# Evaluation of the antiviral effect of exogenous human IFN-lambda mRNA against influenza virus in vitro

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**Short Report**

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# Abstract

**Objective:** Despite the fact that exogenous mRNA has great prospects for the development of therapeutic medicine, its use is still limited. As the immediate protein precursor, positive-stranded mRNA may represent a suitable alternative to prevent of viral infections.

**Results:** Here, we focused our efforts on making the exogenous RNA encoding human interferon lambda (hIFN-λ1). Using the in vitro transcription method, we obtained hIFN-λ1 RNA and showed that it is capable to rapid translation in transfected cells. We compared the translation efficiency of mRNAs containing unmodified and modified (pseudouridine and 5-methyl-cytidine) nucleosides. Our results showed that the level of hIFN-λ1 during translation from containing modified nucleosides mRNA was 10-fold or more times higher compare to unmodified mRNA.

We found that the delivery of exogenous mRNA encoding GFP and hIFN-λ1 in cells resulted in an increase of MDA5, MxA, OAS-1, and IFN-αexpression, which indicate to the activation of innate immune response.

At last it was shown that mRNA encoding hIFN-λ1 significantly reduced the reproduction of A/California/07/09 (H1N1pdm09) in comparison with the nonspecific mRNA encoding GFP.


# Introduction

The technological platform based on the use of exogenous protein-coding mRNAs has been actively developed over the past decade and has successfully realized itself in 2020 when creating COVID-19 mRNA Vaccines [1, 2]. Nevertheless, in vitro transcribed mRNAs have also a great potential for use as therapeutic agents.

Exogenous mRNA is produced by an in vitro transcription reaction, so there is no risk for contamination of any mammalian cells components, viruses or bacteria. The coding mRNA is directly translated into encoded protein in target cells. With this approach, there is no need to optimize complex processes for obtaining and purifying aberrant post-translational protein modifications [3]. The design and production of mRNA takes relatively little time. The main problems of mRNA-based therapy are the delivery and the immunogenicity of exogenous mRNAs [4–6].

The scope of this study was to evaluate of antiviral potential of mRNA coding human IFN lambda 1 type (hIFN-λ1). IFN-λ (IFN type 3) is a group of IFNs related to IFN type 1 and showing similar antiviral activity [7]. We have recently reported about of antiviral activity of protein hIFN-λ1 [8].

In this study, we find that exogenous mRNA coding hIFN-λ1: (1) are rapidly translated in transfected cells and secreted into intercellular spaces, (2) activated the expression of cytosolic sensors and some ISGs (3) specifically inhibit influenza expression *in vitro*.

# Methods

# Cells

The A549 (CCL-185, lot #70018877) and Vero (CCL-81, lot#612626456) cell lines were obtained from the American Type Culture Collection (ATCC,). A549 cells (human type II alveolar epithelial line) were maintained in DMEM medium (Gibco) supplemented with 10% fetal bovine serum (Gibco, USA). Vero cells (African green monkey kidney epithelial line) were maintained in AlphaMEM (Biolot) containing 10% fetal bovine serum (Biowest). Cells were cultured at 37°C (5% CO2 with humidification).

# Design of plasmid and in vitro transcription

The pJet1.2/blunt cloning vector (Thermo Scientific) containing the T7 promoter for *in vitro transcription* (IVT) of cloned insert was used. The coding region of hIFN-λ1 was synthesized via RT-PCR using total RNA extracted from A549 cells as a template.

Plasmids were linearized with NcoI (New England Biolabs) overnight and purified using Cleanup Mini kit (Evrogen) prior to IVT. mRNAs were obtained by IVT using the HighYield T7 ARCA mRNA Synthesis Kit (Jena Bioscience, #RNT-102). One microgram of linearized plasmid template was used for the reaction. Anti-Reverse Cap Analog (ARCA) (Jena Bioscience, #RNT-102) was used for efficient translation of the RNA. To maximize RNA yield and the fraction of capped transcripts, we used ARCA/GTP ratio 4:1. Reaction was performed according to the manufacturer's protocol. For modified RNAs equimolar ratios of ATP and GTP were used alongside pseudouridine-5'-triphosphate (Ψ) and 5-Methylcytidine-5'-triphosphate (m5C).

