



Protein Nanoparticles and Intracellular Infections Induce Interferon Gene Expression

Morozova OV^{1,2,3}, Pritchina TN¹ and Isaeva EI¹

¹Ivanovsky Institute of Virology of the National Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, 16 Gamaleya Street, 123098, Moscow, Russian Federation.

²Federal Research and Clinical Center of Physical-Chemical Medicine of Federal Medical Biological Agency, 1a Malaya Pirogovskaya Street, 119435, Moscow, Russian Federation.

³Moscow Institute of Physics and Technology, 9 Institutsky Per., 141700, Dolgoprudny, Moscow Region, Russian Federation

Corresponding author: Olga V. Morozova, Ivanovsky Institute of Virology of the National Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, 16 Gamaleya Street, 123098, Moscow, Russian Federation. **Tel:** +7 916 421 2628. **E-mail:** omorozova2010@gmail.com

Received: March 27, 2025; **Accepted:** May 23, 2025; **Published:** June 02, 2025

©**Copyright 2025:** Morozova et al. This is an open access article distributed under the terms of the Creative Commons Attribution License [CC-BY 4.0.], which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Abstract

Intracellular delivery of natural and artificial nanomaterials is based on endocytosis, phagocytosis, macropinocytosis and pinocytosis. The endocytosis is the main way for nanomaterials of 50-500 nm even in the absence of receptors. In endocytic course both natural, environmental and artificial nanomaterials become trapped in endosomes and later in lysosomes, where lysosomal enzymes hydrolyze foreign proteins, nucleic acids, carbohydrates and lipids. Antigen presentation of the short hydrolyzed fragments with the major histocompatibility complex (MHC) induces innate immunity with cytokine gene expression.

Objective: analysis of human interferon gene expression in cells and culture media in the presence of protein nanoparticles, extracellular vesicles and post infection with *Mycoplasma* spp., influenza A virus (IAV) and respiratory syncytial virus (RSV).

Methods: Protein nanoparticles (NP) were fabricated by nanoprecipitation of bovine serum albumin (BSA) and human immunoglobulins (Ig) from their solutions in fluoroalcohol. Extracellular vesicles (EV) were isolated from the conditioned culture media of human embryonic and cancer cells by means of differential centrifugation at 21,000 g. Interferon (IFN) α , β , γ and λ RNA were detected using reverse transcription with subsequent quantitative real-time PCR (RT²-PCR) with fluorescent hydrolysis probes. Interferons were also revealed by using ELISA.

Results: NP consisting of non-immunogenic proteins (albumin and human IgG) induced human IFN α , β and λ , but not IFN γ gene expression. Neither IFN RNA of type I and II nor corresponding proteins were detected in the presence of EV due to flexible conformations of lipids and trace amounts of integral transmembrane proteins in cellular membranes. The only exception was IFN λ detected by ELISA with concentrations 60-70 pg/ml in culture media of the human embryonal cells after addition of heterologous EV isolated from human cancer cells. Both viral and mycoplasma infections resulted in IFN gene expression that inhibited subsequent additional viral infections.

Conclusion: Th1 polarized innate immunity with IFN production resulted in protection against further viral infection.

Keywords: Protein nanoparticles; Influenza A virus; Respiratory syncytial virus; *Mycoplasma* spp.; Embryonic and transformed cell lines; Culture media; Fetal calf serum (FCS); Interferons (IFN); Reverse transcription with real-time PCR (RT²-PCR)

