ANTIVIRAL ACTIVITY OF mRNAS ENCODING INTRACELLULAR SCFV ANTIBODIES AGAINST CONSERVED INFLUENZA VIRUS EPITOPES

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Monoclonal antibody therapy is one of the most promising approaches for effective influenza control. In this study, we evaluated the antiviral activity of exogenous mRNA-encoded single-chain variable fragment (scFv) antibodies, which are capable of binding viral antigens inside the cell with high affinity. Two influenza virus proteins, hemagglutinin (antibody FI6) and nucleoprotein (antibody 2/3), were chosen as targets. Each scFv encoded by mRNA was produced in two variants: one containing a signal peptide (SP) to direct secretion into the extracellular space (scFv-SP) and one lacking the signal peptide (scFv-WO) for cytosolic localization and function. These variants showed distinct intracellular localization patterns: scFv-SP localized to regions characteristic of the endoplasmic reticulum and the Golgi complex, whereas scFv-WO was distributed diffusely throughout the cytoplasm. mRNAs encoding scFv-FI6-SP, scFv-2/3-SP, and scFv-2/3-WO exhibited antiviral activity against influenza A virus *in vitro*. The scFv-FI6-SP mRNA showed the strongest antiviral effect, reducing viral load by approximately tenfold compared to the control. For influenza B virus, both scFv-2/3 mRNA variants, with and without the signal peptide, reduced viral load by an average of 50%. These findings highlight the antiviral potential of intracellular antibodies and point to new opportunities for targeting viral components that are not accessible to conventional antiviral therapies.

Keywords: influenza virus, antiviral agents, broadly neutralizing antibodies, passive immunotherapy, mRNA therapeutics, mRNA-encoded antibodies, intrabodies, scFv fragments, virus neutralization

Funding: This study was funded by the Russian Science Foundation, project no. 24-25-00488 'Study of the antiviral potential of intracellular scFv antibodies against influenza virus' (principal investigator — S. A. Klotchenko); https://rscf.ru/project/24-25-00488/

Author contribution: Plotnikova MA — design of structures, conducting experiments, registration and analysis of results, statistical processing, manuscript authoring and formatting; Oleynik VA — conducting experiments, registration of results; Klotchenko SA — study design, preparation and characterization of mRNA preparations, conducting experiments, registration and analysis of results, manuscript editing.

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Received: 27.11.2025 **Accepted:** 10.12.2025 **Published online:** 26.12.2025

DOI: 10.24075/brsmu.2025.077

ПРОТИВОВИРУСНАЯ АКТИВНОСТЬ мРНК, КОДИРУЮЩИХ ВНУТРИКЛЕТОЧНЫЕ SCFV-ФРАГМЕНТЫ АНТИТЕЛ К КОНСЕРВАТИВНЫМ ЭПИТОПАМ ВИРУСА ГРИППА

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Один из перспективных подходов к эффективной борьбе с гриппом — терапия с использованием моноклональных антител. Целью работы было провести оценку противовирусного действия внутриклеточных scFv-фрагментов антител, которые были трансфицированы в клетку в виде экзогенной мРНК, транслировались и были способны к высокоаффинному связыванию с вирусными антигенами непосредственно внутри клетки. В качестве вирусных мишеней были выбраны два белка вируса гриппа — гемагглютинин (антитело FI6) и нуклеопротеин (антитело 2/3). Каждый из scFv-фрагментов, кодируемых мРНК, был получен в двух формах: с сигнальным пептидом (SP) для секреции во внеклеточное пространство (scFv-SP) и без него (scFv-WO) — для внутриклеточного функционирования в цитозоле. Показана различная локализация этих белков в клетке: scFv-SP обнаруживались в областях, характерных для эндоплазматического ретикулума и комплекса Гольджи, в то время как scFv-WO были диффузно распределены по цитоплазме. Продемонстрировано, что мРНК, кодирующие scFv-FI6-SP и scFv-2/3-SP, а также мРНК, кодирующая scFv-2/3-WO, проявляли противовирусное действие в отношении вируса гриппа А на клеточной модели. Наибольшим противовирусным эффектом обладала мРНК scFv-FI6-SP: она приводила к снижению вирусной нагрузки относительно контроля примерно в 10 раз. В отношении вируса гриппа В обе мРНК, кодирующие scFv-2/3 (как с SP, так и без него), приводили к снижению вирусной нагрузки в среднем на 50% относительно контроля. Таким образом, показано, что внутриклеточные антитела обладают высоким противовирусным потенциалом и открывают возможности для воздействия на новые, перспективные вирусные мишени, которые ранее были недоступны для лекарственной терапии.

Ключевые слова: вирус гриппа, противовирусные препараты, антитела широкого спектра действия, пассивная иммунотерапия, технологии терапевтических мРНК, мРНК-кодируемые антитела, внутриклеточные антитела, scFv-фрагменты антител, вируснейтрализующая активность

Финансирование: исследование выполнено при финансовой поддержке Российского научного фонда, Соглашение № 24-25-00488: «Изучение противовирусного потенциала внутриклеточных scFv антител к гриппу» (руководитель — С. А. Клотченко), https://rscf.ru/project/24-25-00488/.

Вклад авторов: М. А. Плотникова — разработка конструкций, проведение экспериментов, регистрация и анализ результатов, статистическая обработка, написание и оформление рукописи; В. А. Олейник — проведение экспериментов, регистрация результатов; С. А. Клотченко — дизайн исследования, получение и характеристика препаратов мРНК, проведение экспериментов, регистрация и анализ результатов, редактирование рукописи.

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Статья получена: 27.11.2025 **Статья принята к печати:** 10.12.2025 **Опубликована онлайн:** 26.12.2025

DOI: 10.24075/vrgmu.2025.077

According to the World Health Organization, influenza was the third leading cause of death from infectious diseases until 2021, accounting for up to 350,000 deaths worldwide [1]. Although the COVID-19 pandemic has changed the epidemiology of respiratory viral infections, influenza remains a frequent cause of severe pneumonia, especially in children and adults over 70 years of age. In addition, influenza viruses have a high zoonotic potential and the capacity to cross species barriers, posing a persistent threat of future pandemics.