Following RNA synthesis DNA template was removed by subsequent digestion with DNase Turbo (Thermo Fisher Scientific, #AM1345). Poly(A) Tailing Enzyme Testkit (Jena Bioscience, #RNT-004) was used to polyadenylate the 3'-termini of transcribed RNA. Transcript was purified by the RNA Clean & Concentrator kit (Zymo Research, #R1017). RNA sample was analyzed using electrophoretic separation under denaturing conditions. Five hundred nanograms of RNA sample was mixed with an equal volume of RNA Gel Loading Dye (2X) (Thermo Fisher Scientific, #R0641) and heated for 10 min at 70°C. Samples were subsequently loaded into wells of 1% agarose gel (containing 0.5 μg/mL ethidium bromide) and run in 1×MOPS buffer at room temperature.

# Cell transfection

RNA transfections were performed using 2X3-DOPE (1:3) Reagent [9]. RNA complexes were formed in serum-free medium by mixing 0.73 μl of 2X3-DOPE (1:3) reagent and 100 ng synthetic mRNA per well of 96-well plate or 300 ng per well of 12-well plate.

For A549 experiments, $1.2\times10^5$ cells/well were seeded overnight into 12-well plates before treatment with the indicated mRNAs. Then medium was replaced with DMEM (Gibco) without serum and cells were transfected by hIFN-λ1-mRNA or GFP-mRNA. IFN-λ level was measured over 24 hpi by ELISA. ISGs expression was examined after 24 hours after transfection by RT-PCR.

For antiviral activity $2.5 \times 10^4$ MDCK cells per well were seeded overnight into 96-well plates before treatment. Cells were then transfected with hIFN-λ1-mRNA$^{\Psi/m5C}$, GFP-mRNA$^{\Psi/m5C}$ or stimulated with IFN-λ1 protein at concentration of 10 ng/ml. After 24 hrs of stimulation, cells were infected with A/California/07/09 (H1N1pdm09) at 0.5, 5 and 50 TCID50 per well. The infectivity of the virus in MDCK cells was $6.0 \times 10^6$ $TCID_{50}$/ml. Viral load was measured at 24 hpi by in situ cellular ELISA with anti-NP antibodies.

# RT-PCR analysis

Total RNA was isolated from A549 cells using TRIZol reagent (Invitrogen, USA) according to the manufacturer's instructions. RNA concentrations and integrity were analyzed using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA).

One microgram of total RNA was treated by DNase (Promega) and then directly reverse transcribed using oligo-$dT_{16}$ primers and RNAscribe RT (BioLabMix, Russia). Complementary DNA synthesis was carried out at 50°C for 50 min. Enzyme was inactivated at 80°C for 5 min. Products were diluted (1:2) and stored at − 20°C until use.

Real-time PCR assays were performed using the CFX96 Real-Time PCR System (Bio-Rad, USA). Multiplex qPCR [10] was performed in 25 µL final reaction volumes containing 12.5 µL BioMaster HS-qPCR mix (2x) (BioLabMix, Russia) and 2 µL cDNA.

# ELISA

For *in situ* cellular ELISA inoculum was removed from cell plates, and cells in monolayer were fixed by cold 80% acetone in DPBS for 30 min in + 4°C. After washing with a 1× PBST solution (0.05% Tween 20), wells were blocked with 5% Blotting-Grade Blocker (Bio-Rad), diluted in 1× PBST, for 1 hour at room temperature. After plate washing, 100 µl of (1 µg/ml) mAb specific to viral NP of IVA was added to the wells, and the plate re-incubated at room temperature for 2 hrs. Binding was detected using GAM-HRP (Bio-Rad) secondary antibodies diluted 1:500 in 1× PBST (30 min incubation at room temperature). Peroxidase reaction was performed using the TMB Peroxidase EIA Substrate Kit (Bio-Rad). After 10 minutes, reactions were stopped by addition of 50 µl of 2N $H_2SO4$ to each well. Optical densities were measured at 450 nm (OD450) on a CLARIOstar plate photometer (BMG LABTECH, Germany).

