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Early Protective Effect of Live Influenza Vaccine against Homologous and Heterologous Influenza Infection at Different Times after Immunization

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Abstract

Live influenza vaccine (LAIV) is an effective tool in the fight against influenza infection. LAIV is easy to administer, economical and fast to produce, and stimulates a systemic and local immune response. The aim of the study was to study the early protective effect of LAIV in mice against homologous and heterologous influenza infection within one week of LAIV immunization and possible pathways of immune system activation. We studied expression of early cytokines and type I interferons in response to LAIV introduction in THP-1 cell line. Mice were immunized intranasally with the vaccine virus A/17/South Africa/2013/01(H1N1)pdm09 at a dose of 6 lg EID50. The production of Interleukin-6 (IL-6), Tumor necrosis factor-α (TNF-α) and type I interferons in the respiratory tract of mice was determined by ELISA. Infection was carried out on the 3rd or 5th day after immunization with the influenza viruses A/South Africa/3626/2013(H1N1)pdm09 or A/Indonesia/5/2005(H5N1) IDCDC-RG2. When both the vaccine virus and the parent virus A/South Africa/3626/2013(H1N1)pdm09 were introduced into THP-1 cell culture, an increase in the expression of type I interferon was observed. Immunization with LAIV lead to increase in the production of early cytokines and type I interferons in the respiratory tract of mice. The mice were completely protected on 5 after immunization against lethal homologous and heterologous influenza infection and partially protected on day 3. The data obtained may indicate the benefit of using LAIV during the seasonal increase in acute respiratory viral infections due to stimulation of innate immune factors.

Keywords: Influenza, Live influenza vaccine, THP-1, Early cytokines, Type I interferons, Early protection, Pandemic

Introduction

In recent years, the World Health Organization (WHO) has identified the problem of vaccination against infections as a priority area for the development of medical and biological science [1]. Acute respiratory infections (ARI) occupy an important place among public health problems due to high morbidity and mortality [2]. The ongoing circulation of the new coronavirus infection (COVID-19) caused by the SARS-CoV-2 coronavirus since December 2019 is an emergency, which also leads to a growing interest in respiratory diseases [3]. Research results show that not only COVID-19, but also influenza vaccination is associated with a lower risk of SARS-CoV-2 infection [4,5]. Leading influenza specialists and WHO experts believe that against the background of a decrease in population immunity to influenza, the risk of infection with bird flu viruses, which have become more virulent and overcome the interspecies barrier, is increasing [6]. In 2022, outbreaks caused by A/H5N1 viruses were registered in Russia among domestic and wild birds. Mortality rates for A/H5N1 influenza viruses reach 60 % [7]. In this regard, the development of universal influenza vaccines that protect against both seasonal and highly pathogenic "bird" flu is in demand.

To prevent seasonal influenza, annual immunization is recommended using inactivated influenza vaccines (IIV) or live attenuated influenza vaccines (LAIV) [8]. IIVs are safe and effective against

homologous strains and are recommended for children aged 6 months and older. The IIVs provide protection through antibodies directed against the surface glycoprotein hemagglutinin (HA) [9]. Intranasal LAIV, recommended for children aged 2 years and older, induces a broader immune response in which protection is mediated not only by systemic antibodies but also by local IgA and cellular immune responses [9,10]. Trivalent LAIV has been used in Russia since the 1980s; in 2003, LAIV was licensed for use in North America and Europe [11]. Modern LAIVs include attenuated reassortant strains of influenza viruses that have a mixed genome: the genes encoding HA and NA are inherited from the antigenically relevant strain, and 6 genes encoding non-glycosylated proteins are inherited from the cold-adapted (ca-) "Master donor virus" (MDV) [11]. In Russia, reassortant vaccine strains of influenza A viruses for LAIV are obtained on the basis of the A/Leningrad/134/17/57(H2N2) MDV [11,12].

