RESEARCH PAPER

Single intranasal immunization with a high dose of influenza vector protects against infection with heterologous influenza virus and SARS-CoV-2 in ferrets and hamsters

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ABSTRACT

BACKGROUND: The challenge of vaccine effectiveness against viruses that undergo constant antigenic changes during evolution is currently being addressed by updating vaccine formulations to match circulating strains. However, this approach proves ineffective if a virus undergoes antigenic drift and shift, or if a new virus, such as SARS-CoV-2, emerges and enters circulation. Hence, there is a pressing need to develop universal vaccines that elicit a T-cell immune response targeting conserved antigenic determinants of pathogens.

OBJECTIVE: To develop a vaccine candidate against influenza virus and coronavirus based on an attenuated influenza vector.

METHODS: In pursuit of this objective, we developed a recombinant influenza vector named FluCoV-N. It incorporates attenuating modifications in the *ns1* and *nep* genes and expresses the N-terminal half of the N protein (N_{1-209}) of the SARS-CoV-2 virus. To assess the vector's protective efficacy against influenza, ferrets were infected with heterologous influenza A/Austria/1516645/2022 (H3N2) virus on the 25th day after a single immunization with 9.4 $\log_{10}EID_{50}$ of the studied vector. To test protection against coronavirus, hamsters were immunized once with the vector at a dose of 8.2 $\log_{10}EID_{50}$ and challenged with SARS-CoV-2 virus 21 days later.

RESULTS: As a result of modifications to the NS genomic segment, the constructed vector acquired a temperature-sensitive (ts) phenotype and demonstrated a heightened ability to induce type 1 interferons. It was harmless to animals when administered intranasally at high doses exceeding 8.0 $\log_{10}EID_{50}$. In ferrets, a single intranasal immunization with FluCoV-N accelerated the resolution of infection caused by heterologous influenza H3N2 virus. Similar immunization in hamsters led to a 10,000-fold reduction in SARS-CoV-2 viral titers in the lungs on the second day after challenge and reduced pathology in the lungs of animals.

CONCLUSION: A single intranasal immunization with the FluCoV-N vector protected from heterologous influenza or SARS-CoV-2 viruses in ferrets and hamsters.

INTRODUCTION

Effective control of respiratory viral pathogens necessitates the development of new vaccines capable of targeting rapidly mutating viruses. The SARS-CoV-2 pandemic underscored the efficacy of mass vaccination using mRNA and adenoviral vector vaccines [1]. However, the COVID-19 outbreak also revealed SARS-CoV-2's ability to evade neutralizing antibodies induced by vaccination or natural infection [2]. With the emergence of SARS-CoV-2 variants such as Delta and Omicron, the diminished efficacy of COVID-19 vaccines has become evident, resulting in breakthrough infections among vaccinated individuals [3, 4]. Continuous circulation of SARS-CoV-2 in the population has led to the emergence of antigenic variants, highlighting the necessity for regular updates to vaccines targeting the S protein.

Prevention of SARS-CoV-2 infection by vaccination in this regard is similar to protection against influenza infection, where influenza vaccines require constant updating due to antigenic drift and shifts in the influenza virus. Considering the potential for global epidemics and pandemics caused by influenza virus, resulting in numerous deaths and severe complications [5, 6], annual vaccination with updated vaccines remains one of the most effective methods for combating this infection [7].

Currently, two types of seasonal vaccines are used to prevent influenza: inactivated vaccines administered intramuscularly, and intranasal live attenuated vaccines. Each vaccine requires a combination of 3−4 contemporary influenza viruses. Most licensed seasonal influenza vaccines are inactivated and produced using various technologies, including whole virion, split, or subunit vaccines. Live vaccines are licensed in the USA and Europe for the vaccination of children and are sublicensed in China and India [8, 9]. Live attenuated cold-adapted vaccines are administered intranasally (i.n.) to ensure the formation of local immunity in the upper respiratory tract of vaccinated individuals. To increase the effectiveness of influenza vaccination, research is underway to create a universal vaccine that provides cross-reactive (heterosubtypic) immunity, protecting against new drift and shift variants of influenza virus [10]. When developing a universal influenza vaccine, the live intranasal vaccine offers more advantages. Unlike inactivated vaccines, it induces mucosal immunity in the respiratory tract mediated by class A secretory immunoglobulins (sIgA) and resident memory T (T_{rm}) cells. This provides broader cross-reactive protection due to interaction with conserved epitopes of viruses [11-14].

Previously, we showed that live attenuated influenza vectors could be constructed by modifying the length of the NS1 protein. [15-17]. Immunization with viruses containing a truncated NS1 protein leads to efficient stimulation of innate immune cells associated with the formation of polyfunctional T lymphocytes recognizing a wide range of conserved influenza epitopes [18, 19]. Vaccine candidates with a modified *ns1* gene have demonstrated protection against both homologous and heterologous influenza viruses in various experimental models [20-25]. A live attenuated vaccine, in which the sequence encoding the receptor-binding domain (RBD) of SARS-CoV-2 was inserted instead of the *ns1* gene, induced systemic and mucosal immune responses and inhibited the replication of SARS-CoV-2 in a mouse model [26]. Phase I and II clinical trials have demonstrated the safety and immunogenicity of this vector in adult volunteers [27].

As part of our endeavor to innovate in the field of influenza virus vaccine development, we engineered a novel vector derived from influenza A/Puerto Rico/8/1934 (H1N1) (PR/8/34) virus. This vector features a partial deletion in the *ns1* gene and incorporates a heterologous *nep* gene from influenza A/Singapore/1/57 (H2N2) virus (PCT patent WO 2014/168522). These genetic modifications render the virus highly attenuated, exhibiting a temperature-sensitive (ts) phenotype and an enhanced cytokine production capacity typical of cold-adapted vaccine strains and NS1 influenza virus mutants, respectively [28, 29]. Moreover, this vector enables the insertion of foreign sequences into the open reading frame of the NS1 protein.