Introduction

Protective systems of vertebrates include immune, nervous and hormonal systems. Regulation of normal physiological processes as well as defense from external pathogens (pathogen-associated molecular patterns (PAMPs)) and internal danger signals (damage-associated molecular patterns (DAMPs) or alarmins that release from cells after stress and necrosis) are mediated by more than 300 known cytokines. Cellular pattern recognition receptors (PRRs) are located on surface of plasma membranes or intracellular vesicles, recognize the PAMPs/DAMPs and trigger cytokine gene expression with their secretion and further activation of other immune cells. The pathogen recognition involves PRRs such as Toll-like receptors (TLR), retinoic acid-inducible gene I (RIG I)-like helicases (RLH), nucleotide-binding oligomerization domain-like receptors, C type lectin-like receptors, intracellular DNA receptors and probably other, not-yet-identified molecules. Among them TLR3 recognizes double-stranded RNA, which are viral replicative intermediates, and after the signal transduction up-regulates the IFN α / β and IFN-stimulated genes (ISG) expression with antiviral activity. TLR9 binds with double-stranded DNA, mainly containing unmethylated CpG motifs that are more prevalent in bacterial than in eukaryotic DNA. The exogenous and endogenous stimuli can also induce epigenetic modifications and metabolic adaptations which may cause chronic inflammation, trained myelopoiesis and unspecific trained immunity [1-3] with subsequent development of immunological disorders (allergic and autoimmune diseases [1]), cardiovascular diseases (atherosclerosis, myocardial infarction or aneurysms) [3] and neurodegenerative disorders [2]. The ubiquitin-proteasome system is the major proteolytic pathway that degrades intracellular proteins. Abnormalities of the ubiquitin-proteasome system lead to DAMPs, cytokine gene expression and autoinflammatory syndromes. Proteasome defects are known to lead to the retention of misfolded proteins in the endoplasmic reticulum [1]. Many signaling ways of the innate immunity result in the interferon (IFN) gene expression. IFN of type I (IFN-I) – IFN α and IFN β are of particular interest because they are induced at high concentrations within hours after exposure to exogenous PAMPs and endogenous DAMPs and possess antiviral properties. Virtually all cells express the IFN-I receptors [4,5].

Currently available pharmacotherapy of various diseases including immunological malfunctions is associated with poor stability, short *in vivo* persistence, decreased intestinal and blood-brain permeation, constrained intracellular transport, and nonselective delivery causing adverse side effects. Unfortunately, majority of conventional macromolecular drugs remain lacking targeting specificity and cellular permeability. The nanostructures ranging in size from a few to hundred nanometers are capable to be accumulated inside cells and to efficiently release the payloads. The main entry ways for nanomaterials including natural, environmental and artificial nanoparticles, vesicles, viruses, intracellular bacteria (such as mycoplasma) and various vaccines are clathrin-mediated and caveola-mediated endocytosis, through which the particles remain trapped in endosomes and lysosomes with induced cytokine and chemokine production and activation of other immune cells [6,7].

Bio-reactivity of NP is based on proteins necessary to compensate possible deficiency of enzymes, growth factors, cytokines and hormones as well as for antibody-dependent targeted delivery [8]. The protein NP advantages include their low (if any) toxicity, natural biodegradation, biological activity as well as amino-, carboxy- and thiol-groups for chemical modifications and

subsequent covalent drug attachment. The protein defined primary structures and developed tools of genetic engineering permit to use their surface functional groups both for the covalent binding with drugs and for targeting of ligands. Moreover, NP from bioactive proteins can be used as therapeutic agents without additional modifications. Current limitations for protein NP implementation in clinical medicine encompass the protein digestion in gastrointestinal tract, poor membrane permeability and tissue penetration. Therefore, despite desirable non-invasive peroral, intranasal and pulmonary administration the proteins are not instilled orally and parenteral injections remain the standard delivery method for proteins. Protein NP exploit natural pathways to selectively deliver drugs to cells [6-9].

Various methods for preparation of NP from natural and synthetic polymers can be applied to pre-formed polymers (solvent evaporation method; spontaneous emulsification/solvent diffusion method; nanoprecipitation, salting out methods, spray drying) or to their monomers (emulsion or interfacial polymerization etc). Anti-solvent precipitation is based on mixing of a polymer solution in its corresponding solvent with a non-solvent resulting in NP nucleation and subsequent growth [8].

Our research was aimed at detection of human IFN gene expression in cells in the presence of protein NP, EV and post infection with *Mycoplasma* spp., IAV and RSV.