The use of intranasal LAIV seems to be a suitable means in the current epidemic situation. Firstly, it is very important to vaccinate against influenza during the rise of SARS-CoV-2, since it has been shown that coinfection with influenza significantly worsens the course of COVID-19 [13]. Secondly, the use of intranasal LAIV allows for the vaccination of large groups of the population. Finally, only LAIV are able to interrupt the transmission of the infectious virus, unlike inactivated vaccines, which has been repeatedly shown in large-scale studies of LAIV [11,14]. One explanation for this effect may be the induction of type I interferon upon intranasal administration of LAIV. Interestingly, vaccine viruses can induce even more significant interferon production than wild-type viruses. It was previously shown that a vaccine candidate based on A/Puerto Rico/8/34(H1N1) (A/PR8) with a deleted NS gene stimulates interferon production in chicken embryos higher than the wild-type A/PR8 virus and can provide protection against infection even before the development of specific adaptive immunity [15]. It has been shown that the use of intranasal LAIV during the period of circulation of seasonal respiratory infections has an indirect effect, protecting against respiratory viruses other than influenza, and this is associated with a change in the cytokine profile [16]. In experiments on vaccination and subsequent heterosubtypic infection (introduction of a wild virus of a different subtype) of mice *in vivo*, it was shown that LAIV reduces the reproduction of wild viruses, which is associated with increased expression of chemokines involved in the recruitment of T lymphocytes to lung tissue [17]. In our previous studies in mice, the early protection provided by LAIV against infection with a homologous influenza virus was assessed [18]. Improved protection was accompanied by a decrease in the content of infectious viruses in the lungs and correlated with an increase in IFN-α expression in the lungs [19]. Activation of early cytokines and their receptors during vaccination affects the subsequent formation of resistance to pathogens. Type I interferons secreted by infected cells shortly after infection with the virus act on the receptors of neighboring cells, preventing further spread of the virus, while the absence of IFN signaling leads to further spread of the virus [20]. In addition to its function as a critical antiviral cytokine, type I IFNs are involved in the development of Th1 immunity [21] and can mediate the sustained expansion of CD8 T cells, increasing their survival in the antigen-dependent proliferative phase, which is critical for the formation of an optimal number of memory cells [22].

The study of innate immunity factors during LAIV immunization is relevant due to the fact that in some cases, for example in old age, the adaptive T- and B-immune response can be partially replaced by the innate function [23]. Therefore, the aim of the present study was to investigate how LAIV immunization protects against homologous and heterologous infection in the 1st week after immunization. The novelty of this study is in the use of LAIV prepared specifically on the A/Leningrad/134/17/57(H2N2) MDV, and also in the fact that protection was studied on mice at the earliest stages of infection.

Materials and methods

Viruses

Live influenza vaccine virus A/17/South Africa/2013/01(H1N1)pdm09, 'wild' type influenza virus A/South Africa/3626/2013(H1N1)pdm09 and recombinant strain A/Indonesia/5/2005(H5N1) IDCDC-RG2 were obtained from the collection of the Department of Virology of the Federal State Budgetary Institution "IEM". All viruses were grown in embryonated chicken eggs (CE) as previously described, aliquoted and stored at –70 °C until use.