To evaluate the efficacy of our vaccine vector platform against COVID-19, we introduced the SARS-CoV-2 N protein sequence into the vector, fused to the C-terminal end of the truncated NS_{1-124} influenza virus protein. Despite significant genome modifications, the vaccine vector exhibited robust growth, reaching up to 9.5 $\log_{10}EID_{50}$/ml (50% embryo infective doses) in 10-day-old embryonated chicken eggs (CE). Notably, the vector's high degree of attenuation enabled intranasal immunization of animals at doses exceeding 8.0 $\log_{10}EID_{50}$/ml without any observed toxic effects.

This study aimed to assess the efficacy of the FluCoV-N vector against heterologous influenza H3N2 virus or SARS-CoV-2 (Wuhan-like strain) infection following a single intranasal immunization in ferrets and hamsters.

MATERIALS AND METHODS

Viruses

A/Austria/1516645/2022 (H3N2) virus was obtained from the collection of the Institute of Virology (Vienna,

Austria). The virus was isolated from an influenza patient and passaged five times on Vero cells in serum-free OptiPRO medium (Gibco, USA) at 37℃ and 5% CO_2 with the addition of 2 mM GlutaMAX™ (Thermo Fisher Scientific, USA) and 1 mg/ml trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone, TPCK-treated, Sigma-Aldrich, USA), as described previously [30].

The laboratory strain of SARS-CoV-2 (Wuhan-like virus; GenBank: MW161041.1), isolated from a nasopharyngeal smear of a COVID-19 patient and propagated on Vero CCL81 cells, was used to challenge vaccinated hamsters. The virus was cultivated at 37℃ in DMEM medium (Gibco, USA) with L-glutamine (300 μg/ml) (Sigma, USA), glucose (4.5 g/l), 5% fetal calf serum (FCS, Gibco, USA), and gentamicin (40 μg/ml) in an atmosphere of 5% CO_2. This strain underwent 20 consecutive passages and caused a pronounced cytopathic effect in the cells. Virus aliquots were prepared and stored at -80℃.

Cell cultures

The epidemic influenza strain was isolated and cultivated in Vero cells obtained from the American Tissue Culture Collection (ATCC). Human alveolar basal epithelial carcinoma cells A549 (provided by J. Seipelt, Austria) were cultured in MEM in the presence of 10% FCS (Gibco, USA) and 2 mM L-glutamine (Sigma, USA).

To titrate the lung suspension of hamsters, we used Vero cells cultivated in DMEM growth medium (Gibco, USA) in the presence of 10% heat-inactivated FCS (PanEko, Russia), 2 mM L-glutamine (Sigma, USA), and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin). The maintenance medium contained all the above ingredients and 2% FCS. The cells were incubated at 37℃ and 5% CO_2.

Vector design

The recombinant influenza virus FluCoV-N was constructed using the reverse genetics method [31, 32]. The following synthetic plasmids (GeneArt, Germany) were used: pHW-PR8-HA, encoding HA of the influenza PR/8/34 (H1N1) virus (GenBank: CY033577); pHW-PR8-NA, encoding NA of PR/8/34 (H1N1) (GenBank: EF467823); pHW-PR8-PB2, encoding the PB2 segment of PR/8/34 (H1N1) (GenBank: AB671295); pHW-PR8-PB1, encoding the PB1 segment of PR/8/34 (H1N1) (GenBank: CY033583); pHW-PR8-PA encoding the PA segment of PR/8/34 (H1N1) (GenBank: AF389117); pHW-PR8-NP encoding the NP segment of PR/8/34 (H1N1) (GenBank AF389119); pHW-PR8-M encoding the M segment of PR/8/34 (H1N1) (GenBank AF389121).

Plasmid pHW-PR8-NN_c contained a chimeric *ns* gene encoding the truncated NS_{1-124} protein of influenza virus and the N-terminal part of the N_{1-209} protein of SARS-CoV-2. Vero cells were transfected with a complete set of plasmids encoding seven genomic fragments of influenza virus (PB2, PB1, PA, HA, NA, NP, and M), with the addition of a plasmid encoding the chimeric NS fragment. Cells for transfection were passaged in the OptiPRO medium (Gibco, USA) (no more than 150 passages) and 2 mM Gluta-Max I (Gibco, USA). The Nucleofector™ I/II/2b kit (Lonza, Switzerland) was used for transfection, and cells were seeded into 6-well plates and incubated at 37℃. The next day, trypsin was added at a final concentration of 1 mg/ml. Ten-day-old CE were infected with the collected supernatant and cultured for two days at 34℃. The genetic stability of the FluCoV-N virus was tested by 10 consecutive passages in CE, followed by control of the insert using polymerase chain reaction (PCR). Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions, followed by reverse transcription and PCR amplification. The QIAGEN® OneStep RT-PCR kit (Thermo Fisher Scientific, USA) was used for PCR. Primers F-NS-372: 5'-GTATCAGAATGGACCAGG and Len R: 5'-CTCTTGTTCCACTTCAAAT were used to control the presence of the transgene, and agarose gel electrophoresis was used for the amplification control. The control virus Flu-NS124, which did not contain the transgene insertion, was obtained using a similar method.

Immunofluorescence

The functional activity of the inserted protein was verified using immunofluorescence after staining with specific antibodies. A fresh monolayer of A549 cells was infected with the FluCoV-N vector at a multiplicity of infection of 2. Cells were incubated in 24-well plates at 34℃ for 24 h, permeabilized with Triton X100, and fixed with 4% paraformaldehyde for 20 min. The cells were then treated with 1% bovine serum albumin (BSA) overnight. SARS-CoV-2 N protein-specific antibodies (2019-nCoV, Nucleocapsid Antibody, Rabbit PAb, Antigen Affinity Purified, SinoBiological, China, Cat: 40588-T62) were diluted 1:200 in the blocking solution and added to the cells for 2 h. Primary antibody binding was visualized after 30 min of incubation using AlexaFluor 488 FITC-labeled anti-rabbit antibodies (Thermo Fisher Scientific, USA).