Human IFN-$\lambda_{1/3}$ concentrations in cell culture supernatants were measured using commercial kit human IL-29/IL-28B (IFN-lambda 1/3) DuoSet ELISA (DY1598B, R&D Systems, USA).

# Statistical data processing

Data processing was carried out in Microsoft Excel. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software).

## Results

## Design Of Exogenous Mrna

To study the potential antiviral effects of exogenic mRNA we used gene of human IFN-λ1 protein (hIFN-λ1) which has antiviral and immunomodulatory effects. According to the presented design (Fig. 1A), the synthetic mRNA encodes hIFN-λ1 together with its signal peptide, which ensures the secretion of the protein into the extracellular space. Using the pJet1.2-based constructs we obtained the mRNAs (Fig. 1B) containing modified (mRNA$^{\Psi/m5C}$) and unmodified (mRNA$^{Nat}$) nucleosides.

As shown in Fig. 1C the mature mRNA was translated into a full-length hIFN-λ1 (NP_742152 [11], Q8IU54 [12]) with a length of 200 aa and molecular mass of 21.9 kDa.

For comparison, the mRNAs encoding green fluorescent protein (GFP) were similarly transcribed from pJet-GFP. Unlike hIFN-λ1, GFP has an intracellular localization, and its fluorescence easily allows visualization of RNA translation.

## Mrna Translation In A549 Cells

At first, we verified the translation of proteins from synthetic mRNAs. By ELISA we detected hIFN-λ1 proteins only in A549 cells transfected with mRNAs coding hIFN-λ1 but not with GFP. Translation of GFP was confirmed by fluorescence microscopy. We evaluated hIFN-λ1 production kinetics at 4, 8, 16, and 24 h post transfection by hIFN-λ1-mRNA$^{\Psi/m5C}$ and hIFN-λ1-mRNA$^{Nat}$. Both, for GFP and hIFN-λ1, translation efficacy from mRNA$^{\Psi/m5U}$ was significantly high then mRNA$^{Nat}$. We found that protein translation from hIFN-λ1-mRNA$^{\Psi/m5C}$ increased during the first day and the hIFN-λ1 level was 250 pg/mL by 24 hours (Fig. 1D).

It is known that the introduction of synthetic exogenous mRNAs can induce an innate immune response by activating cytosolic RNA sensors [13]. To assess the specificity of this response, we have transfected A549 cells by mRNAs coding GFP and hIFN-λ1 and determined the expression of some cellular genes by RT-PCR after 24 hours (Fig. 2). It was found that all RNAs caused a significant increase in the expression of the MDA-5 cytosolic sensor. Rig-1 mRNA level elevated only after transfection with hIFN-λ1-mRNA$^{\Psi/m5C}$. In addition, all the studied RNAs caused a significant increase in the expression of the MxA, OAS-1, PKR and IFN-α. Interestingly, the levels of expression of OAS-1, PKR, IFN-α in the case of GFP-mRNA$^{\Psi/m5C}$ were lower compared to transfection with GFP-mRNA$^{Nat}$ and both mRNAs encoding hIFN-λ1.

## Evaluation Of The Antiviral Effect Of Hifn-λ1 Mrna

To investigate the antiviral effect, we transfected MDCK with hIFN-λ1-mRNA$^{\Psi/m5C}$ and GFP-mRNA$^{\Psi/m5C}$ and then infected cells with influenza virus at doses of 50, 5 and 0.5 TCID50/cell.

