Vaccine xxx (xxxx) xxx

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity

Vladimir Talayev [a,*], Irina Zaichenko [a], Maria Svetlova [a], Alexei Matveichev [b], Olga Babaykina [a], Elena Voronina [a], Andrei Mironov [c]

[a] Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology of Rospotrebnadzor (Russian Federal Consumer Rights Protection and Human Health Control Service), 603950, 71 M. Yamskay str., Nizhny Novgorod, Russia
[b] NPO Petrovax Pharm LLC, 142143, 1 Sosnovaya St., Pokrov Village, Podolsk, Moscow Region, Russia
[c] Lobachevsky State University of Nizhny Novgorod, 603950, 23 Prospekt Gagarina (Gagarin Avenue), Nizhny Novgorod, Russia

ARTICLE INFO

Article history:
Received 31 March 2020
Received in revised form 21 July 2020
Accepted 26 July 2020
Available online xxxx

Keywords:
Influenza vaccine
Adjuvant
Azoximer bromide
Polyoxidonium
Immunity
Cytokines

ABSTRACT

The influenza vaccine Grippol® Quadrivalent (GQ) is a new vaccine, containing the adjuvant Polyoxidonium® and recombinant hemagglutinins from 4 strains of the influenza virus in amount of 5–6 μg of each hemagglutinin per human dose. These doses of antigens are about 3 times less than the standard dose recommended by WHO. We sought to characterize the immune response to the GQ vaccine and to determine the contribution of the adjuvant in this response. BALB/c mice were vaccinated with GQ or with adjuvant-free antigen mixtures (AGs). Then, the antibody response, the number of memory T cells in the spleen, and the functional properties of splenocytes were determined. The vaccine GQ has been shown to induce antibodies to all 4 influenza hemagglutinins. The vaccination with GQ caused a strong increase in the AG-induced proliferation and production of Th2 cytokines ex vivo. These effects were equal to effect achieved by standard dose of antigens. Vaccination also caused the accumulation of $CD4^+$ large lymphocytes with the phenotype of central and effector memory T cells in the spleen. The GQ vaccine enhanced the cytolytic activity of natural killer (NK) cells, whereas the adjuvant-free mixture of AGs in lowered and standard doses did not affect NK activity. We did not find a noticeable response of Th1 and $CD8^+$ T cells to vaccination. In vitro, the GQ vaccine stimulated the maturation of human monocyte-derived dendritic cells (DCs) enhancing the expression of HLA-DR, CD80, CD83, CD86 and ICOSL molecules. Polyoxidonium without AGs also induced expression of ICOSL, which plays an important role in T-dependent humoral immune response. In summary, the low-dose influenza vaccine GQ with Polyoxidonium adjuvant is immunogenic, induces a Th2-polarized T-cell response and $CD4^+$ memory T cells maturation, activates the production of antibodies to influenza hemagglutinins, and increases the activity of NK cells.

1. Introduction

Mass vaccination is one of the most effective means of combating the influenza. It has been estimated that universal influenza vaccination can save the lives of between 250,000 and 500,000 people every year worldwide [1]. Russian Federation National Immunization Schedule approves the use of several vaccines, including vaccines of the Grippol family, for mass immunization against influenza in Russia. The first subunit Russian vaccine Grippol was registered in 1996. Since that time, several Grippol vaccines have been developed and approved based on a single technological platform that implements the principle of using the complex "polymer adjuvant - pure antigens": Grippol, Grippol plus, pandemic MonoGrippol plus and Grippol Quadrivalent. Over the twenty years of use, these vaccines have proven to be safe and effective for adults, children and the elderly, according to an analysis of about 50 million recipients [2].

All vaccines of the Grippol family have two common features: firstly, they contain a reduced dose of antigens, and secondly, they contain adjuvant azoximer bromide from a class of heterochain aliphatic polyamines [3]. Azoximer bromide, marketed under the

* Corresponding author at: 603950, 71 M. Yamskay str., Nizhny Novgorod, Russia.
E-mail address: talaev.v.yu@mail.ru (V. Talayev).

https://doi.org/10.1016/j.vaccine.2020.07.053
0264-410X/

trade name Polyoxidonium®, is a water-soluble copolymer of N-oxidide 1,4-ethylene piperazine and (N-carboxyethyl) −1,4-ethylene piperazinium bromide with a molecular weight of 60–100 kDa [3]. The structure of Polyoxidonium was confirmed in detailed studies performed by the Direction Centrale Recherche & Technologie, Brussels [4]. Biodegradation to nontoxic oligomers with a molecular weight of 1–2 kDa provides easy elimination of Polyoxidonium from the body, its low renal toxicity and a good safety profile [5].

Polyoxidonium is able to bind various antigens both by means of the controlled synthesis of conjugates and due to spontaneous formation of complexes resulting from the interaction of charged functional groups of protein or polysaccharide antigens with anchor groups of Polyoxidonium [4]. Polyoxidonium conjugated with influenza virus hemagglutinins (HAs) enhances the production of anti-HA antibodies in mice of different strains (BALB/c, C57BL/6j, DBA/2, CBA, CC57W, A/Sn, C3H/He and B10CW) [4,6]. However this adjuvant effect of Polyoxidonium is more pronounced in BALB/c, DBA/2, CBA, CC57W and A/Sn mice which have a relatively weak antibody response to immunization with adjuvant free HAs [4,6]. Polyoxidonium mixed with HAs also predominantly enhances production of anti-HA antibody in CBA, CC57W and A/Sn mice with low reactivity to HAs [4]. The conjugate of *M. tuberculosis* HSP70 protein with Polyoxidonium induces enhanced antibody production and proliferation of $CD4^+$ lymphocytes, and administration of Polyoxidonium together with BCG increases the protective effect of the vaccine in mice [4]. Being administered with sheep erythrocytes, Polyoxidonium increases the number of antibody-forming cells not only in healthy mice, but also in animals with immunosuppression caused by old age, radiation, or administration of cyclophosphamide [4]. In vitro human monocytes and neutrophils bind and capture labeled Polyoxidonium [7]. The addition of Polyoxidonium to cell cultures increases the production of interleukine-6 (IL-6) and intracellular H_2O_2, improves the ability of neutrophils and macrophages to capture and process various infectious agents [7–9], stimulates differentiation of monocytes into DCs and enhances T cell proliferation [10]. Due to its safety and ability to stimulate immune responses, Polyoxidonium has been approved in Russia as a vaccine adjuvant and stand alone immune modulator for the treatment of acute and chronic bacterial, viral or fungal infections [3,11] (http://petrovax.-com/medication/catalog/polyoxydonium/).

In this study, we evaluated the humoral and cellular immune responses of mice to the GQ vaccine containing Polyoxidonium adjuvant and recombinant HAs from 4 strains of the influenza virus. The effect of the vaccine on human monocyte-derived immature DCs has also been investigated. The vaccine has been shown to effectively induce an immune response of mice to HAs *in vivo* and stimulates the maturation of human DCs *in vitro*. The article presents results that indicate that adjuvant Polyoxidonium in vaccine enhances the immune response to the HAs of the studied vaccine.

2. Materials and methods

Female BALB/c mice with specific pathogen-free status (Charles River Laboratories) were obtained from the Center for Genetic Collections of Laboratory Animals (Lobachevsky State University of Nizhny Novgorod, Russia) and from Charles River Laboratories (Germany). All animal studies were performed in accordance with Directive 2010/63/EU of the European Parliament and of the Council of the European Union of 22 September 2010 on the protection of animals used for scientific purposes. Investigations were approved by the local Ethics Committee of Lobachevsky State University of Nizhny Novgorod, Russia. Cell lines P-815 and Yac-1 were obtained from the Russian collection of cell cultures (Institute of Cytology RAS, Russia).

2.1. Vaccines, antigens and adjuvant

To evaluate the antibody response to vaccination, we used the Grippol Quadrivalent vaccine (lot № 031115), one dose of which contained 0.55 mg of Polyoxidonium and recombinant HAs of the following viruses: A/H1N1/California/07/09, A/H3N2/Switzerland/9715293/13, B/Phuket/3073/13, B/Brisbane/60/08 (5.6 μg of each HA). In lymphocyte and DC studies, we used the Grippol®Quadrivalent seasonal influenza vaccine (lot № 010818), one dose of which contained 0.49 mg of Polyoxidonium and following recombinant HAs: A/Michigan/45/2015(H1N1)-NYMC X-275A (lot № 63–0518), A/Singapure/INFIMH-16-0019/2016(H3N2)-IVR-186 (lot № 60–78 0518), B/Maryland/15/2016-NYMC-BX-69A (B/Colorado/06/2017-like virus) (lot № 57-0518), B/California/12/2015-NYMC BX-59B (B/Phuket/3073/2013) (lot № 65-0518), 5.9 μg of each HA. The vaccine manufacturer provided us with the recombinant HAs that were used to make these vaccines. Before using HAs in experiments, we prepared a mixture containing 40 μg/ml of each HA and transferred them to PBS using dialysis through the membrane of a 20/32 VISKING dialysis tube (Serva, Germany) (MWCO 12–14 kDa). The dialyzed mixture was sterilized by filtration through a Millipore membrane filter with a pore diameter of 0.22 μm. In experiments with keyhole limpet haemocyanin (KLH), we used a 12.2% aqueous solution of azoximer bromide with a molecular weight of 78 kDa and an oligomer content of 0.48%.