Current strategies for combating influenza rely largely on vaccination. However, its effectiveness is limited, as it is difficult to accurately predict which viral strains will dominate in an upcoming epidemic season. This uncertainty emphasizes the need for new therapeutic approaches.

Monoclonal antibodies, which can specifically bind molecular targets and modulate biological functions — including the disruption of protein-protein interactions — are promising candidates for antiviral drug development. To date, most monoclonal antibody-based therapies have been restricted to extracellular targets. Advances in genetic engineering now make it possible to explore the therapeutic potential of recombinant antibodies or their fragments expressed inside cells. This enables targeting conservative and functionally critical viral antigens that are typically inaccessible to conventional therapies.

Studies from the 1980s and 1990s demonstrated that mature exogenous antibodies can remain stable and functional when introduced directly into the cytosol [2–4], opening new possibilities for suppressing antigen function within living cells. For example, monoclonal antibodies directed against α-tubulin or β-tubulin were shown to induce microfilament destruction and aggregation within 1.5 hours of microinjection into living cells [3].

Nevertheless, the systemic delivery of relatively large proteins, such as antibodies, into cells is still a major challenge [5]. One promising strategy to overcome this limitation is the stable, mRNA-mediated intracellular expression of monoclonal antibodies in non-immune mammalian cells, which enables direct inactivation of target antigens [6].

Proper antibody folding is known to depend on chaperones localized in the endoplasmic reticulum (ER), to which antibody chains are directed by signal sequences that facilitate their translocation [7]. It is during protein synthesis on the ribosome that N-terminal signal peptides (SPs), typically 5–30 amino acids in length, are recognized by the signal-recognition particle, which directs the nascent polypeptide chain into the ER lumen [8, 9]. Upon translocation across the ER membrane, the signal peptide is cleaved by signal peptidase.In the absence of a signal peptide, the heavy and light chains of recombinant immunoglobulins remain in the cytosol, where misfolding is likely to occur, leading to loss of antibody function [10, 11].

More compact antibody formats, such as single-chain variable fragments (scFvs) of ~28 kDa, can retain both functional activity and target specificity. Functional studies have demonstrated that scFvs can fold and assemble correctly in the cytosol, despite the absence of ER-resident chaperones that usually support protein folding, and the reducing environment of the cytosol, which is unfavorable for disulfide bond formation [12].

In this study, we designed synthetic mRNAs encoding high-affinity scFv antibody fragments and evaluated their antiviral activity against influenza virus. Two key viral proteins of different subcellular localizations were selected as targets: the surface hemagglutinin and the internal nucleoprotein. The mRNAs were designed either to promote secretion of scFv fragments for neutralization of viral particles or to drive their accumulation in the cytoplasm to suppress viral replication within infected cells.

METHODS

Design of scFv antibody fragments

We previously developed expression constructs encoding scFv fragments of antibodies FI6 and 2/3 [13]. To generate exogenous mRNAs, DNA sequences encoding scFvs of the desired length were amplified using appropriate primers (Eurogen, Russia) and inserted by restriction cloning at the BstPA I and Bmt I sites (Sibenzyme, Russia). The resulting inserts were then ligated into the pIVTS3 vector system, developed at the Smorodintsev Research Institute of Influenza and based on the PIVOT vector (NovoPro Bioscience, China). The pIVTS3 vector contains a T7 promoter region, 5' and 3' UTRs, and a poly(dA/dT) sequence. Ligation mixtures were used to transform competent *E. coli* cells (NEB Stable strain, New England Biolabs, UK), which were then plated onto selective agar medium supplemented with 100 μg/mL ampicillin. After 24 hours, the resulting colonies were screened by RT-PCR. Plasmids containing inserts of the expected size were propagated in liquid LB medium and purified using the Plasmid Miniprep 2.0 kit (Eurogen, Russia). The sequences of the plasmid constructs used for *in vitro* transcription of exogenous mRNAs were verified by Sanger sequencing performed by Eurogen (Russia).

In vitro transcription (IVT)

Exogenous mRNAs were synthesized using the *in vitro* mRNA Synthesis Kit containing ΨTP, m^5CTP, and m^7GmAmG (Biolabmix, Russia). Plasmid DNA (1 μg) pre-linearized at the AhlI site (Sibenzyme, Russia) served as the template. The IVT reaction was carried out according to the manufacturer's instructions. After completion of transcription, 2 units of TURBO DNase (Thermo Fisher Scientific, USA) were added to the reaction mixture and incubated for 30 minutes at 37°C to cleave residual double-stranded plasmid DNA. The resulting mRNA preparations were purified by lithium chloride precipitation. mRNA concentrations were measured using a NanoDrop ND-1000 spectrophotometer and a Qubit 4 fluorimeter (Thermo Fisher Scientific, USA).

Agarose gel electrophoresis

Plasmid DNA, including linearized forms, was analyzed by electrophoresis on a 0.8% agarose gel prepared in 1× TAE buffer containing 0.5 μg/mL ethidium bromide. DNA samples were mixed with 6× loading buffer and loaded into gel wells.mRNA integrity and quality were assessed by electrophoresis under denaturing conditions in a 1% agarose gel. For sample preparation, 200–500 ng of mRNA was mixed with Gel Loading Buffer II (Invitrogen, USA) and denatured at 70 °C for 5 minutes before loading onto the gel containing ethidium bromide. Electrophoresis was performed in MOPS buffer at room temperature. Gel images were acquired using a Gel Doc EZ Imager (Bio-Rad, USA).

Exogenous mRNA transfection

Established cell lines were used in this study: A549 human lung carcinoma cells (American Type Culture Collection (ATCC), USA; #CCL-185) and Madin-Darby canine kidney (MDCK) cells (International Reagent Resource (IRR), USA; #FR58). A549 cells were cultured in F-12K nutrient medium (Gibco, USA) supplemented with 10% fetal bovine serum, SC-Biol (Gibco, USA). MDCK cells were cultured in α-MEM medium (Biolot, Russia) supplemented with 5% SC-Biol. All cultures were maintained and used in experiments without antibiotics.