Materials and Methods

Cell cultures

Human embryonic fibroblasts and lung cells from the Russian State Tissue Culture Collection (National Research Center of Epidemiology and Microbiology, Moscow, Russia) were grown in Minimum Essential Medium Eagle (MEM) with 7% fetal calf serum (FCS) (HyClone, Thermo Scientific, USA) supplemented with L-glutamine, 50 units/ml penicillin and 50 µg/ml streptomycin until 80% confluent monolayers formation.. Human carcinoma of the cervix cell line HeLa, human larynx carcinoma HEP-2 and oral epithelial carcinoma L41 cells were obtained from the Russian State Tissue Culture Collection (National Research Center of Epidemiology and Microbiology, Moscow, Russia) and grown in culture medium 199 supplemented with 7% FCS in the presence of the same antibiotics and L-glutamine until subconfluent monolayers.

Viruses

Influenza A/Aichi/2/1968 (H3N2) (Genbank accession numbers KC895864 and KC895865 (<https://www.ncbi.nlm.nih.gov>) was kindly provided by Isaeva E.I. (National Research Center of Epidemiology and Microbiology, Moscow, Russia).

Respiratory syncytial virus (RSV) strain 2733 has been isolated from the nasopharyngeal swab of a child in Moscow, Russia in 2012 and deposited to the Russian State Collection of Viruses (D.I. Ivanovsky Institute of Virology of the National Research Center of Epidemiology and Microbiology of N.F. Gamaleya of the Russian Ministry of Health, Moscow, Russia). GenBank accession number is KP713401 (<https://www.ncbi.nlm.nih.gov/nucleotide/KP713401.1>).

Fabrication and analysis of protein NP

Protein NP was constructed by nanoprecipitation as previously described [8]. In brief, BSA and human IgG with concentrations up to 20 mg/ml were dissolved in hexafluoroisopropanol (HFIP). The solutions were slowly added to 40% ethanol until final protein concentration 2 mg/ml with vigorous vortexing. After incubation at 58°C and reduced pressure the water-insoluble particles were pelleted at 15,000 g and washed with deionized water. The protein NP morphology was analyzed by atomic force and scanning electron microscopy. Their sizes were additionally evaluated by dynamic light scattering (DLS) [8]. The protein NP were sonicated for 20 min and added in culture media of human embryonic and cancer cells.

Isolation of EV

EV were isolated from the conditioned culture media of the human embryonic and cancer cell lines by means of two-step differential centrifugations at 9,000 g for 15 min to remove membrane debris and 20,800 g for 30 min at +2°C to precipitate EV [9,10]. Their structures were confirmed by scanning transmission electron microscopy (STEM) after standard contrasting with 2% uranyl acetate solution [10].

Reverse transcription with real time PCR (RT²-PCR)

Total nucleic acids were isolated from 100 µl of control intact and experimental cells using “Proba-NK” kit (“DNA-technology”, Russia). Then the reverse transcription with random hexamer primer was performed using “Reverta-L” kit (AmpliSens, Russia). The specific primers and fluorescent hydrolysis probes to detect *Mycoplasma* spp. DNA as well as IAV and RSV RNA are shown in the Table. RT²-PCR to detect mRNA of human interferons IFN α , β , γ , λ , interleukin (IL)4, IL6 was performed as previously described [11]. Quantitations of genome-equivalents were based on Lukyanov-Matz’s equation and on calibration curve with the standards.

Table: Structures of primers and fluorescent hydrolysis probes (5’- 3’ end) for RT²-PCR.

Targets	Forward primer	Reverse primer	TaqMan probe
Influenza A virus (IAV)	TCTGTCTGGCTCTCGG CC	GATTGTTGCATATTTTCCCCG	SyberGreen I
Respiratory syncytial virus A (RSA) [12]	AGATCAACTTCTGTCA TCCAGCAA	TTCTGCACATCATAATTAGGAGTA TCAAT	R6G- CACCATCCAACGGAGCACA GGAGAT-BHQ2
<i>Mycoplasma</i> spp.	CGCTAAACATCATCGC CTTGGTA	GGCGAATGGGTGAGTAACACG	R6G- ACCAACTAGCTAATGTTCCG CACCCCG-BHQ2

Sequencing and phylogenetic analysis

Sequencing of RT-PCR products was performed in "Syntol" (<http://syntol.ru>) using an automatic DNA analyzer of the ABI 310 model (Applied Biosystems, USA) and the BigDye 3.1 kit. Phylogenetic analysis of the nucleotide sequences of the RSV N gene fragment was carried out by the Mega 6.06 software (<http://www.megasoftware.net/>) using 5 alternative algorithms with 1000 replications [13].