Vaccines, their antigens and an aqueous solution of azoximer bromide were provided by NPO Petrovax Pharm, LLC (Russia). Grippol® and Polyoxidonium® are trademarks of NPO Petrovax Pharm LLC.

2.2. Hemagglutination inhibition (HI) test

Groups (n = 16 each) of female BALB/c mice aged 6–7 weeks, which weighed 21 ± 1 g, were immunized twice with GQ vaccine (0.2 μg of each HA and 19.8 μg of Polyoxidonium in 0.5 ml of PBS per mouse) or with vaccine antigens (0.2 μg of each HA in 0.5 ml of PBS). Control mice (n = 18) were injected with 0.5 ml of PBS. The substances were administered intraperitoneally with an interval of 2 weeks. Blood serum was collected 4 weeks after the start of immunization. Before the HI test, serum samples were treated with heat [12]. A series of serum dilutions was prepared and the diluted samples were mixed with 8 hemagglutinating units of the viruses from the kits for HI test (LLC "PPDP", Institute of Influenza, Russia). The mixtures were incubated at room temperature for 1 h and then mixed with an equal volume of a 0.5% chicken erythrocyte suspension. Agglutination patterns were read after complete sedimentation of erythrocytes in control wells without serum and viruses.

2.3. Immunization and cell preparation to assess the effect of the vaccine on lymphocyte properties

Three groups (n = 10 each) of female BALB/c mice aged 6 to 8 weeks, which weighed 22 ± 2 g, were immunized twice with the following substances: 0.2 human dose of GQ vaccine (1.18 μg of each HA and 98 μg of Polyoxidonium in 0.5 ml of PBS), a mixture of vaccine antigens in an equivalent dose (1.18 μg of each HA in 0.5 ml of PBS), a mixture of vaccine antigens in a high dose (3 μg of each HA in 0.5 ml of PBS). Control mice (n = 10) were injected with 0.5 ml of PBS. The substances were administered intraperitoneally at 2 week intervals. Spleens were aseptically removed from mice 5 weeks after the start of immunization and were gently dissociated by glass homogenizer into cold DME medium (Gibco, UK). Cell suspensions were filtered through cell dissociation sieve and splenocytes were collected by centrifugation for 10 min at

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

450g in a Megafuge 16R (Thermo Scientific, Germany). For red blood cells lysis, we resuspended the splenocytes in 3 ml of RBC Lysis Buffer (BioLegend, USA) and incubated on a shaker for 5 min at + 4° C. Lysis was stopped by the addition of PBS to a volume of 12 ml. Then, splenocytes were washed twice by centrifugation and counted using a hemocytometer. For *ex vivo* experiments, 3.5×10^7 splenocytes from each spleen were resuspended in complete cultural medium (CCM) of the following composition: RPMI-1640 medium (Gibco, UK) with 292 mg/1 of L-glutamine (PanEko, Russia) and 10% inactivated fetal bovine serum (FBS) (Invitrogen, USA). For flow cytometry analysis, 3×10^6 splenocytes from each spleen were resuspended in PBS with 0.09% NaN_3.

2.4. Splenocyte proliferation assays

Suspensions of splenocytes in CCM were plated at 4×10^5 cells per well in 96-well flat-bottom plates (Costar, USA). Mixture of recombinant HAs was added to evaluate the response to vaccine antigens. The final concentration of each HA was 1 μg/ml. Mitogen concanavalin A (ConA) was added to evaluate the response to the polyclonal stimulation of T cells. The final concentration of ConA was 7.5 μg/ml. An equivalent volume of PBS was added to control wells containing unstimulated cells. All culture variants were triplicated. The final volume in all wells was 200 μl. Cells were cultured for 72 h at +37 °C and 5% CO_2, in a CO_2 incubator Heracell 150i (Thermo Scientific, Germany). Then, 20 μl of 100 μM BrdU solution from Cell Proliferation ELISA, BrdU (colorimetric) kit (Roche, Germany) was added to each well, and the cells were cultured for the next 24 h. The medium was then removed from the wells, the cells were dried and fixed, and the incorporation of BrdU into DNA was evaluated by colorimetric immunoassay using an ELISA cell proliferation, BrdU (colorimetric) kit, in accordance with the manufacturer's instructions. The optical density (OD) of samples was measured at 450 nM and reference wavelength of 620 nM on Sunrise-Basic Tecan platereader (Tecan Austria GmbH, Austria).

2.5. Cytokine production assays

Splenocytes in CCM were plated at 2×10^6 cells per well in 24-well plates (Costar, USA). Cells were stimulated with a mixture of vaccine antigens (final concentration of each HA was 1 μg/ml) or ConA (final concentration was 7.5 μg/ml). An equivalent volume of PBS was added to control wells containing unstimulated cultures. The final volume in all wells was 1 ml. Cells were incubated at + 37 °C and 5% CO_2 for 72 h. Cell culture supernatants were then harvested and frozen at −70 °C until enzyme-linked immmunosorbent assay was performed. The concentration of interferon-γ (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-4 (IL-4) and IL-5 in the culture medium was determined using ELISA MAX™ Deluxe kits (BioLegend, USA) in accordance with the manufacturer's instructions.

2.6. Determination of NK cell activity

NK activity of splenocytes was measured by the cytolytic effect on target cells Yac-1, as described previously [13]. Splenocytes and target cells were seeded in a round bottom 96-well plate as follows. Experimental wells contained 2×10^5 of splenocytes and 10^4 of Yac-1 cells per well in 200 μl of CCM. Different control wells contained: CCM without cells ("background"); target cells without splenocytes ("control 1"); splenocytes without targets ("control 2"); target cells destroyed by Triton X-100 at the end of the cultivation period ("control 3"). The plates were incubated at +37 °C and 5% CO_2 for 72 h. After incubation, the plates were centrifuged at 250g for 10 min, and samples of supernatants (100 μl) from each well were transferred to fresh optically clear 96-well flat-bottom plate. The concentration of lactate dehydrogenase leaving the dead cells was determined colorimetrically using the Cytotoxicity Detection Kit (LDH) (Roche, Germany) according to the instructions provided by the manufacture. Briefly, 100 μl of reconstituted substrate mixture was added to each well. The plates were incubated for 30 min at room temperature in the dark. The reaction was stopped by adding 50 μl of 1 N HCl to the wells. The absorbance of the samples was measured at 492 nM and reference wavelength of 620 nM on a Sunrise-Basic Tecan plate reader. The percentage of specific lysis was calculated as follows: ((experimental LDH release − control 2) – control 1) / (control 3 – control 1)) × 100.

2.7. Determination of activity of cytotoxic T lymphocyte (CTL)

CTL activity was measured by the cytolytic effect of splenocytes on antigene-loaded, haplotype-matched target cells P-815 [14]. Before the test, splenocytes were cultured at a concentration of 4×10^6 cells/ml in CCM supplemented with 50 units/ml of IL-2 for 3 days to expand the pool of *in vivo* activated T cells. Then the splenocytes were collected, washed by centrifugation and resuspended in fresh CCM. Target cells were loaded with vaccine antigens using incubation of P-815 cells in serum-free RPMI-1640 medium supplemented with a mixture of recombinant HAs at +37° C and 5% CO_2 for 2 h [15]. The final concentration of each vaccine antigen was 1 μg/ml [16]. Then target cells were washed twice by centrifugation and resuspended in fresh CCM. Splenocytes (2×10^5 cells per well) together with loaded target cells (10^4 cells per well) were seeded into the experimental wells of round-bottom 96-well plates. Control wells, similar to those used in the NK cell activity test, were also seeded. The final volume in all wells was 200 μl. The plates were incubated at +37° C and 5% CO_2 for 4 h. After incubation, the plates were centrifuged and supernatants were taken from the wells to determine the percentage of specific lysis using the Cytotoxicity Detection Kit (LDH) as described above for the NK cell activity test.

2.8. Flow cytometry

Suspensions of splenocytes were divided into samples containing 5×10^5 cells in 50 μl PBS with 0.09% NaN_3. Titrated fluorescent conjugates of monoclonal antibodies CD4-FITC, CD8b-PerCP-Cy5.5, CD62L-PE and CD44-APC (BioLegend, USA) were added to the cell samples. Cells were incubated on a shaker for 20 min at +4 °C in the dark. Then, cells were washed in PBS with 0.09% NaN_3, fixed in 1% paraformaldehyde and stored at +4 °C until analysis, which was performed using an FACS Calibur flow cytometer (BD, USA). The whole sample was acquired and analysis was performed using CellQuest software (BD, USA). We gated small lymphocytes and large lymphocytes (lymphoblasts) according to the forward scatter (FSC) / side scatter (SSC) profiles and divided each of these cell groups into $CD4^+CD8^-$ T-helper cells and $CD8^+CD4^-$ T-killer cells. Then, we measured the expression of CD62L and CD44 molecules into each group of cells. Memory T cells were identified as $CD62L^+$-$CD44^{hi}$ central memory T cells and $CD62L^-CD44^{hi}$ effector memory T cells [17–19].