Study of the expression of early cytokines in cell culture THP-1

We used the human monocyte-macrophage cell line (THP-1) to assess early cytokine expression *in vitro*. THP-1 cells were cultured in 24-well cell culture plates at 3.0×106 cells per well with RPMI medium (Roswell Memorial Institute, Buffalo, NY, USA) containing 10 % calf serum, 100 IU/ mL penicillin and 100 μg/mL streptomycin. Before the experiment, cells were incubated at 37 °C and 5 % CO2 for 48 h. Cells were inoculated with 106 EID50/mL viruses A/17/South Africa/2013/01(H1N1)pdm09 or the 'wild'-type parent strain A/South Africa/3626/2013(H1N1)pdm09. As a positive control, we used the Toll -like receptor (TLR) agonist polyinosinic:polycytidylic acid (Poly I:C) (Sigma, St. Louis, MO, USA) at a final concentration of 1 μg/mL. After 24 h of incubation, the contents of the wells were collected for PCR analysis as described earlier [19]. The levels of cytokine gene expression were determined using real-time reverse transcriptase PCR (rRT-PCR). RNA extraction was performed using the RNeasy Mini kit (QIAGEN, Hilden, Germany). RNA was eluted in 50 mL RNase-free water and used as a template for rRT-PCR. For cDNA synthesis, reverse transcription (RT) was performed from RNA using a mixture of oligo (dt) primers and random hexamers and the SuperScript III kit (Invitrogen, Kalsbad, USA). RT-PCR was performed in a CFX96 thermal cycler (Biorad, Hercules, USA) using the intercalating dye SybrGreen in 25 mL reactions containing 5 mL of cDNA sample and primers developed for this purpose using Primer Bank sequences (https://pga.mgh.harvard.edu/primerbank/) (**Table 1**).

Table 1 Oligonucleotide primers for studying mRNA expression of early cytokines in THP-1 cells.

Primers	GenBank accession	Primer and probe sequence (F - forward, R - reverse)
GAPDH	NM_002046	F-CATGAGAAGTATGACAACAGCCT R- AGTCCTTCCACGATACCAAAGT
IFN-alpha	NM_024013.2	F-TCAAAGACTCTCACCCCTGC R-CAGTGTAAAGGTGCACATGACG
IFN-beta	NM_002176.2	F-GCGACACTGTTCGTGTTGTC R-GCCTCCCATCAATTGCCAC
IL-6	NM_000600	F-ACTCACCTCTTCAGAACGAATTG R-CCATCTTTGGAAGGTTCAGGTTG
TNF-alpha	NM_000594	F-CCTCTCTCTAATCAGCCCTCTG R- GAGGACCTGGGAGTAGATGAG

At the end of the reaction, melting curve analysis of the PCR products was performed to confirm the amplification specificity of each primer pair. Data were analyzed using the comparative threshold cycle (ΔΔCt) method, normalized to the expression of the housekeeping gene GAPDH and negative control (medium).

Mouse study

CBA mice (Rappolovo nursery, Leningrad region) were kept in accordance with GOST 33216-2014 "Rules for working with laboratory rodents and rabbits". The study was approved by the Local Committee on the Ethics of the Care and Use of Animals at the Institute of Experimental Medicine, St. Petersburg, protocol 3/23 dated September 20, 2023. Randomized groups of animals (20 mice in each group) were immunized intranasally with 50 μL, distributing the volume evenly between the nostrils, under light ether anesthesia. were immunized intranasally with the LAIV vaccine strain. We used LAIV strain containing 1×10^6 50 % embryonic infectious doses (EID_{50}) of the vaccine strain A/17/South Africa/2013/01(H1N1)pdm09. Animals in the control group were inoculated with PBS in the same way. Nasal turbinated were obtained on days 3 and 5 after intranasal immunization. The samples from 5 animals per group were disrupted in 1 ml PBS containing 100 U/mL penicillin, 100 μg/mL

streptomycin as stated above, followed by centrifugation for 10 min at 6,000×g. Early cytokines levels in the tissue homogenates were assessed using ELISA kits (eBioscience, Frankfurt am Main, Germany).

Challenge study

Experimental infection was carried out on day 3 and day 5 after immunization using the A/Indonesia/5/2005(H5N1) IDCDC-RG2 virus or A/South Africa/3626/2013(H1N1)pdm09 at an infectious dose of 5 50 % mouse lethal doses (MLD_{50}) of each virus. To determine the virus titer in the lungs after challenge, samples from 5 animals from the group were homogenized in 1 mL PBS containing 100 units/mL penicillin, 100 μg/mL streptomycin as stated above. To determine the virus titer, samples were titrated in CE using serial dilutions starting from 1:10, as described previously [24]. Virus titers were expressed as log10 EID_{50}. Animal survival was observed for 2 weeks after the onset of viral challenge. All samples were transferred to the researchers in an anonymized form. The scheme of the experiments on mice is shown in **Figure 1**.

