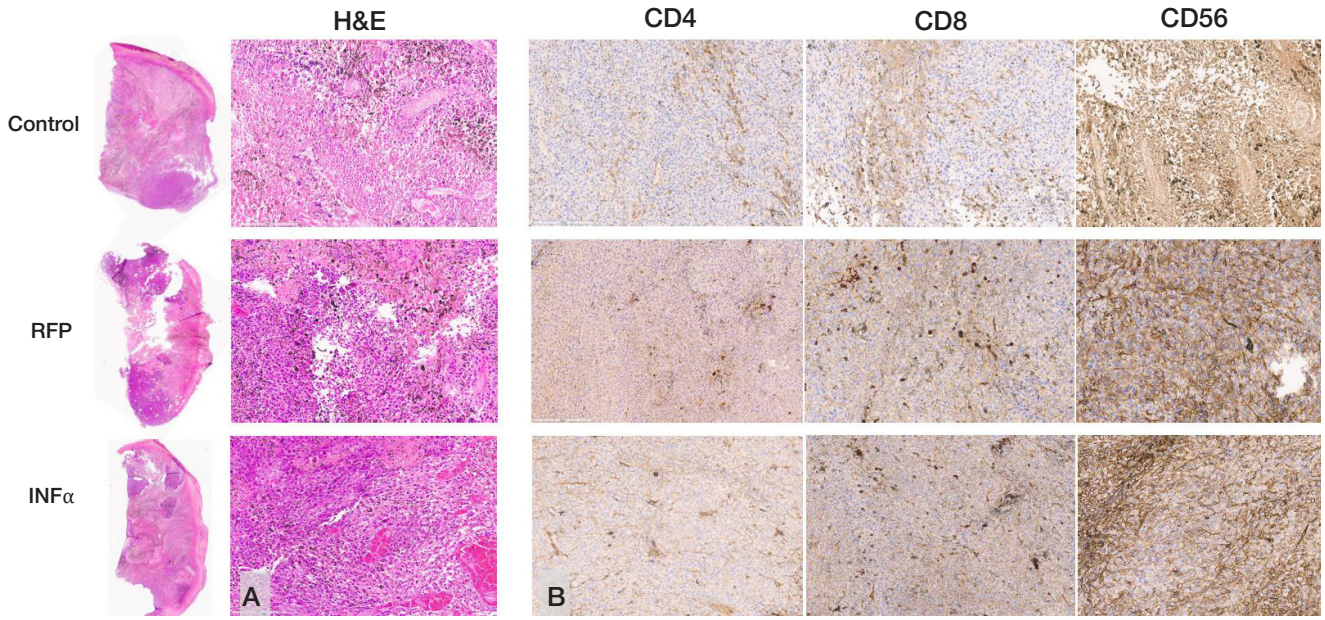

Fig. 7. Histological and immunohistochemical examination of tumor preparations 24 days after inoculation. **A**. Hematoxylin and eosin staining (general view of the tumor on the left side images and fragments at ×200 magnification on the right side images). **B**. Immunohistochemical staining for differentiation clusters: CD4, CD8, CD56. Representative preparations of animals from the control group, the group with LIVP-RFP, and the LIVP-mIFNα group, respectively, are shown from top to bottom.

activating antitumor immune response and recruiting cytotoxic immune cells to tumor have been created and successfully tested in preclinical and clinical studies [7, 26, 27].

A recombinant JX-594 virus strain with a deletion in the thymidine kinase (TK) gene containing the granulocyte-macrophage colony-stimulating factor (GM-CSF) sequence and lac-Z transgenes showed very encouraging results in phase II clinical trials of oncolytic therapy of colorectal cancer and melanoma liver metastases. JX-594 virus injected intravenously was detected in tumor tissue which was accompanied by IFNα secretion, induction of chemokines and activation of antitumor immune response [26].