Vector propagation in CE

Pathogen-free CE were obtained from VALIO Biomedia (Munich, Germany). The virus was cultivated in 10-day

CE at 34°C for 48 h. Virus reproduction was determined by hemagglutinating activity with chicken erythrocytes. The virus was purified from the allantoic fluid and concentrated using tangential filtration in sucrose buffer, and then aliquoted and frozen. The final virus infectious titer was 9.4 $\log_{10}EID_{50}$/ml.

IFNα/β activity

Created by stable transfection of HEK293 cells with human *STAT2* and *IRF9* genes to produce an active IFN type I signaling pathway, HEK-Blue™ IFNα/β cells enable the detection of biologically active human IFN I by monitoring ISGF3 pathway activation. Cells were additionally transfected with the *SEAP* gene under the control of the IFNα/β-inducible ISG54 promoter. Stimulation of HEK-Blue™ IFNα/β cells with human IFNα or IFNβ activates the JAK/STAT/ISGF3 pathway and subsequently induces SEAP production. SEAP levels in the supernatant were determined using QUANTI-Blue™ (InvivoGen, France). The reporter cell line was maintained and cultured in DMEM (Gibco, USA) in the presence of 10% FCS (Gibco, USA) and 30 μg/ml blasticidin (100 μg/ml, Zeocin™, InvivoGen, France) and passaged until the monolayer reached 70-80%. A549 cells were infected with the viruses at a multiplicity of infection of 2. At 24 h post-infection, supernatants were collected and the presence of IFNα/β in reporter cell lines was determined.

Protective efficacy of the FluCoV-N vector against heterologous influenza virus in ferrets

The ferret study was conducted at VOXCAN (France). Specific pathogen-free (SPF) male ferrets (*Mustela putorius furo*), 11 weeks old, were obtained from Marshall Bioresources (USA). Animals were microchipped and tested for antibodies against the current influenza A/Noway/16606/2021(H3N2), A/Victoria/1/2020 (H1N1), B/Austria/1359417/2021, and B/Phuket/3073/2013 viruses. Animals were kept in special cages for ferrets in biosafety level 2 (BSL2) and BSL3 premises before and after infection, respectively. Room temperature was maintained at 18°C, and the light cycle was 12/12 h. The animals were provided ferret food and water ad libitum.

On the -25th day, ferrets were divided into groups of 6 and immunized i.n. with the FluCoV-N virus in a volume of 1 ml (9.4 $\log_{10}EID_{50}$/animal) under light isoflurane anesthesia. The animals in the control group received PBS. Two animals served as negative control. Twenty-five days later (Day 0), the animals were infected with 0.5 ml of the epidemic influenza A/Austria/1516645/2022 (H3N2) virus at a dose of 5×10^4 $TCID_{50}$/animal (50% tissue culture infective dose). After infection, ferrets

were assessed for clinical symptoms, including activity (1-3 points), sneezing (1-2 points), nasal discharge (1-2 points), discharge from the eyes (1-2 points), difficulty breathing (1-3 points), condition of the mucous surfaces, temperature (1-3 points), and weight. On Day -1 blood samples were collected from the animals, and nasal swabs were collected on Days -20, -18, -16, 2, 4, and 6 to check for the presence of the virus. Titration of nasal washes was performed on Vero cells, and the titer was expressed as $TCID_{50}$ as previously described [30]. On Day 6 of the study, animals were weighed and euthanized, and the lungs and spleens were collected for macroscopic monitoring and detection of the virus in homogenates.

Protective efficacy against SARS-CoV-2 in hamsters

The experiment was performed on Syrian golden hamsters (females and males in equal proportions) from one batch, weighing 50-60 g, obtained from the Pushchino nursery (Russia). The study was conducted at the Department of Virology of the Mechnikov Scientific Research Institute of Vaccines and Sera (Moscow, Russia). All the animals were examined before testing. Hamsters with external signs of malaise (impaired coordination, lethargy, or decreased appetite) were excluded from the study. Each animal was assigned an individual number ranging from 1 to 10 in the corresponding group. Animals were randomized based on their weight. Hamsters were kept in accordance with the rules of the European Convention for the Protection of Vertebrate Animals used for experimental and other scientific purposes (Strasbourg, 1986) and the guidelines for accommodation and care of animals in experimental biological clinics in Russia (Russian State Standard 33215-2014). The following conditions were maintained in the facility during the experiment: temperature of 20-24°C, 12/12 h light cycle, relative humidity of 45-65%, and ventilation without recirculation with an air exchange of 7-12 room volumes per hour.

The experiment included three groups of animals immunized with the FluCoV-N vector (Group 1), with the insert-free Flu-NS124 vector (Group 2), or with PBS (Control). Vaccination with both vectors was performed i.n. in a volume of 100 μl at an equivalent dose of 8.2 $\log_{10}EID_{50}$/animal under light anesthesia. Animals in the control group were administered PBS. On the 21st day after vaccination, the animals were infected i.n. with 50 μl of SARS-CoV-2 at a dose of 100 $TCID_{50}$/animal under light anesthesia. After vaccination and throughout the experiment, the animals were examined for clinical signs, including general condition, appetite for water and food, and activity. On the 2nd and 5th days after infection

with SARS-CoV-2, half of the animals were euthanized, and the lungs were collected. To analyze lung pathology, histological sections of the lungs were prepared and stained with hematoxylin and eosin (H&E). Collected organs were homogenized and centrifuged, and the supernatant was used for virus titration.

