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Factors of early protective action of live influenza vaccine combined with recombinant bacterial polypeptides against homologous and heterologous influenza infection

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Abstract

We are developing an associated vaccine based on live influenza vaccine (LAIV) and streptococcal recombinant peptides. The recombinant group B streptococcus (GBS) peptides P6 and ScaAB demonstrated a distinguished immunomodulating effect in THP-1 cells. The increase in IFN 1-alpha expression after ScaAB inoculation was similar to that against LAIV. We immunized mice intranasal using of A/H7N3 LAIV or/and ScaAB peptide. At day 5 after immunization, we detected serum IgM which reacted with non-vaccine influenza viruses. Associated vaccination of mice using LAIV and GBS peptide was the most effective against sub-lethal infection with A/H7N9 influenza virus and against lethal challenge with A/H1N1pdm virus at day 5 after immunization. Not only LAIV but also the ScaAB protected about 20% of the immunized animals against lethal challenge with A/H1N1pdm virus. The early protection was related to increasing type 1 interferons expression in the lungs.

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Our results in mice have shown that successful protection against homologous and heterologous influenza infections can be achieved soon after vaccination with either LAIV or LAIV in combination with GBS recombinant peptide. Presumably, such protection may be mediated by non-specific IgM antibodies and an increase in the expression of early cytokines in the airway.

Keywords: Immunology, Microbiology, Vaccines, Virology

1. Introduction

Associated viral-bacterial intranasal immunization based on live influenza vaccines (LAIV) and recombinant bacterial polypeptides (either by simple mixing of preparations or by creating genetically engineered constructions) is a new direction of vaccine prophylaxis, which allows to reach the protective effect against post-influenza bacterial complications by one vaccination. This effect is achievable both due to the reduction of the primary viral infection, and through the formation of specific antibodies against bacterial polypeptides [1]. For the prevention of respiratory infections, it is important to develop mucosal vaccines to stimulate not only systemic but also local humoral immunity. Previously, the immunogenicity, including local IgA formation, and protective efficacy of recombinant group B streptococcus (GBS) proteins in combination with LAIV, were demonstrated in mouse experiments after intranasal administration [2]. The protective effect of associated vaccination against double influenza and GBS infections correlated with serum and local antibody response against vaccine compounds and increased expression of type II interferon – interferon-gamma – in the lungs of mice [2]. Unlike the adaptive immune response, which is very specific, the agents of innate immunity provide protection against a wide range of pathogens. Due to the fact that we are developing an associated virus-bacterial vaccine on the basis of LAIV, we evaluate the early protective effect of individual components of the associated virus-bacterial vaccine and some of the mediators of innate immunity which may provide such protection.

Influenza viruses infect the epithelial cells of the respiratory tract, tissue macrophages, monocytes and dendritic cells, which produce various antiviral, proinflammatory and regulatory cytokines and chemokines in response to the infection [3]. In a number of studies on animals and humans, it was shown that early cytokines such as interferon (IFN) 1-alpha, tumor necrosis factor (TNF) - alpha, and interleukin-6 (IL-6) are key mediators that trigger the immune response and determine the course and outcome of the disease [4, 5]. IFN 1-alpha has extremely strong antiviral properties [6], TNF- alpha and IL-6 cytokines are responsible for the development of inflammation at the site of infection and are involved in immune activation and recruitment of macrophages, T- and B-lymphocytes [7, 8]. Thus, these agents may play a role of a link between innate and adaptive immune response.

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On the other hand, the same cytokines can respond to acute influenza symptoms and pathologies associated with influenza infection, being powerful pyrogens and inducers of eicosanoid production. Thus, a high level of TNF- alpha production plays a key role in the pathogenesis of endothelial cell damage [9].

The aim of our study was to evaluate protective causes against sub-lethal and lethal influenza reinfection during the early stages after combined vaccination based on LAIV and GBS surface proteins.

2. Methods

2.1. Viruses and vaccine preparation

The reassortant A/17/Mallard/Netherlands/00/95 (H7N3) influenza virus [10] was provided by the Virology Department of the Institute of Experimental Medicine collection of viruses. The A/Shanghai/2/2013(H7N9) CDC-RG virus was provided by Centers for the Diseases Control and Prevention, USA. The A/South Africa/3626/2013 (H1N1)pdm influenza virus was obtained from the National Institute for Biological Standards and Control (NIBSC, UK) repository. All viruses were propagated in 10-day old chicken embryos (CE) and stored at -70 °C.

GBS recombinant polypeptides P6 (30-kDa) and ScaAB (35-kDa) expressed in *E. coli* and purified as described earlier [11] were provided by Molecular Microbiology Department, Institute of Experimental Medicine.