The results of the reproduction of the virus were examined 24 hours after infection (single-cycle reproduction).As shown in Fig. 3A, a downward trend in viral reproduction was observed in infected

MDCK cells previously treated with hIFN-λ1-mRNAΨ/m5U. The hIFN-λ1-mRNA$^{\Psi/m5C}$ transfection significantly reduced the level of viral proteins in cells at 50 TCID50 ($p_{value}$ = 0.0007), 5 TCID50 ($p_{value}$ < 0.0001), and 0.5 TCID50 ($p_{value}$ = 0.0004) per well. We found also that transfection reagent alone without mRNA (Mock-TR) significantly reduced the virus replication (for example Fig. 3B) compared to infected untreated cells (Mock).

# Discussion

Here, we show that hIFN-λ1 coded by exogenous mRNA suppress influenza reproduction in MDCK cells. We show that simultaneous introduction of modified nucleosides such as pseudouridine and 5-methylcytidine into hIFN-λ1-mRNA increases the lifespan of the protein and the efficiency of its translation. It is known that nucleoside modification is an effective approach to enhance stability and translational capacity of mRNA while diminishing its immunogenicity *in vivo* [14]. Our results showed that the level of hIFN-λ1 during translation from exogenous mRNA containing modified Ψ/m5C nucleosides was 10-fold or more times higher compare to unmodified mRNA.

So transfection of 2×10$^5$ A549 cells with 600 ng hIFN-λ1-mRNA$^{\Psi/m5C}$ resulted in stable production of its encoded protein after 4 hours. It should be noted that transfection of mRNAs encoding hIFN-λ1 leads to significant production of IFN-λ1 in the supernatant, both in comparison with intact cells and in comparison with mRNAs encoding GFP, which eliminates the possibility of nonspecific production in response to the introduction of exogenous mRNA.

Since the antiviral activity of synthetic mRNAs may be due to the activation of the innate immune system, in our work we evaluated the effect of hIFN-λ1-mRNA$^{\Psi/m5C}$ and hIFN-λ1-mRNA$^{Nat}$ on cytosolic sensors, which play a key role in the detection of viral RNA molecules.

We found that the delivery of exogenous mRNA in cells resulted in an increase of MDA5 expression, which we speculate is indicate to the activation of innate immune response. A weak increase in RIG-I expression was shown only in the case of hIFN-λ1-mRNA$^{\Psi/m5C}$. We believe that this may be due to the activity of the hIFN-λ1 protein product. The induction of interferons is closely associated with the activation of pattern-recognition receptors, the leading role among which is played by RIG-I-like receptors (RLR) [15, 13]. We also noticed that the expression levels of OAS-1, PKR, and IFN-α in the case of GFP-mRNA$^{\Psi/m5C}$ were lower compared to other RNAs. Probably, the presence of modified bases in GFP-mRNA$^{\Psi/m5C}$ makes it less immunogenic. On the other hand in the case of hIFN-λ1-mRNA$^{\Psi/m5C}$, hIFN-λ1 secretion may further stimulate the expression of these genes.

To assess the antiviral activity, we used the MDCK (canine kidney) cell culture permissive against influenza viruses. We have previously shown that human hIFN-λ1 is able to bind to its receptor and cause antiviral effect [10].

We found that the reagent we used for transfection slightly suppressed the translation of influenza viral proteins (NP protein). Presumably the transfection reagent may interfere of viral entry by agglutinating the influenza virion. Nevertheless, the prophylactic transfection of hIFN-λ1-mRNA$^{\Psi/m5C}$ significantly reduced the reproduction of the virus both in comparison with the nonspecific GFP-mRNA$^{\Psi/m5C}$ and in comparison with the hIFN-λ1 protein.

It is important to note that a stable high level of hIFN-λ1 remained in the supernatants of infected cells two days after transfection (not presented). We assume that our results indicate a high potential for the use of mRNA as therapeutic agents.

Nevertheless, the ease and simplicity of obtaining mRNA preparations encoding antiviral proteins makes this approach extremely promising. Thus, our future studies we plan to devote to assessing the therapeutic potential of exogenous mRNAs encoding intracellular proteins that have a direct antiviral effect, such as MxA.