Figure 1 The scheme of the experiments on mice.

Statistical analysis

Statistical data processing was performed using Prism 8 (GraphPad Software, San Diego, USA). Means and standard errors of means (SE) were used to present data. Comparisons between 2 independent groups were performed using Wilcoxon - Mann - Whitney non-parametric tests. The log-rank (Mantel - Cox test) was used to compare the survival distributions. A p-value < 0.05 was considered statistically significant.

Results and discussion

Figure 2 presents the results of the expression of mRNA of early cytokine genes and type 1 interferons in a cell culture of monocyte-macrophage origin (THP-1). When influenza viruses introduced to THP-1 cells, the level of stimulation of mRNA of type I interferons was not inferior to wells with a positive control (**Figures 2(A)** and **2(B)**); and in the case of IL-6, the wild-type virus even exceeded the m-RNA levels caused by Poly I:C (**Figure 2(A)**). In all cases, the mean levels of mRNA expression of early cytokines and type 1 interferons were statistically significantly higher after stimulation with influenza viruses compared to control unstimulated wells (p < 0.05). The introduction of the vaccine virus caused an increase in the expression of interferon alpha mRNA at a level no lower than that observed with the introduction of the wild-type virus (**Figure 2(A)**).

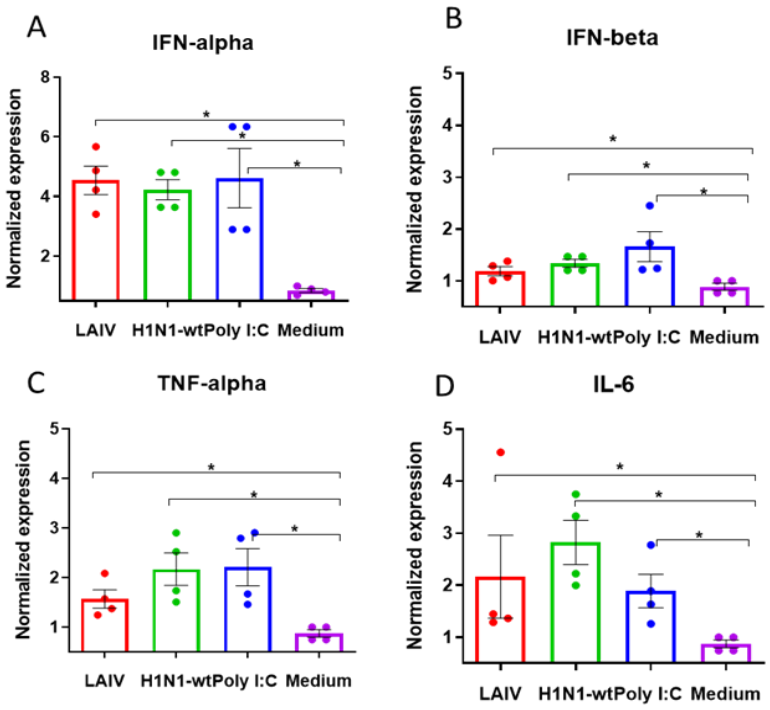

Figure 2 A-D. Type 1 interferons and early cytokines and chemokine mRNA expression in THP-1 cell line after stimulation with influenza viruses. GAPDH gene was used for normalization. The mRNA expression was assessed on 24 h after stimulation. Data from 2 independent experiments performed in duplicate are presented. The bars represent means and standard errors. * - significant at $p < 0.05$.