2.2. Early cytokines expression and secretion in THP-1 cells

The THP-1 cells were seeded onto 24-well tissue culture plates, at 3.0×10^6 cells per well with RPMI (Roswell Park Memorial Institute) medium supplemented with 10 % fetal calf serum, 100 IU/ml of penicillin, and 100 μg/ml of streptomycin. The culture plates were incubated at 37 °C and 5 % CO2 for 48 h prior to the experiment. Cells were inoculated with 10^6 fifty percent egg infectious doses (EID_{50}) per ml of A/17/Mallard/Netherlands/00/95 (H7N3) LAIV virus, the P6 or ScaAB GBS polypeptides in a concentration of 5 μg/ml or the mixed LAIV, the P6 and ScaAB. The cells were further incubated for 3, and 24 hours in RPMI. Cell lysates were collected at 3 and 24 hours for cytokine and viral load assays. We performed three independent experiments. The levels of cytokines genes expression were determined by real-time reverse transcription polymerase chain reaction (rRT-PCR). RNA extraction was performed using RNeasy Mini Spin Column (QIAGEN, Hilden, Germany). The RNA was eluted in 50 μl of RNAse-free water and was used as the template for rRT-PCR. For cDNA synthesis, reverse transcription (RT) with 100 pg of total RNA was performed using oligo(dt) primers and random hexamers mix and the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). The rRT-PCR was performed in a

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CFX96 (Biorad, Hercules, CA, USA) thermocycler using SybrGreen as fluorogenic probe in 25 µl reactions containing 5 µl cDNA sample, 10 supermix (Thermo Scientific, Waltham, MA, USA), 50 pMol of forward and reverse primer and nuclease free water (Applied Biosystems, USA).

RT-qPCR assays for IFN 1-alpha; IFN 1-beta; IL-6; Macrophage Inflammatory Protein-1 (MIP-1 alpha, CCL-3); MIP-1 beta (CCL-4); Regulated on Activation, Normal T Expressed and Secreted (RANTES, CCL-5), TNF-alpha m-RNA expression was performed with primers listed in Table 1. We used Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and Hypoxanthine-guanine Phosphoribosyltransferase (HPRT) for normalization [12]. Melting curve analysis was performed for each primer pair at the end of the reaction to confirm the specificity of the assay. All rRT-PCR assays were performed in duplicates. Data were analyzed using the comparative Ct method, normalized to GAPDH and HPRT, and presented as the fold changes in gene expression of treated cells relative to the control non-treated cells.

The enzyme-linked immunosorbent assay (ELISA) with culture supernatants was carried out using commercial test system manufactured by eBioscience (San Diego, CA, United States) according to the manufacturer's instructions. The optical density (OD) measurement results were obtained on a microplate reader (ELx800, BIO-TEK INSTRUMENTS, Inc, Winooski, VT, USA) at a wavelength of 450 nm.

Table 1. Primers compositions for the detection of early cytokines expression in THP-1 cells.

Gene	Primers	Primer bank ID
Housekeeping genes		
GAPDH	F- TGTGGGCATCAATGGATTTGG R- ACACCATGTATTCCGGGTCAAT	126012538c3
HPRT	F- CCTGGCGTCGTGATTAGTGAT R- AGACGTTCAGTCCTGTCCATAA	164518913c1
Tested genes		
IFN-alpha	F-TCAAAGACTCTCACCCCTGC R-CAGTGTAAAGGTGCACATGACG	6754304a1
IFN-beta	F-GCGACACTGTTCGTGTTGTC R-GCCTCCCATCAATTGCCAC	6754304a1
IL-6	F-ACTCACCTCTTCAGAACGAATTG R-CCATCTTTGGAAGGTTCAGGTTG	224831235c1
MIP-1 alpha (CCL-3)	F-AGTTCTCTGCATCACTTGCTG R-CGGCTTCGCTTGGTTAGGAA	4506843a1
MIP-1 beta (CCL-4)	F-CTGTGCTGATCCCAGTGAATC R-TCAGTTCAGTTCCAGGTCATACA	4506845a1
RANTES (CCL-5)	F- CCAGCAGTCGTCTTTGTCAC R- CTCTGGGTTGGCACACACTT	22538813c1
TNF-alpha	F-CCTCTCTCTAATCAGCCCTCTG R- GAGGACCTGGGAGTAGATGAG	25952110c1

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2.3. Virus titration in MDCK using RT-PCR

The virus RNA was isolated by means of the QIAamp Viral RNA Mini Kit (Qiagen, Venlo, Netherlands) from 80 μl of MDCK supernatants at 3 and 24 hours after inoculation. One-step rRT-PCR was performed using SuperScript III Platinum® One-Step Quantitative RT-PCR System (Invitrogen, Carlsbad, CA, USA) with primers and Taqman probes for influenza A M-gene designed by Centers for Diseases Control and Prevention (Atlanta, GA, USA): universal influenza A virus forward primer -GAC CRA TCC TGT CAC CTC TGA C; universal influenza A virus reverse primer -AGG GCA TTY TGG ACA AAK CGT CTA; universal influenza A virus probe -(FAM)-TGC AGT CCT CGC TCA CTG GGC ACG-(BHQ1). Primers and probes were synthesized and provided by the "Beagle" Company (Saint Petersburg, Russia). To generate standard curve RNAs were extracted from MDCK supernatants after inoculation with virus in allantoic fluid with a known multiplicity of infection (MOI). The standard curve was made by plotting cycle threshold (Ct) values against log10 dilutions of the virus and viral titers were determined using linear regression analysis.

2.4. Immunization of mice

The 8 to 10-week-old female CBA mice were provided by the laboratory breeding nursery of the Russian Academy of Sciences (Rappolovo, Leningrad Region, Russia). Groups of mice (60 animals in group) were lightly anesthetized with ether and intranasally (i.n.) inoculated with 50 μL divided equally per nostril using the following preparations: 1) live influenza vaccine (LAIV) containing 10^6 50% egg infectious dose (EID_{50}) of the A/17/Mallard/Netherlands/00/95 (H7N3) vaccine virus; 2) GBS vaccine (ScaAB) containing the ScaAB recombinant polypeptide (20 μg); 3) mixed LAIV+ ScaAB vaccine; 4) control animals were inoculated by PBS. All procedures involving animals were performed according to the "Rules Laboratory Practice" of Ministry of Health of the Russian Federation № 708 n. The study was approved by the Local Ethics Committee for Animal Care and Use at the Institute of Experimental Medicine, Saint-Petersburg, Russia.