# Limitations

We speculate that hIFN-λ1 is probably not a perfect choice for mRNA therapy. This is an extracellular protein and there are no delivery problems for it. There are also some concerns regarding the use of interferons as antivirals [16].

Data for antiviral activity of exogenous RNA encoding hIFN-λ1 were obtained using only an in vitro cell model.

# Declarations


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### Authors' contributions

MAP: Validation, Investigation, Visualization, Writing – original draft; SAK: Conceptualization, Investigation, Resources; AAL: Validation, Investigation, Writing – original draft; OAD: Investigation, Visualization; EAR-R: Investigation; VVV: Investigation, Visualization; AVV: Conceptualization, Resources.

### Competing interests

The authors declare that there are no conflicts of interests regarding the publication of this paper.

### Availability of data and materials

All data relevant to the study are included in the article.

### Ethics approval and consent to participate

Not applicable.

### Consent for publication

Not applicable.

# References


1. E. E. Walsh et al., “Safety and Immunogenicity of Two RNA-Based Covid-19 Vaccine Candidates,” New England Journal of Medicine, vol. 383, no. 25, pp. 2439–2450, Dec. 2020, doi: 10.1056/nejmoa2027906.
2. “COVID-19 vaccines: comparison of biological, pharmacological characteristics and adverse effects of Pfizer/BioNTech and Moderna Vaccines.”
3. L. van Hoecke and K. Roose, “How mRNA therapeutics are entering the monoclonal antibody field,” Journal of Translational Medicine, vol. 17, no. 1. BioMed Central Ltd., Feb. 22, 2019. doi: 10.1186/s12967-019-1804-8.
4. N. Pardi, M. J. Hogan, F. W. Porter, and D. Weissman, “mRNA vaccines-a new era in vaccinology,” Nature Reviews Drug Discovery, vol. 17, no. 4. Nature Publishing Group, pp. 261–279, Mar. 28, 2018. doi: 10.1038/nrd.2017.243.
5. J. Probst et al., “Spontaneous cellular uptake of exogenous messenger RNA in vivo is nucleic acid-specific, saturable and ion dependent,” Gene Ther, vol. 14, no. 15, pp. 1175–1180, 2007, doi: 10.1038/sj.gt.3302964.
6. T. Schlake, M. Thran, K. Fiedler, R. Heidenreich, and B. Petsch, “mRNA : A Novel Avenue to Antibody Therapy ?,” Molecular Therapy, vol. 27, no. 4, pp. 773–784, 2019, doi: 10.1016/j.ymthe.2019.03.002.
7. E. A. Hemann, M. Gale, and R. Savan, “Interferon lambda genetics and biology in regulation of viral control,” Front Immunol, vol. 8, no. DEC, Dec. 2017, doi: 10.3389/fimmu.2017.01707.
8. M. Plotnikova et al., “IFN-λ1 displays various levels of antiviral activity in vitro in a select panel of RNA viruses,” Viruses, vol. 13, no. 8, Aug. 2021, doi: 10.3390/v13081602.
9. V. Vysochinskaya, S. Shishlyannikov, Y. Zabrodskaya, E. Shmendel, S. Klotchenko, O. Dobrovolskaya, N. Gavrilova, D. Makarova, M. Plotnikova, E. Elpaeva, A. Gorshkov, D. Moshkoff, M. Maslov, A. Vasin Influence of lipid composition of cationic liposomes 2X3-DOPE on mRNA delivery into eukaryotic cells. Pharmaceutics. 2022; 14:xx-xx
10. A. A. Lozhkov *et al.*, “Simultaneous Detection of RIG-1, MDA5, and IFIT-1 Expression Is a Convenient Tool for Evaluation of the Interferon-Mediated Response,” *Viruses*, vol. 14, no. 10, pp. 1–12, 2022, doi: 10.3390/v14102090.
11. NCBI's database; www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene
12. UniProt database; http://www.uniprot.org

