Chapter 5

The Impact of Adjuvanted and Non-Adjuvanted Influenza Vaccines on the Innate and Adaptive Immunity Effectors

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Additional information is available at the end of the chapter

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Abstract

To date, the advantage of adjuvanted over non-adjuvanted vaccines in the specific antibodies formation is proved. However, cellular mechanisms, including parameters of the innate immunity, involved in the vaccine-induced immune response are not well studied. The human study of inactivated vaccines showed that both subunit vaccine and split vaccine induced cellular immune response, but adjuvanted vaccine containing Polyoxidonium had the greatest potential. Despite the fact that influenza vaccines must activate endosomal receptors, they cause non-specific activation of the surface TLRs. They can trigger intracellular signals leading to the induction of antiviral mechanisms and to the activation of the body's protective resources against microbial infections. To assess the immunological efficacy of adjuvanted vaccines and humoral reactions to vaccination it is necessary to evaluate activation of cellular mechanisms of innate and adaptive immunity.

Keywords: influenza vaccines, adjuvanted influenza vaccine, lymphocyte subpopulations, toll-like receptors

1. Introduction

Vaccination is the most effective means of preventing influenza and consequently reducing incidence and severity of complications. Modern influenza vaccines include a live attenuated, inactivated (whole-virion, split-virion and subunit) vaccines. Currently, inactivated split and subunit vaccines are used for influenza prevention as the safest ones and stimulating the production of a protective level of strain-specific virus-neutralizing antibodies to the globular domain of hemagglutinin protein and neuraminidase protein of contemporary serotypes of the influenza virus. These vaccines protect against infection with the appropriate antigenic variants of influenza virus. Not all inactivated vaccines have been reported to be effective enough for certain categories of vaccinated people [1–7]. Some of them are not able to protect against drift variants of influenza virus [8–11].

Due to the continuous antigenic drift of influenza viruses and the emergence of pandemic influenza viruses, the study of influenza vaccines causing broader protective immunity is of great interest [12]. In this regard, before influenza pandemic of 2009–2010 vaccination with adjuvanted vaccines began, aiming to enhance the synthesis of specific antibodies. In addition, given poor population health in the modern era, there is the need to enhance the efficacy of vaccines meant to activate all the components of the immune system. According to the literature data, adjuvanted vaccines seem to have such effect. However, a small number of human studies to investigate, how adjuvanted vaccine influence cellular immunity and activate of not only adaptive, but also innate immunity, have been conducted. In addition, unlike foreign adjuvanted influenza vaccines developed in 2009–2010, the National Immunization Calendar of the Russian Federation for more than 20 years applies polymer-subunit influenza vaccine containing immunomodulator PO as the adjuvant. Furthermore, immunomodulators have long been used in vaccination practice for immunocompromised patients in the Russian Federation. Immunomodulator use to support the vaccination was shown to promptly enhance the synthesis of specific antibodies and significantly decrease the incidence of respiratory infections in the postvaccinal period [13–15].

To date, the vaccine immunogenicity is assessed according to the requirements of the European Committee for influenza vaccines [16], and must meet at least one of the three criteria:

- seroconversion (percentage of subjects with a fourfold increase in antibody titers after vaccination)—at least 40%;
- seroprotection (percentage of subjects with a protective antibody titers before and 21–28 days after vaccination)—at least 70% and
- multiplicity factor for the increase of antibody titers compared to baseline—at least 2.5.

Taking into account a new type of vaccine (adjuvanted), not only humoral, but also cellular immune response is important for the evaluation of immunological efficacy. The activation of cellular immunity parameters, important to the formation of immunological memory, may differ from that of non-adjuvanted vaccines.

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The aim of the study was to examine the effect of immunoadjuvant-containing and non-adjuvanted influenza vaccines on the immunophenotype of healthy donor lymphocytes and the number of cells with toll-like receptor expression *in vitro.*

2. Materials and methods

2.1. Clinical characteristics of patients

An open-label non-randomized monocenter study enrolled 27 healthy women of childbearing potential (aged 18–40 years) without co-morbidities who were not influenza-vaccinated within the previous 3 years and acquired no influenza or influenza-like illnesses within the previous 6 months.

2.2. Legal basis of the study

Once the signed informed consent for study participation was obtained, venous blood samples were drawn from volunteers with all applied aseptic and antiseptic techniques met and in accordance with the Study Protocol approved in 2015 by the Ethics Committee at the Mechnikov Research Institute of Vaccines and Sera. The study was conducted at the certified laboratory of the Mechnikov Research Institute of Vaccines and Sera (Moscow) using modern reagents and equipment.

2.3. Distribution pattern of lymphocyte subpopulations

The distribution pattern of peripheral blood lymphocyte subpopulations *in vitro* in healthy women exposed to influenza vaccine was tested by flow cytometer FC-500 (Beckman Coulter, USA), using anti-CD45/CD3, anti-CD45/CD3/CD4, anti-CD45/CD3/CD8, anti-CD16/56, anti-CD3/CD16/56, anti-CD45/CD20, anti-CD8/HLA-DR, anti-CD3/HLA-DR, anti-CD45/CD25, and anti-CD4/CD25/Foxp3 FITC- and PE-labeled monoclonal antibodies mAbs (Beckman Coulter, USA).

2.4. Toll-like receptors

The concentration of granulocytes with TLR expression was evaluated by flow cytometer FC-500 (Beckman Coulter, USA) using anti-TLR2, anti-TLR 3, anti-TLR4, anti-TLR6, anti-TLR8, and anti-TLR9 mAbs (eBioscience, USA).

Mononuclear WBCs were isolated from the whole blood using Ficoll-Urografin density gradients. We incubated 10^6 cells/mL in RPMI-1640 complete growth medium (PanEco, Russia) with 10% FBS (PanEco, Russia) and antibiotic (streptomycin) in the presence of 10 μL of a corresponding vaccine for 72 hours.

2.5. Study vaccines

Influvac ("Abbott biologicals" B.V., Netherlands) – inactivated subunit influenza vaccine, Vaxigrip ("Sanofi Pasteur", France)– inactivated split-virion influenza vaccine for influenza

prevention. These vaccines contain hemagglutinin of the influenza virus type A subtypes A/H1N1 и A/H3N2 (15 μg each) and hemagglutinin of the influenza virus type B (15 μg). Grippol plus (LLC "NPO Petrovax Pharm," Russia) – trivalent polymer subunit inactivated influenza vaccine. It contains hemagglutinin of the influenza virus type A subtypes A/H1N1 и A/H3N2 and hemagglutinin of the influenza virus type B (5 μg each), and immunoadjuvant Polyoxidonium (500 μg). All the vaccines contained current influenza virus strains for epidemiological seasons 2015–2016 and 2016–2017.

Anti-influenza virus A/H1N1/California/07/09, p.149, A/H3N2/Switzerland/9715293/13 (subunit antigen), B/Phuket/3073/13, p. 25 (season 2015–2016); A/H1N1/California/07/09 p.124 till 01.17, A/H3N2/Hong Kong/4801/14 p.200, and B/Brisbane/60/08 p. 27 (season 2016–2017) **baseline serum antibody levels** were studied in volunteers using the standard method (MU 3.3.2 1758–03) for HAI assay. The 4+ system was applied to HAI assay: an antigen titer, i.e., 1 HAU, was highest antigen dilution giving complete hemagglutination of RBCs (3+ or 4+). In HAI assay the antigen working dose was the antigen dilution containing 4 hemagglutination units (4 HAU) in 0.2 mL.

2.6. Statistical analysis

Cell percentage difference between test groups was measured by a robust dispersion analysis of repeated measures (R Statistical Software, WRS2 package, rmanova function) with subsequent pairwise comparisons (R Statistical Software, WRS2 package, rmmcp function), the obtained significance level was corrected by Holm method [17]. Benjamini-Hochberg method was used to account for multiple comparison (false discovery rate control) [18]. The obtained data were described with the median and interquartile range.

3. Study results

First, we estimated vaccine effect on distribution pattern of lymphocyte subpopulations in PBMC cultures. Volunteers were divided into three groups according to the baseline antibody (AB) titers against the hemagglutin of the influenza virus A/H1N1, A/H3N2, and B: low AB titers (20–40 U) in the first group, medium AB titers (80–160 U) in the second group, and high AB titers (≥320 U) in the third group. Such differences in AB level indicate that influenza infection in the unvaccinated volunteers could have been masked under the guise of another infection, as all volunteers did not report previous influenza infection.