2.5. Receptor-destroying enzyme (RDE) treatment

The serum samples were collected on day 4 after immunization and treated with receptor destroying enzyme (RDE) (Denka Seiken) to remove nonspecific inhibition. A 20-μl volume of mouse serum was added to 60 μl of RDE. The mixture was incubated at 37 °C for 18 h. RDE was inactivated at 56 °C for 30 min. Finally, 120 μl of PBS was added to give a final serum sample dilution of 1:10.

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2.6. ELISA with mouse sera

The 20 hemagglutinating units (HAU) of purified tested viruses A/17/Mallard/Netherlands/00/95 (H7N3), A/Shanghai/2/2013 (H7N9) CDC-RG, A/South Africa/3626/2013 (H1N1)pdm or 2 μg/ml of recombinant peptide ScaAB in phosphate-buffered saline (PBS) were adsorbed onto flat-bottomed 96-well microplates (Jet Biofil, Madrid, Spain). Nonspecific binding sites were blocked by incubation with 4% bovine serum albumin (BSA) PBS. The 4-fold serial dilutions of RDE-treated sera were loaded starting from 1:40 dilution in duplicates. The reaction mixture was developed with goat anti-mouse HRP-labled IgM (1:2000 dilution, Sigma-Aldrich, St. Lois, USA) and TMB substrate (Sigma-Aldrich, St. Lois, USA) and absorbance read at 450 nm by a microplate reader (ELx800, BIO-TEK INSTRUMENTS, Inc, Winooski, VT, USA). The endpoint cutoffs for test sera was calculated as the reciprocal of the highest dilution that gave an OD at 450 nm (OD_{450}) value greater than the mean OD_{450} of three to six serum-free control wells plus 3 standard deviations (SD). A titer of $>$1:40 was the threshold of the method sensitivity.

2.7. Viral challenge study

On day 5 after vaccination the mice from all groups were inoculated intranasally. For this we used 300 fifty percent mouse infectious doses (MID_{50}) of A/Shanghai/2/2013 (H7N9) CDC-RG which was lower than 1 50% lethal dose (LD_{50}). Another infectious virus was A/South Africa/3626/2013 (H1N1)pdm, which we used in a dose of 50% LD_{50}. To determine viral titers in the lungs, the samples were collected from mice at 48 h and 72 h after viral infection and the supernatants were centrifuged for 10 min. The viral titers were calculated as the 50% egg infectious doses (EID_{50}) using hemagglutination as the endpoint as described previously [13].

2.8. M-RNA expression analysis in the mouse lungs after viral challenge

Total RNA was isolated from lung homogenates using GeneJet RNA Purification Kit (Thermo Scientific, Waltham, MA USA). For cDNA synthesis, reverse transcription (RT) with 100 pg of total RNA was performed using oligo(dt) primers and random hexamers mix and the SuperScript III kit (Invitrogen, Carlsbad, CA, USA). The rRT-PCR was performed as described earlier using the thermal cycler CFX96 (Bio-Rad, Hercules, CA, USA) with the following forward and reverse primers: GAPDH (NM_008084, Primer Bank ID: 126012538c3) – TGGCCTTCCGTGTTCCTAC, GAGTTGCTGTTGAAGTCGCA; interferon

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(IFN) 1-alpha (NM_206975, Primer Bank ID: 6754304a1) CAGCTCCAA GAAAGGACGAAC, GGCAGTGTAACTCTTCTGCAT; interferon (IFN) 1-beta (NM_206975, Primer Bank ID: 6754304a1) CAGCTCCAAGAAAGGAC GAAC, GGCAGTGTAACTCTTCTGCAT.

Melting curve analysis at the end of the reaction was performed to confirm the specificity of amplification of each pair of primers. Data were analyzed using the comparative Ct method, normalized to GAPDH, and presented as the fold changes in gene expression of vaccinated and infected mice relative to the control non-treated animals.

2.9. Statistics

Data were processed using Statistica software, version 6.0 (StatSoft, Inc. Tulsa, Oklahoma, USA). All data are given as mean ± standard errors of means (SEM). Geometric mean titers (GMT) were calculated and used to represent the antibody levels. To compare two independent groups we used a Mann-Whitney U-test. To compare multiple independent groups we used a Kruskal-Wallis ANOVA and post-hoc Tukey-HSD. The p-value <0.05 were considered to be statistically significant.

3. Results

The recombinant GBS peptides belonging to different peptide families (the immunodominant IgA-binding peptide - P6, and the major surface lipoprotein - ScaAB) demonstrated pronounced immunomodulating effect in the THP-1 cell line. Thus, the ScaAB peptide was more effective in stimulation of IFN 1-alpha m-RNA expression compared to P6 (Fig. 1A) as it was shown in rRT-PCR analysis. A 10−100-fold excess of IFN 1-alpha m-RNA expression after stimulation using ScaAB peptide was demonstrated as compared to P6 ($P = 0.03$) at 3 hours after stimulation (Fig. 1A). An increase in IFN 1-alpha m-RNA expression after the administration of mixed LAIV with peptides in comparison with LAIV alone ($P < 0.05$) was also noted. We demonstrated a short-term increase in the level of cytokines expression after introducing recombinant polypeptides, as after 24-hour contacts m-RNA expression decreased compared to 3 hours (Fig. 1B). Just like IFN 1-alpha, expression of IL-6 was more pronounced after the administration of ScaAB compared to P6 at 3 hours after stimulation ($P = 0.02$). The IFN 1-alpha expression data at 24 hours after stimulation were confirmed by determining the corresponding protein product in ELISA-test with the supernatants collected at the same time (Fig. 1C). It was shown that 24 hours after stimulation with ScaAB, the expression of IFN 1-alpha was still just as elevated as with the stimulation of the LAIV. In regard to IL-6, the most important mediators of the acute phase of inflammation, its expression at