To determine the infectious virus titer in the lungs of hamsters, Vero CCL81 cells were seeded in 96-well plates (Costar, Fisher Scientific, UK) at an average density of 20,000 cells per well and grown in MEM in the presence of 5% FCS, 10 mM glutamine, and antibiotics (penicillin 100 units/ml and streptomycin 100 µg/ml) for 3 days until a complete monolayer was formed. Before infection with the virus, the cells were washed twice with serum-free DMEM. Ten-fold dilutions of each lung sample were prepared and 200 µl of each dilution was added to cells, followed by incubation at 37°C and 5% CO_2 for 5 days until the development of cytopathic effects in viral control cells. The virus titer was determined using the quantitative colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay and expressed as $\log_{10}TCID_{50}$/ml.

RESULTS

Structure and properties of the recombinant FluCoV-N vector

The FluCoV-N vector was engineered based on the PR/8/34 (H1N1) strain of influenza virus through truncation of the NS1 protein to 124 amino acids to ensure viral attenuation and immunogenicity (Fig. 1A) [33]. Additionally, the gene encoding the NEP protein of the PR/8/34 virus was substituted with the corresponding gene from another virus subtype, A/Singapore/1/57 (H2N2), resulting in 6 amino acid substitutions. The final vector had a ts phenotype, rendering the virus ineffective at replicating at 39°C (Fig. 1B), and exhibited high interferonogenic activity compared to the wild-type control PR/8/34 strain (Fig. 1C). The N-terminal region of the SARS-CoV-2 N protein gene, spanning 209 amino acid residues, was inserted into the NS1 protein's reading frame as a transgene. Expression of the transgene was confirmed by immunofluorescence using specific antibodies targeting the N protein (Fig. 1D, E). Intriguingly, the chimeric protein NS_{1-124}-N_{1-209} was predominantly detected in the cytoplasm of infected cells, in contrast to the NS1 protein and its previously obtained chimeric variations, which exhibited a more pronounced nuclear localization [34].

For preclinical studies, the FluCoV-N vector was amplified in 10-day-old CE. The virus-containing allantoic fluid was then purified from ovalbumin and concentrated using tangential flow filtration, as detailed in the Materials and Methods section. The resulting concentrate contained 9.4 $\log_{10}EID_{50}$/ml of the virus, which was diluted to working concentrations immediately before animal inoculation.

The protective efficacy of the FluCoV-N vector was evaluated against the heterologous strain of influenza A/Austria/1516645/2022(H3N2) virus in ferrets and against SARS-CoV-2 in hamsters.

Protective efficacy of the FluCov-N vector against influenza A/Austria/1516645/2022 (H3N2) virus in ferrets

Intranasal immunization of ferrets with the FluCoV-N vaccine vector at a dose of 9.4 $\log_{10}EID_{50}$/animal caused neither bodyweight changes, nor an increase in temperature, nor other clinical symptoms. In 25 days after immunization, the animals were challenged with a heterologous strain of influenza virus, A/Austria/1516645/2022 (H3N2). Ferrets immunized with the FluCoV-N vector did not show any significant weight loss after challenge. The body weight of the immunized ferrets continued to increase evenly until the last, 6th day of observation (Fig. 2). In contrast, the average weight of animals administered with PBS was significantly lower on the 2nd day post-challenge (both in the morning and evening): 3.2±0.8% (p<0.05) and 2.2±1.2% (p<0.001), respectively (Fig. 2).

The ferrets in the control group (PBS) also exhibited mild nasal discharge beginning on Day 2 post-challenge. A similar discharge was noted in animals vaccinated with the FluCoV-N virus, which disappeared by the 5th day of observation. However, starting on Day 5, three out of the six control animals showed an increase in clinical signs, including lethargic behavior, tremors, sneezing, nasal discharge, and rapid breathing (tachypnea) (Fig. 3). The clinical symptom indexes in the group of ferrets that received PBS were 2.3±2.9 and 1.8±2.6 on Days 5 and 6, respectively. In the group of animals vaccinated with the FluCoV-N virus, clinical manifestations disappeared from Day 5 (0.0±0.0).