Immunophenotypic analysis showed changes in the number of T lymphocytes (CD45+/CD3+), NK cells (CD16+/56+), NKT cells (CD3 + CD16/56+), B lymphocytes (CD45+/CD20+), and activated cells (**Table 1**).

There were statistically significant differences ($F = 8.00$, $p < 0.001$, $q = 0.001$) in T lymphocytes (CD3+) distribution after incubation with different types of vaccines (**Figure 1**). It should be noted that regardless of the AB level vaccines did not have a significant effect on T lymphocyte number except subunit vaccine, which caused a decrease in the percent of T lymphocytes compared to control (PBMC culture without vaccine) while the absolute number did not change. These results may indicate a shift in the number of cells due to an increase in the number of other subpopulations.

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Lymphocyte subpopulations	N	% in comparison groups – Me(Q1–Q3)				F	p	q
		Control	Subunit V	Adjuvanted V	Split-product V			
T lymphocytes (CD45/CD3+)	18	79.85 (74.17–83.35)	71.25 (64.7–79.75)	74.6 (66.38–79.17)	73.91 (66.92–78.22)	8.00	<0.001	0.001
Helper T cells (CD45/CD3/CD4+)	21	43.5 (41–49.8)	37.5 (32.7–43.8)	40.2 (31.8–46.5)	41.9 (35.6–47.7)	2.50	0.071	0.107
Cytotoxic T lymphocytes, CTL (CD45/CD3/CD8+)	21	23.5 (17.3–24.7)	21.2 (17.4–23.6)	22.5 (16.9–26.9)	21.5 (18.4–5.8)	0.64	0.533	0.601
Natural killer cells, NK cells (CD16/56+)	24	4.85 (4.175–5.9)	13.2 (11.15–14.85)	17.25 (15.93–18.25)	15 (13.8–16.25)	180.28	<0.001	<0.001
Natural killer T cells, NKT (CD3/CD16/56+)	24	1.6 (1.3–2.25)	3.6 (2.775–5.825)	7.5 (6.675–8.225)	5 (4.625–6.75)	57.52	<0.001	0.00001
B lymphocytes (CD45/CD20+)	24	5.15 (4.475–6.725)	16.36 (15.47–17.7)	21.15 (18.93–22.9)	18.1 (15.88–19.62)	167.44	<0.001	<0.001
Activated cytotoxic T lymphocytes, CTL(CD8/HLA-DR+)	20	0.4 (0.275–0.5)	0.7 (0.3–1.2)	1.6 (1.2–2.4)	1.35 (0.4875–1.975)	13.36	<0.001	<0.001
Activated T lymphocytes (CD3/HLA-DR+)	12	1.05 (0.65–1.65)	2.7 (1.875–3.375)	4.95 (3.775–7.1)	2.6 (1.9–3.575)	8.92	<0.001	0.002
Activated lymphocytes (CD45/CD25+)	16	1.45 (1–1.775)	3.7 (2.6–4.85)	4.15 (3.2–9.075)	4.15 (3.075–5.275)	12.94	<0.001	0.001
Regulatory T cells, Tregs (CD4/CD25/Foxp3+)	13	2.7 (1.7–2.9)	3.5 (3.2–4.9)	3.7 (3.2–5.5)	4.2 (2.2–4.5)	4.27	0.017	0.032
IRI (CD4/CD8)	20	1.825 (1.5–3.275)	1.85 (1.45–2.325)	1.85 (1.4–2.5)	1.65 (1.475–.525)	1.26	0.300	0.389

Note. Aliquots of 10 μL vaccines were added to cell suspensions (PBMC, 10^6 cells/mL). Cells were incubated for 72 hours at 37°C in 5% CO_2. The cells were then washed with RPMI-1640 at 1500 g for 10 min. Monoclonal antibodies against studied cell receptors were added in accordance with the manufacturer's instructions. The number of cells (%) in each sample was determined by flow cytometry.

Table 1. Distribution pattern of peripheral blood lymphocyte subpopulations incubated with influenza vaccines.

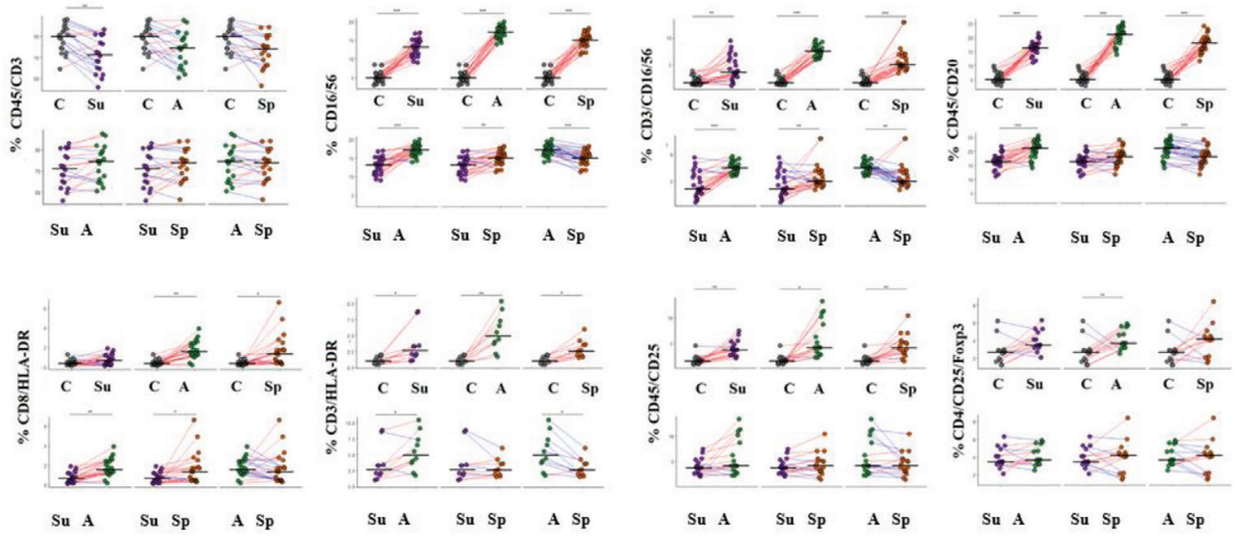

Figure 1. Lymphocyte count in PBMC culture after incubation with influenza vaccines. C = control; Su = inactivated subunit influenza vaccine; A = trivalent inactivated polymer-subunit influenza vaccine; Sp = inactivated split-product influenza vaccine.

The comparison of the T lymphocyte count between vaccines showed a significant decrease in the number of cells after incubation with subunit vaccine only (71.2% vs. 79.8% in control, p = 0.008) (**Figure 1**). However, the changes in the T lymphocyte (CD3+) number after incubation with different types of vaccines were observed only in women with medium AB level (F = 6.40, p = 0.004, q = 0.007). In this group, statistically significant differences were found for subunit vaccine (72 vs. 82.6% in control, p = 0.022) and split-product vaccine (74.8 vs. 82.6% in control, p = 0.022) (**Figure 2**).

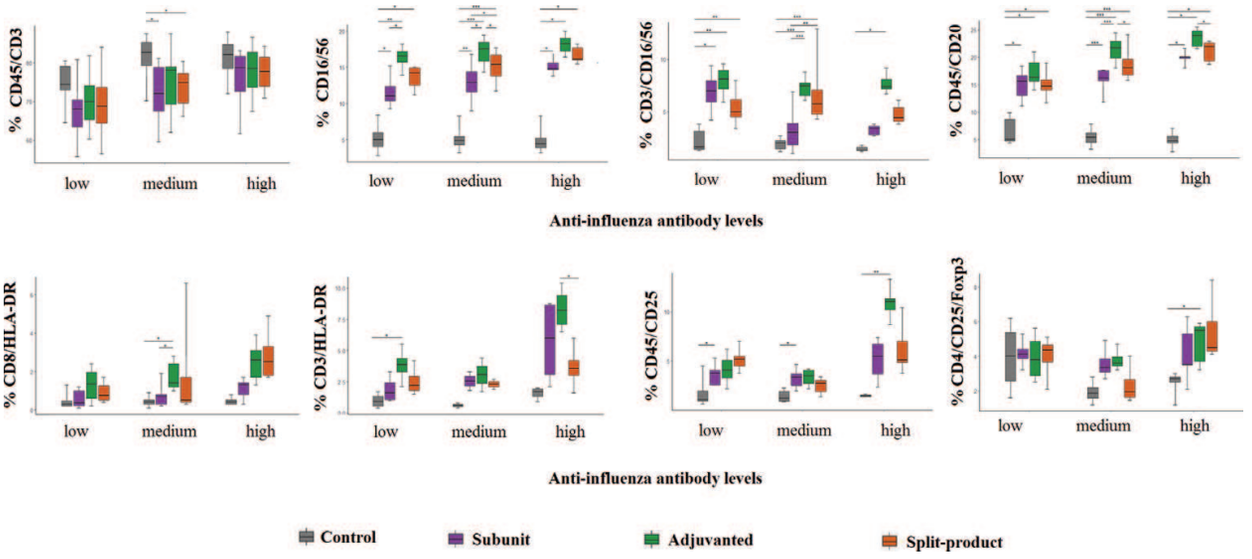

Figure 2. The impact of influenza vaccines on the lymphocyte count in PBMC cultures from volunteers with different antibody titers against the hemagglutinin of the influenza virus a/H1N1, a/H3N2, and B. Significant differences: *** p < 0.001, ** p < 0.01, * p < 0.05.