2.9. Stimulation of antibody response to KLH

We immunized mice once with a predetermined suboptimal dose of KLH (0.1 mg per mouse), intraperitoneally on day 0. Azoximer bromide was administered in doses from 0.085 to 2.72 mg/kg (1.7–54.4 μg per mouse), intraperitoneally, daily for 5 days, starting from day 0. Negative control mice received a suboptimal dose of KLH and 5 PBS injections instead of azoximer bromide. Positive control mice received the optimal dose of KLH (2 mg per mouse)

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

and 5 PBS injections. No substances were administered to the intact control mice. In the experiment, 7 groups of 10 mice were used. Blood serum was collected on day 6. The concentration of anti-KLH IgM was determined using KLH IgM (Mouse) ELISA Kit (Abnova, USA) in accordance with the manufacturer's instructions.

2.10. In vitro stimulation of human DCs maturation

DCs were derived from venous blood monocytes of 13 healthy adult donors (5 women and 8 men). Donors were from 25 to 43 years old. The average age was 32.2 ± 2.2 years. All donors gave written informed consent for blood sampling. DCs were generated as described in [20] with modifications [21]. Briefly, peripheral blood mononuclear cells from healthy adult donors were isolated by centrifugation with Hystopaque-1077 (Sigma-Aldrich, USA), plated in 24-well plate (Costar, USA) at 5×10^6 cells per well and incubated at 37 °C and 5% CO_2. After 2 h, non-adherent cells were removed and adherent cells were cultured in RPMI-1640 with 10% FCS, 100 ng/ml granulocyte–macrophage colony-stimulating factor (GM-CSF) and 20 ng/ml IL-4 (R&D Systems, USA). On day 3, IL-4 and GM-CSF were again added in the same concentration. On day 7, monocyte-derived immature DCs (iDCs) were collected and placed in 24-well plate in RPMI-1640 with 10% FCS at 3×10^5 cells per well. iDCs were stimulated with vaccine GQ, adjuvant-free mixture of vaccine AGs or Polyoxidonium adjuvant alone. Final concentrations of the vaccine were 0.001–0.2 doses/ml. Concentrations of AGs and Polyoxidonium were equivalent 0.2 doses/ml of vaccine. Maturation of reference mature DCs was induced by stimulation with a cocktail consisting of 25 ng/ml IL-1β, 25 ng/ml IL-6, 50 ng/ml TNF-α and 1 μg/ml prostaglandin E_2 (PGE_2). After 48 h, DCs were harvested, spinned, and resuspended in PBS with 0.09% NaN_3. Aliquots of cells were stained with fluorescent conjugates of antibodies to HLA-DR, CD14, (Sorbent, Russia), CD80, CD83, CD86, ICOSL (CD275), CCR5 (CD195), or CCR7 (CD197) (eBioScience, USA) for 20 min at +4 °C in the dark. Then, cells were washed in PBS with 0.09% NaN_3, fixed in 1% paraformaldehyde and stored at +4 °C until analysis, which was performed using an FACS Calibur flow cytometer and CellQuest software.

2.11. Statistical analysis

Statistical analyses were performed using Mann–Whitney *U* test, Newman-Keuls, Dunnett's tests. Data are presented as mean ± standard error of the mean.

3. Results

3.1. Antibody response

Mice were immunized twice with adjuvant-free mixture of vaccine AGs or with GQ vaccine containing the Polyoxidonium adjuvant. Doses of AGs in the adjuvant-free mixture and in the vaccine were identical (0.2 μg of each HA per mouse). The dose was selected based on previous studies of Gripol vaccines (unpublished data). Immunization with both the vaccine and AGs alone significantly increased the HI titer to all strains of influenza viruses (Fig. 1). Polyoxidonium in the vaccine caused a strong increment in the HI titer of antibody specific for the influenza A/H3N2/Switzerland/9715293/13 virus ($\alpha < 0.01$ compared with adjuvant-free AGs) and statistically significant increase in the titer of antibodies to viruses A/H1N1/California/07/09 and B/Phuket/3073/13 (both, $\alpha < 0.05$ compared with adjuvant-free AGs).

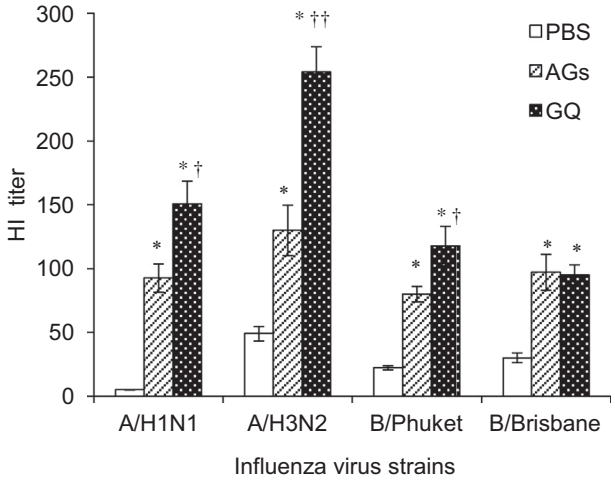

Fig. 1. HI titer of serum antibody to the influenza virus strains measured 4 weeks after start of immunization. Mice were immunized twice with GQ vaccine (0.2 μg of each HA and 19.8 μg of Polyoxidonium), with mixture of vaccine AGs (0.2 μg of each HA), or with PBS. The substances used for immunization are indicated in the legend. Mann–Whitney U was used for comparison to PBS group (*$\alpha < 0.05$) or to AG group (†$\alpha < 0.05$, ††$\alpha < 0.01$).

3.2. Action of the GQ vaccine and its components on functional properties of murine lymphocytes

We immunized mice twice with a higher dose of the vaccine GQ to evaluate the functional and phenotypic properties of lymphocytes. The dose was selected based on the results of previous studies [13]. At each immunization, 0.2 dose of vaccine (1.18 μg of each HA and 98 μg of Polyoxidonium) was administered per mouse. Two other groups of mice obtained nonadjuvanted mixtures of antigens in an equivalent dose (1.18 μg of each HA) or in a high dose (3 μg of each HA). A high dose of antigens corresponded to their content in 0.2 doses of the standard vaccine, based on the recommendation of WHO. Five weeks after the start of immunization, the splenocytes were stimulated with vaccine antigens or ConA to evaluate the proliferative response and cytokine production.

The proliferative response of splenocytes to *ex vivo* stimulation with AGs was significantly higher in group of mice immunized with GQ vaccine compared to the control group and group immunized with an equivalent dose of AGs without adjuvant (both $\alpha' < 0.01$, Fig. 2A). In addition, splenocytes from GQ-immunized mice showed a high level of proliferation after polyclonal activation with mitogen ConA. The level of ConA-induced proliferation in this group was significantly higher than in the control group and the group immunized with an equivalent dose of AGs (both $\alpha' < 0.01$). Mice immunized with the vaccine and mice immunized with a high dose of AGs did not have significant differences in proliferative responses to antigen-specific or polyclonal stimulations. The proliferation of splenocytes taken from mice immunized with an equivalent dose of AGs did not differ from that of non-immunized control mice.

After immunization of mice with GQ, splenocytes acquired the ability to produce large amounts of T helper type 2 (Th2) cytokine IL-5 in response to antigen-specific stimulation (Fig. 2D). The production of this cytokine by antigen-activated splenocytes in the group of vaccine immunized mice was 978.9 times greater than in the control group ($\alpha' < 0.01$). Immunization of mice with a high dose of AGs induced a similar level of IL-5 production ($\alpha' < 0.01$ compared with the control group), while immunization with an equivalent dose of AGs caused a slightly lesser effect ($\alpha' < 0.05$ compared with the control group). It should be noted that the powerful antigen-induced production of IL-5 in mice immunized with a