In our study, in order to enhance tumor selectivity, we used a LIVP strain with a defective gene encoding thymidine kinase (TK), an enzyme essential for virus DNA synthesis which is hardly expressed during the normal cell cycle but is highly expressed in low-differentiated tumor cells [28]. We hypothesized that insertion of IFNα sequence into recombinant vaccinia virus could enhance the selectivity of virus replication in tumor cells where interferon signaling is often defective, and would also promote the activation of NK cells, macrophages, and cytotoxic T cells in response to tumor-specific virus replication and thus contribute to effective tumor eradication.

$TCID_{50}$ experiments with tumor and normal cell lines confirmed the enhanced tumor selectivity of LIVP-hIFNα and showed that normal cells have less sensitivity to vaccinia virus expressing IFNα than to virus expressing tagRFP. Decreased CPE of vesicular stomatitis virus after treatment of human glioblastoma and mouse adenocarcinoma cells with supernatants from LIVP-hIFNα and LIVP-mIFNα-infected cells, respectively, confirmed that IFNα expressed by infected cells is functionally active. HeLa cell line was shown to be the most sensitive to LIVP-hIFNα assuming the potential applicability of the recombinant virus for the therapy of adenocarcinomas. The observed sensitivity of U251-MG glioblastoma cells to LIVP-hIFNα seemed to be interesting as regards possible practical application of this virus in the therapy of glial tumors. The assumption was confirmed for five other lines of the tumor including primary glioblastoma cultures available. The data suggest that LIVP-hIFNα may be a promising oncolytic virus for glioblastoma therapy.

The results of *in vivo* experiments using a murine model of melanoma B16 confirmed our assumption of high tumor selectivity and enhanced oncolytic activity of LIVP-mIFNα. Decreased tumor volumes in animals administered intravenously with LIVP-mIFNα by 64% compared to control animals demonstrated the advantages of the recombinant VV inducing the secretion of functionally active IFNα by infected cells. Immunohistochemical assays showed a high level of infiltration of tumors treated with LIVP-mIFNα by $CD8^+$ T cells and NK cells. The mechanisms of the increased oncolytic activity of LIVP-mIFNα need to be further investigated however, according to the up-to-date publications and our own data related to other recombinant strains [7, 10] containing cytokine sequences, it can be assumed that there is an increased activation of NK cells and cytotoxic T cells around IFNα-secreting tumors.

CONCLUSIONS

We constructed new recombinant strains of vaccinia virus expressing mouse and human IFNα having oncolytic activity similar to the parent strain, with significant tumor selectivity shown for LIVP-hIFNα due to decreased sensitivity of normal cells to the virus. The functional activity of interferons expressed by recombinant LIVP-hIFNα and LIVP-mIFNα strains was confirmed *in vitro*. A significantly higher oncolytic activity of LIVP-mIFNα *in vivo* was demonstrated in a murine model of melanoma B16.