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Analysis revealed significant changes ($F = 180.28$, $p < 0.001$, $q < 0.001$) in percent of natural killer cells (NK, CD16/56+) after incubation with different types of vaccines (**Table 1**, **Figure 1**). Regardless of the AB level there was an increase in number of NK cells from 4.8 (control) to 13.2% (subunit vaccine), 17.2% (adjuvanted vaccine), and 15% (split vaccine). There were statistically significant differences for subunit (13.2 vs. 4.8%, $p < 0.001$), adjuvanted (17.2 vs. 4.8%, $p < 0.001$), and split vaccines (15 vs. 4.8%, $p < 0.001$) compared to control, for subunit vaccine compared to adjuvanted (13.2% vs. 17.2%, $p < 0.001$) and split vaccines (13.2 vs. 15%, $p = 0.003$), and for adjuvanted vaccine compared to split vaccine (17.2 vs. 15%, $p < 0.001$). That means that incubation with influenza vaccines increased the number of NK cells in all cultures.

However, the changes in number of NK cells (CD16/56+) after incubation of PBMC with different types of vaccines were observed in all groups of volunteers, regardless of the baseline anti-influenza AB level ($F = 48.88$, $p < 0.001$, $q < 0.001$ – low AB level, $F = 103.04$, $p < 0.001$, $q < 0.001$ – medium AB level, $F = 89.09$, $p < 0.001$, $q < 0.001$ – high AB level) (**Figure 2**).In women with low anti-influenza AB level, percent of NK cells (CD16/56+) was significantly higher after incubation with subunit (11 vs. 5%, $p = 0.045$), adjuvanted (16.5% vs. 5%, $p = 0.001$), and split vaccines (14.2 vs. 5%, $p = 0.01$) compared to control. Immunoadjuvant-containing vaccine had a higher potential for elevation of NK cell number (3.3-fold increase) compared with subunit vaccine (2.2-fold increase) ($p = 0.017$).

In women with medium anti-influenza AB level, percent of NK cells (CD16/56+) was significantly higher after incubation with subunit (12.8 vs. 4.8%, $p = 0.001$), adjuvanted (17.5 vs. 4.8%, $p < 0.001$), and split vaccines (15.3 vs. 4.8%, $p < 0.001$) compared to control. This corresponds to a 2.6- to 3.6-fold increase. Immunoadjuvant-containing vaccine produced more pronounced increase compared to subunit vaccine (17.5 vs. 12.8%, $p = 0.029$) and split vaccine (17.5 vs. 15.3%, $p = 0.011$), and number of NK cells was significantly higher after incubation with split vaccine compared to subunit vaccine (15.3 vs.12.8%, $p = 0.029$).

In women with high anti-influenza AB level, percent of NK cells (CD16/56+) was significantly higher after incubation with subunit (14.8 vs. 4.4%, $p = 0.023$), adjuvanted (18.2 vs. 4.4%, $p = 0.046$), and split vaccines (16.1 vs. 4.4%, $p = 0.035$) compared to control. This corresponds to a 3.3-, 4.1-, and 3.6-fold increase, respectively. There were no statistically significant differences between various types of vaccines.

For NKT cells (natural killer T cells, CD3 + CD16/56+), following findings were revealed. Regardless of the AB level there were statistically significant changes ($F = 57.52$, $p < 0.001$, $q < 0.001$) in NKT cells distribution after incubation with different types of vaccines: for subunit (3.6 vs. 1.6%, $p = 0.006$), adjuvanted (7.5 vs. 1.6%, $p < 0.001$), and split vaccines (5 vs. 1.6%, $p < 0.001$) compared to control, for subunit vaccine compared to adjuvanted (3.6 vs. 7.5%, $p < 0.001$) and split vaccines (3.6 vs. 5%, $p = 0.007$), and for adjuvanted vaccine compared to split vaccine (7.5 vs. 5%, $p = 0.006$). Therefore, subunit vaccine caused a 2.2-fold increase in NKT cell number, adjuvanted vaccine caused a 4.6-fold increase, and split vaccine caused a 3.1-fold increase compared to control (**Table 1**, **Figure 1**).

An increase of NKT cell (CD3 + CD16/56+) number in all cultures was dependent of baseline anti-influenza AB level ($F = 22.08$, $p < 0.001$, $q < 0.001$ – low AB level, $F = 20.02$, $p < 0.001$, $q < 0.001$ – medium AB level, $F = 65.92$, $p < 0.001$, $q < 0.001$ – high AB level) (**Figure 2**).

In women with low anti-influenza AB level, NKT cell (CD16/56+) number was significantly higher after *in vitro* incubation with subunit (7 vs. 1.6%, p = 0.033), adjuvanted (8.1 vs. 1.6%, p = 0.007), and split vaccines (5 vs. 1.6%, p = 0.005) compared to control. There were no statistically significant differences between various types of vaccines.

In women with medium anti-influenza AB level, NKT cell number in PBMC cultures was significantly higher after incubation with adjuvanted vaccine compared to control (7.4 vs. 1.3%, p < 0.001) and subunit vaccine (7.4 vs. 3%, p < 0.001) (5.7- and 2.48-fold increase, respectively) and after incubation with split vaccine compared to control (4.4 vs. 1.3%, p < 0.001) and subunit vaccine (4.4 vs. 3%, p = 0.009) (3.38- and 1.46-fold increase, respectively).

In women with high anti-influenza AB level, percent of NKT cells (CD3 + CD16/56+) was significantly (4.6-fold) higher after incubation with adjuvanted vaccine compared to control (7.4 vs. 1.6%, p = 0.043).

Analysis also revealed statistically significant differences (F = 167.44, p < 0.001, q < 0.001) in B lymphocytes (CD45/CD20+) distribution after incubation of PBMC with different types of vaccines (regardless of the AB level): for subunit (16.3 vs. 5.1%, 3.1-fold increase, p < 0.001), adjuvanted (21.1 vs. 5.1%, 4.1-fold increase, p < 0.001), and split vaccines (18.1 vs. 5.1%, 3.5-fold increase, p < 0.001) compared to control, and for adjuvanted vaccine compared to subunit (21.1 vs. 16.3%, 1.3-fold increase, p < 0.001) and split vaccines (21.1 vs. 18.1%, 1.1-fold increase, p < 0.001). Therefore, adjuvanted vaccine was the most effective (**Table 1**, **Figure 1**).

Regardless of the AB level there was a significant increase in B lymphocyte number after incubation with different types of vaccines (F = 24.09, p < 0.001, q < 0.001 – low AB level, F = 181.14, p < 0.001, q < 0.001 – medium AB level, F = 150.61, p < 0.001, q < 0.001 – high AB level) (**Figure 2**). In women with low anti-influenza AB level, percent of B lymphocytes (CD20+) was significantly higher after incubation with subunit (15.6 vs. 5%, p = 0.017), adjuvanted (16.3 vs. 5%, p = 0.046), and split vaccines (14.7 vs. 5%, p = 0.014) compared to control. There were no statistically significant differences between various types of vaccines.

In women with medium anti-influenza AB level, percent of B lymphocytes (CD20+) was also significantly higher after incubation with all types of vaccines: subunit (16.2 vs. 5.3%, p < 0.001), adjuvanted (21.6 vs. 5.3%, p < 0.001), and split vaccines (18.1 vs. 5.3%, p < 0.001) compared to control. Immunoadjuvant-containing vaccine had the greatest potential for elevation of B lymphocyte number (21.6%) compared with subunit vaccine (16.2%, 1.3-fold increase) (p < 0.001) and split vaccine (18.1%, 1.2-fold increase) (p = 0.013).