ELISA

IFN α , IFN γ , IL4 and IL10 were detected using ELISA kits «Vector-Best» (Russia). IFN β and INF λ was revealed by using the corresponding kits DIFNB0 (R&D Systems, Inc., USA) and ELH- IL29-1 (RayBiotech, Inc., Russia).

Results and Discussion

Innate immunity induction with protein nanoparticles

Nanomaterials may be ingested by cells and recognized by PRRs resulting in immunomodulatory stimuli. To determine inherent immunogenicity of protein NP, lacking any known immunostimulatory components, BSA NP and human IgG NP were added to human embryonic (fibroblast and lung) and cancer (HeLa, HEp-2 and L41) cells as well as to donor blood leukocytes. In 1 day posttreatment RNA transcription of canonical pro-inflammatory IFN of I and III types but not IFN γ , IL 4 and IL10 was revealed

by RT²-PCR (Figure 1). Production of human IFN α , β and λ was additionally confirmed by ELISA of conditioned cell culture media. Polarization indexes calculated as ratio of Th2:Th1 cytokine RNA amounts were near 0. Accordingly, the protein NP induced Th1 polarized innate immunity. Protein NP in the range of 100-300 nm were previously shown to remain non-immunogenic [14]. The absence of detectable antibodies in the presence of protein NP could be caused by Th1 polarized mainly T-cellular immune response [7, 9]. Despite IFN α , β and λ gene expression in the presence of the BSA NP and IgG NP the antiviral properties against the IAV and RSV were not observed (data not shown). It could be explained by the lack of the interferon-stimulated genes (ISG) expression, in particular, MX1 gene encoding human myxovirus resistance protein 1 (MxA) [15]. MX1 gene expression is regulated by IFN type I and III and is not inducible directly by viruses or other external and internal stimuli [15]. Besides the meaningful antiviral response includes different PRRs capable to recognize both virion surface glycoproteins and nucleic acids. But the artificial protein NP lacks any nucleic acids.

Low immunogenic extracellular vesicles

In the presence of homologous EV neither IFN α , β , γ RNA nor corresponding cytokines were detected by RT²-PCR and ELISA, respectively, because of flexible conformations of lipids in solutions and trace amounts of the integral transmembrane proteins in cellular membranes [9]. The only exception was IFN λ with its significant concentration growth in the culture medium of the human lung embryonic cells up to 70 pg/ml after treatment with heterologous EV (Figure 1).