For transfection with exogenous mRNAs, cells were grown to a 90–100% confluent monolayer, and the growth medium was replaced with serum-free medium immediately before mRNA delivery. Transfections were performed using GenJector-U (Molekta, Russia) according to the manufacturer's instructions. Lipoplexes containing 100 ng mRNA and 0.3 µL transfection reagent were prepared and added to wells of a 96-well plate at a volume of 10 µL per well. Depending on the experimental design, transfected cells were incubated for 2–48 hours at 37 °C in a humidified atmosphere containing 5% CO_2.

Immunofluorescence

Immunofluorescence staining was performed 24 hours after transfection of eukaryotic cells with exogenous mRNAs. Cells were seeded onto Lab-Tek II chamber slides (Nunc, USA). Monolayers were washed with DPBS, fixed with 4% paraformaldehyde for 10 minutes, and permeabilized with 0.1% Triton X-100 (Sigma-Aldrich, USA). Blocking was performed overnight at 4 °C in DPBS containing 1% bovine serum albumin. The nuclei were stained with DAPI (AppliChem, USA), and the actin cytoskeleton was visualized using rhodamine-conjugated phalloidin (Thermo Fisher Scientific, USA). scFv antibody fragments were detected using primary mouse monoclonal against the 6×His-tag sequence (Penta-His; Qiagen, USA) at a dilution of 1 : 1000, followed by secondary goat anti-mouse antibodies conjugated to Alexa Fluor 488 at a final concentration of 0.5 µg/mL. Cells were imaged sequentially using a Cytell cell imaging system (GE Healthcare, USA) with blue, green, and orange fluorescence channels.

ELISA

Levels of scFv antibody fragments in cell culture medium were quantified by ELISA. Purified viral concentrates of influenza A/California/07/09 (H1N1pdm) and influenza B/Phuket/3073/13 (Yamagata), diluted in PBS to a final concentration of 2 µg/mL, were used as capture ligands. Aliquots of 100 µL of ligand solution were added to wells of a 96-well Microlon High Binding plate (Greiner Bio-One, Germany) and incubated overnight (12–18 hours) at 4 °C. Plates were then washed three times with PBST (PBS containing 0.05% Tween 20) using an ELx405 automatic plate washer (BioTek, USA). Wells were blocked with 200 µL per well of a 5% skimmed milk in PBST (Blotting-Grade Blocker; Bio-Rad, USA) using an MB100-4A thermoshaker (Allsheng, China) at 37 °C for 1 hour. After washing, 100 µL of test culture medium, diluted 1 : 2 in blocking reagent, was added to each well and incubated at 37 °C for 2 hours. Plates were then washed, and bound scFv fragments were detected using horseradish peroxidase-conjugated anti-6×His-tag antibodies (His17-HRP; Hytest, Russia), diluted 1 : 2000 in blocking reagent, and incubated at 37 °C for 1 hour (100 µL per well). After washing, the peroxidase reaction was initiated by adding 100 µL of tetramethylbenzidine (TMB) substrate (Khema, Russia) to each well. The reaction was stopped by adding 100 µL of 2H H_2SO_4, and absorbance (optical density, OD) was measured at 450 nm (OD_{450}) and 620 nm (OD_{620}) using a Multiskan SkyHigh Microplate Spectrophotometer (Thermo Fisher Scientific, USA). Primary data were analyzed in Microsoft Office Excel 2010 (Microsoft, USA) and GraphPad Prism 8 (GraphPad Software, USA).

Western blotting

MDCK cell lysates were prepared using a two-component extraction system consisting of Extraction Buffer 5× PTR and Extraction Enhancer Buffer 50× (Abcam, USA). Fifty microliters of freshly prepared, ice-cold 1× extraction buffer were added to each well containing a cell monolayer pre-washed with DBPS. Plates were incubated on ice for 30 minutes, after which lysates were collected into microcentrifuge tubes and clarified by centrifugation at 13,800×g for 30 minutes at 4 °C using a 5415R centrifuge (Eppendorf, Germany). Clarified supernatants were mixed with Laemmli sample buffer, heated at 95 °C for 10 minutes, and separated under denaturing conditions on Any kD Mini-PROTEAN TGX Stain-Free Protein Gels (Bio-Rad, USA). Next, proteins were transferred to 0.2 µm nitrocellulose membranes using a Trans-Blot Turbo semi-dry transfer system and Trans-Blot Turbo Mini 0.2 µm nitrocellulose transfer packs (Bio-Rad, USA). After transfer, membranes were blocked overnight at 4 °C and incubated for 2 hours at 37 °C with mouse monoclonal anti-6×His-tag antibodies (Penta-His; Qiagen, USA) diluted 1:2000 in blocking reagent. After washing with PBST, membranes were incubated for 1 hour at 37 °C with horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (GAM-HRP; Bio-Rad; USA) diluted 1 : 2000 in blocking reagent. Protein bands were visualized using Clarity Western ECL substrate (Bio-Rad, USA) and imaged with a ChemiDoc MP system (Bio-Rad, USA). Lysates from cells transfected with mRNA encoding the heavy chain of an immunoglobulin specific to an irrelevant antigen were used as a negative control.

Antiviral activity assessment

A combined prophylactic–therapeutic protocol was used to evaluate the antiviral activity of exogenous mRNAs encoding scFv antibody fragments. Confluent MDCK cell monolayers were washed with sterile DPBS to remove serum components that inhibit viral replication. Fresh α-MEM medium (Biolot; Russia; 100 µL) containing 10 µL of lipoplexes prepared with the commercial transfection reagent GenJector-U (Molekta; Russia) was then added to each well according to the manufacturer's instructions. Each well of a 96-well plate contained 100 ng of mRNA and 0.3 µL of transfection reagent (approximately 5×10^4 cells). Next, cells were incubated for 6 hours at 37 °C in a humidified atmosphere containing 5% CO_2.