The level of stimulation interferon I beta mRNA by both influenza viruses and Poly I:C was noticeably lower compared to interferon alpha ($p = 0.019 - 0.021$, data not shown). This is consistent with the data that blood cells are predominantly sources of IFN-alpha, while viral induction of interferon beta mRNA, which originates from both mononuclear cells and epithelial cells, occurs later than induction of interferon alpha mRNA [25]. With regard to early cytokines such as IL-6 and IFN-alpha, it was shown that the influenza vaccine virus stimulated mRNA less than the wild virus and the positive control (**Figures 2(C)** and **2(D)**).

Mouse study demonstrated that after intranasal introduction of the vaccine virus A/17/South Africa/2013/01(H1N1)pdm09 to animals, LAIV caused a noticeable increase in the mean levels of TNF-α, IL-6 and IFN-α in the nasal turbinates on day 3 and day 5 compared to PBS-vaccinated mice ($p < 0.01$; **Figure 3(B)**).

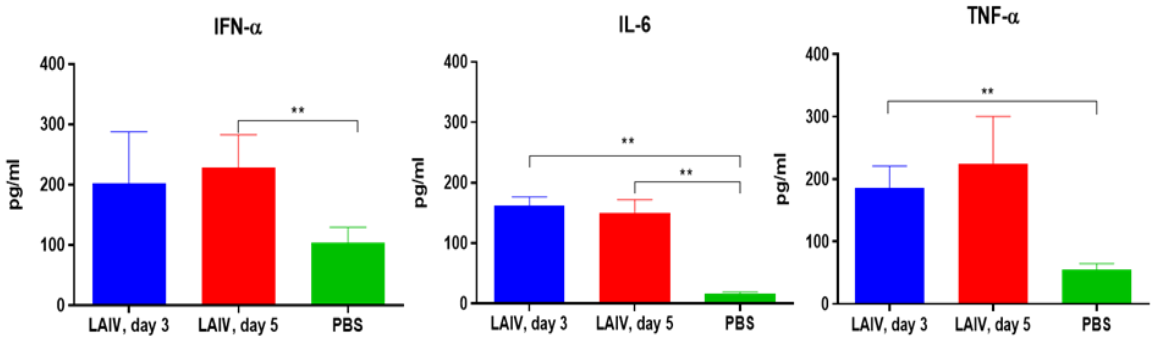

Figure 3 Production of early cytokines in the nasal turbinates of mice on day 3 and day 5 after intranasal immunization (n = 5), ELISA. Means and standard errors are presented. ** - significant at $p < 0.01$.

When LAIV-immunized mice were infected with the homologous A/South Africa/3626/2013(H1N1) pdm09 virus on day 3 after immunization, the mice were partially protected from lethal infection (**Figure 4(A)**). When infected on day 5 after immunization, the protection from lethal infection was complete, while in the group of mock-immunized animals the lethality was 70 % (**Figure 3(A)**). In the early stages of homologous influenza infection, mice immunized with LAIV lost more weight after infection on day 3 after immunization compared to infection on day 5 after immunization ($p < 0.05$; **Figure 4(B)**). Whenever the experimental infection occurred, on the 3rd or 5th day, LAIV immunization had a positive effect, as the survival rate of animals increased and the viral load in the lungs decreased 25 - 125 times ($p < 0.01$; **Figures 4(A)** and **4(C)**).