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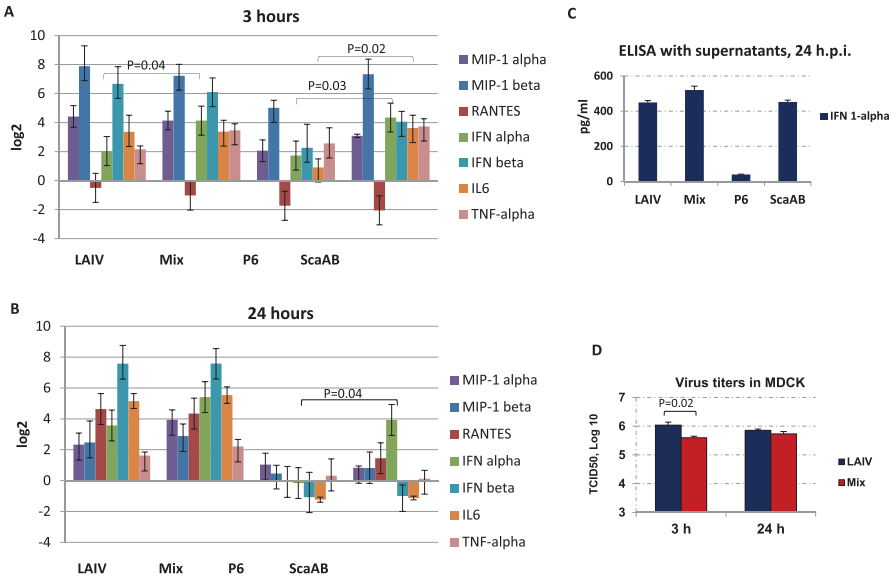

Fig. 1. (A, B) Cytokines and chemokines m-RNA expression in THP-1 cell line after stimulation with the A/17/Mallard/Netherlands/00/95 (H7N3) or GBS polypeptides (P6 or ScaAB) or mixed LAIV+P6+ScaAB. The THP-1 cells were seeded 3×10^6 cells/ml, and treated 48 hours later with LAIV(6 EID_{50}/1 ml), of P6 or ScaAB(10 μg/ml of each) or LAIV mixed with both polypeptides. The GAPDH and HPRT were used as normalizing genes. The m-RNA-expression was estimated at 3 and 24 hours post inoculation (h.p.i.). Data presented for three independent experiments performed in duplicate. (C) ELISA with supernatants on 24 h.p.i. (D) Mean viral load on 3 and 24 h.p.i.

24 hours after stimulation of both ScaAB and P6 was reduced to almost zero. IFN 1-beta expression was up-regulated following inoculation with LAIV or LAIV merged with peptide mix at 3 hours compared to P6 or ScaAB peptides separately (Fig. 1A), and in contrast to IFN 1-alpha, the elevated level of expression was not registered at 24 hours after ScaAB stimulation (Fig. 1A and B).

Both LAIV and GBS recombinant peptides equally stimulated m-RNA expression of MIP 1-alpha and beta, also known as CCL3 and CCL4 at 3 hours after inoculation. After 24 hours, the elevated level of expression of these chemokines was preserved only in the presence of LAIV.

RANTES or CCL5 as a later cytokine [14] was not overexpressed 3 hours after inoculation, and its expression was elevated at 24 hours after the LAIV infection and weak after administration of the GBS peptides (Fig. 1A and B).

The viral load did not increase 24 hours after inoculation of the vaccine virus, either alone or in mixtures with polypeptides, which confirms the absence of viral reproduction in macrophages. At 3 hours after the introduction of the LAIV in a mixture with polypeptides, the decrease in the viral load differed significantly compared to LAIV alone ($P < 0.05$; Fig. 1D), which may indicate some competition, possibly

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associated with increased expression of Type 1 interferons. After 24 hours, no such difference was noted.

For intranasal immunization of mice, we used the A/17/Mallard/Netherlands/00/95 (H7N3) vaccine virus and the ScaAB recombinant peptide. On day 4 after immunization, we measured IgM content in the sera of immunized animals. In the sera of mice immunized with LAIV we have noticed IgM reacting not only with vaccine virus A/17/Mallard/Netherlands/00/95 (H7N3) but also with A/Shanghai/2/2013 (H7N9) CDC-RG and A/South Africa/3626/13 (H1N1)pdm influenza viruses, as well as with the ScaAB peptide (Fig. 2A). The IgM titers against influenza viruses were increased in the sera of immunized animals compared to controls (PBS-vaccinated animals), but these differences were not statistically significant. In the sera of mice after immunization with the ScaAB peptide, a certain amount of antibodies reacting with the tested viruses was detected.