13. Rehwinkel, J.; Gack, M.U. RIG-I-like receptors: Their regulation and roles in RNA sensing. Nat. Reviews. Immunol. **2020**; 20: 537–551.
14. D. Weissman, "mRNA transcript therapy," *Expert Rev Vaccines*, vol. 14, no. 2, pp. 265–281, 2014, doi: 10.1586/14760584.2015.973859.
15. Thompson, M.R.; Kaminski, J.J.; Kurt-Jones, E.A.; Fitzgerald, K.A. Pattern recognition receptors and the innate immune response to viral infection. Viruses **2011**, 3, 920–940.
16. Busnadiego, I., Fernbach, S., Pohl, M. O., Karakus, U., Huber, M., Trkola, A., ... & Hale, B. G. (2020). Antiviral activity of type I, II, and III interferons counterbalances ACE2 inducibility and restricts SARS-CoV-2. MBio, 11(5), e01928-20.

# Figures

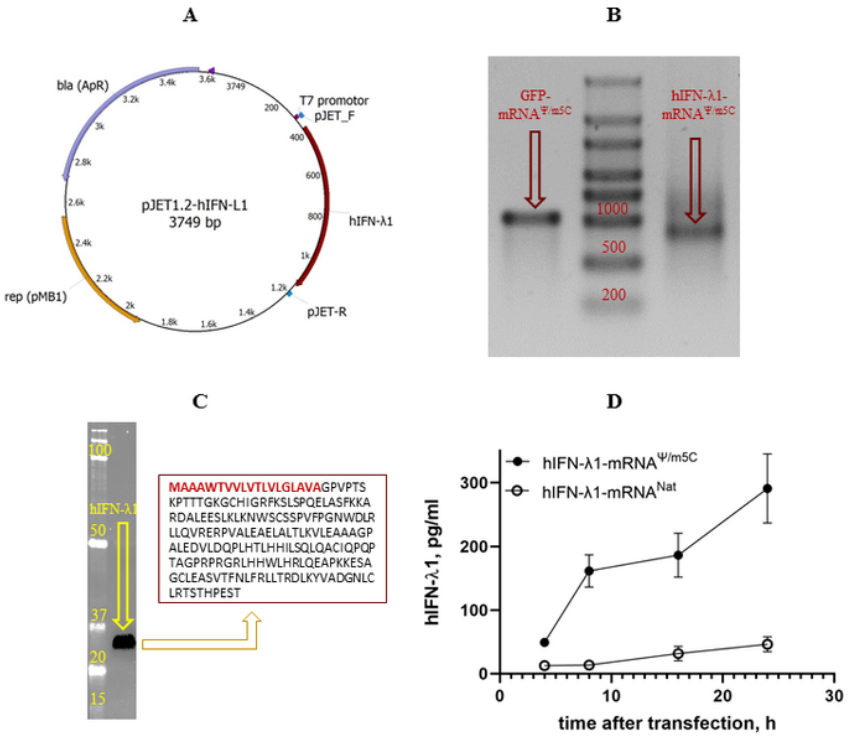
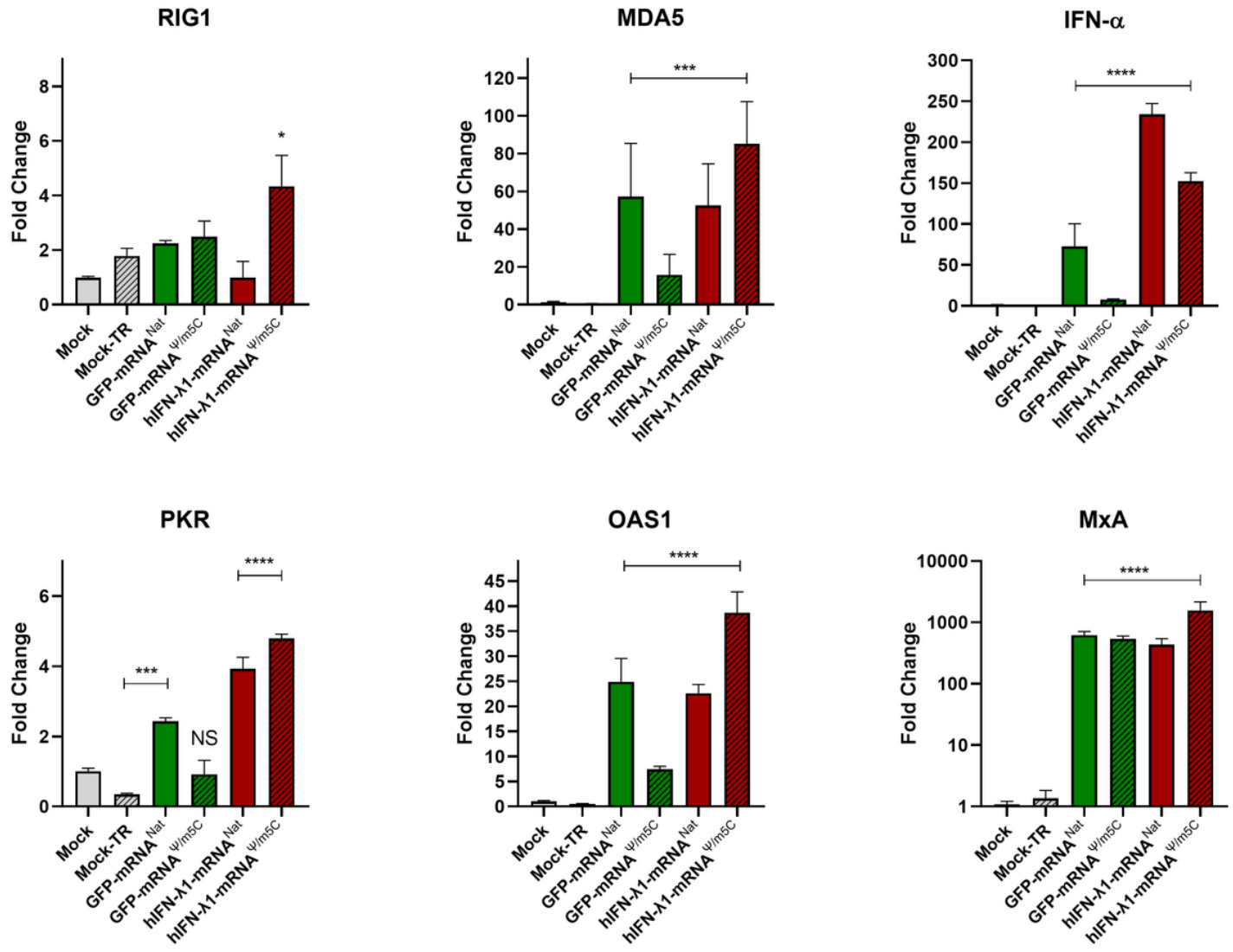