Cells were subsequently infected by exposure to 50 µL of virus-containing medium for 1 hour at 37 °C and 5% CO_2. Reference strains of human influenza viruses from the collection of the Smorodintsev Research Institute of Influenza were used: A/California/07/09 (H1N1pdm), A/Cambodia/e0826360/2020 (H3N2), B/Phuket/3073/13 (Yamagata), and B/Malaysia/2506/2004 (Victoria), with initial titers of 6×10^8 TCID50/mL, 3.16 × 107 $TCID_{50}$/mL, 3.16×10^6 $TCID_{50}$/mL, and 1.0×10^8 $TCID_{50}$/mL, respectively. Cells were infected at MOIs ranging from 0.1 to 1, corresponding to virus dilutions of up to 1:1000.

After incubation, the virus-containing medium was removed and replaced with the original transfection medium containing lipoplexes. Cells were then incubated for an additional 24 hours at 37 °C and 5% CO_2. Viral particles were subsequently detected in culture medium using a hemagglutination assay performed according to a standard protocol [14] and in infected cells using in-cell ELISA.

In-cell ELISA

Viral particles in infected cells were detected using the ELISA protocol described above, with minor modifications. Briefly, infected cells in 96-well plates were fixed with 80% acetone

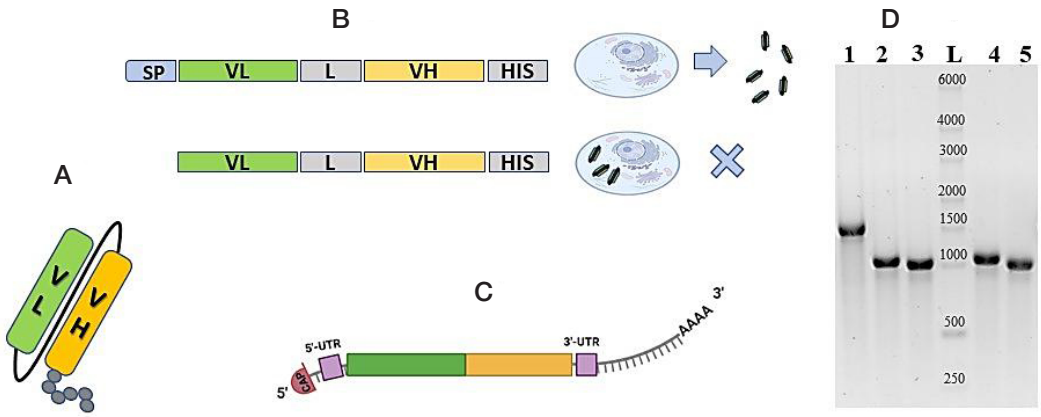

Fig. 1. Basic structure of the developed scFv antibody fragments against influenza A and B viruses and the exogenous mRNAs encoding them. **A**. Structure of scFvs composed of variable domains of the light (green) and heavy (orange) chains. The black line indicates the linker peptide $(G_4S)_4$. Gray circles indicate the C-terminal 6×His-tag sequence. **B**. Structure of the coding regions of secreted and cytosolic scFv antibodies. The key difference is the presence of a signal peptide (SP) at the N terminus in the secreted variant. VL and VH denote the variable domains of the light and heavy chains, respectively; L, the linker peptide; HIS, the 6'His tag sequence. **C**. Structure of the exogenous mRNA used for intracellular scFv translation. The 5' cap (m^7GmAmG) is shown at the 5' terminus; the 5' UTR and 3'UTR regulatory regions are shown in lilac; green and yellow blocks represent the scFv coding region; the extended gray line with notches represents the poly(A) tail. **D**. Agarose gel electrophoretic analysis of purified exogenous mRNA preparations: lane 1, negative control mRNA encoding the heavy chain of an immunoglobulin against an irrelevant antigen (NC-RNA), estimated length 1792 nt; lane 2, mRNA encoding scFv-FI6-SP, 1219 nt; lane 3, mRNA encoding scFv-FI6-WO, 1162 nt; lane 4, mRNA encoding scFv-2/3-SP, 1201 nt; lane 5, mRNA encoding scFv-2/3-WO, 1138 nt. L, RNA molecular weight marker (RiboRuler High Range RNA Ladder; Thermo Fisher Scientific, USA). Marker sizes (nt) are indicated on the lane

in DPBS (50 µL per well) for 30 minutes at 4 °C. After washing with PBST, wells were incubated with blocking reagent (200 µL per well) for 1 hour at 37 °C and then washed. Mouse monoclonal antibodies against the nucleoprotein of influenza A and B viruses [15] were added at a final concentration of 0.5 µg/mL in blocking reagent (100 µL per well) and incubated for 1 hour at 37 °C. Plates were washed, and bound antibodies were detected using horseradish peroxidase-conjugated goat anti-mouse secondary antibodies (GAM-HRP; Bio-Rad, USA), diluted 1 : 2000 in blocking reagent (100 µL per well) and incubated for 1 hour at 37 °C. After washing, signal development and data acquisition were performed as described for the ELISA protocol above.

Statistical data analysis

Statistical analyses were performed using GraphPad Prism 8 (GraphPad Software, USA). Differences between groups were evaluated using one-way ANOVA followed by Holm-Šídák multiple comparison test. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Design and production of exogenous mRNAs encoding scFv antibodies

This study further explored antigen-binding regions of antibodies in the scFv format that we had developed previously [13]. One of these fragments, scFv-FI6, specifically recognized the stem region of hemagglutinin (HA), which is conserved among influenza A viruses (IAV) of both major phylogenetic groups [16]. The second fragment, scFv-2/3, specifically binds the nucleoprotein (NP) of influenza B viruses (IBV) from both genetic lineages [17].