We also evaluated virus-specific IgM and IgG dynamics in the sera of mice on day 5 and day 21 after LAIV or LAIV+ScaAB immunization (Fig. 2B). On the 5th day after immunization, the response of IgM antibodies in the vaccine groups was not

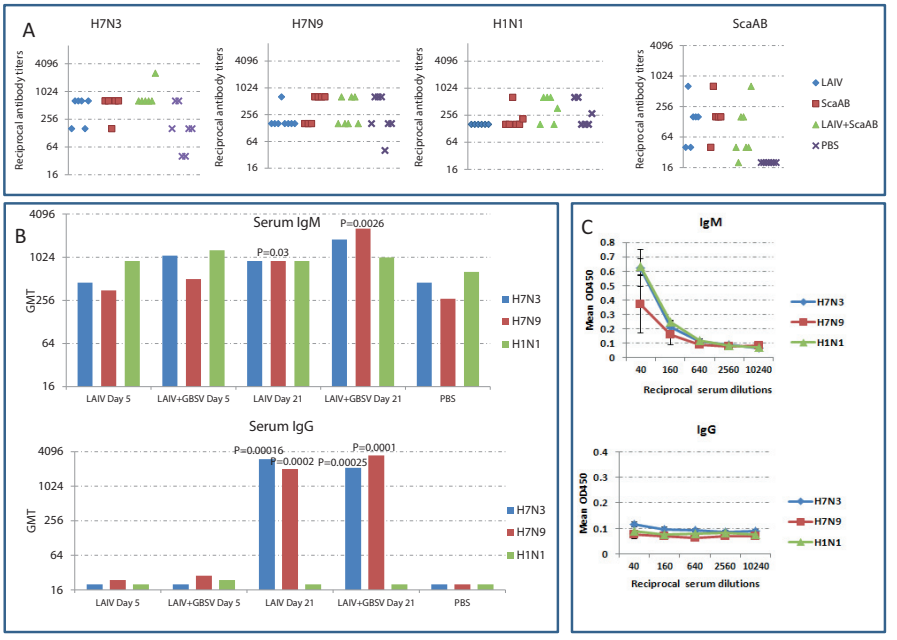

Fig. 2. Antibody response determined in ELISA with the sera of immunized mice. A. IgM antibodies against whole viruses A/17/Mallard/Netherlands/00/95 (H7N3), A/Shanghai/2/2013 (H7N9) CDC-RG, A/South Africa/3626/13 (H1N1)pdm or recombinant peptide ScaAB on day 4 after immunization (6−10 mice per group). Each dot represents a separate mouse. B. Serum IgM and IgG GMT on day 5 and day 21 after immunization (8 mice per group). P-values the level of significance of the differences between antibody titers obtained on day 21 compared with day 5. C. The binding activity of IgM and IgG in normal mouse sera to influenza viruses of different subtypes *in vitro*. Data plotted to represent mean values from 6 mice and the standard error of the mean (SEM).

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significantly different from the control group. The titers of serum virus-specific IgM slightly increased by 21 days after immunization compared to day 5, while only A/H7N9-specific IgM levels increased statistically significantly ($P = 0.03-0.0026$; Fig. 2B). The titers of IgG antibodies to the viruses A/H7N3 and A/H7N9 but not to A/H1N1 significantly increased by the 21st day after immunization, compared with day 5 ($P < 0.001$). This data confirm the previously obtained results on the immunogenicity of an associated vaccine based on LAIV and recombinant GBS polypeptides [1, 2]. Fig. 2C shows the binding curves of IgM and IgG with influenza viruses of different subtypes in the sera obtained from normal mice. Nonspecific IgM binding to all three influenza viruses was shown at serum dilution <1:640. In this case, the highest level of binding was observed for viruses of the A/H7N3 and A/H1N1 subtypes, while the binding to A/H7N9 virus was weaker. With respect to IgG in the sera of control mice, there was no non-specific binding to influenza viruses (Fig. 2C).

On day 5 after vaccination, challenge with 300 MID_{50} of A/Shanghai/2/2013 (H7N9) CDC-RG influenza virus was not lethal, so animals in all the vaccine groups survived. The curves of body mass dynamics did not differ between vaccine groups and the placebo group (Fig. 3A). Both LAIV and mixed vaccine decreased viral load in the lungs after infection with 300 MID_{50} of A/Shanghai/2/2013 (H7N9) CDC-RG