References

1. Fukuhara H, Ino Y, Todo T. Oncolytic virus therapy: A new era of cancer treatment at dawn. Cancer Sci. 2016; 107 (10): 1373–9.
2. Shakiba Y, et al., Recombinant Strains of Oncolytic Vaccinia Virus for Cancer Immunotherapy. Biochemistry (Mosc). 2023; 88 (6): 823–41.
3. Shvalov AN, et al. Complete Genome Sequence of Vaccinia Virus Strain L-IVP. Genome Announc. 2016; 4 (3).
4. Zonov E, et al. Features of the Antitumor Effect of Vaccinia Virus Lister Strain. Viruses. 2016; 8 (1).
5. Hamad A, et al. Recent Developments in Glioblastoma Therapy: Oncolytic Viruses and Emerging Future Strategies. Viruses. 2023; 15 (2).
6. Naik S, Russell SJ. Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. Expert Opin Biol Ther. 2009; 9 (9): 1163–76.
7. Shakiba Y, et al. Oncolytic therapy with recombinant vaccinia viruses targeting the interleukin-15 pathway elicits a synergistic response. Mol Ther Oncolytics. 2023; 29: 158–68.
8. Kirn DH, et al. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. PLoS Med. 2007; 4 (12): e353.
9. Park BH, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. Lancet Oncol. 2008; 9 (6): 533–42.
10. Shakiba Y, et al. Oncolytic Efficacy of a Recombinant Vaccinia Virus Strain Expressing Bacterial Flagellin in Solid Tumor Models. Viruses. 2023; 15 (4).
11. Kochneva G, et al. Apoptin enhances the oncolytic properties of vaccinia virus and modifies mechanisms of tumor regression. Oncotarget. 2014; 5 (22): 11269–82.
12. Tysome JR, et al. Lister vaccine strain of vaccinia virus armed with the endostatin-angiostatin fusion gene: an oncolytic virus superior to dl1520 (ONYX-015) for human head and neck cancer. Hum Gene Ther. 2011; 22 (9): 1101–8.
13. Crouse J, Kalinke U, Oxenius A. Regulation of antiviral T cell responses by type I interferons. Nat Rev Immunol. 2015; 15 (4): 231–42.
14. Gastl G, Huber C. The biology of interferon actions. Blut. 1988; 56 (5): 193–9.
15. Matveeva OV, Chumakov PM. Defects in interferon pathways as potential biomarkers of sensitivity to oncolytic viruses. Rev Med Virol. 2018; 28 (6): e2008.
16. Pikor LA, Bell JC, Diallo JS. Oncolytic Viruses: Exploiting Cancer's Deal with the Devil. Trends Cancer. 2015; 1 (4): 266–77.
17. Stojdl DF, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med. 2000; 6 (7): 821–5.
18. Feola S, et al. Oncolytic ImmunoViroTherapy: A long history of crosstalk between viruses and immune system for cancer treatment. Pharmacol Ther. 2022; 236: 108103.
19. Li R, et al. Using oncolytic viruses to ignite the tumour immune microenvironment in bladder cancer. Nat Rev Urol. 2021; 18 (9): 543–55.
20. Kaufman HL, Kohlhapp FJ, Zloza A. Oncolytic viruses: a new class of immunotherapy drugs. Nat Rev Drug Discov. 2015; 14 (9): 642–62.
21. Davola ME, Mossman KL. Oncolytic viruses: how "lytic" must they be for therapeutic efficacy? Oncoimmunology. 2019; 8 (6): e1581528.

22. Kirn DH, Thorne SH, Targeted and armed oncolytic poxviruses: a novel multi-mechanistic therapeutic class for cancer. Nat Rev Cancer. 2009; 9 (1): 64–71.
23. Vorobyev PO, et al. Comparative Efficiency of Accessible Transfection Methods in Model Cell Lines for Biotechnological Applications. Bulletin of RSMU. 2022; 3: 11–18.
24. Kochneva G, et al. Engineering of double recombinant vaccinia virus with enhanced oncolytic potential for solid tumor virotherapy. Oncotarget. 2016; 7 (45): 74171–88.
25. Lin AH, et al. Blockade of type I interferon (IFN) production by retroviral replicating vectors and reduced tumor cell responses to IFN likely contribute to tumor selectivity. J Virol. 2014; 88 (17): 10066–77.
26. Samson A, et al. Neoadjuvant Intravenous Oncolytic Vaccinia Virus Therapy Promotes Anticancer Immunity in Patients. Cancer Immunol Res. 2022; 10 (6): 745–56.
27. Vasileva N, et al. The Recombinant Oncolytic Virus VV-GMCSF-Lact and Chemotherapy Drugs against Human Glioma. Int J Mol Sci. 2024; 25 (8).
28. Shakiba Y, et al. Comparison of the oncolytic activity of recombinant vaccinia virus strains LIVP-RFP and MVA-RFP against solid tumors. Bulletin of RSMU. 2023; 2: 4–11.