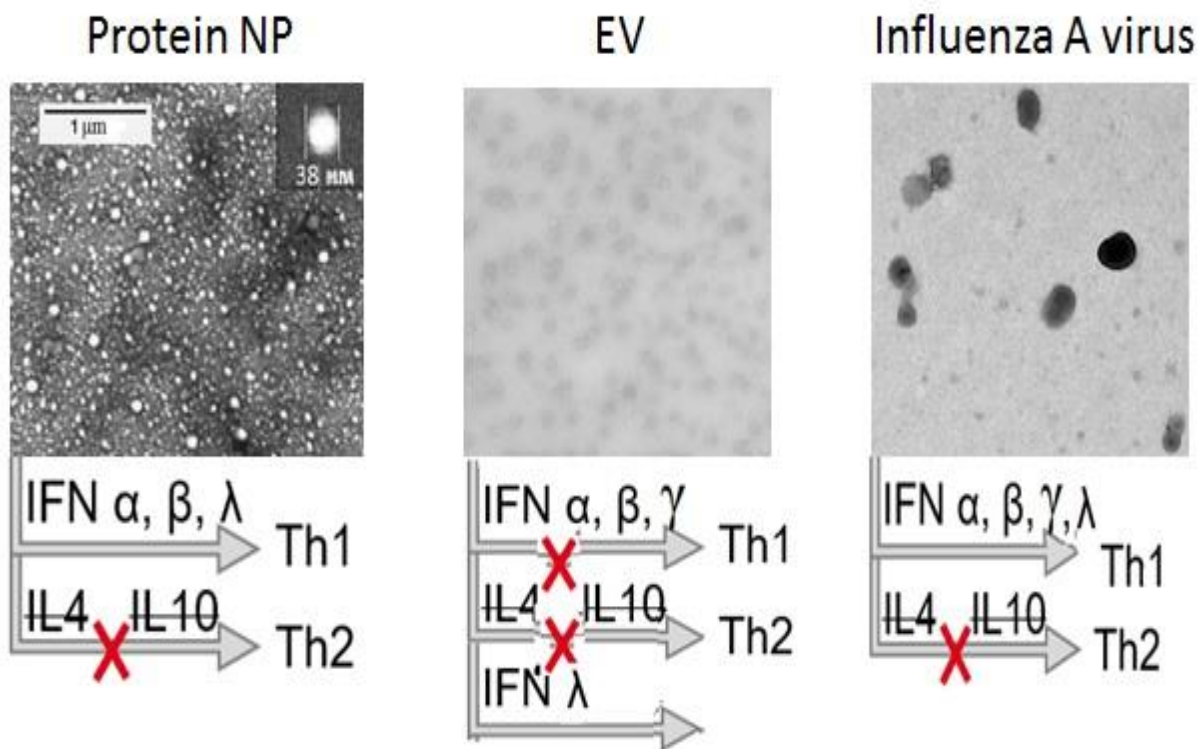


Figure 1: Scheme of innate immunity induced by the protein nanoparticles, extracellular vesicles and infection with the influenza virus A.

Both viral and mycoplasma infections resulted in IFN gene expression that inhibited viral infections.

Respiratory viral infections

Infection with influenza A virus H3N2 Aichi/1/68 strand of the permissive HEp-2 cells caused Th1 cytokine gene expression including all IFN genes of 3 types during the first 7 passages (Figure 1). The viral loads calculated on the base of threshold

cycles (Ct) of RT²-PCR with maximal values for the passage 4 reached 10 genome equivalents of influenza A virus RNA per cell (Figure 2).