The animals were euthanized on Day 6 post-challenge. A/Austria/1516645/2022 (H3N2) was detected in the lungs of half of the control animals (Fig. 4). It is important to mention that these animals showed an increase in clinical symptoms starting from the 5th study day. In only one of these animals, the virus was detected in the spleen with a titer of $1.59 \times 10^3 TCID_{50}$/ml. No virus was detected in the lungs or spleen of ferrets immunized with the FluCoV-N vector.
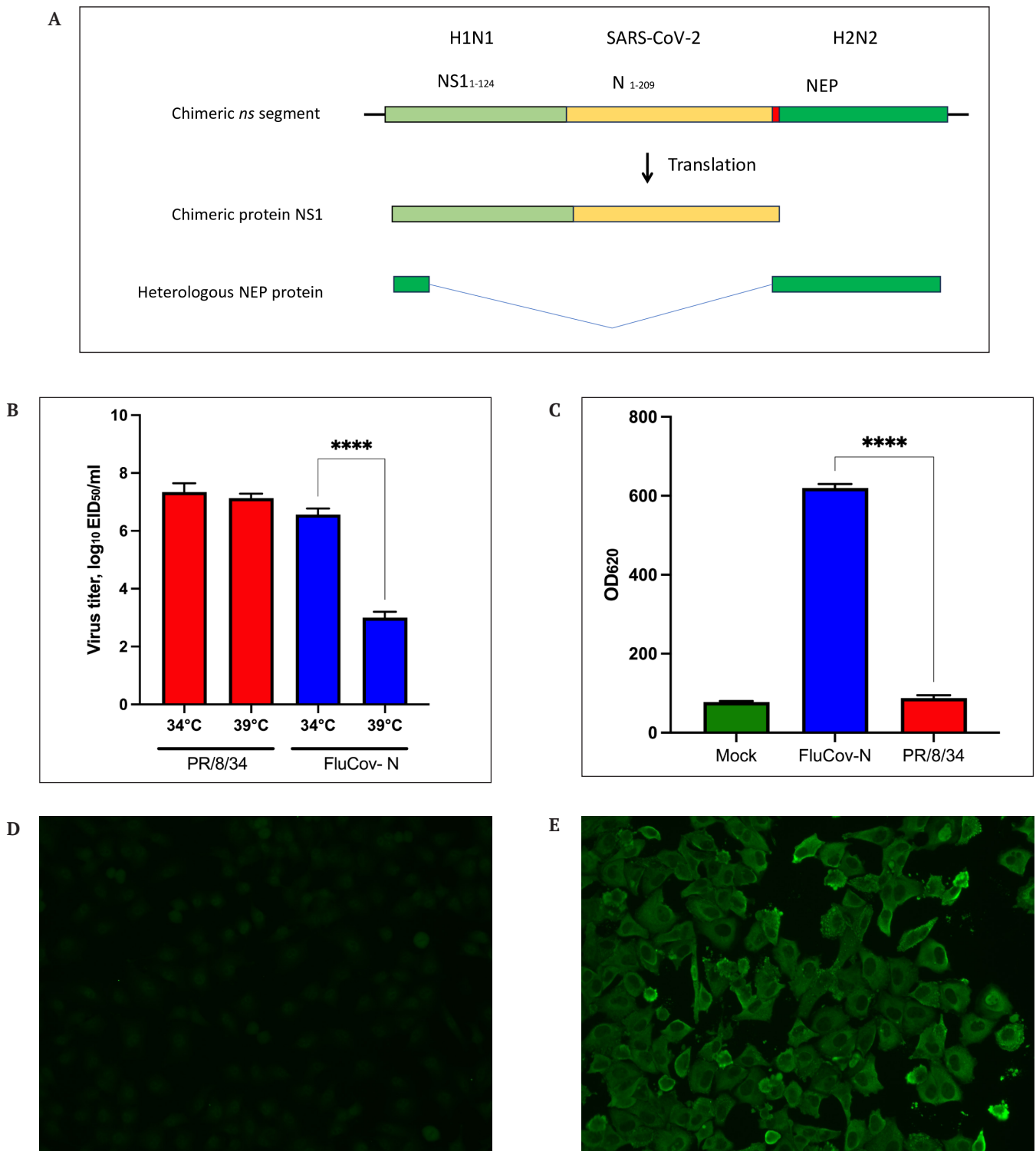
Fig. 1. Structure and properties of the FluCoV-N vector. **A.** Schematic representation of the structure of the recombinant *ns* segment of FluCoV-N. **B.** Reproduction activity of FluCoV-N and the PR/8/34 virus in CE at 34℃ and 39℃. **C.** Induction of type I IFN by FluCoV-N and PR/8/34 viruses in A549 cells, assessed in the cell supernatant 24 h post-infection by stimulation of HEK-Blue™ reporter cells. All data are presented as the mean of three experiments plus the standard deviation (mean±SD); (****) indicates a significant difference between the corresponding values according to Student's t-test (p<0.0001). **D, E**. Specific staining of intact Vero cells (**D**) or cells infected with the FluCoV-N vector (**E**) with antibodies to the SARS-CoV-2 N protein.
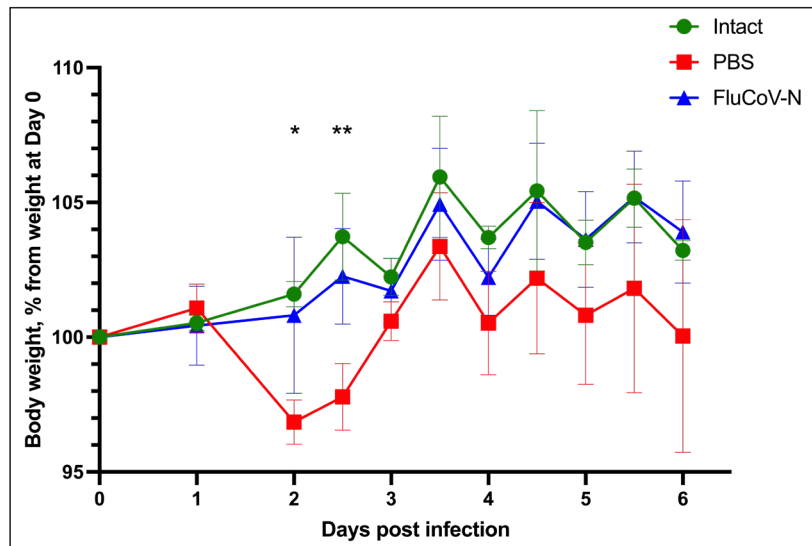
Fig. 2. Average body weight of immunized ferrets after challenge with the heterologous epidemic virus A/Austria/1516645/2022 (H3N2). Each experimental group included 6 animals, and the intact group – 2 animals. Data are presented as the % of average weight variation of six animals from their weight on Day 0 (mean±SD). Ferrets were immunized on Day -25 and challenged with the epidemic A/Austria/1516645/2022 (H3N2) virus on Day 0. The significant difference in the weight of ferrets immunized with PBS compared to the intact group according to two-way ANOVA is shown by stars, where (*) indicates $p<0.05$ and (**) indicates $p<0.01$.
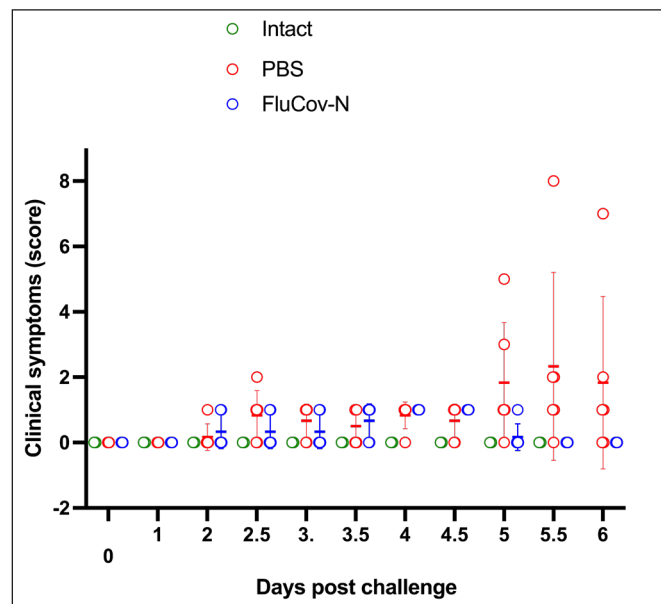