In women with high anti-influenza AB level, there was a significant increase in B lymphocyte number after incubation with subunit (20 vs. 4.7%, p = 0.021), adjuvanted (23.6 vs. 4.7%, p = 0.030), and split vaccines (21.9 vs. 4.7%, p = 0.030) compared to control. Immunoadjuvant-containing vaccine induced higher (fivefold) increase of B lymphocyte number than split vaccine (4.6-fold, p = 0.011).

Analysis revealed statistically significant differences (F = 13.36, p < 0.001, q < 0.001) in the distribution of activated cytotoxic T lymphocytes (CD8/HLA-DR+) after incubation of PBMC with different types of vaccines (regardless of the AB level) (**Table 1**, **Figure 1**).

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Immunoadjuvant-containing and split vaccines more effectively increased the number of this type of cells. There were statistically significant changes for subunit (1.6 vs. 0.4%, $p < 0.001$), adjuvanted (1.3 vs. 0.4%, $p = 0.050$), and split vaccines (1.6 vs. 0.7%, $p = 0.002$) compared to control, for adjuvanted vaccine compared to subunit vaccine (1.3 vs. 0.7%, respectively, $p = 0.046$), and for adjuvanted vaccine compared to split vaccine (4.9 vs. 2.6%, respectively, $p = 0.044$).

However, changes in the number of activated cytotoxic T lymphocytes *in vitro* between vaccine types were significant only in women with medium anti-influenza AB level ($F = 5.16$, $p = 0.020$, $q = 0.035$) (**Figure 2**). Incubation with adjuvanted vaccine caused significant increase of the number of activated cytotoxic T lymphocytes compared to control (1.4 vs. 0.4%, $p = 0.049$) and subunit vaccine (1.4 vs. 0.7%, $p = 0.047$).

Regardless of the AB level there were significant changes in the number of T lymphocytes with late activation marker (CD3/HLA-DR+) after incubation with different types of vaccines ($F = 8.92$, $p < 0.001$, $q = 0.002$) (**Table 1**, **Figure 1**). There were statistically significant changes for subunit (2.7 vs. 1%, $p < 0.044$), adjuvanted (4.9 vs. 1%, $p = 0.006$), and split vaccines (2.6 vs. 1%, $p = 0.010$) compared to control, and for adjuvanted vaccine compared to subunit (4.9 vs. 2.7%, $p = 0.015$) and split vaccines (4.9 vs. 2.6%, $p = 0.044$).

Statistically significant changes in the number of this type of cells were demonstrated only in women with low ($F = 30.17$, $p < 0.001$, $q < 0.001$) and high ($F = 12.49$, $p = 0.001$, $q = 0.003$) anti-influenza AB level (**Figure 2**). In women with low serum AB level, analysis of activated T lymphocytes showed significant activation by adjuvanted vaccine compared to control (3.8 vs. 0.9%, $p = 0.047$). In women with high serum AB level, the number of activated T lymphocytes was significantly higher after incubation with adjuvanted vaccine compared to split vaccine (8.2 vs. 3.5%, $p = 0.027$) (**Figure 2**).

For lymphocytes with early activation marker (CD45/CD25+), there was statistically significant increase ($F = 12.94$, $p < 0.001$, $q = 0.001$) after incubation of PBMC with different types of vaccines, regardless of the AB level (**Table 1**, **Figure 1**).All types of vaccines increased number of cells with early activation marker. Furthermore, there were statistically significant changes for subunit (3.7 vs. 1.4%, $p = 0.007$), adjuvanted (4.1 vs. 1.45%, $p = 0.049$), and split-product vaccines (4.1 vs. 1.4%, $p = 0.003$) compared to control. There were no statistically significant differences between various types of vaccines.

Regardless of the AB level there was a significant changes in the number of activated CD45/CD25+ lymphocytes. It was dependent of the vaccine type in all groups of volunteers ($F = 9.96$, $p = 0.002$, $q = 0.006$ – low AB level, $F = 7.92$, $p = 0.002$, $q = 0.005$ – medium AB level, $F = 25.89$, $p < 0.001$, $q < 0.001$ – high AB level) (**Figure 2**).

In women with low and medium AB level, percent of T lymphocytes with early activation marker (CD45/CD25+) was significantly increased after incubation of PBMC with subunit vaccine (3.8 and 3.3%, respectively) compared to control (1.1 and 1.2%, respectively) ($p = 0.024$ and $p = 0.036$). At the same time, in women with high AB level, the number of these cells was increased after incubation of PBMC with adjuvanted vaccine (11%) compared to control (1.5%) ($p = 0.009$).

Analysis also revealed significant changes (F = 4.27, p = 0.017, q = 0.032) in regulatory T cell (T-regs) number with CD4/CD25/Foxp3+ phenotype after incubation of PBMC with different types of vaccines, regardless of the AB level (**Table 1**, **Figure 1**). Immunoadjuvant-containing vaccine increased T-regs number compared to control (3.7 vs. 2.7%, 1.3-fold increase, p = 0.005). Other types of vaccines did not have a significant effect on these cells.

Significant changes in the number of T-regs between vaccine types were noted only in women with high AB level against influenza viruses A/H1N1, A/H3N2, and B (F = 8.15, p = 0.003, q = 0.006) (**Figure 2**).Incubation of PBMC with adjuvanted vaccine induced significant increase of T-regs count (CD25/CD4/Foxp3+) compared to control (5.5 vs. 2.7%, p = 0.049).

At the next step of the study we evaluated number of TLR-expressing granulocytes in PBMC cultures incubated with influenza vaccines.

All types of vaccines had immunostimulating effect on TLR-expressing cells by increasing the number of granulocytes expressing TLR 2,3,4,6,8, and 9, as shown in **Table 2**.

We found significant differences (F = 270.16, p < 0.001, q < 0.001) in the percent of granulocytes expressing TLR2 (**Table 2**, **Figure 3**) after incubation of PBMC with different types of vaccines, regardless of the AB level against the hemagglutinin of the influenza virus A/H1N1, A/H3N2 and B.. Subunit vaccine increased number of TLR2+ cells in PBMC culture from 16.6 (in control) to 38.2% (p < 0.001), adjuvanted vaccine—to 39.8% (p < 0.001), and split vaccine—to 37.5% (p < 0.001). However, there were no significant differences in TLR2 cell number between vaccine types.

Incubation of cell culture in the presence of influenza vaccines induced an increase in the number of TLR2+ granulocytes regardless of the baseline anti-influenza AB level (F = 53.25, p < 0.001, q < 0.001 – low AB level, F = 169.63, p < 0.001, q < 0.001 – medium AB level, F = 103.89, p < 0.001, q < 0.001 – high AB level) (**Figure 4**). In women with low AB level, the number

TLR	N	TLR-expressing granulocytes, %, Me (Q1-Q3)				F	p	q
		Control	Subunit	Adjuvanted	Split			
2	24	16.6 (14.2–18.38)	38.2 (36.45–40.05)	39.35 (37.73–42.4)	37.5 (35.38–39.27)	270.16	<0.001	<0.001
4	24	22.3 (19.75–25.4)	26.85 (25.23–29.43)	24.45 (22.15–26.9)	23.35 (21.5–25.35)	10.62	<0.001	<0.001
3	24	20.2 (18.23–22.95)	20 (18.02–24.05)	21.7 (19.5–23.05)	24.15 (21.95–25.95)	6.90	<0.001	<0.001
9	24	11.95 (9.825–12.85)	19.85 (17.95–25.2)	25.45 (24–26.32)	26.4 (24.48–28.23)	86.57	<0.001	<0.001
8	24	20.6 (18.68–22.4)	32.7 (30.12–35)	42.5 (37–45.1)	34.4 (29–37)	138.59	<0.001	<0.001
6	23	4.3 (4.05–5.15)	6.5 (5.95–7)	5.7 (5.2–6.9)	6.9 (5.95–7.55)	18.04	<0.001	<0.001

Table 2. Number of TLR-expressing granulocytes after incubation with influenza vaccines.