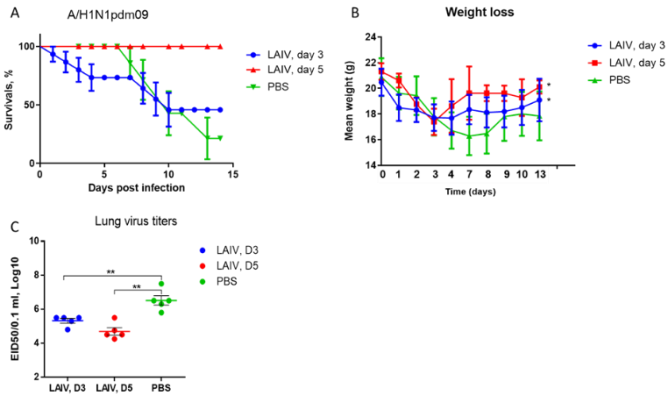

Figure 4 Challenge using 5 MLD50 of A/South Africa/3626/2013(H1N1)pdm09 influenza virus on day 3 and day 5 after A/17/South Africa/2013/01(H1N1)pdm09immunization.. A. Survival proportions (n = 10 per group). Weight loss (n = 10). * - significant at $p < 0.05$ compared to PBS-immunized group. C. Infectious virus isolation from the lungs on day 3 after virus challenge (n = 5). * - significant at $p < 0.05$. ** - significant at $p < 0.01$

When challenged with heterologous A/Indonesia/ 5/2005(H5N1) IDCDC-RG2 virus on day 5 after immunization, 100 % of immunized animals survived; and when challenged on day 3 after immunization, 80 % of immunized animals survived (differences with the non-immunized group are significant at $p < 0.05$; **Figure 5(A)**). Mice immunized with LAIV lost significantly less weight than non-immunized animals (differences with the non-immunized group are significant at $p < 0.001$; **Figure 5(B)**). Viral load in the lungs was statistically significantly lower when LAIV-immunized mice were challenged on day 3 after intranasal immunization ($p < 0.01$; **Figure 5(C)**).

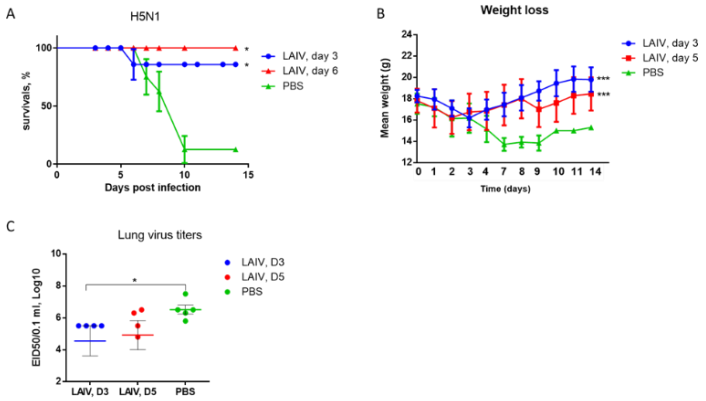

Figure 5 Challenge using 5 MLD50 of A/Indonesia/5/2005(H5N1) IDCDC-RG2 influenza virus on day 3 and day 5 after A/17/South Africa/2013/01(H1N1)pdm09 immunization. * - significant at $p < 0.05$ compared to PBS-immunized group. A. Survival proportions (n = 10 per group). B. Weight loss (n = 10). *** - $p < 0.001$ compared to PBS-immunized group. C. Infectious virus isolation from the lungs on day 3 after virus challenge (n = 5). * - significant at $p < 0.05$.

LAIV has been well studied, has been used for many years, and has a well-established safety and efficacy profile. Clinical studies have shown that LAIV is effective, particularly in children, in preventing influenza infection and reducing illness severity [26]. Intranasal immunization with live attenuated virus may induce a more natural immune response compared to inactivated vaccines [27] mediated by both the induction of systemic antibodies and the formation of immune memory cells, which may provide long-lasting protection [28]. Local protection by LAIV, mediated by the production of secretory IgA antibodies, which are induced separately from blood antibodies, targets the mucosal surfaces of the respiratory tract, which are the entry points for infection [29].