Fig. 3. Clinical symptoms in ferrets after challenge with the A/Austria/1516645/2022 (H3N2) virus. The experimental groups included six animals, and the intact group – two animals. Symptoms are expressed in points; data are presented as the mean±SD.

Protective efficacy of the FluCoV-N vector against SARS-CoV-2 in hamsters

Hamsters were immunized i.n. with 100 µl of FluCoV-N or Flu-NS124 (vector without the inserted transgene) at a dose of 8.2 $\log_{10}EID_{50}$/animal under light ether anesthesia. The animals in the control group received PBS. Vaccination caused no signs of malaise, weakness, decreased appetite, or other clinical symptoms of allergic or inflammatory origin. After vaccination, the animals steadily gained weight and no local reactions or deaths were observed.

On the 21st day after vaccination, animals were challenged i.n. with 50 µl of SARS-CoV-2 (Wuhan-like strain) at a dose of 100 $TCID_{50}$/animal under light anesthesia. On Days 2 and 5 after infection, the animals were euthanized, and their lungs and spleens were collected for virus isolation. On the 2nd day after challenge, the virus titer in the lungs of the control unvaccinated animals reached 6.45 $\log_{10}TCID_{50}$/0.1 ml,
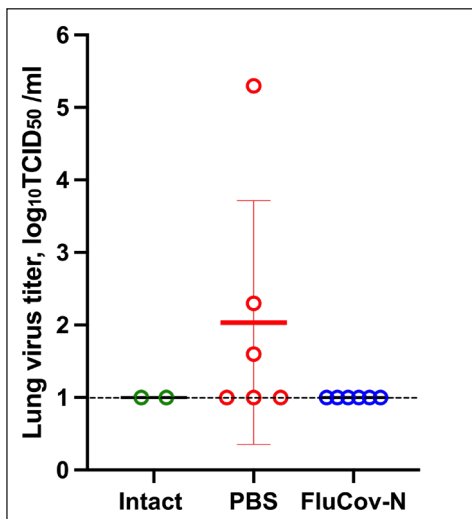
Fig. 4. Titers of the A/Austria/1516645/2022 (H3N2) virus in the lungs of ferrets on the 6th day after challenge. The experimental groups included six animals, and the intact group – two animals. Data are presented as mean±SD. The dotted line indicates the limit of titration (1.0 $\log_{10}TCID_{50}$/ml).

Fig. 5. Reproduction of SARS-CoV-2 in the lungs of hamsters after challenge with SARS-CoV-2 (Wuhan-like). Virus titers are presented as mean±SD. Number of animals in groups: control group – 5; group vaccinated with FluCoV-N – 4 (one of the hamsters was withdrawn from the study for reasons unrelated to the experiment); group vaccinated with Flu-NS124 – 5. The dotted line indicates the limit of titration equal to 1.5 $\log_{10}TCID_{50}$/0.1 ml. (****) indicates a significant difference compared to the control group immunized with PBS, according to the Student's t-test, $p < 0.0001$.

indicating acute infection. Immunization with FluCoV-N led to the lung titer of challenge virus decreasing significantly to 2.25 $\log_{10}TCID_{50}$/0.1 ml on Day 2 (Fig. 5). Immunization with the Flu-NS124 vector without transgene insertion did not prevent the reproduction of coronavirus in the lungs of hamsters, indicating the absence of nonspecific protection caused by the influenza vector. On the 5th day after infection, virus titers in the lungs of animals from all experimental groups (non-immunized and immunized with both vectors) did not differ significantly and were in the range of 4.0-4.5 $\log_{10}TCID_{50}$/0.1 ml.

Thus, vaccination with the FluCoV-N virus led to a significant ($p < 0.0001$) decrease in coronavirus titers in the lungs of hamsters on the 2nd day after challenge. However, on the 5th day, the titers of SARS-CoV-2 were equalized with those in the lungs of the control animals.

A histological analysis of the lungs of Syrian hamsters in the control group revealed pronounced inflammatory changes in all lobes, corresponding to viral interstitial pneumonia characteristic of SARS-CoV-2 infection according to morphological signs. The area of extensive confluent airless zones of pneumonia, along with adjacent areas of alveolitis, ranged from 30 to 60% of the total resection area of the lung lobe (Fig. 6A, B).