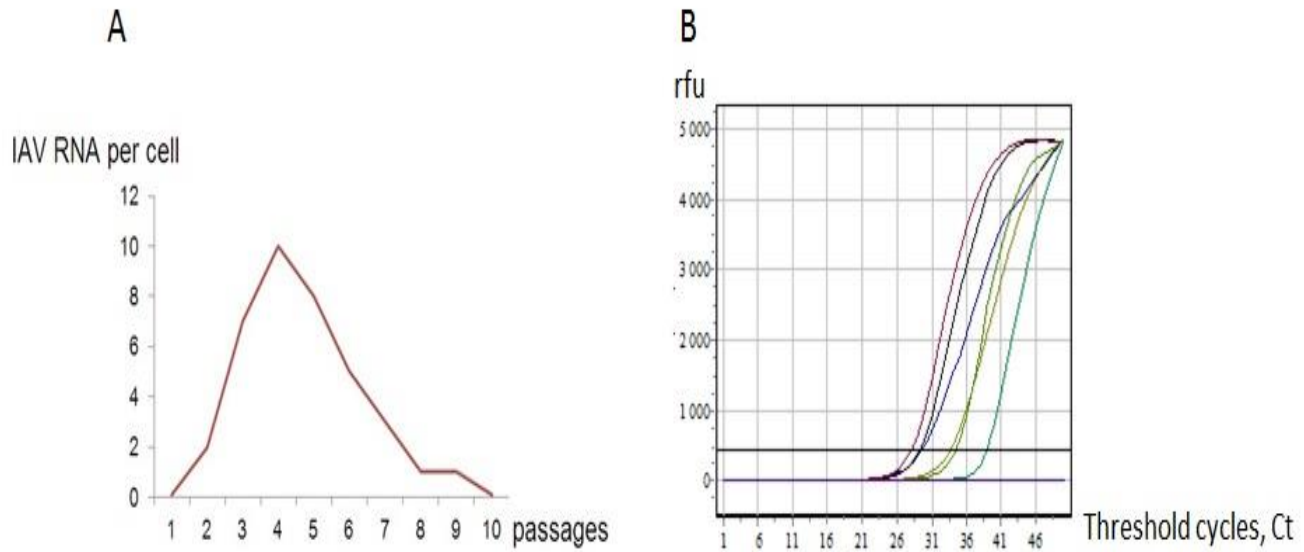


Figure 2: Inhibition of the influenza A virus infection in human cell lines contaminated with *Mycoplasma* spp.

Respiratory syncytial virus (RSV) type A strain 2733 (GenBank access number (<http://www.ncbi.nlm.nih.gov> KP713401) was isolated from nasopharyngeal swab of a child in Moscow in 2012. Phylogenetic analysis using Mega 6.06 software and nucleotide sequences of the RSV N gene fragments revealed similar topologies of phylogenetic trees constructed by means of 5 alternative algorithms (Maximum likelihood, Neighbor-Joining, Minimum-Evolution, UPGMA and Maximum Parsimony) and excellent bootstrap support (100). Phylogenetic analysis of the RSV N gene fragment nucleotide sequences (Figure 3) showed that the strain 2733, isolated from the nasopharyngeal lavage of a patient in Moscow in 2012, belongs to type A and is most similar to RSV strains isolated in USA in 1991 and 2005, but not to isolates from Europe. Noteworthy that the RSV nucleoprotein N gene structure remained very stable over the past 37 years (from 1988 to 2025) (Figure 3). Homology levels of the nucleotide sequences were 96-98% and amino acid sequences 98-99% for RSV type A isolates from different continents. The evolution stability of the RSV nucleoprotein N with variations between RSV types A and B in the range 2-7% suggested its numerous functions and lethal mutant variants.

One should note that the RSV-induced innate immune response was weaker than for infection with the IAV in spite of both respiratory viruses are RNA-containing with double stranded RNA in their replication cycles that can be recognized by TLR3 [16]. RSV replication and budding *in vitro* are inefficient; therefore, its infectivity is unstable [16]. The RSV nonstructural proteins were shown to suppress the IFN-mediated innate immunity by degrading or inhibiting multiple cellular factors required for either IFN induction or response pathways, including RIG-I, IRF3, IRF7, TBK1 and STAT2 [17].