Each scFv consisted of variable domains derived from the corresponding full-length recombinant antibody, with the variable regions of the heavy and light chains connected by a linker composed of four tandem repeats of the G_4S motif. To enable secretion into the extracellular space, the N terminus of each scFv contained a signal peptide (SP) derived from the light chain of the original antibody; these constructs are referred to as scFv-SP. The following signal peptide sequences were used: MKSQTQVLVFLLCVSGAHG for scFv-FI6-SP and MDFQVQIFSFLLISASVIISRG for scFv-2/3-SP. To promote intracellular accumulation, we also generated versions of each scFv without the signal peptide (scFv-WO). In these constructs, the ORF begins with an ATG codon encoding methionine, immediately followed by the sequence of the light-chain variable domain.

The scFv-SP and scFv-WO constructs differed only in the presence or absence of the N-terminal signal peptide. All recombinant scFv antibodies were engineered with C-terminal hexahistidine tags to facilitate detection (Fig. 1A, B). On the basis of this sequence design, plasmid constructs containing a T7 promoter were generated for *in vitro* transcription of mRNA.

mRNA technology was used to translate intracellular scFv antibodies in non-immune eukaryotic cells. The mRNAs was generated by *in vitro* transcription and consisted of five standard elements: an optimized cap analogue at the 5' terminus (CleanCap m7GmAmG; TriLink BioTechnologies, USA), followed by a 5' untranslated region (UTR), the scFv ORF, a 3' UTR, and a poly(A) tail encoded by a poly(dA/dT) sequence embedded in the plasmid (Fig. 1B). To reduce immunogenicity and increase stability, the mRNAs incorporated modified nucleotides: pseudouridine (ΨTP) and 5-methylcytidine (m5CTP).

The resulting purified preparations included four mRNAs encoding scFv antibody fragments FI6 and 2/3 against IAV and IBV, each produced with or without a signal peptide, as well as a control mRNA encoding the heavy chain of an immunoglobulin specific to an irrelevant antigen. All mRNAs were characterized by spectrophotometry and agarose gel electrophoresis (Fig. 1G). The observed transcript lengths matched the predicted sizes, and the purity and integrity of the mRNA preparations were sufficient to support intracellular translation of the target proteins.

Functional properties of scFv antibodies encoded by exogenous mRNAs

We evaluated the translational activity of exogenous mRNAs encoding scFv fragments of antibodies in the eukaryotic cell lines MDCK and A549. The mRNA-containing culture medium was removed 24 hours after transfection, and the cells were