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

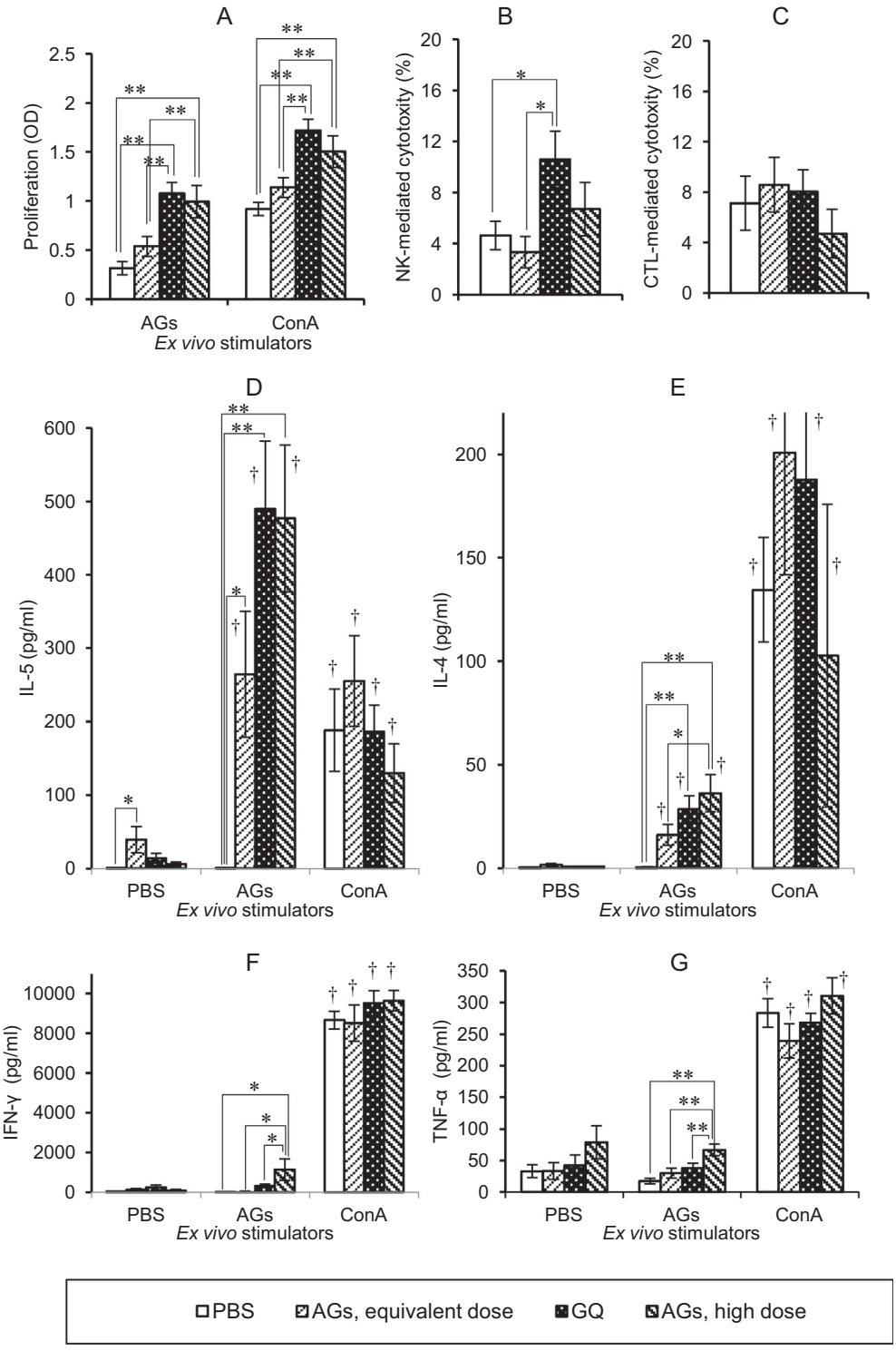

Fig. 2. Assessments of (A) proliferation, (B) NK cell activity, (C) CTL activity and production of (D) IL-5, (E) IL-4, (F) IFN-γ and (G) TNF-α by splenocytes from immunized mice. Mice were immunized twice with the following substances: GQ vaccine (1.18 μg of each HA and 98 μg of Polyoxidonium), a mixture of vaccine AGs in an equivalent dose (1.18 μg of each HA), a mixture of vaccine AGs in a high dose (3 μg of each HA), PBS. The substances used for immunization are indicated in the legend. Properties of splenocytes were evaluated in *ex vivo* tests 5 weeks after the start of immunization. The substances used for *ex vivo* stimulation are indicated below the histograms. Newman-Keuls test was used for comparison between groups of mice (*$\alpha' < 0.05$, ** $\alpha' < 0.01$) and between *ex vivo* stimulated and unstimulated splenocytes into each group (†$\alpha' < 0.05$).

GQ vaccine or a high dose of antigens was so great that it significantly exceeded ConA-induced production in the same groups of mice ($\alpha' < 0.05$).

Immunization with the vaccine also caused the appearance of AG-specific producers of IL-4 (Fig. 2E) in the spleen. The concentration of IL-4 in cultures of antigen-activated splenocytes in mice immunized with the vaccine was 50.5 times higher than the corresponding parameter in the control group ($\alpha' < 0.01$). Immunization with a high dose of AGs caused a 64-fold increase in IL-4 production ($\alpha' < 0.01$). The concentration of IL-4 in cultures of antigen-activated splenocytes in mice immunized with an equivalent dose of AGs did not significantly differ from control group.

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

The immunization with GQ or with equivalent dose of AGs did not lead to augmentation in the production of IFN-γ and TNF-α by splenocytes in response to stimulation with AGs or ConA (Fig. 2F, G). Immunization with high doses of AGs induced a slight increase in the production of these Th1 cytokines in AG-stimulated splenocyte cultures compared to AG-stimulated cultures in other groups of mice. However, differences between antigen-induced and spontaneous production were not found in this group.

Using YAC-1 cells as standard targets of NK cells, we showed that the NK activity of splenocytes increased in mice immunized with a GQ vaccine, while administration of AGs without adjuvant did not affect NK activity (Fig. 2B). All used test substances were unable to increased the CTL activity of splenocytes against antigen-loaded P-518 cells (Fig. 2C).

3.3. Action of GQ vaccine on maturation of mice lymphocytes in vivo

The phenotype of splenocytes was determined 5 weeks after the start of immunization with the vaccine or mixtures of its AGs. Maturation of memory T cells was evaluated by expression of CD62L and CD44 molecules, sequentially gating small lymphocytes and large lymphocytes (lymphoblasts), then $CD4^+CD8^-$ T-helper cells and $CD8^+CD4^-$ T-killer cells (Fig. 3A).

Although it has previously been shown that effector T cells can transform into memory T cells during the contraction phase of the immune response, there is growing evidence that events during antigen presentation may determine the early programming of cells to differentiate into memory T cells (reviewed in [18]). We hypothesized that memory T cells generated shortly after antigen presentation can retain the large size of the activated cells for some time after maturation. Indeed, $CD4^+CD8^-$ large lymphocytes compared to $CD4^+CD8^-$ small lymphocytes contained a bigger proportion of $CD62L^+CD44^{hi}$ central memory T cells (Tcm) and $CD62L^-CD44^{hi}$ effector memory T cells (Tem) after vaccination (Fig. 3B). $CD8^+CD4^-$ large lymphocytes also contained a bigger proportion of Tem and, especially, Tcm compared to small lymphocytes.

The administration of the GQ vaccine or mixtures of AGs led to a significant increase in the number of both $CD4^+CD8^-$ and $CD8^+CD4^-$ large lymphocytes in the spleen of mice (Fig. 3C-E). The increment in the number of $CD4^+CD8^-$ large lymphocytes was due to an increase in the content of the least mature $CD62L^+CD44^{lo}$ T cells as well as mature Tcm and Tem (Fig. 3D). In a subpopulation of $CD8^+$ T cells, the effect of the vaccine and its AGs in the equivalent dose extended only to $CD62L^+CD44^{lo}$ large lymphocytes (Fig. 3E). Immunization with high dose of AGs increased the number of $CD8^+CD62L^+CD44^{lo}$ T cells in both the subset of large and small lymphocytes (Fig. 3E, H). Neither the vaccine nor the AGs had a significant effect on the content of $CD8^+$ T-memory cells (Fig. 3E), $CD4^+$ small lymphocytes (Fig. 3G) and common number of small lymphocytes (Fig. 3F).

3.4. The effect of azoximer bromide on the production of anti-KLH antibodies

We investigated how azoximer bromide (the active substance of Polyoxidonium) as a therapeutic immunostimulating agent affects the production of antibodies to the KLH model antigen. In this experiment, we immunized mice with a suboptimal dose of KLH once and administered various doses of azoximer bromide for 5 days. Azoximer bromide dose-dependently increased the production of anti-KLH IgM in the concentration range of 0.085–1.36 mg/kg, raising it to the level of response to the optimal dose of KLH (Fig. 4).

The findings suggest that the Polyoxidonium in the GQ vaccine stimulates a humoral immune response to influenza antigens, increasing the production of antibodies and Th2 cytokines.

3.5. Action of the GQ vaccine and its components on maturation of human DCs

Immature DCs were obtained by cultivation of monocytes with IL-4 and GM-CSF for 7 days. Obtained iDCs were stimulated with vaccine GQ, vaccine AGs or Polyoxidonium adjuvant for 2 days. Development of reference mature DCs was induced by stimulation with a cocktail of proinflammatory cytokines and PGE_2. Unstimulated iDCs expressed the major histocompatibility complex class II molecule HLA-DR, had a low expression of CD83 and the costimulating molecule ICOSL and lacked the monocytic marker CD14 and chemokine receptors CCR5 and CCR7 (Fig. 5B). About half of the iDCs carried costimulatory molecules CD80 and CD86 on the outer membrane. Reference mature DCs had the typical phenotype $HLA\text{-}DR^{hi}CD14^-CD80^+CD83^+CD86^+CCR5^-$. 24.5% of these cells acquired the expression of CCR7, which is involved in the migration of mature DCs into the T-cell zones of lymphoid organs. Maturation of DC induced with proinflammatory cytokines and PGE_2 did not increase ICOSL expression (Fig. 5 B).