In the present work, we demonstrated that the LAIV caused the expression of early cytokines and type I interferon *in vitro* and induced production of corresponding proteins in the respiratory tract of mice. A number of animal and clinical studies have shown that early cytokines such as IFN1-α, TNF-α and IL-6 are key mediators that trigger the immune response and determine the course and outcome of the disease [30-32]. Attenuated influenza viruses, when they enter the nasal mucosa, generate a number of immune reactions, leading to the formation of both systemic (humoral) immunity and local (mucous) immunity. Thus, the secretion of IL-33 by the mucous membrane in the first 8 h after inoculation is associated with the divergence of CD8+ and circulating T-follicular helper (cTfh) T cells 7 days after inoculation [33]. In the initial stages of influenza virus infection, the main cells producing type I IFN are epithelial cells and alveolar macrophages [34-36]. Type I interferons secreted by infected cells shortly after infection with the virus act on receptors of neighboring cells, preventing further spread of the virus, while the absence of IFN signaling leads to further spread of the virus [20,22]. This helps reduce viral replication in the upper respiratory tract and reduce transmission among people, especially in community settings.

Thus, it can be hypothesized that LAIV may modulate innate immune pathways, thereby contributing to the reduction of primary viral infection. Our data in a mouse model have shown that a single intranasal administration of the pandemic A/H1N1pdm09 LAIV vaccine strain completely protected animals from lethal homologous and heterologous influenza infection as early as on day 5 post-immunization. Since LAIV induced the expression and production of type I interferons *in vitro* and in the respiratory tract of mice, it can be assumed that protective effect may be associated with the priming effect of type I interferons. Recently, there is growing evidence that the innate immune system can be pre-stimulated, resulting in an enhanced response to secondary infections. This state of enhanced innate immunity, called “pre-stimulated immunity,” is mediated by prototypical cells of the innate immune system, such as natural killer cells and monocytes/macrophages. In this case, protection from reinfection, carried out in a T/B cell-independent manner, is mediated by both specific mechanisms and non-specific epigenetic reprogramming [37]. This process can lead to a more rapid and robust immune response in case of re-infection [38]. Priming and tolerance regulate similar ‘signature’ inflammatory genes such as TNF, IL6 and IL1B and use overlapping epigenetic mechanisms [39]. Early cytokines, such as TNF-alpha and IL-6, not only involved in the primary non-specific response at the early stage of viral infection, but also participating in the activation of the immune system and in the launch of a ‘specific’ immune response [40]. Activation of cytokine and cytokine receptor genes at the early stage of vaccination can be markers of the subsequent humoral response and its duration [41].

Thus, early cytokines and type I interferons are essential for the initial immune response against influenza, acting as the 1st line of defense to control viral replication and promoting the recruitment and activation of immune cells. This response limits viral spread, provides immediate antiviral effects, and primes the adaptive immune system for a more robust response. Explanation these mechanisms is of interest for the development of effective vaccines and therapeutic strategies against influenza and other viral infections [42-45].

Vaccination against influenza is limited to the period of seasonal increase in the incidence of the disease. Considering that specific antibodies are formed 2 - 4 weeks after immunization [46], the effect of early non-specific protection provided by LAIV is especially

important during vaccination in conditions of circulation of not only influenza viruses, but also etiological agents of other respiratory infections. Studying the expression of early cytokines and type I interferons during viral infection and vaccination will contribute to a better understanding of the mechanisms of interaction of immune factors and the study of independent functions of cytokines in the sequential regulation of non-specific protection against respiratory infections.

Study limitations. The study was performed on mice, an existing, proven and accessible model of influenza infection. Further studies are needed, including the use of primary human cell lines and other models of influenza infection closer to humans.

Conclusions

In summary, the data obtained may indicate the advantage of using a live influenza vaccine due its ability to induce early protection. This may justify the use of LAIV during the seasonal rise of ARI due to stimulation of innate immune factors. It can be hypothesized that in cases of unexpected influenza outbreaks, seasonal flu seasons, or pandemics, the use of a live attenuated vaccine may provide more rapid responses compared to traditional inactivated influenza vaccines. Future research directions could include investigating the pathways by which the human immune system is activated by LAIV immunization.

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