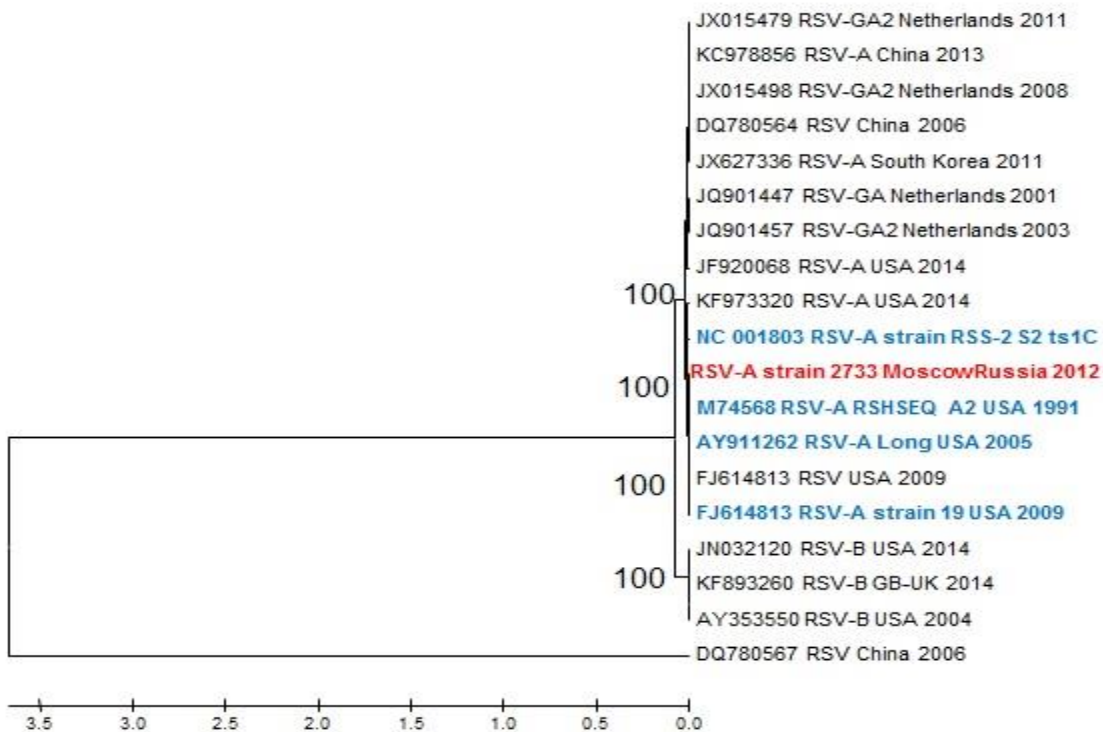


Figure 3: Phylogenetic analysis of the RSV N gene fragment nucleotide sequences using Mega 6.06 software (UPGMA, 1000 replications). Reference prototype strains are shown in blue. The strain 2733 isolated in Moscow, 2012 is in red.

Antiviral activity of interferons induced by intracellular *Mycoplasma* spp.

Mycoplasmas are the smallest bacteria of 0.3 - 0.8 μm in diameter. So small sizes along with membrane flexibility permit them to pass through antibacterial filters with pore diameters of 0.45 μm . Due to resistance of mycoplasmas to most antibiotics employed in cell cultures their extracellular variants survive in culture media, in sera, whereas the intracellular location may protect them from antibacterial drugs [18]. Infection rates of cell cultures with *Mycoplasma* spp. are in the range of 15–35%, but sometimes up to 80% [18]. Quantitative real time PCR revealed the fluorescent thresholds cycles (Ct) in the range 27.6 – 39.7 (Figure 2B) that corresponded up to 5.4×10^3 genome-equivalents in reaction mixtures or less than 1 mycoplasma per 1 human cell. Despite slow growth *in vitro* one mycoplasma cell can grow to 10^6 colony forming units per ml during several days [18].

Infection of human embryonic and cancer cells with *Mycoplasma* spp. resulted in IFN types of I, II and III gene expression. IFN are known to possess antiviral properties. Therefore, subsequent viral infections of *Mycoplasma*-contaminated human cells with respiratory viruses IAV and RSVA resulted in gradual decline of viral loads until undetectable levels in 10 passages (Figure 2A). Moreover, all attempts of modelling of mixed bacterial and viral infections caused trained immunity with fast and enhanced IFN response [2, 3]. Malignant transformation of normal cells including model embryonic cells may be caused by physical and chemical factors as well as cellular and viral oncogens.

Thus, the first cell line HeLa is characterized to contain human papillomavirus [18]. Other viral oncogens are known to be from Epstein-Barr virus, hepatitis B virus (HBV) and hepatitis C virus (HCV), human immunodeficiency virus, human herpes virus and others. Innate immunity induced by natural viruses as well as artificial protein NP and EV cannot prevent RNAemia, widespread intracellular persistence of RNA-containing viruses and oncogenesis.

Conclusion

Interferon gene expression induced by the protein nanoparticles and extracellular vesicles without internal nucleic acids does not prevent infections with RNA-containing viruses. However, *Mycoplasma* contamination of culture media and human tissue cultures may protect cells from further additional infection with respiratory RNA-containing enveloped viruses.

Acknowledgements

This work was supported by the Ministry of Science and Higher Education of the Russian Federation (Goszadaniye) (project No. FSMG-2023-0015; agreement 075-03-2025-662 from 2025-01-17).

Conflicts of Interest

The author declares no conflict of interest.