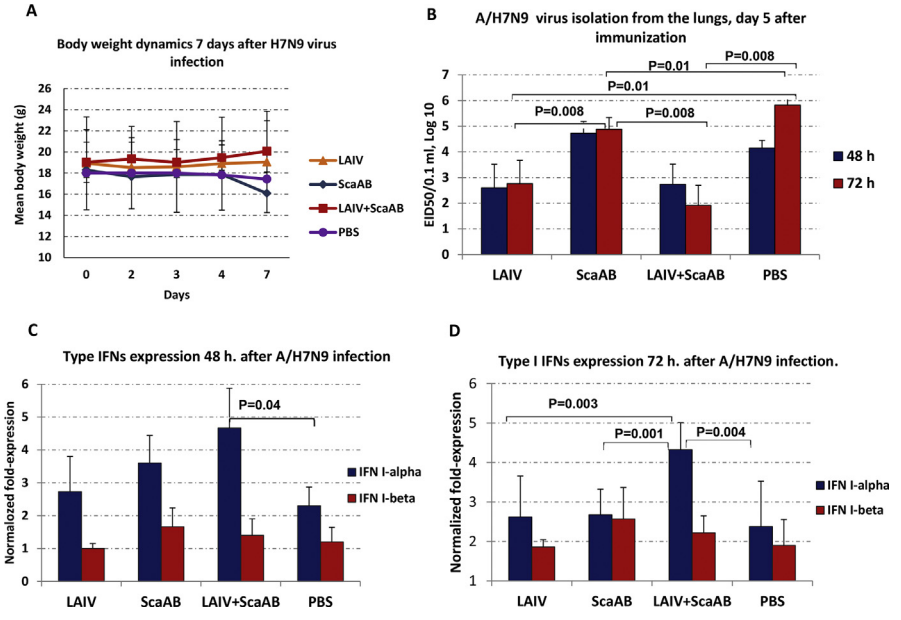

Fig. 3. Protection against A/Shanghai/2/2013 (H7N9) CDC-RG virus infection on day 5 after intranasal immunization using 7 log10 EID_{50} of A/17/Mallard/Netherlands/00/95 (H7N3) or/and 20 μg of ScaAB. (A) Body weight dynamics. (B) The A/Shanghai/2/2013 (H7N9) CDC-RG virus reproduction in the lungs on 48 and 72 hours on day 5 post infection (n = 6).* - lung viral titers were significantly lower compared to controls ($P < 0.01-0.001$). (C, D) Type 1 interferon expression in the lungs after the challenge on day 5 after vaccination (48 and 72 h.p.i; n-6).

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influenza virus; the ScaAB-only also reduced influenza virus reproduction on day 5 after vaccination (Fig. 3B). Associated LAIV+ScaAB vaccine better than other vaccine preparations reduced viral reproduction in the lungs (Fig. 3B), and this was accompanied by an increase in expression of IFN 1-alpha in the lungs at 48 and 72 hours post infection (Fig. 3C and D).

We further studied the early protection against lethal challenge with heterologous A/South Africa/3626/13 (H1N1)pdm influenza virus on day 5 after immunization. It was shown that associated vaccination using LAIV and ScaAB was the most effective in prevention of mortality and weight loss when 50% of the animals were protected from infection with 10 LD_{50} of the A/South Africa/3626/13 (H1N1)pdm influenza virus (Fig. 4A and B). The vaccine preparations containing LAIV-only or the ScaAB-only protected about 20% of the immunized animals on day 5 after the first vaccination (Fig. 4B). After A/H1N1 challenge, the virus reproduction in the lungs significantly decreased only in LAIV+ScaAB vaccine groups compared to mock-immunized animals (Fig. 4C), this correlated with elevated IFN 1-beta expression in the lungs (Fig. 4D). It is interesting that after viral challenge, the type 1 interferons expression was also elevated in the case of immunization with ScaAB protein alone, although the differences from the control group were not statistically significant.

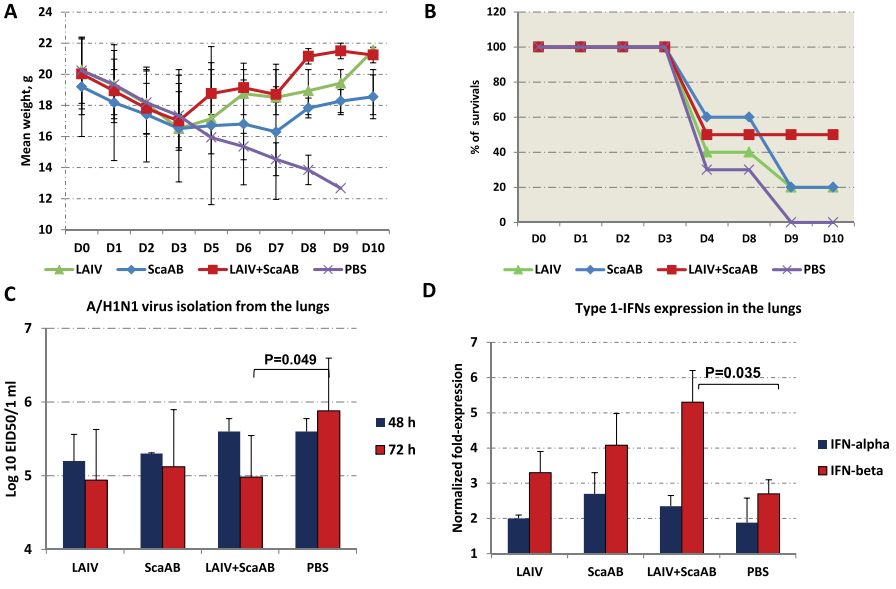

Fig. 4. Protection against A/South Africa/3626/13 (H1N1)pdm infection. The mice were intranasally immunized using 7 log 10 EID_{50} of A/17/Mallard/Netherlands/00/95 (H7N3) and 20 μg of ScaAB. Infection with 10 LD_{50} of A/South Africa/3626/2013 (H1N1)pdm influenza virus was done on day 5 after vaccination. The lungs were collected on 48 and 72 hours post infection. (A) Weight dynamics. (B) Lethality rate. (C) Virus isolation from the lungs (n = 6). (D) Type 1 interferons m-RNA expression in the lungs on 48 hours post infection (n = 6).