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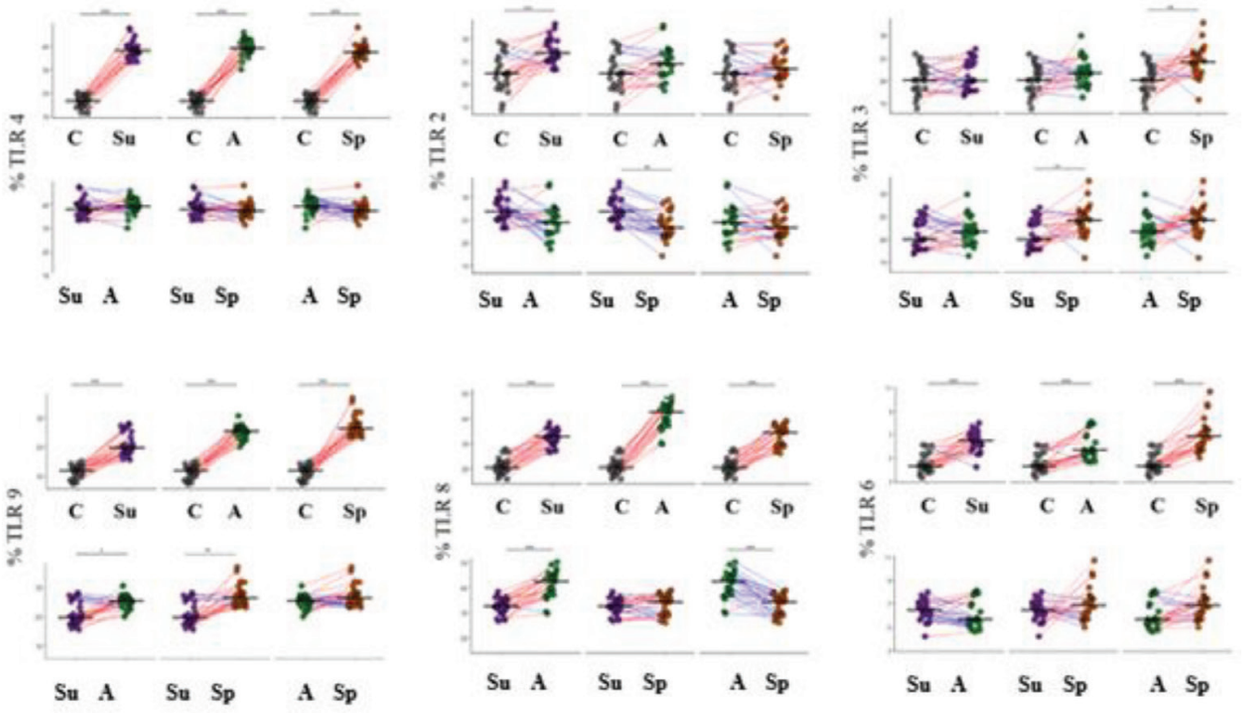

Figure 3. Number of TLR-expressing granulocytes in PBMC cultures incubated with influenza vaccines. C = control; Su = inactivated subunit influenza vaccine; A = trivalent inactivated polymer-subunit influenza vaccine; Sp = inactivated split-product influenza vaccine.

of TLR2-expressing granulocytes increased 2.4-fold after incubation with subunit vaccine ($p_h = 0.019$), 2.3-fold after incubation with adjuvanted vaccine ($p_h = 0.019$), and 2.2-fold after incubation with split vaccine ($p_h = 0.003$) compared with control.

In women with medium AB level, there was similar increase in the number of these cells: 2.3-fold for subunit and split vaccines ($p_h < 0.001$), and 2.4-fold for adjuvanted vaccine ($p_h = 0.001$) compared with control.

In women with high AB level, the number of TLR2-expressing granulocytes increased 2.6-fold after incubation with subunit vaccine ($p_h = 0.031$), 2.8-fold after incubation with adjuvanted vaccine ($p_h = 0.029$), and 2.7-fold after incubation with split vaccine ($p_h = 0.029$) compared with control.

Analysis revealed significant differences ($F = 10.62$, $p < 0.001$, $q < 0.001$) in the percent of granulocytes expressing TLR4 after incubation of PBMC with different types of vaccines, regardless of the AB level against the hemagglutinin of the influenza virus (**Table 2**, **Figure 3**). Subunit vaccine increased number of TLR4+ cells 1.2-fold compared to control ($p < 0.001$) and 1.1-fold compared to split vaccine ($p < 0.001$).

Statistically significant changes in the number of TLR4+ cells (**Figure 4**) between vaccine types were demonstrated only in women with medium AB level ($F = 5.24$, $p = 0.008$, $q = 0.010$): the number of these cells increased 1.1-fold after incubation with subunit vaccine compared to control ($p_h = 0.047$) and 1.2-fold compared to split vaccine ($p = 0.007$).

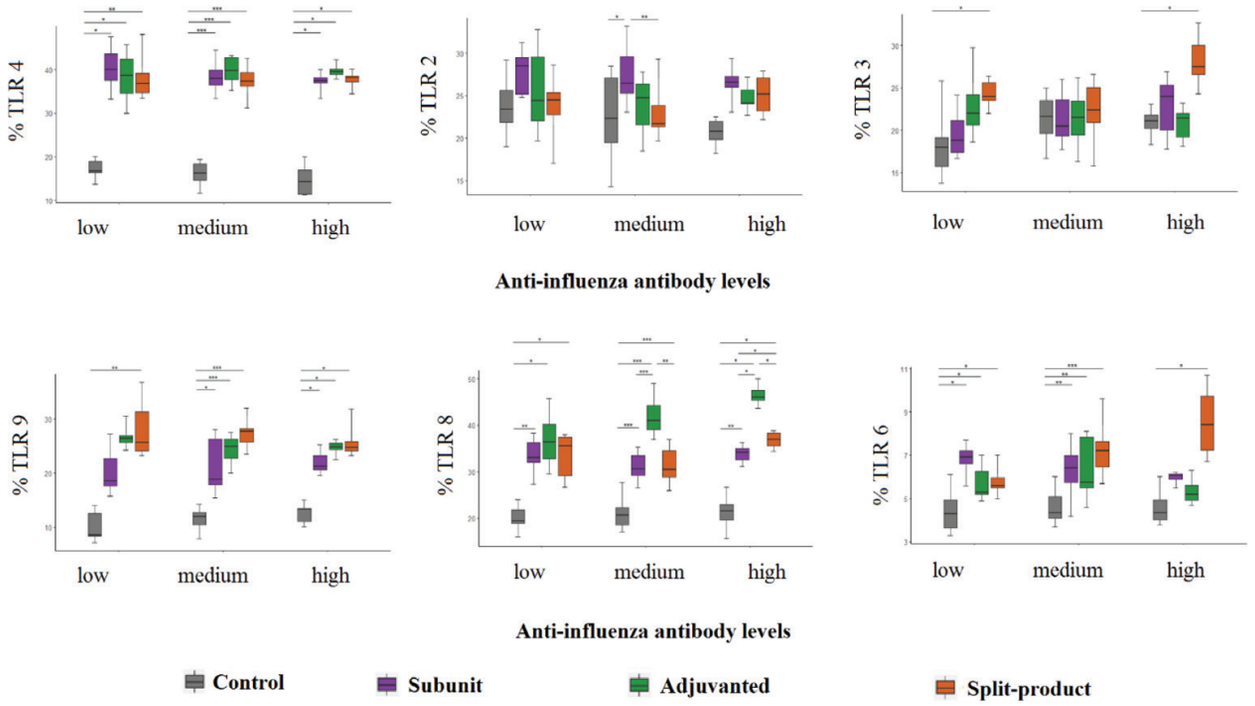

Figure 4. The impact of influenza vaccines on TLR-expressing granulocytes in PBMC cultures from volunteers with different AB titers against the hemagglutinin of the influenza virus A/H1N1, A/H3N2 and B.

Analysis of TLR3-expressing granulocytes (**Table 2**, **Figure 3**) revealed significant differences ($F = 6.90$, $p < 0.001$, $q < 0.001$) between groups, meaning that activation of the innate immunity effectors was dependent of the vaccine type, but not baseline AB level. There were significant differences for split vaccines compared to control (1.2-fold increase, $p = 0.001$) and subunit vaccine (1.2-fold increase, $p = 0.008$). That means that split vaccine had higher activity.

In women with low and high AB level, there were significant changes in the number of TLR3-expressing cells (**Figure 4**). The significance of differences was ($F = 6.05$, $p = 0.025$, $q = 0.030$) for low AB level and ($F = 6.45$, $p = 0.008$, $q = 0.010$) for high AB level. In women with low and high AB level, percent of TLR3-expressing granulocytes significantly increased after incubation with split vaccine (1.3-fold, $p_h = 0.042$ and $p_h = 0.050$, respectively) compared to control.