Incubation of iDCs with the GQ vaccine enhanced the expression of HLA-DR, CD86 and a marker of mature DCs CD83 in a dose-dependent manner (Fig. 5 A). In addition, GQ vaccine at a concentration of 0.2 doses/ml increased the expression of CD80 and ICOSL compared to unstimulated iDCs (Fig. 5 B). Adjuvant-free mixture of vaccine AGs significantly enhanced the expression of HLA-DR, CD80 and CD86, but caused a weak and statistically unreliable increase in the expression of CD83, ICOSL and CCR5 (Fig. 5 B). Polyoxidonium without AGs enhances ICOSL expression (Fig. 5 B).

4. Discussion

The immunogenicity of influenza virus antigens allows the use of adjuvant-free subunit vaccines to prevent seasonal flu worldwide. However, the antigens of some influenza viruses, including highly pathogenic strains of H5 and H7, have relatively poor immunogenicity. Vaccines against these virus strains, such as the recombinant H5 vaccine [22], the surface antigen influenza A/H5N3 vaccine [23], the subvirion vaccine A/H5N1 [24], and the surface antigen A/H7N9 vaccine [25], require large doses and/or at least two vaccinations for effective protection if used without adjuvants. Adjuvants in vaccines against seasonal influenza can also improve the antibody response, enhance efficacy, and reduce the antigen dose required to induce protection. However, the effectiveness of the combination of various adjuvants and antigens varies significantly. The classic adjuvant Alum enhanced the response to low doses of the A/H9N2 avian influenza virus vaccine [26], increased antibody production and the Th2 shift in response to the A/H7N9 vaccine [13], but in other influenza vaccines Alum induced only marginal improvements [27,28]. The oil-in-water adjuvant MF59 significantly improved immune responses to A/H5N1 vaccines [23,29], surface antigen A/H5N3 vaccine [24] and subunit A/H9N2 vaccine [25]. Combination of MF59 with A/H7N9 vaccine more strongly induced humoral immune response and Th2 cytokine production compared to Alum [13]. Clinical trials have shown that MF59 helps to achieve a protective antibody titer in response to extremely low dose of split A/H7N9 vaccine [30]. MF59 has an acceptable safety profile when paired within influenza vaccines [22,31], but it can increase local reactogenicity [23,32].

In the present study, we evaluated the effect of the low-dose Polyoxidonium-adjuvanted influenza tetravalent vaccine GQ. Poly-

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

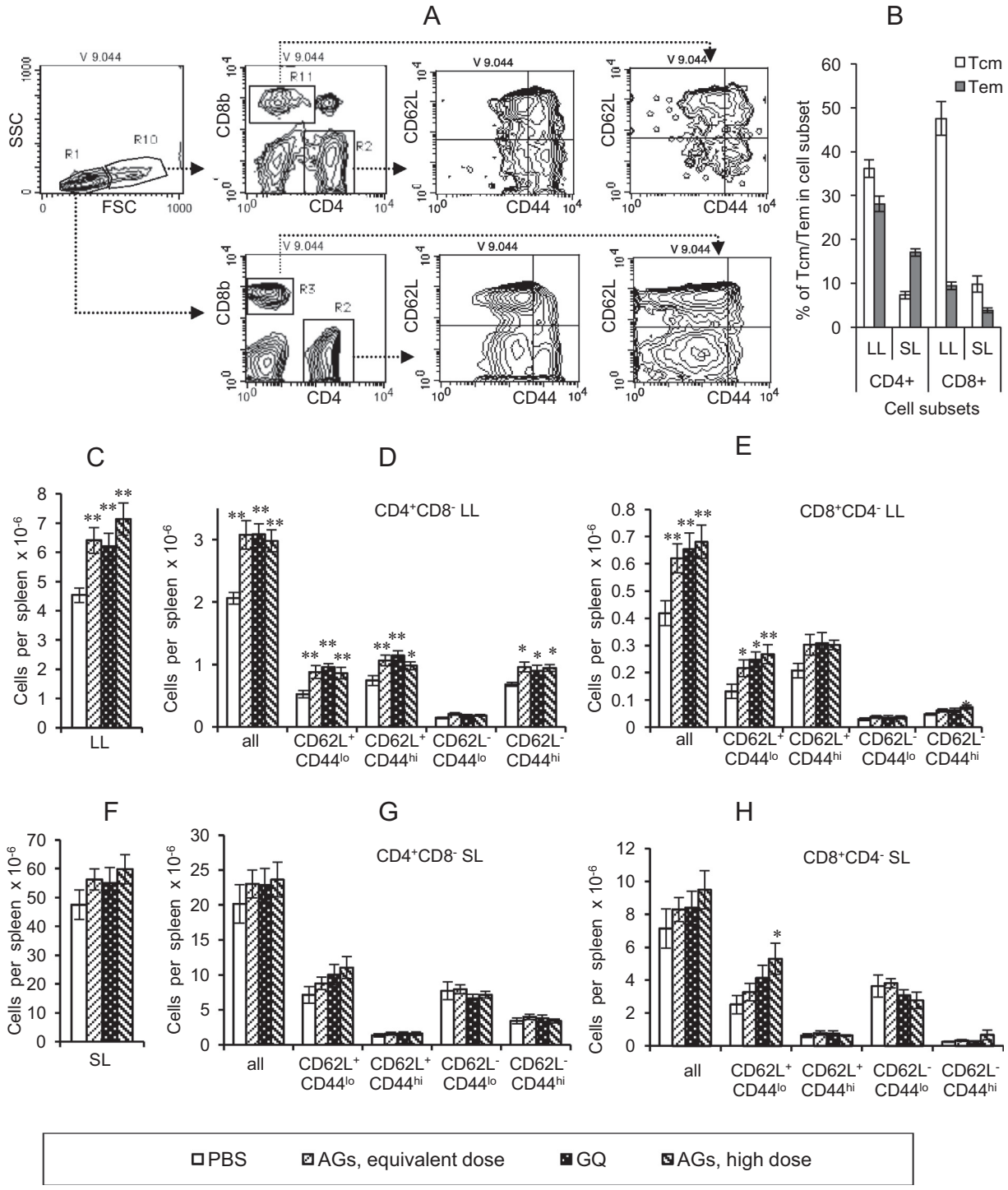

Fig. 3. Action of vaccination on maturation of splenocytes. (A) Gating of splenocytes. Small lymphocytes (SL) localized in R1, large lymphocytes (LL) localized in R10. (B) Content of Tcm and Tem in $CD4^+$ and $CD8^+$ subpopulation of LL and SL after vaccination with GQ. (C–H) Content of LL, SL and cells with different phenotypes in the spleens of mice 5 weeks after start of immunization. Mice were immunized twice with the following substances: GQ vaccine (1.18 μg of each HA and 98 μg of Polyoxidonium), a mixture of vaccine AGs in an equivalent dose (1.18 μg of each HA), a mixture of vaccine AGs in a high dose (3 μg of each HA), PBS. The substances used for immunization are indicated in the legend. Newman-Keuls test was used for comparison to PBS group (*α' < 0,05, ** α' < 0,01).

oxidonium has been described as an immune adjuvant and immunomodulator that is clinically used with excellent tolerance [2,4,5]. The vaccine GQ has been shown to induce antibody response to all 4 influenza hemagglutinins during immunization of mice. Polyoxidonium causes an additional increase in antibody titer. An experiment with model T-dependent antigen KLH [33–35] showed that the stimulating effect of Polyoxidonium is not restricted to immune response to influenza HAs.

Our studies of the response of T cells to vaccination have revealed the predominant involvement of T helper cells in the response. Immunization of mice with GQ vaccine caused the accumulation of $CD4^+$ large lymphocytes with the phenotype of mem-

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

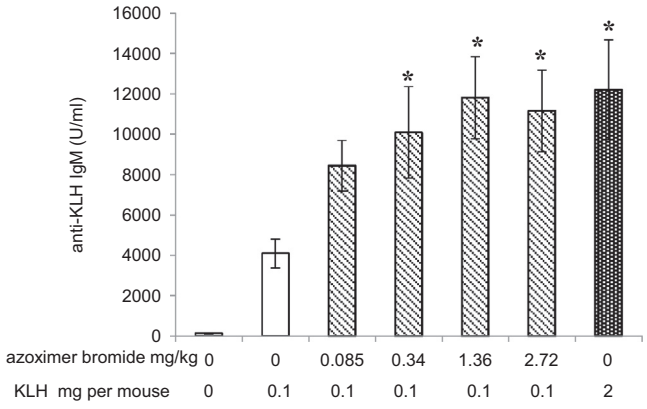

Fig. 4. Action of azoximer bromide on the production of anti-KLH antibodies. Mice were immunized once with a suboptimal (0.1 mg per mouse) or optimal (2 mg per mouse) dose of KLH, intraperitoneally on day 0. Azoximer bromide was administered in doses from 0.085 to 2.72 mg/kg, intraperitoneally, daily for 5 days, starting from day 0. Blood serum was collected on day 6. Dosage of azoximer bromide and KLH are indicated below the histogram. Dunnett's test was used for comparison to control group immunized with 0.1 mg of KLH without azoximer bromide (*α' < 0,05).

ory T cells in the spleen. Vaccination also caused a strong increase in the proliferation and production of Th2 cytokines by splenocytes activated with vaccine antigens *ex vivo*. As a result of vaccination, the antigen-induced production of IL-5 was increased by almost 1000 times and the production of IL-4 by 50.5 times. Strong IL-5 production and a relatively weak IL-4 response have been described previously in antigen-stimulated cultures of lymphocyte from vaccinated animals or humans [36–38]. However, it was unexpected for us that the powerful antigen-induced production of IL-5 in mice immunized with the GQ vaccine or a high dose of AGs exceeded the production induced by polyclonal activator ConA. We suggest that the production of Th2 cytokines in ConA stimulated cultures may be limited by the increased activity of other cells, such as Th1 [39]. At the same time, the activity of Th1 in all cultures stimulated by HAs was minimal and could not have a significant effect on strong Th2 response.