When studying the lung morphology in hamsters immunized with the FluCoV-N vector, a tendency for reduced tissue damage was observed regarding the severity of inflammatory changes in the respiratory and air-conducting parts of the lungs (Fig. 6). In two animals, the histological picture was significantly better than
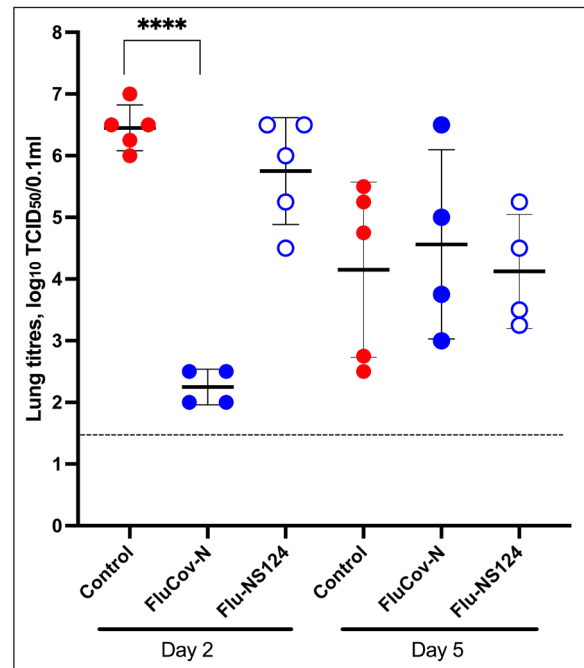
that of the control group hamsters (Fig. 6C, D.). Loci of dystrophic ciliated epithelial cells in the bronchi and bronchioles were rare, and damage to the interalveolar septa, as well as hemodynamic disturbances, were less pronounced compared to the control group. The number of apoptotic cells was also decreased in pneumonia foci. The degree of infiltration of interalveolar septa by macrophages and lymphocytes was moderate. Diffuse peribronchial and perivascular lymphoid-histiocytic infiltrates were scanty or, less often, moderate.

Thus, vaccination of Syrian hamsters with the FluCoV-N vector resulted in a reduction of coronavirus reproduction in the lungs during the early stage of infection. This was reflected in an improvement in the histological picture of the lungs on the 5th day after infection.

DISCUSSION

The effectiveness of vaccinations against viruses that undergo constant evolutionary antigenic changes is currently being addressed through the monitoring of viral isolates and updating the formulation of vaccines
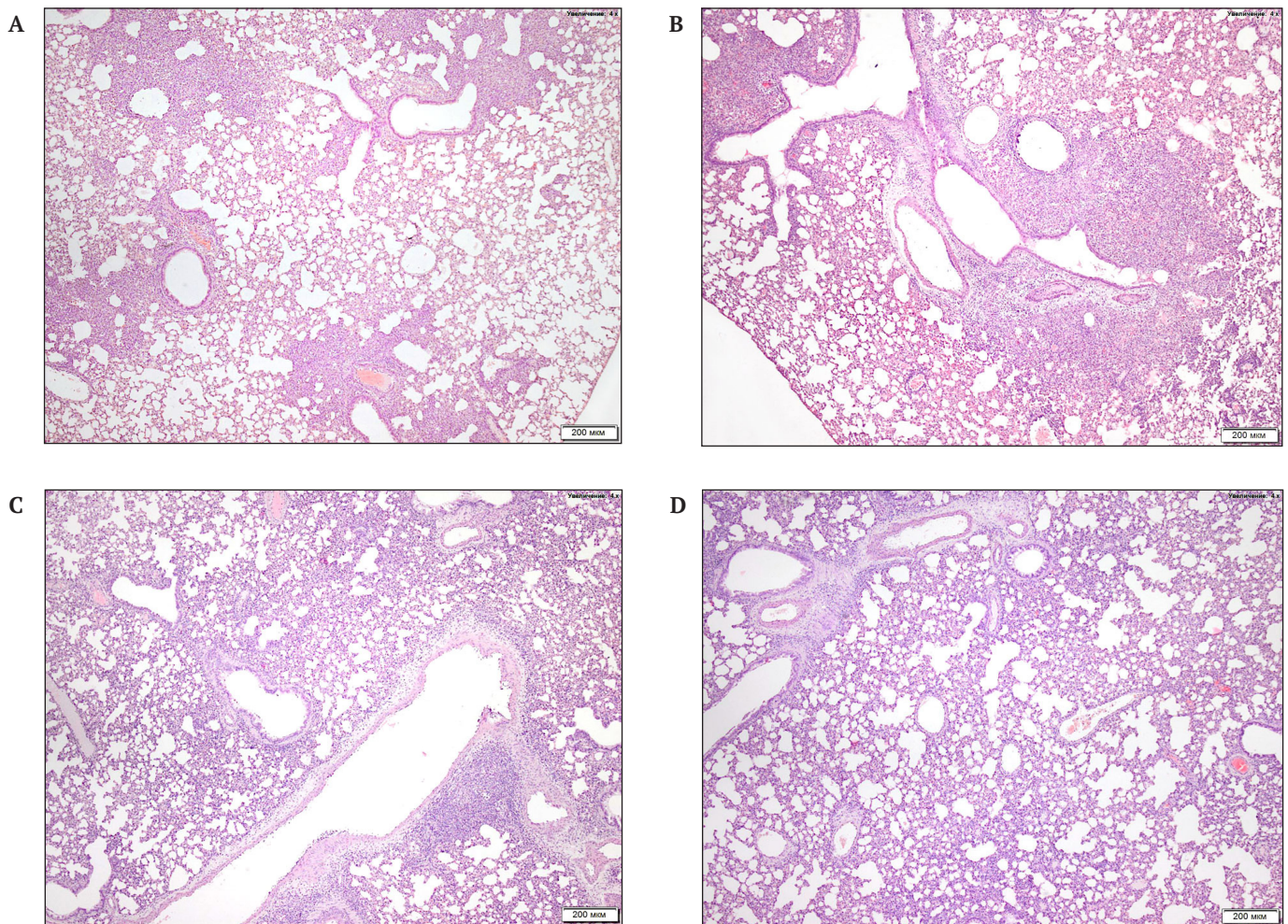
Fig. 6. Morphological changes in the lungs of hamsters on the 5th day after SARS-CoV-2 challenge. **A**, **B**. Lungs of hamsters from the control group immunized with PBS show pronounced inflammatory changes caused by SARS-CoV-2. **C**, **D**. Lungs of hamsters immunized with FluCoV-N after SARS-CoV-2 challenge. H & E staining, 40× magnification.