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4. Discussion

When developing virus-associated bacterial vaccines, it may be useful to explore an appropriate *in vitro* model for studying individual immunostimulatory properties of the immunogenic polypeptides and their interaction with live vaccine viruses. Cytokines are the key mediators that not only regulate the immune response to vaccination but also mediate protective mechanisms in viral reinfection. Different types of cells can synthesize cytokines in response to a variety of stimuli. In this case, the spectrum of cell-produced cytokines depends on the nature, duration, and intensity of the inducer's action, as well as on the presence of additional mediators: other cytokines, hormones, and intercellular interactions [15]. The influenza A viruses cause the production of chemokines (MIP-1α, RANTES, IP-10), pro-inflammatory cytokines (IL-6, TNF-α) and interferons during the early stages of infection [3]. At the local level, cytokines are responsible for all consecutive stages of an antiviral response to infection with influenza viruses, ensuring virus limitation and removal, and then restoring the damaged tissue structure.

Profiling gene expression is considered to be a promising molecular genetic analysis approach to determine the impact of vaccines or other environmental stimuli, as gene transcription is a dynamic process that allows cells to adapt quickly to changes in the homeostasis. We used a set of the above cytokines to evaluate the factors of innate immunity during initial contact of the associated viral-bacterial formulation with the cells of the immune system.

To simulate the expression of early cytokines in response to the vaccine influenza virus and bacterial recombinant peptides introduction, we used THP-1 cell line of human monocyte-macrophage origin [16]. Unlike epithelial cells, the macrophages form the second line of protection against infection and provide an important contribution to the secretion of antiviral and immunostimulatory cytokines for influenza infection. Previously it was shown that in the early stages of infection the macrophages produce significant amounts of antiviral cytokines IFN 1-alpha (more than epithelial cells) [17] and chemokines involved in the migration process of leukocytes from circulation to the focus of inflammation. Our early investigations demonstrated that human macrophages after treatment with both live and inactivated influenza A viruses produced type 1 interferon at 24 hours after inoculation [18]. Thus, viral replication was not a necessary condition in this system, although macrophages treated with inactivated virus produced less interferon, compared to macrophages treated with live virus.

The system of interferons presents the main humoral factor of innate antiviral protection [20]. Type 1 interferons (IFN- alpha, IFN- beta) are produced directly in response to the virus introduction, after which they partially suppress viral replication and limit the spread of viral progeny. Synthesis of type 1 interferons leads to the

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formation of IL-6, IL-12, IL-18, and TNF-α in cells. Type 1 interferons alpha/beta provide an antiviral effect in both infected and uninfected cells, and in various ways regulate innate and adaptive immunity not only against viral but also bacterial and numerous other pathogens [19].

Bacterial pathogen-associated molecular patterns (PAMPs) can also activate type 1 IFN signaling in macrophages which possess broad panel of pattern recognition receptors allowing the detection of multiple surface determinants such as components of the bacterial cell wall or flagella [20]. Previously it was shown that type 1 IFNs can be induced in phagocytic cells by protein A, a staphylococcal virulence factor [21] or *Salmonella* LPS recognized by TLR4 [22].

The consequences of induction of type 1 interferons by bacteria and viruses differ. Bacteria increase virulence by inducing type 1 IFNs, making a gap in the immune cell system. Viral infections usually do not lead to a similar result, despite the production of significant amounts of type 1 IFN. The exact mechanism by which bacterial pathogens modulate the expression of type 1 IFN to their advantage remains to be studied. In addition, these data also imply that simultaneous infections with bacterial and viral pathogens can lead to an increase in bacterial infection [23].

Previously published data about the antiviral effect of interferons are have not explored the impact of the combined viral and bacterial vaccination on innate immunity factors in the subsequent viral infection. In our study, the individual GBS recombinant peptides − the P6 (an immunodominant peptide containing IgA-binding motif) or ScaAB (the major surface lipoprotein) - demonstrated a distinguished immunomodulating effect on the innate immune system in THP-1 cell line on 3 hours after inoculation. Also, the P6 and ScaAB differed by expression of the IFN 1-alpha for 24 hours. An increased and prolonged level of interferon 1-alpha expression was observed when the ScaAB was administered, on a par with the live virus and mixed virus-bacterial preparation. Based on this observation, it can be assumed that not only immunization with a living virus, but also an immunogenic bacterial peptide will provide a positive effect on the course of the viral infection, which was confirmed later in a mouse model. An important observation was that the effect of peptides on the cells of the immune system was very short-lived and practically did not manifest after a day. Previously, it was shown that dysregulated continual production of such host response modulators to infection as IL-6 or TNF−alpha may have a pathological effect on chronic inflammation and autoimmunity [24, 25].

In a study in mice, we estimated early protection against challenge with homologous and heterologous influenza viruses after vaccination with a combination of LAIV and recombinant bacterial peptides. Using sub-lethal infection with 300 MID_{50} of A/Shanghai/2/2013 (H7N9) CDC-RG influenza virus we have shown the enhanced effect of associated vaccination on viral reproduction in the lungs on day 5 after vaccination. In case of lethal infection with A/South Africa/3626/13 (H1N1)pdm

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influenza virus, viral reproduction in the lungs did not differ significantly between vaccine groups. However, in this case, it was possible to demonstrate the advantage of the associated vaccine in preventing mortality and to assess the differences in weight dynamics, which were not expressed in the case of sub-lethal influenza infection. Thus, it has been shown that combining LAIV with recombinant peptides of the GBS had the best protective effect against re-infection with both a homologous A/H7N9 virus and heterologous A/H1N1 influenza virus. Improved protection was related to a lowered release of infectious viruses from the lungs, reduced clinical manifestations, determined by the dynamics of body weight, a decrease in mortality. Data of increased levels of IFN 1-alpha expression correlating with the enhanced protection against A/Shanghai/2/2013(H7N9) CDC-RG among LAIV+ScaAB vaccinated mice on day 5 post immunization may suggest previously reported anti-viral action of such kind of interferon [26]. The ScaAB immunization to some extent reduced the reproduction of the A/H7N9 virus in the mouse lungs and prevented lethality after infection with A/South Africa/3626/13 (H1N1)pdm on the 5th day after immunization which might be attributed to short-term induction of type 1 interferon.