Analysis also revealed (**Table 2**, **Figure 3**) that different vaccines influenced ($F = 86.57$, $p < 0.001$, $q < 0.001$) the number of TLR9-positive cells regardless of the AB level. All types of vaccines increased the number of TLR9-expressing granulocytes in PBMC culture. Subunit vaccine caused 1.6-fold increase ($p < 0.001$), adjuvanted vaccine caused 2.1-fold increase ($p < 0.001$), and split vaccine caused 2.2-fold increase ($p < 0.001$) compared to control. Subunit vaccine was 1.2-fold less effective than adjuvanted vaccine ($p = 0.012$) and 1.3-fold less effective than split vaccine ($p = 0.003$).

Analysis showed that effect of different types of vaccines on TLR9-positive cells depended on the baseline AB level ($F = 26.93$, $p < 0.001$, $q < 0.001$ – low AB level; $F = 39.81$, $p < 0.001$, $q < 0.001$ – medium AB level; $F = 29.41$, $p < 0.001$, $q < 0.001$ – high AB level) (**Figure 4**). In women with low AB level, split vaccine induced threefold increase in the number of TLR9+ granulocytes

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compared to PBMC culture without stimulation ($p_h = 0.002$). However, in women with medium and high AB level, other types of vaccines stimulated TLR9+ granulocytes. There were following differences between vaccine types: subunit vaccine caused 1.6-fold increase ($p_h = 0.017$, $p_h = 0.050$), adjuvanted vaccine caused 2- and 1.8-fold increase ($p_h < 0.001$, $p_h = 0.040$), and split vaccine caused 2.3- and 1.8-fold increase ($p_h < 0.001$, $p_h = 0.050$) compared to control, respectively, in women with medium and high AB level.

Analysis of TLR8-expressing cells showed interesting results (**Table 2**, **Figure 3**). This receptor plays important role in recognition of viral single-stranded RNA. Analysis revealed a significant increase in the number of these cells in PBMC culture dependent on vaccine type ($F = 138.59$, $p < 0.001$, $q < 0.001$). All vaccines induced increase in the number of TLR8-positive granulocytes. This parameter increased 1.6-fold after incubation with subunit vaccine ($p < 0.001$), twofold after incubation with adjuvanted vaccine ($p < 0.001$), and 1.7-fold after incubation with split vaccine ($p < 0.001$) compared to control. Adjuvanted vaccine was 1.3-fold more effective than subunit vaccine ($p < 0.001$) and 1.2-fold more effective than split vaccine ($p < 0.001$).

Differences in the number of TLR8+ cells dependent on vaccine type were detected in all groups of volunteers, regardless of the baseline anti-influenza AB level ($F = 35.99$, $p < 0.001$, $q < 0.001$ – low AB level, $F = 76.10$, $p < 0.001$, $q < 0.001$ – medium AB level, $F = 116.13$, $p < 0.001$, $q < 0.001$ – high AB level) (**Figure 4**). In women with low, medium and high serum AB level, subunit vaccine induced 1.7-fold ($p_h < 0.001$), 1.5-fold ($p_h < 0.001$), and 1.6-fold ($p_h = 0.002$) increase, respectively, adjuvanted vaccine caused 1.9-fold ($p_h < 0.014$), twofold ($p_h < 0.001$), and 2.1-fold ($p_h = 0.014$) increase, respectively, and split vaccine caused 1.8-fold ($p_h < 0.029$), 1.5-fold ($p_h < 0.001$), and 1.7-fold ($p_h = 0.042$) increase of TLR8-expressing granulocyte number, respectively, compared to control. In women with medium and high serum AB level, immunoadjuvant-containing vaccine was, respectively, 1.3- and 1.2-fold more effective than split vaccine ($p_h = 0.002$ and $p_h = 0.042$), and 1.3-fold more effective than subunit vaccine ($p_h < 0.001$ и $p_h = 0.042$). In women with medium and high serum AB level, immunoadjuvant-containing vaccine was, respectively, 1.3- and 1.2-fold more effective than split vaccine ($p_h = 0.002$ and $p_h = 0.042$), and 1.3-fold more effective than subunit vaccine ($p_h < 0.001$ и $p_h = 0.042$).

Changes in the distribution of TLR6-expressing granulocytes were similar (**Table 2**, **Figure 3**). Analysis showed significant increase in the number of these cells in PBMC cultures dependent on vaccine type ($F = 18.04$, $p < 0.001$, $q < 0.001$). TLR6-expressing granulocyte number increased 1.5-fold after incubation with subunit vaccine, 1.3-fold after incubation with adjuvanted vaccine, and 1.6-fold after incubation with split vaccine compared to control ($p < 0.001$). However, there were no statistically significant differences between various types of vaccines.

Analysis also showed that effect of different types of vaccines on TLR6-positive cells depended on the baseline AB level ($F = 26.38$, $p < 0.001$, $q < 0.001$ – low AB level; $F = 11.71$, $p < 0.001$, $q < 0.001$ – medium AB level; $F = 16.57$, $p = 0.001$, $q = 0.001$ – high AB level) (**Figure 4**). In women with low and medium serum AB level, subunit vaccine induced 1.6-fold ($p_h = 0.043$) and 1.5-fold ($p_h = 0.004$) increase, respectively, adjuvanted vaccine caused 1.2-fold ($p_h = 0.032$) and 1.3-fold ($p_h = 0.004$) increase, respectively, and split vaccine caused 1.3-fold ($p_h = 0.027$) and 1.6-fold ($p_h < 0.001$) increase of TLR6-expressing granulocyte number, respectively, compared to control.

In women with high serum AB level, the number of TLR6-expressing granulocytes increased only after incubation with split vaccine ($p_h = 0.050$).

4. Discussion

Considering that inactivated influenza vaccines have a number of drawbacks (lack of efficacy in certain patients [1–7], no protection against drift influenza viruses [8–11]), there is a need for next generation vaccines to be developed. Besides, the effect many influenza vaccines have on the cellular and molecular immunologic mechanisms remains poorly studied.

The effects of inactivated influenza vaccines on key effectors of innate and acquired immunity are being investigated at the Mechnikov Research Institute of Vaccines and Sera (Moscow). Various types of influenza vaccines were selected for the study. First, their effect on distribution pattern of lymphocyte subpopulations was estimated *in vitro*.

Analysis of the vaccine effect on the immunophenotype of lymphocytes cultured for 72 hours, showed activation of the innate and acquired immunity effectors: NK cells (CD16/56), NKT cells (CD3/CD16/56), B lymphocytes (CD45/CD20), cells with early activation marker (CD45/CD25), T lymphocytes with late activation marker (CD3/HLA-DR), and regulatory T cells (Tregs, CD4/CD25/Foxp3). In view of this, below are characteristics of the cells that most actively responded to influenza vaccines added to PBMC culture.

Natural killer cells are essential to the innate immunity in influenza. Their function is to lyze tumor and virus-infected cells and to regulate innate and adaptive immune responses [19, 20]. Natural killer cells have been reported to identify influenza-infected cells through the NKp44 and NKp46 receptors that bind influenza hemagglutinin. Natural killer cells have also been reported to stimulate cellular immune response, regulate eosinophil maturation, and protect respiratory epithelium [21]. When interacting with peripheral mononuclear cells, PO, a component of the adjuvanted vaccine, significantly increases NK cells' cytotoxic effect on target cells. The phenomenon was observed almost in all donors examined, with the increased effect being especially pronounced in patients with the baseline activity of NK cells at the lower normal limit or decreased [22].

Being phenotypically heterogenous, NKT cells duplicate the functions of NK cells and link innate and acquired immunity [23].

Cytotoxic T lymphocytes identify and kill virus-infected cells. Infected cells present virus core antigens coupled to MHC class I molecules, which ensures their identification and subsequent killing by cytotoxic T lymphocytes [24, 25].

Specific cytotoxic lymphocytes cannot prevent cells from being initially infected with the virus, but they can restrict virus reproduction and enhance virus elimination out of the body. In unvaccinated adults, cytotoxic lymphocytes are crucial for clearing the body from influenza. They release perforin and stimulate apoptosis of virus-infected cells [26, 27].