It should be noted that the hemagglutinins of the vaccine caused the accumulation of Th2 in the spleen without the aid of an adjuvant. However, the Polyoxidonium adjuvant significantly enhanced the proliferative response and increased the production of IL-4 and IL-5 to the level of action of a triple dose of AGs.

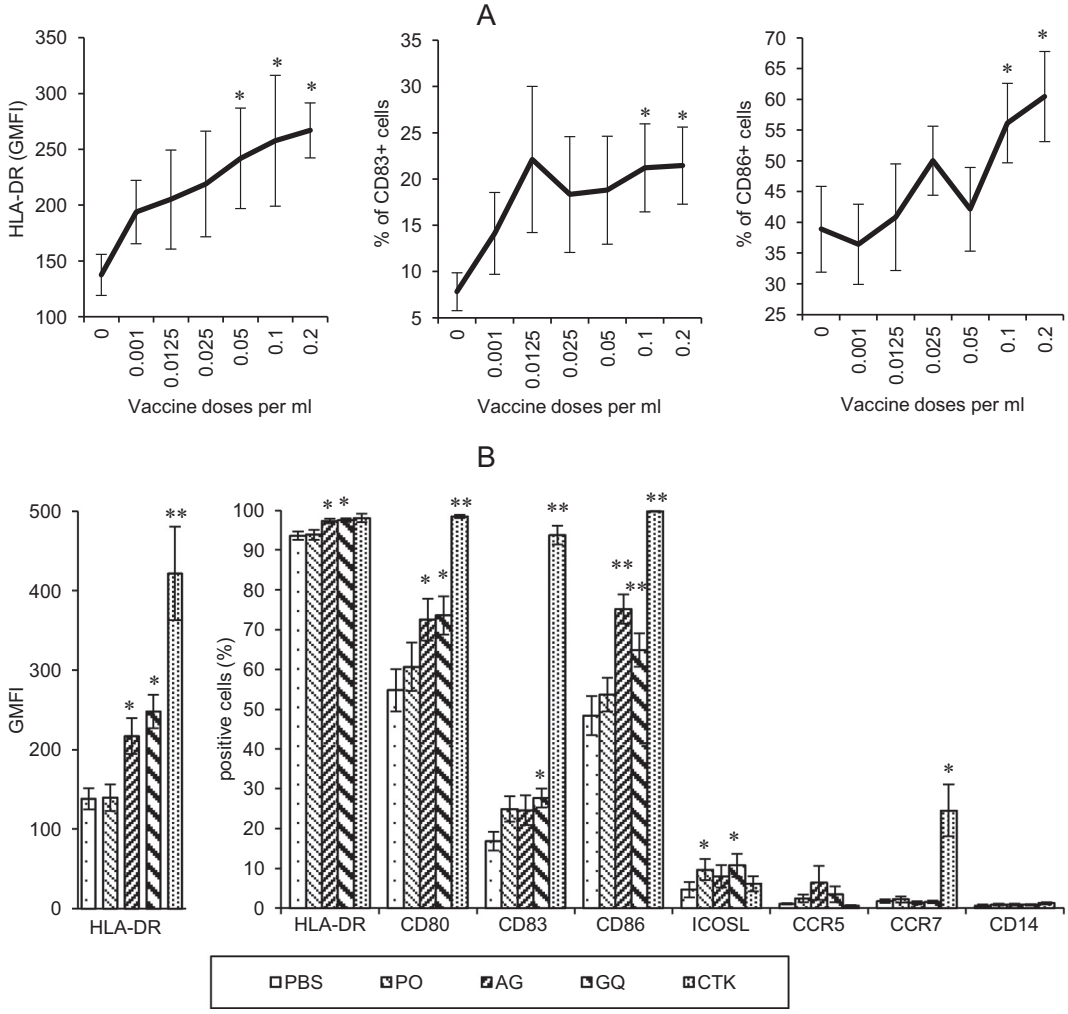

Fig. 5. Action of the GQ vaccine and its components on maturation of human monocyte derived DC. (A) Dose-dependent effect of the vaccine on the expression of HLA-DR, CD83 and CD86 (n = 7). Vaccine concentrations (doses/ml) are indicated on the X axis. *p < 0,05 in comparison to iDC (pair *t*-test). (B) DC phenotype after 2 days of incubation with GQ vaccine (0.2 doses /ml) and equivalent amounts of AGs and polyoxidonium (PO) (n = 13). Substances introduced into cell cultures are indicated in the legend. Stimulator-free iDC (PBS) served as a negative control. DCs stimulated with a mixture of prostaglandin E2 and pro-inflammatory cytokines (CTK) were a positive control for maturation. Dunnett's test was used for comparison to negative control (*α' < 0,05, ** α' < 0,01).

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

The hemagglutinins of the vaccine, both without adjuvant and with Polyoxidonium, could not induce a significant response of Th1 and $CD8^+$ T cells. Vaccination of mice did not increase the production of IFN-γ and TNF-α by splenocytes. In a subpopulation of $CD8^+$ T cells, the vaccine and its antigens apparently stimulated the relatively early stages of cell maturation. As a result, vaccination induced the generation of immature $CD8^+CD62L^+CD44^{lo}$ lymphocytes, but did not cause the maturation of antigen-specific CTLs and $CD8^+$ memory T cells.

The release of Th2-type cytokines, including IL-5, is usually associated with B-cell stimulation and antibody production. IL-5 stimulates the differentiation of B cells into IgM producing plasmocytes, induces IgG1 isotype switch recombination, and also enhances the production of antibodies, especially IgM and IgA [40–45]. In turn, IgA is an important factor in the protection of the mucosa, which is the entrance gate to the influenza virus. In contrast, the release of IFN-γ and TNF-α have typically been associated with cellular immunity, which is responsible for stimulating inflammation, phagocytes, and CTLs [46]. Although IFN-γ producing Th1 cells can stimulate the synthesis of IgG2a antibodies in mice and IgM, IgA, IgG1, IgG2 and IgG3 antibodies in humans [47], the high Th1 activity generally suppresses the humoral immune response. Such suppression can be associated both with the negative influence of Th1 on the maturation of other T-helper types, and with the direct cytotoxic effect of Th1 on activated B cells [39]. Thus, the type of cytokine production induced by the vaccine in our experiment seems to be favorable for the induction of a T-dependent humoral immune response to vaccine antigens.

In cell cultures of human iDCs, the vaccine induced the expression of molecules associated with the presentation of antigens and costimulation of T-lymphocytes. DCs incubated with the GQ vaccine significantly increased the expression of the major histocompatibility complex class II molecule HLA-DR and co-stimulating molecules CD80, CD83, CD86 and ICOSL. It should be noted that DCs stimulated with GQ vaccine were inferior to DCs stimulated with inflammatory cytokines in terms of expression of HLA-DR, CD80, CD83 and CD86, but were superior in expression of ICOSL molecule. Polyoxidonium also enhances ICOSL expression. The costimulating molecule ICOSL is weakly expressed on unstimulated human blood DCs, but is found in such subpopulations of skin DC as classical DC 2 cells and Langerhans cells [48]. In vitro, DCs increase ICOSL expression after stimulation with LPS or thymic stromal lymphopoietin [49]. Interaction of ICOSL with the ICOS receptor on naïve activated $CD4^+$ T cells plays a key role in inducing the maturation of follicular T-helper cells [50,51]. In turn, follicular T-helpers stimulate important processes of the T-dependent humoral immune response: isotype switching and antibody affinity maturation, generation of long-lived plasma cells and memory B cells [52–54]. Homozygous loss of ICOS in humans leads to common variable immunodeficiency with a predominant lesion of the humoral immune response [55]. In addition, $ICOSL^+$ DCs can stimulate Th17 and regulatory T cells, but inhibit Th1 [56]. Finally, the ICOS/ICOSL interaction is necessary for the functioning and survival of NK cells and $CD4^+$ invariant natural killer T cells [57,58].

The stimulating effect of Polyoxidonium on innate immune cells, such as neutrophilic granulocytes, monocytes, macrophages, and dendritic cells, has been described previously [7–10]. We have shown that the GQ vaccine containing Polyoxidonium enhances the cytolytic activity of mouse NK cells, whereas the adjuvant-free vaccine antigens do not affect NK activity. However, Polyoxidonium did not exhibit such activity when directly acting on NK cells *in vitro* [10]. Apparently, the stimulating effect of the adjuvant is mediated by factors formed in response to combined action of influenza hemagglutinins and Polyoxidonium.