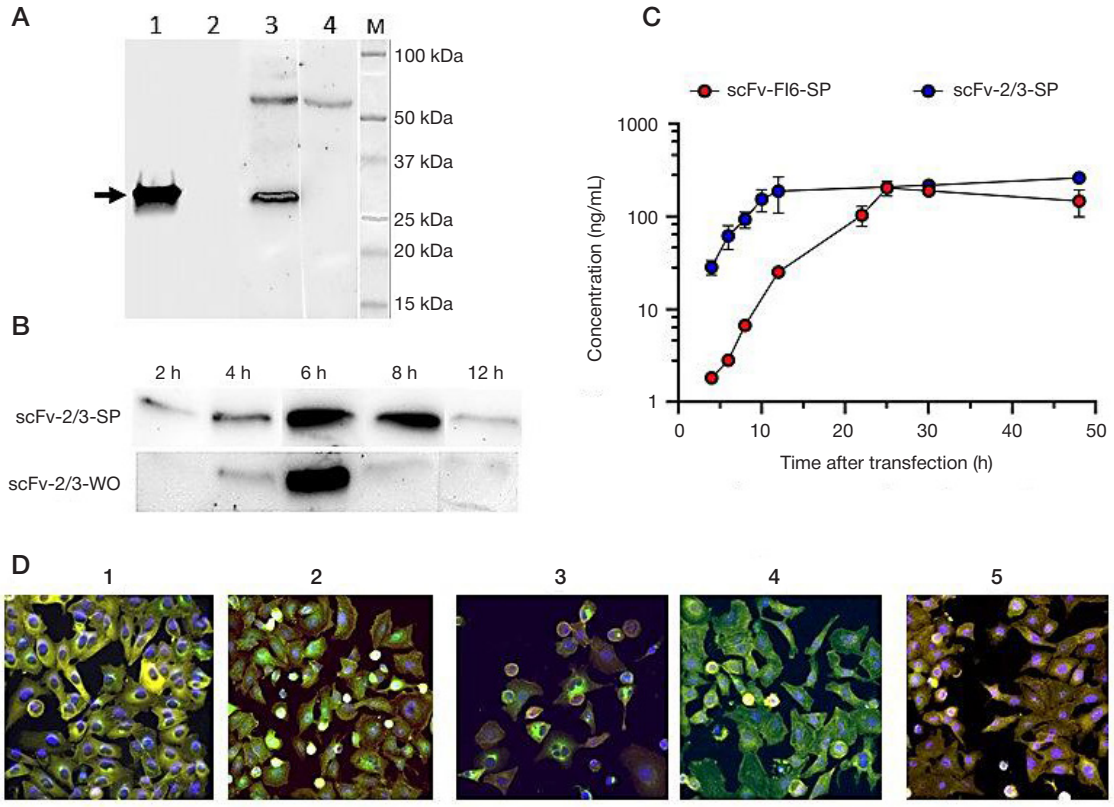

Fig. 2. Translational activity of exogenous mRNAs encoding cytosolic (WO) and secreted (SP) forms of scFv antibody fragments against influenza A and B viruses. **A**. Western blot analysis of scFv-2/3 antibody fragments in culture medium and MDCK cell lysates using antibodies specific to the 6'His tag sequence. Similar results were obtained for scFv-FI6 but are not shown. Lanes 1 and 2 show culture medium from MDCK cells transfected with mRNAs encoding scFv-2/3-SP and scFv-2/3-WO, respectively; lanes 3 and 4 show cell lysates from MDCK cells transfected with mRNAs encoding scFv-2/3-SP and scFv-2/3-WO, respectively. M, molecular weight marker (Precision Plus Protein Kaleidoscope Preferred Protein Standards; Bio-Rad, USA); corresponding molecular weights (kDa) are indicated to the right. All samples were collected 24 h after mRNA cell transfection. **B**. Western blot analysis of scFv-2/3 antibody fragments in MDCK cell lysates at different time points using anti–6'His-tag antibodies. The upper panel shows intracellular production of scFv-2/3-SP, and the lower panel shows scFv-2/3-WO. Sampling times after mRNA transfection are indicated at the top (hours). **C**. ELISA analysis of scFv concentrations in culture medium after transfection of A549 cells with mRNAs encoding scFv-FI6-SP (*red*) and scFv-2/3-SP (*blue*). Data points represent mean values from two biological replicates ± SD. **D**. Representative fluorescence microscopy images of fixed A549 cells transfected with exogenous mRNAs encoding: (1) scFv-FI6-SP, (2) scFv-FI6-WO, (3) scFv-2/3-SP, (4) scFv-2/3-WO, and (5) NC-RNA. The actin cytoskeleton (yellow) was visualized using rhodamine-conjugated phalloidin, and nuclei (blue) were stained with DAPI. scFv fragments (*green*) were detected using primary mouse monoclonal anti-6'His-tag antibodies followed by Alexa Fluor 488-conjugated goat anti-mouse secondary antibodies. Images were acquired and processed using a Cytell cell imaging system (40× objective); individual channels were pseudocolored in RGB during image processing. Enlarged views of the processed images are shown

then lysed, and the levels of histidine-tagged proteins in the lysates were analyzed by Western blotting.

At 24 hours after transfection of MDCK cells with mRNAs encoding scFv-SP, proteins of the expected molecular weight (~28 kDa), corresponding to monomeric scFv antibody fragments, were readily detected using antibodies against the 6×His tag (Fig. 2A). By contrast, detection of scFv products in cell lysates proved challenging. For both scFv-SP and scFv-WO constructs, nonspecific high-molecular-weight bands (~55 kDa) were observed, which were also present in the negative control. Enzymatic signal amplification combined with extended exposure time allowed us to identify intracellular scFv-SP (Fig. 2A, lane 3), whereas scFv-WO remained undetectable (Fig. 2A, lane 4). Thus, transfection with mRNA encoding scFv-SP resulted in the secretion of mature monomeric scFv fragments within 24 hours, which were detected both in the culture medium and in cell lysates.

Assuming that intracellular scFvs may be unstable and have short half-lives of only a few hours, we next examined their expression kinetics in cell lysates at 2, 4, 6, 8, and 12 hours after transfection with exogenous mRNAs. For both SP and WO variants, maximal translation was observed at 6 hours post-transfection (Fig. 2B). Notably, intracellular stability of scFvs varied significantly depending on the presence or absence of the signal peptide. scFv-SP antibodies were detectable as early as 2 hours after transfection, and their production increased by 6 hours and then dropped by 12 hours. In contrast, scFv-WO was first detected in cells at near-threshold levels at 4 hours after transfection, reached peak abundance at 6 hours, and decreased to the detection limit by 8 hours.

Next, we evaluated the kinetics of scFv-SP accumulation in the culture medium of A549 cells transfected with the corresponding exogenous mRNAs (Fig. 2B). To this end, we performed an ELISA using purified inactivated influenza A and B virus concentrates as capture ligands and horseradish peroxidase–conjugated antibodies to detect the 6×His tags. scFv fragment concentrations were quantified using a calibration curve generated with purified recombinant scFv preparations [13].

Functional scFv fragments of antibodies were detected in the culture medium as early as 2 hours after transfection of A549 cells with scFv-SP mRNA, at analytically detectable levels. scFv levels increased steadily over the subsequent 24 hours, reaching approximately 200 ng/mL for both scFv-FI6-SP and scFv-2/3-SP. Saturation of protein accumulation occurred at around 24 hours post-transfection, after which scFv levels reached a plateau maintained throughout the rest of the 48-hour observation period.

To assess the intracellular localization of proteins encoded by scFv-SP and scFv-WO constructs, we performed immunofluorescence staining of A549 cells transfected with the corresponding mRNAs (Fig. 2D). Immunocytochemical analysis showed that scFv-FI6-WO and scFv-2/3-WO were diffusely