Efficacy of influenza vaccines is currently assessed from their ability to activate the humoral immune response, as recommended in WHO guidelines. We think that this assessment

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does not adequately reflect the mechanisms of immune response to viruses. Therefore, it is essential to also study the cellular immunity. Immunodominance, which means that the immune system chooses one or more key epitopes for recognition, is an important factor for the development of vaccines stimulating the cellular immune response [28]. Vaccines aimed at producing cytotoxic T lymphocytes specific for an immunodominant epitope can significantly narrow the cross-reactive range of immune response to various virus strains. The role of antigen delivery route and presentation should also be considered when developing such vaccines. To stimulate a strong cytotoxic immune response, an antigen should be processed and presented by dendritic cells and coupled to MHC class I molecules. These may occur either at the moment dendritic cells are being infected or transduced or when dendritic cells engulf apoptotic bodies from other infected cells. Thus, the induction of cytotoxic immune response varies from strong one (with live attenuated vaccines) to a weaker, lower one (with inactivated whole-virion and subunit vaccines) [21].

B lymphocytes are among the key adaptive immunity effectors in influenza, since they produce anti-hemagglutinin (HA) (mainly against its globular domain) virus-neutralizing antibodies that prevent hemagglutinin from interacting with cellular receptors. Moreover, their Fc portion contributes to virion phagocytosis and to stimulation of antibody-dependent cellular cytotoxicity. HA amino acid sequence homology is about 80% between different strains within one subtype and 40–70% between strains of different subtypes. Besides, anti-neuraminidase antibodies have protective properties. They do not offer virus-neutralizing activity but they can inhibit neuraminidase enzymatic activity, which prevents the virus from spreading. Anti-neuraminidase antibodies also stimulate antibody-dependent cellular cytotoxicity. In addition, anti-neuraminidase antibodies have been shown to protect mice from H5N1 influenza virus [29].

Our study showed high stimulating effect of all studied influenza vaccines on B cell counts in PBMC culture. Adjuvanted vaccine was 1.3-fold more effective than subunit vaccine and 1.1-fold more effective than split vaccine. That means that adjuvanted vaccine activated B cell proliferation more effectively than the inactivated vaccines studied.

B cells were found to produce IgA, IgG, and IgM antibody isotypes in primary infection, while no production of IgM antibodies was observed in secondary infection. IgM antibodies are capable of activating the complement cascade as well as of neutralizing the virus [21, 29]. Secretory immunoglobulins A protect respiratory mucosae, through which influenza enters the body, and are indicative of recent virus exposure. Immunoglobulins G ensure the longest protection against influenza [21, 30].

Comparative analysis of the vaccines studied showed that adjuvanted vaccine is more effective in stimulating NK, NKT cells and Tregs, as well. The vaccine was 1.3- and 1.1-fold more effective than subunit and split vaccines in increasing NK cell count, 2.1- and 1.5-fold for NKT cell count, 1.3- and 1.16-fold for B lymphocyte count, and 1.5- and 1.2-fold for Treg count, respectively. The studied vaccines were not found to activate other cell types.

Natural thymus-derived regulatory cells (nTreg) of CD4 + CD25+ surface phenotype with constitutive expression of Foxp3 transcription factor responsible for their regulatory activity are one of the best documented cell population. Increased Treg number can possibly be explained by the immunoregulatory effect of PO (adjuvant)-containing vaccine. Immunoregulatory function of

nTreg is implemented both through cytokine secretion, such as TGF-β and IL-10, and through contact interaction with the effector T lymphocytes and antigen-presenting cells [31, 32].

Innate immune mechanisms are key to protection against pathogens, since they ensure prompt inflammatory reactions including detection of highly conservative structures, which are common to many microorganisms, through special receptors of broad specificity. These are signal PRRs, and TLRs are the most important of them [33–36].

Having recognized a specific pattern, PRRs initiate a series of signal cascades, which make the first line of defense against microorganisms. Besides, these signals initiate maturation of dendritic cells, which prepare the second line of immune response to the infection, known as acquired immunity. Thus, TLRs contribute to the regulation of innate and acquired immunity. Currently known are 11 types of TLRs in humans and 13 types in mice [37, 38]. Four of them (TLR3, TLR7, TLR8, and TLR9) recognize virus RNA and DNA. TLRs have an established role in physiological regulation of pro-inflammatory cytokine production, which are required for immune response to infections caused by bacteria, fungi, and viruses [39]. Inflammation is known to be directly associated primarily with neutrophils, which express almost all identified TLRs, as it has been shown recently. This explains the importance of TLRs in neutrophil activity regulation: LPS-induced TLR4 activation induces the production of pro-inflammatory cytokines and chemokines (IL-1β, IL8, and TNFα); TLR2, TLR4, and TLR9 stimulation is accompanied by respiratory burst and changed expression of adhesion molecules [40, 41].

The study of the effect influenza vaccine has on TLR-positive cell (granulocyte) expression gave the following results.

Patients with initially different anti-influenza AT titers *in vitro* showed statistically significant differences in TLR3, TLR8, and TLR9-expressing cell counts, depending on the type of influenza vaccine added to leukocyte culture.

All the influenza vaccines studied, caused a statistically significant ($p < 0.05$) increase in TLR2-, TLR6-, TLR8-, and TLR9-positive granulocyte counts in PBMC culture, compared to non-stimulated cells.

Subunit vaccine showed statistically significant ($p < 0.001$) stimulating effect on the expression of TLR4-positive granulocytes, compared to control group and split vaccine. TLR4 is known to be an important regulator of neutrophil survival [40–42].

Split vaccine provided better increase in TLR3- ($p = 0.008$) and TLR9- ($p = 0.001$) positive cell counts, compared to subunit vaccine. Both vaccines had similar effect on TLR8+ granulocyte proliferation. TLR3 is an important receptor in recognition of viral double-stranded RNA generated during replication [43]. TLR3 expression by CD4+ и CD8+ lymphocytes is known to be accompanied by their activation, which allows them to get directly involved in various types of immune response [44].

Dendritic cell activation has been reported to occur predominantly with TLR2, TLR3, TLR4, TLR7, and TLR9. TLRs are effective contributors to APC activation, not only because they induce pro-inflammatory cytokine production, but also because they enhance expression of various co-stimulating molecules required for effective antibody recognition [45, 46]. Moreover, TLRs control dendritic cell maturation and antigen-presenting function [47].

Influenza vaccines have been reported to activate innate effectors—the first line of defense to infection—dendritic cells, both myeloid and lymphoid lineages [48]. TLR3 plays an important part in cross-priming of naive CD8 T cells that differentiate to cytotoxic T cells [49, 50]. They are key to killing virus-infected cells. TLR3 expressed on dendritic cells is also essential for NK cell activation via INAM molecule [51].

Adjuvanted vaccine showed high induction potential with respect to TLR9- and TLR8-expressing cells, compared to subunit vaccine ($p = 0.012$ and $p < 0.001$, respectively) and split vaccine ($p = 0.003$ and $p < 0.001$, respectively). TLR8 has been found to recognize viral single-stranded RNA and to be a specific receptor responsible for influenza virus recognition [45, 52]. The increased TLR8-positive cell count in this study can be attributed to the co-stimulating effect of the adjuvant in the adjuvanted vaccine.

TLR9 along with TLR2 and TLR4 are involved in the regulation of B lymphocyte activation, proliferation, differentiation, and survival (this is considered an alternative pathway of B lymphocyte activation) [53]. TLR9 is also supposed to be a PRR key to influenza identification and binding, while recognition of influenza virions by TLR7/8 is significant for the induction of protective immune response to main antigens (hemagglutinin) [54].

Two different intracellular signaling systems are generally recognized at the moment. One of them involves TLR2, TLR4, TLR5, TLR7, TLR9 and intracellular molecules MyD88, IRAK, TRAF, NFkB. This intracellular signaling system usually activates an early pro-inflammatory response. The other intracellular system involves TLR3, TLR4, (might involve TLR7 and TLR8), adaptor protein TRIF and intracellular proteins TRAM, TBK1, and IRF3. This signaling system ensures the activation of anti-virus response. TLR3 is the key component of this signaling pathway, since it interacts with double-stranded viral RNA. TLR4 is equally effective in the activation of both intracellular signaling systems. Thus, there are two important types of innate immune responses. The first type activates antibacterial protection along with the tissue inflammation. The second type provides type I interferon-mediated antiviral response, with interferon being the primary antiviral mediator in innate immunity [55].

5. Conclusion

Thus, the studies have shown that influenza vaccines activate cellular immunity effectors as well as induce humoral immune response. PO-containing adjuvanted vaccine showed the strongest capability of inducing the cellular response, among the three vaccines studied.