5. Conclusion

In summary, the influenza vaccine GQ with Polyoxidonium adjuvant is immunogenic, induces a Th2-polarized T-cell response and the production of antibodies to all influenza hemagglutinins of GQ. The effect of low-dosed GQ vaccine upon AG-induced proliferation, IL-4 and IL-5 production was equal to effect of standard dose of hemagglutinins. The GQ vaccine enhanced the cytolytic activity of natural killer (NK) cells, whereas the adjuvant-free mixture of AGs in lowered and standard doses did not affect NK activity. GQ vaccine induces partial maturation of human DC. Polyoxidonium alone stimulates DCs to enhance expression of ICOSL, which is crucial for inducing of humoral immune response.

Funding

This work was supported by a contract between NPO Petrovax Pharm, LLC (Moscow region, Russia) and Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology of Russian Federal Consumer Rights Protection and Human Health Control Service (Nizhny Novgorod, Russia). NPO Petrovax Pharm, LLC was not involved in the collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This work was supported by a contract between NPO Petrovax Pharm, LLC (Moscow region, Russia) and Academician I.N. Blokhina Nizhny Novgorod Scientific Research Institute of Epidemiology and Microbiology of Russian Federal Consumer Rights Protection and Human Health Control Service (Nizhny Novgorod, Russia). NPO Petrovax Pharm, LLC was not involved in the collection, analysis, interpretation of data, the writing of this article or the decision to submit it for publication. A.V. Matveichev is an employee of NPO Petrovax Pharm, LLC, and participated in study design. Other authors declare no conflict of interests.

References

[1] Poland GA, Morse D. Improving the public health: the U. S. recommendation for universal influenza immunization. Vaccine 2010;28(16):2799–800. https://doi.org/10.1016/j.vaccine.2010.03.002.

[2] Karaulov AV, Bykov AS, Volkova NV. Review of grippol family vaccine studies and modern adjuvant development. Epidemiol Vaccinal Prevent 2019;18(4):101–19. https://doi.org/10.31631/2073-3046-2019-18-4-101-119 (In Russ.).

[3] Powell BS, Andrianov AK, Fusco PC. Polyionic vaccine adjuvants: another look at aluminum salts and polyelectrolytes. Clin Exp Vaccine Res 2015;4:23–45. https://doi.org/10.7774/cevr.2015.4.1.23.

[4] Ivanova AS, Puchkova NG, Nekrasov AV, Gurkovskaya YaI, Apryatina VA, Paschenkov MV, Dagil YA, Masternak TB, Pinegin BV, Abramova MA. Polyoxidonium adjuvant effect mechanisms. Hæmatopoïesis Immunol 2015;13:30–92.

[5] Pružinec P, Chirun N, Sveikata A. The safety profile of Polyoxidonium in daily practice: results from postauthorization safety study in Slovakia. Immunotherapy 2018;10:131–7. https://doi.org/10.2217/imt-2017-0116.

[6] Manko VM, Rudneva TB, Razvalyaeva NA, Puchkova NG, Khaitov RM. Phenotyping correction of gene control of immune response to influenza vaccine. Immunologiya 1997;18:36–9 (in Russian).

[7] Dyakonova VA, Dambaeva SV, Pinegin BV, Khaitov RM. Study of interaction between the polyoxidonium immunomodulator and the human immune system cells. Int Immunopharmacol 2004;4:1615–23. https://doi.org/10.1016/j.intimp.2004.07.015.

[8] Dambaeva SV, Mazurov DV, Golubeva NM, D'yakonova VA, Pinegin BV, Khaitov RM. Effect of polyoxidonium on the phagocytic activity of human peripheral blood leukocytes. Russ J Immunol 2003;8:53–60.

[9] Dambaeva SV, Mazurov DV, Golubeva NM, D'yakonova VA, Pinegin BV. The effect of Polyoxidonium, a novel immunomodulator, on the phagocytic activity of human peripheral blood leukocytes. Centr Eur J Immunol 2003;28(2):127–33.