References

1. Funes SC, Rios M, Fernández-Fierro A, Di Genaro MS, Kalergis AM (2022) Trained Immunity Contribution to Autoimmune and Inflammatory Disorders. *Front Immunol* 13: 868343.
2. Domínguez-Andrés J, Dos Santos JC, Bekkering S, Mulder WJM, van der Meer JWM, et al, (2023) Trained Immunity: Adaptation Within Innate Immune Mechanisms. *Physiol Rev* 103(1): 313-346.
3. Kleimann P, Irschfeld LM, Grandoch M, Flögel U, Temme S (2024) Trained Innate Immunity in Animal Models of Cardiovascular Diseases. *Int J Mol Sci* 25(4): 2312.
4. Kalinke U, Prinz M (2012) Endogenous, or Therapeutically Induced, Type I Interferon Responses Differentially Modulate Th1/Th17-Mediated Autoimmunity in the CNS. *Immunol. Cell Biol* 90(5): 505-509.
5. Sengupta P, Chattopadhyay S (2024) Interferons in Viral Infections. *Viruses* 16(3): 451.
6. Oh N, Park J-H (2014) Endocytosis and Exocytosis of Nanoparticles in Mammalian Cells. *Int J Nanomedicine* 9: 51-63.
7. Morozova OV, Sokolova AI, Pavlova ER, Isaeva EI, Obraztsova EA, et al. (2020) Protein Nanoparticles: Cellular Uptake, Intracellular Distribution, Biodegradation and Induction of Cytokine Gene Expression. *Nanomedicine* 30: 102293.
8. Morozova OV, Pavlova ER, Bagrov DV, Barinov NA, Prusakov KA, (2018) Protein Nanoparticles with Ligand-Binding and Enzymatic Activities. *Int J Nanomedicine* 13: 6637-6646.
9. Morozova OV, Golubinskaya PA, Obraztsova EA, Ereemeev AV, Klinov DV (2024) Structures, Stability and Cellular Uptake of Protein Nanoparticles (NP) and Extracellular Vesicles (Evs). *Current Drug Delivery* 21.
10. Morozova OV, Obraztsova EA, Klinov DV (2024) Features of Protein Nanoparticles Surrounded By Cell Membrane Shells. *Biotechnology*. 40(7): 144-145. DOI: 10.56304/S0234275824071001 (In Russian).
11. Ospelnikova TP, Morozova OV, Isaeva EI, Andreeva SA, Lyzogub NV, et al (2016) Respiratory Viruses and Proinflammatory Cytokines Imbalance in Adults and Children with Bronchial Asthma. *J Infect Dis Prev Med* 4(2): 138.
12. Tan L, Lemey P, Houspie L, Viveen MC, Jansen NJ, et al. (2012) Genetic Variability among Complete Human Respiratory Syncytial Virus Subgroup A Genomes: Bridging Molecular Evolutionary Dynamics and Epidemiology. *PLoS One* 7(12): e51439.
13. Tamura K, Stecher G, Peterson D, Filipinski A, Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Mol Biol Evol* 30(12): 2725-2729.
14. Perumal OP, Podaralla SK, Kaushik RS (2017) Method of Forming Non-Immunogenic Hydrophobic Protein Nanoparticles, and Uses Therefor, USOO9616021B2.
15. Haller O, Kochs G (2011) Human MxA Protein: An Interferon-Induced Dynamin-Like GTPase with Broad Antiviral Activity. *J Interferon Cytokine Res* 31(1): 79-87.

16. Collins PL, Fearn R, Graham BS (2013) Respiratory Syncytial Virus: Virology, Reverse Genetics, and Pathogenesis of Disease. *Curr Top Microbiol Immunol* 372: 3-38.
17. Goswami R, Majumdar T, Dhar J, Chattopadhyay S, Bandyopadhyay SK, (2013) Viral Degradosome Hijacks Mitochondria to Suppress Innate Immunity. *Cell Res* 23(8): 1025-1042.
18. Drexler HG, Uphoff CC (2002) Mycoplasma Contamination of Cell Cultures: Incidence, Sources, Effects, Detection, Elimination, Prevention. *Cytotechnology* 39(2): 75-90.

-