Influenza vaccines *in vitro* induced an increase in the number of the innate and acquired immunity effectors: NK cells, NKT cells, B lymphocytes, cells with early activation marker, T lymphocytes with late activation marker, and regulatory T cells.

Despite the fact that influenza vaccines must activate endosomal receptors, they cause non-specific activation of the surface TLRs. This might be due to the influence exerted by antigen complexes contained in influenza vaccines of various types and due to the presence of an adjuvant in one of the vaccines studied. These vaccines activate TLR signaling cascade and, thus, can probably stimulate key effectors of the innate (DC, NK, and NKT cells) and adaptive

(CTL, B lymphocytes) immunity, which provide antiviral effect and induce body's own defense mechanisms against microbial infection.

6. Vaccination against influenza: the prospect of using adjuvants

The flu is widespread around the world and causes seasonal epidemics, which result in the death of hundreds of thousands of people each year [56]. Complications leading to morbidity and mortality following infection are predominantly observed in high-risk groups: children of early age, people with chronic diseases and pregnant women [57]. According to WHO, globally annual epidemics result in 3,000,000–5,000,000 cases of severe disease and approximately 250,000–500,000 deaths [58].

The vaccination is the most effective tools for preventing of influenza and, as a consequence, reducing the number and severity of post-infectious complications. Inactivated influenza vaccines received the most widely used, due to its high efficiency and low reactogenicity. But, at the same time, inactivated influenza vaccines, including seasonal trivalent vaccines, used for the annual prevention of influenza in the autumn-winter period, are not without some limitations. These vaccines are not enough (effective) immunogenic in vaccinating a number of population groups - small children, pregnant women, the elderly, people with various chronic diseases that are considered to be influenza risk groups. Also, inactivated influenza vaccines are not sufficiently protected against antigenically different strains (drift and heterologous) of the influenza virus that are not contained in the vaccine. In addition, the capacity of all manufacturers may not be sufficient to provide mass vaccine prevention around the world, especially in the event of a pandemic [59–61].

To increase the immunogenicity of inactivated influenza vaccines, adjuvants (immunoadjuvants) have been proposed. With the use of adjuvants, it is possible to increase the immunogenicity of influenza vaccines against a set of antigenically different strains. Adjuvants in the influenza vaccine can also provide efficacy in the immunization of various population groups, including at-risk groups. In addition, a significant increase in the immunogenicity of the vaccine due to the adjuvant will allow the transition to simple (single) immunization regimens, as well as reduce the dose of the antigen (hemagglutinin). This is especially important for pre-pandemic vaccines, because with the same production capacity, more vaccines will be obtained - and as a result, more people are immunized [59, 61].

The action of most adjuvants is based on the prolongation of the AG action, which is provided by the creation of a "depot" of the AG, which slows its absorption. Due to the sorption of AG on certain carriers, the antigen is held in places necessary for exposure of the antigen to antigen presenting cells and lymphocytes. Such an effect occurs when using aluminum alum, immunostimulating complexes, an oil microemulsion [62].

The effect of deposition is also achieved through the use of liposomes [63]. Adjuvants that primarily affect the phagocytic link of the immune system include polyelectrolytes, including PO. Structural association of the AG and polymer-immunostimulant enhances the migration of phagocytes, the functional activity of macrophages in tissues and increases their processing activity [64].

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The action of adjuvants depends on the initial immune status of the organism preceding the vaccination. Adjuvants accelerate development and increase the level of immune response, increase the duration of its retention. Long rise and a slow decrease in the intensity of post-vaccination immunity is characteristic of adjuvanted vaccines. At the same time, a reliable immune response is achieved with the help of small doses of AG and a small number of injections of the vaccine [63].

PO possesses expressed immune modulating effects acting first of all on the innate immunity factors such as monocytic-macrophagal system cells, neutrophils and NK-cells and inducing their activation under initially reduced functions. Flow cytochemistry data showed that PO does interact with three lymphocyte subclasses, predominantly binds with monocytes and neutrophils and to a lesser extent with lymphocytes, enhancing intracellular H2O2 production. Hydrogen peroxide being the secondary messenger activates the transcriptional NF-kB factor that is the participant of the cytokines synthesis regulation. The enhancement of the pro-inflammatory cytokines IL-1β, IL6, TNF-α synthesis takes place. Activation by PO cells of monocytic-macrophagal cluster and natural killers promotes mobilization of both cellular and humoral immunity. Finally, all immunity starts up for adequate response development similarly to that as it occurs in natural way [65].

Besides its own clinical application as independent drug, Polyoxidonium is used as immunoadjuvant in new generation vaccines and is a compound in subunit adjuvanted Grippol family vaccines since 1997 when first Grippol® vaccine was registered in Russian market. Due to Polyoxidonium, all Grippol family vaccines contain 3-times lower antigen content in one immunizing dose - 5 mcg per strain, in comparison to 15 mcg per strain in other subunit and split influenza vaccines. This provides Grippol family vaccines with higher safety profile. Today Grippol vaccines are approved and especially recommended for vaccination of cohorts that previously were considered to be not vaccinated (patients with allergic conditions, subjects with chromic somatic diseases, individuals with different immune deficiencies), and children from 6 months of age, and pregnant women. These recommendations were made based on relevant clinical trials results followed by many years practical mass vaccine application experience [66, 67].

Annual vaccination with the "yearly adapted vaccine" is an effective means of prevention and control of influenza in immunocompetent individuals, even in those with a known poor antibody response. In addition to the development of protective antibodies after vaccination, the induction of cell-mediated immunity is considered to be of critical importance [68]. Recent researches concerning the response to influenza vaccination in patients with CVID and unclassified antibody deficiency have shown that while the humoral immune response was strongly impaired, a T cell response against the vaccine was detected in most patients [69].

Seasonal vaccines primarily work through the induction of neutralizing antibodies against the principal surface antigen HA. This important role of HA-specific antibodies explains why previous pandemics have emerged when new HAs have appeared in circulating human viruses. It has long been recognized that influenza virus-specific CD4(+) T cells are important in protection from infection through direct effector mechanisms or by providing help to B cells and CD8(+) T cells. However, the seasonal influenza vaccine is poor at inducing CD4(+) T cell responses and needs to be combined with an adjuvant facilitating this response [70].

Protective immunity induced by SF-10 (synthetic human pulmonary surfactant with a carboxy vinyl polymer as a viscosity improver) against lethal influenza virus infection was partially and predominantly suppressed after depletion of CD8+ and CD4+ T cells (induced by intraperitoneal injection of the corresponding antibodies), respectively, suggesting that CD4+ T cells predominantly and CD8+ T cells partially contribute to the protective immunity in the advanced stage of influenza virus infection [71]. These results suggest that adjuvants can promote effective antigen delivery to antigen presenting cells, activates CD8+ T cells via cross-presentation, and induces cell-mediated immune responses against antigen.

Influenza infection elicits high-affinity IgA in the respiratory tract and virus-specific IgG, which correlates with protection. Long-lived influenza-specific T cells have also been shown to ameliorate disease [72]. Activation of the parameters of innate immunity is critical for the recognition of infection, as well as for the effectiveness of vaccination, which allows not only eliminating pathogens and cells with altered antigenic properties, but also having a significant effect on the formation of adaptive immunity [73].

Development of a universal influenza vaccine currently seems to be quite workable and promising task. Such universal vaccines are expected to contain both antibody production stimulants and inductors of cellular immune response with effectors of innate and adaptive immunity being involved. Adjuvants may play an important part, their functions being aimed both at enhancing immune response to an antigen and at regulating that response [74]. Thus, due to the emergence of a new type of vaccine (adjuvant), in assessing the immunological efficacy is important not only humoral but also cellular immune response.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

CD	cluster of differentiation
PO	polyoxidonium
TLR's	toll-like receptors
WBCs	leukocytes (white blood cells)
FBS	fetal bovine serum
HAI	hemagglutination inhibition
HAU	hemagglutination unit
RBCs	red blood cells

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V	Vaccine
PBMC	peripheral blood mononuclear cell
AB	Antibody
RNA	Ribonucleic acid
MHC	Major histocompatibility complex
HA	hemagglutinin
nTreg	natural thymus-derived regulatory cells
Foxp3	Forkhead box p3
TGF-β	transforming growth factor beta
IL	interleukin
PRRs	pathogen-recognize receptors
LPS	lipopolysaccarides
AG	antigen
DNA	deoxyribonucleic acid
CVID	common variable immunodeficiency
APC	antigen-presenting cell

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