to ensure their consistency with circulating strains. Such a procedure is complex and expensive, making it unsuitable when new viruses suddenly appear in the human population, as seen recently with SARS-CoV-2 or influenza A viruses that cause pandemics. Therefore, there is an urgent need to develop vaccines that can serve as reserve vaccines. Such preparations should induce an immune response to conserved antigenic determinants and thus be universal. It is generally accepted that developing a universal vaccine based solely on an antibody-neutralizing response is challenging because viruses can easily overcome this barrier through conformational changes of the surface glycoproteins due to mutations or glycosylation [35]. The role of T cross-protective cellular response in controlling viral respiratory infections has been demonstrated in animal experiments [18, 19] and epidemiological observations in humans [36].

Resident memory T_{rm} cells associated with mucosal surfaces, particularly in the respiratory tract, play a special role in protection against respiratory infections.

Upon recognition of a conserved epitope of the pathogen at the gate of infection, these cells rapidly produce IFNγ, which stimulates the production of chemokines – CXCR3 receptor agonists. Therefore, various subpopulations of lymphocytes capable of destroying infected cells are attracted to the primary site of infection [37]. At a low multiplicity of infection, as occurs under natural conditions, these lymphocytes can eradicate the infected cells before the virus spreads widely in the respiratory tract [38]. During parenteral immunization, the formation of tissue-resident T_{rm} is challenging, prompting a continuous quest for intranasal vaccines to induce cross-protective mucosal immunity [39].

Another problem in modern vaccinology is the need for multiple vaccinations. Thus, vaccines based on mRNA or adenoviral vectors have been used up to 4-5 times to protect against the mutating virus during the SARS-CoV-2 pandemic [40].

The goal of this study was to create a vaccine against influenza virus and coronavirus suitable for

immunization in emergency conditions during a pandemic. Immunization with such a vaccine should create cross-protection and protect against a newly emerging virus. To achieve this, we constructed the FluCoV-N vector based on the PR/8/34 (H1N1) strain with modification in the *ns1* and *nep* genes. The high safety level of this virus enabled the vaccination of animals with doses exceeding 8.0 $\log_{10}EID_{50}$, without toxic effects or lung damage. This vector was supplemented with the sequence encoding the N-terminal half of the N protein of SARS-CoV-2, containing B and T cell epitopes [41]. The FluCoV-N vector can grow in CE to high titers while exhibiting genetic stability. Immunofluorescence analysis confirmed the intensive accumulation of the coronavirus N antigen in the cytoplasm of infected cells. The N protein is of increased interest among coronavirus proteins capable of inducing cross-reactive response because it is one of the most abundant proteins produced during viral replication and it shares a high degree of homology among coronaviruses [42]. The N protein is an important target of SARS-CoV-2-specific T cell responses during COVID-19. At the same time, SARS-CoV-2 N-specific CD8^{+} T cells are associated with the protection from severe disease, control of viral replication, and maintenance of antiviral efficacy against multiple virus variants (Alpha, Beta, Gamma, and Delta) for at least six months post-infection [43]. Therefore, an immune response against the N protein may protect against disease induced by different SARS-CoV-2 variants.

When studying the preventive effectiveness against influenza and SARS-CoV-2, we aimed to induce a protective immune response after a single i.n. immunization. For this purpose, the maximum dose of the viral vector for the particular species was used to immunize animals. To model protection against influenza, we used ferrets challenged with a modern strain of influenza virus, A/Austria/1516645/2022 (H3N2), which is evolutionarily distant from the H1N1 subtype vaccine virus. After challenge, ferrets rapidly resolved the infection, while half of the control group animals had increased clinical symptoms of influenza within six days before euthanasia. The virus was isolated from the lung and spleen tissue of the control group animals. It should be noted that infection with H3N2 was mild and did not cause febrile reactions in ferrets. This protective effect against heterologous strains should be confirmed in the future using a more virulent influenza virus, such as a highly pathogenic strain of avian influenza.

The most interesting aspect appears to be the protective effect of the FluCoV-N vaccination against the SARS-CoV-2 challenge in hamsters. The immunization with a high dose (8.2 $\log_{10}EID_{50}$) of the vaccine vector also did not cause any significant clinical manifestations of influenza infection in hamsters. In contrast to the ferret model of influenza infection, SARS-CoV-2 infection in hamsters was accompanied by an extremely high viral load in the lungs two days after challenge. Surprisingly, a single vaccination with the influenza vector reduced virus titers in the lungs of animals by approximately 10,000-fold on Day 2 after challenge. Histological studies on the 5th day after challenge revealed a tendency for a less pronounced lung pathology in vaccinated hamsters compared to the control animals. However, it should be noted that viral titers in the lungs of infected hamsters from control and vaccinated groups leveled off on the 5th day after infection, reaching approximately 4.0 $\log_{10}TCID_{50}$/0.1 ml of lung tissue. Thus, in contrast to the ferret model of influenza challenge, the decreased viral load induced by vaccination dissipated over time in coronavirus-infected hamsters. This phenomenon can be explained by the coronavirus carrying a whole set of nonstructural proteins that can suppress the cytotoxic activity of lymphocytes [44].

The discovered effect requires further research to optimize this vaccine vector by including genes encoding the protective epitopes of early viral proteins. A possible alternative may be the use of mucosal adjuvants to enhance the formation of immunity associated with the respiratory tract.

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