**Figure 1**

**Expression of exogenous mRNA encoded hIFN-λ1 in A549 cells. A.** Scheme of pJet1.2-based hIFN-λ1 construction containing the T7 promoter for *in vitro transcription*. **B.** Analysis of transcribed mRNA (with polyA+) encoded GFP (960 bp) and hIFN-λ1 (670 bp) by denaturing agarose gel electrophoresis. **C.** Immunoblot analysis of hIFN-λ1 expressed in A549 cells after 24 hours post transfection with hIFN-λ1-mRNA$^{\Psi/m5C}$. **D.** Dynamics of hIFN-λ1 accumulation in A549 cell supernatants.




## Figure 2

**Exogenous mRNAs increase the expression of the MDA5, IFN-α, PKR, OAS1 and MxA in A549 cells.** Gene expression was analyzed via the ΔΔCt method (relative to GAPDH). Statistical significance (p-value) was determined by Kruskal-Wallis test, followed by Dunn's multiple comparisons test; **** — adjusted $P_{value}$ < 0.0001; *** < 0.001; **— < 0.01; * — < 0.05 compared to Mock-TR (cells transfected with transfection reagent without mRNA). Data are represented as median ±SD.

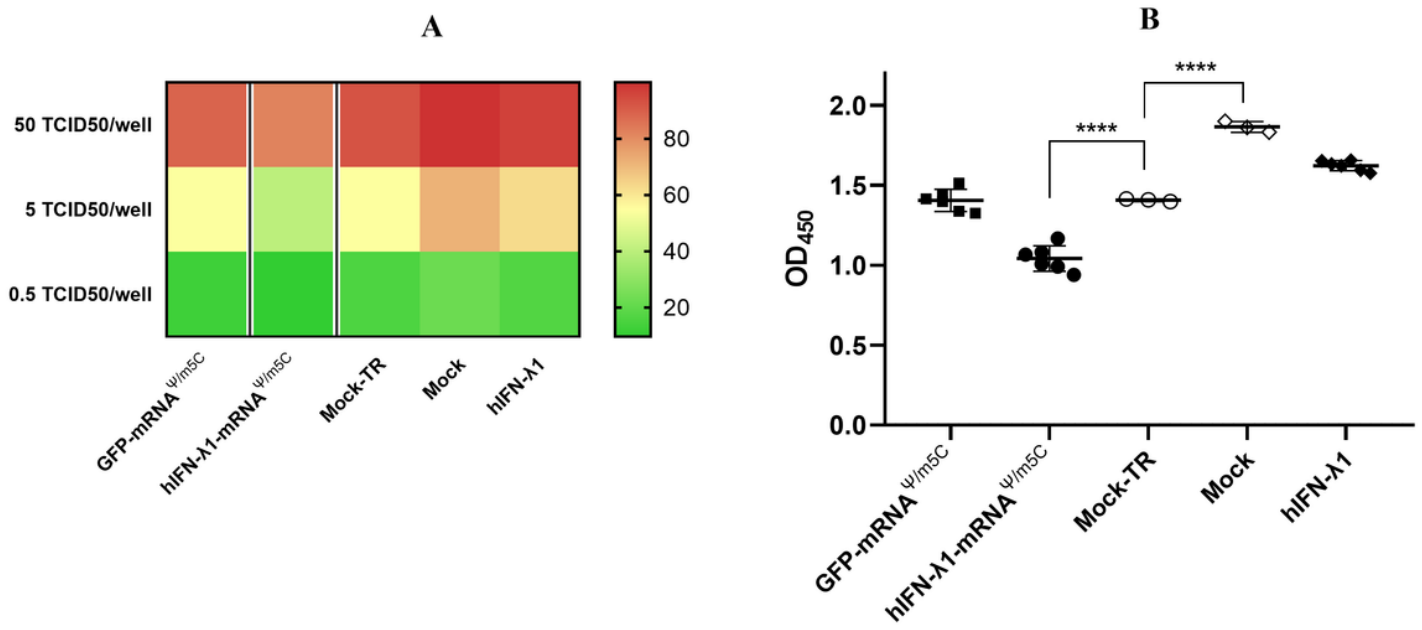



**Figure 3**

**Inhibition of influenza A/California/07/09 (H1N1pdm09) reproduction in mRNA treatment.** Viral antigen was measured at 24 hpi by in-cell ELISA with anti-NP antibodies **A.** Visualization of viral NP levels in *MDCK cells* after mRNAs transfection and infection with A/California/07/09 (H1N1pdm09) at 0.5, 5 and 50 TCID50 per well. **B**. The hIFN-λ1-mRNA$^{\Psi/m5C}$ transfection significantly reduces the level of NP protein in MDCK cells (data presented for 5 TCID50 per well). P-$_{value}$ significance levels were obtained by comparing groups using the Kruskal-Wallis test. ****—Adjusted $p_{value} < 0.0001$. Data are represented as mean ± SD.

# Supplementary Files

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