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

[10] Alexia C, Cren M, Louis-Plence P, Vo D-N, El Ahmadi Y, Dufourcq-Lopez E, et al. Polyoxidonium® activates cytotoxic lymphocyte responses through dendritic cell maturation: clinical effects in breast cancer. Front Immunol 2019;10:2693. https://doi.org/10.3389/fimmu.2019.02693.
[11] Toptygina A, Semikina E, Alioshkin V. Influence of an immunopotentiator Polyoxidonium on cytokine profile and antibody production in children vaccinated with Priorix. Arch Physiol Biochem 2012;118:197–203. https://doi.org/10.3109/13813455.2012.659669.
[12] Manual for the laboratory diagnosis and virological surveillance of influenza, WHO, 2011 http://whqlibdoc.who.int/publications/2011/9789241548090_eng.pdf?ua=1.
[13] Ou H, Yao H, Yao W, Wu N, Wu X, Han C, et al. Analysis of the immunogenicity and bioactivities of a split influenza A/H7N9 vaccine mixed with MF59 adjuvant in BALB/c mice. Vaccine 2016;34(20):2362–70. https://doi.org/10.1016/j.vaccine.2016.03.037.
[14] Luciani MF, Brunet JF, Denizot F, Golstein P. P815 mastocytoma cells, classical targets for cytotoxic T lymphocytes, exert natural cytotoxicity. Ann Inst Pasteur Immunol 1987;138(1):37–43. https://doi.org/10.1016/s0769-2625(87)80094-6.
[15] Zhou X, Liu B, Yu X, Zha X, Zhang X, Wang X, et al. Enhance immune response to DNA vaccine based on a novel multicomponent supramolecular assembly. Biomaterials 2007;28(31):4684–92. https://doi.org/10.1016/j.biomaterials.2007.07.002.
[16] Cristillo AD, Wang S, Caskey MS, Unangst T, Hocker L, He L, et al. Preclinical evaluation of cellular immune responses elicited by a polyvalent DNA prime/protein boost HIV-1 vaccine. Virology 2006;346(1):151–68. https://doi.org/10.1016/j.virol.2005.10.038.
[17] Sallusto F, Lenig D, Forster R, Lipp M, Lanzavecchia A. Two subsets of memory T lymphocytes with distinct homing potentials and effector functions. Nature 1999;401(6754):708–12. https://doi.org/10.1038/44385.
[18] Opata MM, Stephens R. Early decision: effector and effector memory T cell differentiation in chronic infection. Curr Immunol Rev 2013;9:190–206. https://doi.org/10.2174/15733955096661311262312O9.
[19] Rosenblum MD, Way SS, Abbas AK. Regulatory T cell memory. Nat Rev Immunol 2016;16(2):90–101. https://doi.org/10.1038/nri.2015.1.
[20] Sallusto F, Lanzavecchia A. Efficient presentation of soluble antigen by cultured human dendritic cells is maintained by granulocyte/macrophage colony-stimulating factor plus interleukin 4 and downregulated by tumor necrosis factor alpha. J Exp Med 1994;179:1109–18. https://doi.org/10.1084/jem.179.4.1109.
[21] Talayev VYu, Talaeva MV, Voronina EV, Babaykina ON. Migration of human dendritic cells in vitro induced by vaccines stimulating humoral and cellular immunity. Sovremennye tehnologii v medicine [Modern Technol Med] 2016;8(3):91–9. https://doi.org/10.17691/stm2016.8.3.10.
[22] Treanor JJ, Wilkinson BE, Masseoud F, Hu-Primmer J, Battaglia R, O'Brien D, et al. Safety and immunogenicity of a recombinant hemagglutinin vaccine for H5 influenza in humans. Vaccine 2001;19:1732–7. https://doi.org/10.1016/s0264-410x(00)00395-9.
[23] Nicholson KG, Colegate AE, Podda A, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. Lancet 2001;357:1937–43. https://doi.org/10.1016/S0140-6736(00)05066-2.
[24] Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. N Engl J Med 2006;354:1343–51. https://doi.org/10.1056/NEJMoa055778.
[25] Atmar RL, Keitel WA, Patel SM, Katz JM, She D, El Sahly H, et al. Safety and immunogenicity of nonadjuvanted and MF59-adjuvanted influenza A/H9N2 vaccine preparations. Clin Infect Dis: Off Publ Infect Dis Soc Am 2006;43:1135–42. https://doi.org/10.1086/508174.
[26] Hehme N, Engelmann H, Kunzel W, Neumeier E, Sanger R. Pandemic preparedness: lessons learnt from H2N2 and H9N2 candidate vaccines. Med Microbiol Immunol (Berl) 2002;191:203–8. https://doi.org/10.1007/s00430-002-0147-9.
[27] Bresson JL, Perronne C, Launay O, Gerdil C, Saville M, Wood J, et al. Safety andimmunogenicity of an inactivated split-virion influenza A/Vietnam/1194/2004(H5N1) vaccine: phase I randomised trial. Lancet 2006;367:1657–64. https://doi.org/10.1016/S0140-6736(06)68656-X.
[28] Bernstein DI, Edwards KM, Dekker CL, Belshe R, Talbot HK, Graham IL, et al. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. J Infect Dis 2008;197:667–75. https://doi.org/10.1086/527489.
[29] Baras B, Stittelaar KJ, Simon JH, Thoolen RJ, Mossman SP, Pistoor FH, et al. Cross-protection against lethal H5N1 challenge in ferrets with an adjuvanted pandemic influenza vaccine. PLoS ONE 2008;3:. https://doi.org/10.1371/journal.pone.0001401e1401.
[30] Mulligan MJ, Bernstein DI, Winokur P, Rupp R, Anderson E, Rouphael N, et al. Serological responses to an avian influenza A/H7N9 vaccine mixedat the point-of-use with MF59 adjuvant: a randomized clinical trial. JAMA 2014;312:1409–19. https://doi.org/10.1001/jama.2014.12854.
[31] Banzhoff A, Haertel S, Praus M. Passive surveillance of adverse events of anMF59-adjuvanted H1N1v vaccine during the pandemic mass vaccinations. Human Vaccines 2011;7:539–48. https://doi.org/10.4161/hv.7.5.14821.
[32] Podda A. The adjuvanted influenza vaccines with novel adjuvants: experience with the MF59-adjuvanted vaccine. Vaccine 2001;19:2673–80. https://doi.org/10.1016/s0264-410x(00)00499-0.
[33] Gore ER, Gower J, Kurali E, Sui J-L, Bynum J, Ennulat D, et al. Primary antibody response to keyhole limpet hemocyanin in rat as a model for immunotoxicity evaluation. Toxicology 2004;197:23–35. https://doi.org/10.1016/j.tox.2003.12.003.
[34] Plitnick LM, Herzyk DJ. The T-Dependent antibody response to keyhole limpet hemocyanin in rodents. In: Dietert RR, editor. Immunotoxicity testing: methods and protocols, methods in molecular biology, vol. 598, 2010; 159–72. (1) doi:10.1007/978-1-60761-401-2_11.
[35] Kawai R, Ito S, Aida T, Hattori H, Kimura T, Furukawa T, et al. Evaluation of primary and secondary responses to a T-cell-dependent antigen, keyhole limpet hemocyanin, in rats. Immunotoxicol 2013;10(1):40–8. https://doi.org/10.3109/1547691X.2012.691122.
[36] Dillon PM, Olson WC, Czarkowski A, Petroni GR, Smolkin M, Grosh WW, et al. A melanoma helper peptide vaccine increases Th1 cytokine production by leukocytes in peripheral blood and immunized lymph nodes. J Immun Ther Cancer 2014;2:23. https://doi.org/10.1186/2051-1426-2-23.
[37] García-Piñeres A, Hildesheim A, Dodd L, Kemp TJ, Williams M, Harro C, et al. Cytokine and chemokine profiles following vaccination with human papillomavirus type 16 L1 virus-like particles. Clin Vaccine Immunol 2007;14(8):984–9. https://doi.org/10.1128/CVI.00090-07.
[38] Skibinski DAG, Jones LA, Zhu YO, Xue LW, Au B, Lee B, Naim ANM, Lee A, Kaliaperumal N, Low JGH, Lee LS, Poidinger M, Saudan P, Bachmann M, Ooi EE, Hanson BJ, Novotny-Diermayr V, Matter A, Fairhurst A, Hibberd ML, Connolly John E. Induction of human T-cell and cytokine responses following vaccination with a novel influenza vaccine. Sci Rep 2018;8(1):18007. https://doi.org/10.1038/s41598-018-36703-7.
[39] Mosmann TR, Coffman RL. Two types of mouse helper T-cell clone: implicationsfor immune regulation. Immunol Today 1987;8:223–7. https://doi.org/10.1016/0167-5699(87)90171-X.
[40] Matsumoto R, Matsumoto M, Mita S, Hitoshi Y, Ando M, Araki S, Yamaguchi N, Tominaga A, Takatsu K. Interleukin-5 induces maturation but not class switching of surface IgA-positive B cells into IgA-secreting cells. Immunology 1989;66:32–8. PMCID:PMC1385116.
[41] McIntyre TM, Kehry MR, Snapper CM. Novel in vitro model for high-rate IgA class switching. J Immunol 1995;154:3156–61. PMID: 7897205.
[42] Sonoda E, Matsumoto R, Hitoshi Y, et al. Transforming growth factor b induces IgA production and acts additively with interleukin 5 for IgA production. J Exp Med 1989;170:1415–20. https://doi.org/10.1084/jem.170.4.1415.
[43] Mizoguchi C, Uehara S, Akira S, Takatsu K. IL-5 induces IgG1 isotype switch recombination in mouse CD38-activated sIgD-positive B lymphocytes. J Immunol 1999;162:2812–9. PMID: 10072528.
[44] Bertolini JN, Sanderson CJ, Benson EM. Human interleukin-5 induces staphylococcal A Cowan 1 strain-activated human B cells to secrete IgM. Eur J Immunol 1993;23:398–402. https://doi.org/10.1002/eji.1830230215.
[45] Ochoa MT, Teles R, Haas BE, Zaghi D, Li H, Sarno EN, Rea TH, Modlin RL, Lee DJ. A role for interleukin-5 in promoting increased immunoglobulin M at the site of disease in leprosy Immunology 2010; 131(3):405–14. Doi:10.1111/j.1365-2567.2010.03314.x
[46] Thomas PG, Keating R, Hulse-Post DJ, Doherty PC. Cell-mediated protection ininfluenza infection. Emerg Infect Dis 2006;12:48–54. https://doi.org/10.3201/eid1201.051237.
[47] Annunziato F, Romagnani S. Heterogeneity of human effector CD4+ T cells. Arthrit Res Therapy 2009;11(6):257–65. https://doi.org/10.1186/ar2843.
[48] Alcántara-Hernández M, Leylek R, Wagar L, Engleman EG, Keler T, Marinkovich MPr, Davis Mark M, Nolan GP, Idoyaga J. High-dimensional henotypic mapping of human dendritic cells reveals interindividual variation and tissue specialization. Immunity 2017;47(6):1037–50. https://doi.org/10.1016/j.immuni.2017.11.001.
[49] Pattarini L, Trichot C, Bogiatzi S, Grandclaudon M, Meller S, Keuylian Z, et al. TSLP-activated dendritic cells induce human T follicular helper cell differentiation through OX40-ligand. J. Exp. Med. 2017;214(5):1529–46. https://doi.org/10.1084/jem.20150402.
[50] Akiba H, Takeda K, Kojima Y, Usui Y, Harada N, Yamazaki T, et al. The role of ICOS in the CXCR5+ follicular B helper T cell maintenance in vivo. J Immunol 2005;175:2340–8. https://doi.org/10.4049/jimmunol.175.4.2340.
[51] Choi YS, Kageyama R, Eto D, Escobar TC, Johnston RJ, Monticelli L, et al. ICOS receptor instructs T follicular helper cell versus effector cell differentiation via induction of the transcriptional repressor Bcl6. Immunity 2011;34:932–46. https://doi.org/10.1016/j.immuni.2011.03.023.
[52] Breitfeld D, Ohl L, Kremmer E, Ellwart J, Sallusto F, Lipp M, et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000;192:1545–52. https://doi.org/10.1084/jem.192.11.1545.
[53] Schaerli P. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med 2000;192:1553–62. https://doi.org/10.1084/jem.192.11.1553.
[54] Ma CS, Deenick EK, Batten M, Tangye SG. The origins, function, and regulation of T follicular helper cells. J Exp Med 2012;209(7):1241–53. https://doi.org/10.1084/jem.20120994.
[55] Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Dräger R, Eibel H, Fischer B, Schäffer AA, Mages HW, Kroczek RA, Peter HH. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol 2003;4(3):261–8. https://doi.org/10.1038/ni902.
[56] Gao Xiaoling, Zhao Lei, Wang Shuhe, Yang Jie, Yang Xi. Enhanced Inducible Costimulator ligand (ICOS-L) expression on dendritic cells in interleukin-10

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053

deficiency and its impact on T-cell subsets in respiratory tract infection. Mol Med 2013;19(1):346–56. https://doi.org/10.2119/molmed.2013.00035.

[57] Akbari O, Stock P, Meyer EH, Freeman GJ, Sharpe AH, Umetsu DT, et al. ICOS/ICOSL interaction is required for $CD4^+$ invariant NKT cell function and homeostatic survival. J Immunol 2008;180(8):5448–56. https://doi.org/10.4049/jimmunol.180.8.5448.

[58] Montes-Casado M, Ojeda G, Aragoneses-Fenoll L, Lorpez D, de Andrers B, Gaspar ML, et al. ICOS deficiency hampers the homeostasis, development and function of NK cells. PLoS ONE 2019;14(7):. https://doi.org/10.1371/journal.pone.0219449e0219449.

Please cite this article as: V. Talayev, I. Zaichenko, M. Svetlova et al., Low-dose influenza vaccine Grippol Quadrivalent with adjuvant Polyoxidonium induces a T helper-2 mediated humoral immune response and increases NK cell activity, Vaccine, https://doi.org/10.1016/j.vaccine.2020.07.053