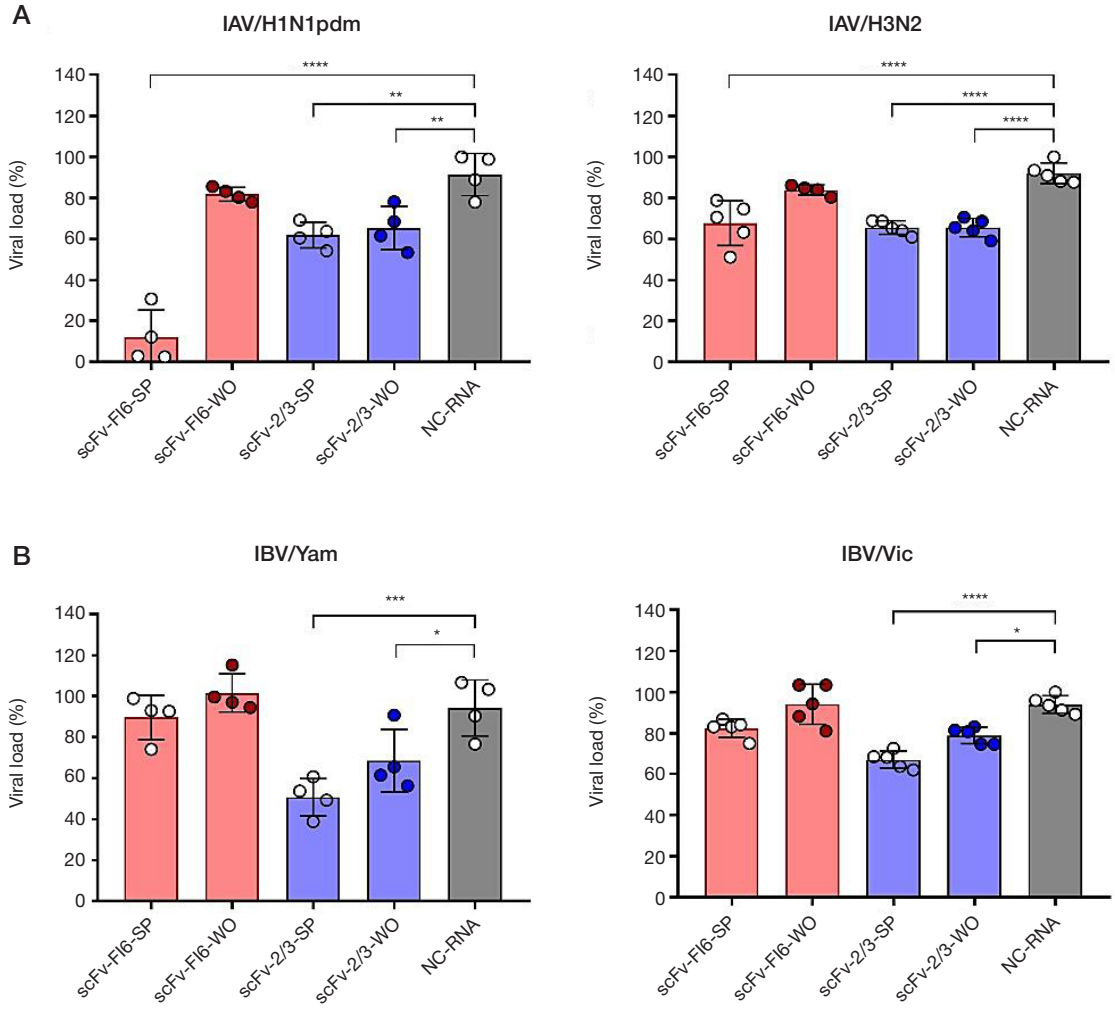

Fig. 3. In-cell ELISA analysis of the antiviral activity of exogenous mRNAs encoding cytosolic (WO) and secreted (SP) forms of scFv antibody fragments against influenza A and B viruses. **A**. Intracellular levels of influenza A virus (IAV) after infection with A/California/07/09 (H1N1pdm) (left) and A/Cambodia/e0826360/2020 (H3N2) (right) strains. **B**. Intracellular levels of influenza B virus (IBV) after infection with B/Phuket/3073/13 (Yamagata) (left) and B/Malaysia/2506/2004 (Victoria) (right) strains. Data points represent mean values for four samples ± SD. Red bars indicate therapeutic administration of exogenous mRNAs encoding scFv fragments against IAV hemagglutinin; blue bars indicate administration of exogenous mRNAs encoding scFv fragments against IBV nucleoprotein. Gray bars show mean values for cells transfected with exogenous mRNA encoding the heavy chain of an immunoglobulin specific to an irrelevant antigen (negative control). Statistical significance was determined using one-way ANOVA followed by Holm-Šídák multiple comparison test. *Asterisks* indicate significant differences compared with the negative control (NC-RNA): * — p_{value} < 0.0332; ** — p_{value} < 0.0021; *** — p_{value} < 0.0002; **** — p_{value} < 0.0001

distributed throughout the cytoplasm, with no distinct subcellular localization. scFv-FI6-SP and scFv-2/3-SP, on the contrary, were distinctly localized in the perinuclear region, consistent with their accumulation primarily in the ER (not labeled directly).

In the final stage of the study, we evaluated the antiviral activity of the exogenous mRNAs against influenza A and B viruses. As a negative control, we used an mRNA encoding the heavy chain of an immunoglobulin specific to an irrelevant antigen (NC-RNA).

Experiments were conducted using the combined prophylactic–therapeutic protocol involving mRNA transfection both before and after a 1-hour exposure to the virus. Three mRNAs — encoding scFv-FI6-SP, scFv-2/3-SP, and scFv-2/3-WO — were found to have antiviral activity against IAV (Fig. 3A). For the H1N1pdm strain, scFv-FI6-SP mRNA produced the strongest antiviral effect, decreasing viral load by approximately tenfold relative to the control. No such enhancement was observed in the case of the H3N2: all three mRNAs showed comparable antiviral effects, reducing viral load to 60–70% of control levels. It is noteworthy that scFv-FI6-WO mRNA, which specifically binds IAV hemagglutinin, did not affect IAV replication.

For IBV, both mRNAs encoding scFv-2/3 (SP and WO) demonstrated antiviral activity. Prophylactic transfection decreased viral load to 50–70% of that observed in control samples, with similar effects against IBV strains of the Yamagata and Victoria lineages. Importantly, neither the original full-length monoclonal antibody 2/3 [17] nor the derived scFv-2/3 fragment was previously shown to neutralize IBV [13].

DISCUSSION

Modern passive immunotherapy based on neutralizing monoclonal antibodies can provide broad protection against infections caused by influenza viruses [16, 18, 19]. Most currently available therapeutic immunoglobulins target hemagglutinin or neuraminidase, the major surface antigens of influenza virus. Yet, reliance on antibodies directed against these highly variable proteins has inherent limitations and risks [20, 21], underscoring the value of strategies that target more conserved viral antigens.

This study aimed to evaluate the potential of intracellular therapeutic antibodies directed against two distinct influenza virus targets: surface hemagglutinin, which is accessible to neutralizing antibodies, and the internal nucleoprotein, which is shielded from antibody recognition in mature virions. Efforts to introduce monoclonal antibodies into the cytosol of living cells

and to study their functional activity date back to the 1970s [22–24]. However, the practical therapeutic application of intracellular antibodies has only become feasible in the past decade, supported by advances in mRNA technologies that enable direct intracellular expression of antibody constructs.

Full-length immunoglobulins comprise not only antigen-binding regions but also an Fc domain that mediates effector functions through interactions with immune cells [25, 26]. Such functions are unnecessary for specific antigen recognition within the intracellular environment. Accordingly, to evaluate intracellular antibody activity, we chose the scFv antibody format [27, 28] that comprises only the variable domains.