Differences in the patterns of the IFN 1-alpha and IFN 1-beta expression after challenge with A/H7N9 compared to A/H1N1 can be followed in the mock-vaccinated mice, when IFN 1-alpha was prevalent in the case of A/H7N9 infection (Fig. 3C and D), and IFN 1-beta in the case of A/H1N1 infection (Fig. 4D). Such differences could be explained by the features of the innate immune response modulation by these two viruses. Previously it was shown that A/H1N1 NS1 affects the production of IFN 1-beta. In mice, there were demonstrated decreased expression levels of IFN 1-beta in the lung after infection with NS1-expressing influenza A/H1N1 virus [27] compared to the NS1-deficient virus. The A/Shanghai/2/2013(H7N9) CDC-RG challenge virus contains the genes of internal and non-structural proteins from the strain of A/Puerto-Rico/8/34 (H1N1), in which PB1 and PA are responsible for modulating the type 1 IFN response [28]. Since in the study of cytokine expression we used lungs from intact mice for normalization, it was seen that when the unvaccinated mice were infected, there was an increase in type 1 IFNs. But with the preliminary introduction of an associated vaccine, such an increase was even more pronounced, which can be explained by the effect of priming [29] when respiratory cells of vaccinated mice treated with certain doses of interferon produced more interferon than in unvaccinated mice. It should be noted that in all the cases we observed, though such an increase was statistically significantly different, it was very modest which may indicate a potential safety of such an increase due to the absence of a risk of immunopathology.

One more factor of nonspecific protection is the presence of the polyreactive IgM antibodies representing another humoral component of the innate immune system [30]. Primary interaction of the natural antibody, mainly represented by polyreactive IgM

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with pathogens serve as one of the earliest components of protection before the formation of adaptive immunity, mediated by antigen-specific antibodies [27]. Unlike strictly specific mono-reactive antibodies, the antigen-binding site of polyreactive antibodies is not so rigid that it provides binding to a variety of different antigens [31].

It was shown previously in mice, that in case of influenza infection an effective adaptive immune response including virus-specific IgM begins to develop around day 5 after infection [32]. In our study, it was shown that in the case of LAIV implementation, on the 4th day after vaccination, nonspecific IgM reacting with homologous and heterologous viruses were detected. It has been shown previously that natural polyreactive IgM antibodies secreted by B-1 cells have broad cross-reactivity against various influenza viruses. The broad specificity of the B-1 cell−derived antibodies might suggest their binding to carbohydrate patterns on the virus [33]. These antibodies are increased after influenza immunization in the tissue where the B-1 cells persist rather than in sera [34]. The increase of serum IgM by day 7 after influenza infection is attributed to B-2 cells-derived antibodies which were much less cross-reactive with different influenza viruses in contrast to natural IgM produced by B-1 cells [33]. In our study, serum IgM detected on the 4th day after immunization are most likely to be natural polyreactive antibodies. This can explain the absence of any statistical significance between immunized and mock-vaccinated mice (Fig. 2A). Moreover, we observed non-specific binding of IgM with influenza viruses of A/H7N3, A/H7N9 and A/H1N1 subtypes in the sera obtained from naïve mice. The stronger binding to viruses A/H7N3 and A/H1N1 compared to A/H7N9 presumably can be explained by strain-specific features of influenza viruses.

5. Conclusion

Taken together, this data suggests that intranasal immunization using LAIV and ScaAB may modulate innate immunity pathways, thus reducing the primary viral infection.

Influenza vaccination is limited to the period of the seasonal influenza epidemic. Our data on the mouse model clearly show that the use of LAIV has a protective effect against homologous and heterologous influenza in the first days after intranasal administration. The addition of a recombinant polypeptide to the vaccine has an additional protective effect against influenza infection. The ScaAB peptide from lipoprotein family not only showed an adjuvant effect on LAIV after intranasal administration, but it also provided protection against influenza viruses possibly due to type 1 interferon stimulation.

Given that specific antibodies are formed on 2−4 weeks after immunization [35], the effect of early nonspecific protection provided by LAIV and an associated vaccine on the basis of LAIV is especially important during vaccination under conditions

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of not only influenza viruses circulation, but also the etiologic agents of other respiratory infections.

Examination and expression of early cytokines in viral infection and vaccination will promote a better understanding of the interaction of immune mechanisms and the study of independent functions of cytokines in the consistent regulation of nonspecific protection against viral and bacterial infections. In vitro studied cytokine response not only can reflect the immunomodulatory properties of viral or peptide vaccines but can also mimic a viral-bacterial competitive pathway.

Declarations

Author contribution statement

Yulia A. Desheva: Conceived and designed the experiments; Analyzed and interpreted the data; Wrote the paper.

Galina F. Leontieva: Performed the experiments; Analyzed and interpreted the data.

Tatiana A. Kramskaya, Galina O. Landgraf, Andrey R. Rekstin: Performed the experiments.

Ivan A. Sychev: Performed the experiments; Wrote the paper.

Alexander N. Suvorov: Conceived and designed the experiments.

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Competing interest statement

The authors have no conflict of interests to declare.

Additional information

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