Литература

1. Fukuhara H, Ino Y, Todo T. Oncolytic virus therapy: A new era of cancer treatment at dawn. Cancer Sci. 2016; 107 (10): 1373–9.
2. Shakiba Y, et al., Recombinant Strains of Oncolytic Vaccinia Virus for Cancer Immunotherapy. Biochemistry (Mosc). 2023; 88 (6): 823–41.
3. Shvalov AN, et al. Complete Genome Sequence of Vaccinia Virus Strain L-IVP. Genome Announc. 2016; 4 (3).
4. Zonov E, et al. Features of the Antitumor Effect of Vaccinia Virus Lister Strain. Viruses. 2016; 8 (1).
5. Hamad A, et al. Recent Developments in Glioblastoma Therapy: Oncolytic Viruses and Emerging Future Strategies. Viruses. 2023; 15 (2).
6. Naik S, Russell SJ. Engineering oncolytic viruses to exploit tumor specific defects in innate immune signaling pathways. Expert Opin Biol Ther. 2009; 9 (9): 1163–76.
7. Shakiba Y, et al. Oncolytic therapy with recombinant vaccinia viruses targeting the interleukin-15 pathway elicits a synergistic response. Mol Ther Oncolytics. 2023; 29: 158–68.
8. Kirn DH, et al. Targeting of interferon-beta to produce a specific, multi-mechanistic oncolytic vaccinia virus. PLoS Med. 2007; 4 (12): e353.
9. Park BH, et al. Use of a targeted oncolytic poxvirus, JX-594, in patients with refractory primary or metastatic liver cancer: a phase I trial. Lancet Oncol. 2008; 9 (6): 533–42.
10. Shakiba Y, et al. Oncolytic Efficacy of a Recombinant Vaccinia Virus Strain Expressing Bacterial Flagellin in Solid Tumor Models. Viruses. 2023; 15 (4).
11. Kochneva G, et al. Apoptin enhances the oncolytic properties of vaccinia virus and modifies mechanisms of tumor regression. Oncotarget. 2014; 5 (22): 11269–82.
12. Tysome JR, et al. Lister vaccine strain of vaccinia virus armed with the endostatin-angiostatin fusion gene: an oncolytic virus superior to dl1520 (ONYX-015) for human head and neck cancer. Hum Gene Ther. 2011; 22 (9): 1101–8.
13. Crouse J, Kalinke U, Oxenius A. Regulation of antiviral T cell responses by type I interferons. Nat Rev Immunol. 2015; 15 (4): 231–42.
14. Gastl G, Huber C. The biology of interferon actions. Blut. 1988; 56 (5): 193–9.
15. Matveeva OV, Chumakov PM. Defects in interferon pathways as potential biomarkers of sensitivity to oncolytic viruses. Rev Med Virol. 2018; 28 (6): e2008.
16. Pikor LA, Bell JC, Diallo JS. Oncolytic Viruses: Exploiting Cancer's Deal with the Devil. Trends Cancer. 2015; 1 (4): 266–77.
17. Stojdl DF, et al. Exploiting tumor-specific defects in the interferon pathway with a previously unknown oncolytic virus. Nat Med. 2000; 6 (7): 821–5.
18. Feola S, et al. Oncolytic ImmunoViroTherapy: A long history of crosstalk between viruses and immune system for cancer treatment. Pharmacol Ther. 2022; 236: 108103.
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21. Davola ME, Mossman KL. Oncolytic viruses: how "lytic" must they be for therapeutic efficacy? Oncoimmunology. 2019; 8 (6): e1581528.
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23. Vorobyev PO, et al. Comparative Efficiency of Accessible Transfection Methods in Model Cell Lines for Biotechnological Applications. Bulletin of RSMU. 2022; 3: 11–18.
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25. Lin AH, et al. Blockade of type I interferon (IFN) production by retroviral replicating vectors and reduced tumor cell responses to IFN likely contribute to tumor selectivity. J Virol. 2014; 88 (17): 10066–77.
26. Samson A, et al. Neoadjuvant Intravenous Oncolytic Vaccinia Virus Therapy Promotes Anticancer Immunity in Patients. Cancer Immunol Res. 2022; 10 (6): 745–56.
27. Vasileva N, et al. The Recombinant Oncolytic Virus VV-GMCSF-Lact and Chemotherapy Drugs against Human Glioma. Int J Mol Sci. 2024; 25 (8).
28. Shakiba Y, et al. Comparison of the oncolytic activity of recombinant vaccinia virus strains LIVP-RFP and MVA-RFP against solid tumors. Bulletin of RSMU. 2023; 2: 4–11.