For each antibody — scFv-FI6 targeting IAV hemagglutinin and scFv-2/3 targeting IBV nucleoprotein, we designed two types of constructs. One included a signal peptide (SP) sequence to direct nascent polypeptide into the ER, followed by transport through the Golgi apparatus and secretion into the extracellular space. The second construct lacked the signal peptide or any additional targeting sequences, such as nuclear localization signals or ER retention motifs (for example, KDEL) [8, 9]. We reasoned that the protein product translated from such exogenous mRNA would remain in the cytosol, where it could interact effectively with target proteins, thus suppressing viral replication.

Consistent with the design principles, observed distinct intracellular distribution patterns for the two scFv variants. scFv-SP fragments were mainly concentrated in regions with highly curved smooth membranes characteristic of the ER-Golgi interface. In contrast, scFv-WO fragments had no distinct subcellular localization and were distributed diffusely throughout the cytoplasm. This likely reflects cytosolic retention of this variant and a lack of involvement in vesicular transport.

Notably, Western blot analysis of cell lysates 24 hours after transfection with mRNA encoding scFv-WO failed to detect monomeric scFv antibody fragments (~28 kDa), in contrast to scFv-SP. At the same time, after transfection with either scFv-FI6-SP or scFv-2/3-SP mRNAs, proteins of the expected molecular weight were readily detected in the culture medium.

We further established that intracellular scFv antibodies have a short half-life. Products encoded by mRNA lacking a signal peptide (scFv-WO) remained detectable for only 4 hours, which indicates their rapid degradation, likely mediated by the ubiquitin-proteasome system. Numerous studies have shown that cytosolic scFv fragments often have low stability and are prone to dimerization and degradation [29]. This instability can be attributed to suboptimal conditions for antibody folding in the cytosol, including a lack of chaperones, low pH, and a reducing environment that prevents the formation of stabilizing disulfide bonds [11, 12, 30]. As a result, the effectiveness of scFvs operating in the cytosol can be substantially limited by impaired folding and the inability to adopt a stable, functional conformation [31]. Importantly, scFv antibodies containing a secretion signal showed an earlier and more sustained intracellular production, suggesting that routing through the secretory pathway can enhance both folding efficiency and protein stability.

We next assessed the antiviral activity of the exogenous mRNAs encoding scFv antibody fragments in a cellular model of influenza infection. It is known that exogenous mRNAs can suppress viral replication due to their intrinsic immunogenicity and activation of cellular nucleic-acid-sensing pathways [32, 33]. To control for these non-specific effects, we used an mRNA encoding the heavy chain of an immunoglobulin targeting an irrelevant antigen and containing a signal peptide sequence, which was produced and purified using the same protocol as the experimental mRNAs.

Of the two constructs encoding scFv-FI6, which specifically binds IAV hemagglutinin, only the mRNA encoding the secreted form (scFv-FI6-SP) showed antiviral activity. The therapeutic application of this mRNA reduced viral load after infection with the H1N1pdm strain by nearly tenfold compared to the control treatment. We detected no antiviral activity of the cytosolic form, scFv-FI6-WO. These findings suggest that intracellular antibodies are more effective when their intracellular trafficking mirrors that of their viral targets. Indeed, an intracellular human scFv antibody against hemagglutinin, expressed from plasmid DNA, has been shown to suppress H5N1 influenza virus replication *in vitro*. Moreover, its *in vivo* efficacy significantly increased when combined with an extracellular IgG1 containing identical variable domains [34].

In contrast, the results obtained with scFv-2/3, which targets IBV nucleoprotein and lacks neutralizing activity [13], were unexpected. Both the cytosolic and secreted variants showed antiviral activity, reducing IBV viral load by approximately twofold relative to the control. In addition, transfection of exogenous mRNAs encoding scFv-2/3 also suppressed IAV replication. As the original full-length monoclonal 2/3 antibody does not exhibit neutralizing activity [17], it is challenging to identify its epitope on the IBV nucleoprotein. It is therefore plausible that this epitope is highly conserved between influenza A and B viruses. Overall, expression of exogenous mRNAs encoding scFv-2/3 reduced replication of both IAV and IBV by an average of ~50% relative to the control.

The use of intracellular antibodies targeting IAV nucleoprotein has been described previously. Such antibodies inhibit viral replication by blocking interactions between nucleoprotein and the viral polymerase subunits PB1 and PB2, which are essential for viral transcription and replication [35]. In another study, single-domain llama antibodies directed against various IAV nucleoprotein epitopes were shown to suppress viral replication by inhibiting nuclear import of ribonucleoprotein complexes responsible for nuclear transport, transcription, replication, and packaging of the viral genome into newly formed virions [36]. It is important to emphasize that, in both cases, intracellular antibodies were expressed via transfection with plasmid constructs rather than through the delivery of exogenous mRNAs.

Our findings indicate that the antiviral efficacy of structurally distinct intracellular scFv antibody fragments depends on the intracellular trafficking of their viral target proteins. The data suggest that effective inhibition of certain viral functions requires antibody-antigen interactions to occur within specific cellular compartments.

Thus, the use of exogenous mRNAs encoding high-affinity scFv antibody fragments to conserved intracellular viral proteins, particularly influenza virus nucleoprotein, is a promising strategy for suppressing viral replication. The main advantage of this approach is its ability to target internal viral components, including intracellular viral replication-transcription complexes, which are substantially more conserved and less susceptible to antigenic drift than viral surface glycoproteins [37].

CONCLUSIONS

In this work: (1) single-chain variable fragments (scFv) of antibodies targeting influenza A hemagglutinin and influenza B nucleoprotein were constructed; (2) exogenous mRNAs encoding these scFvs in both cytosolic and secreted forms were generated; (3) efficient translation of scFv proteins was confirmed following transfection of exogenous mRNAs into eukaryotic cells; (4) the cytosolic forms showed diffuse

intracellular distribution, whereas the secreted forms localized predominantly within the endoplasmic reticulum; and (5) the antiviral activity of exogenous mRNAs encoding scFv fragments against influenza A and B viruses was demonstrated.

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