Characteristics and immunostimulating properties of chemical and microbial vaccine adjuvants

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Abstract

Introduction. Currently, vaccination is the most cost-effective and efficient tool against infectious diseases helping reduce global morbidity and mortality. To increase the effectiveness of vaccines and ensure a stronger immune response in vaccinated people, various components – adjuvants - are added to them. This review focuses on the analysis of the immunological and characteristics of adjuvants of different nature.

Materials and methods. The modern scientific publications of leading periodicals on the topic of the use of adjuvants and their systems were studied: immunostimulating effect, efficiency, review of manufacturers, biotechnological production methods and the possibility of integration into the composition of tested vaccines.

Results and discussion. In contrast to the use of immunopotentiating agents of chemical origin, more and more scientific studies are appearing confirming the advisability of using adjuvants of microbial origin. These are vesicles of the outer membrane of bacteria *Escherichia coli*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Bacillus anthracis*, *Enterococcus faecium*; the structural protein flagellin of the flagella of gram-negative bacteria; monophosphoryl lipid A (MPL-A R595), adjuvant systems based on a combination of liposomes from *Salmonella typhi* in the form of nanoparticles using the model antigen ovalbumin, as well as polysaccharides isolated from the fungi *Aspergillus oryzae*, yeast *Saccharomyces cerevisiae*, *Candida albicans*, bacteria *Xanthomonas campestris* and *Bacillus natto*. The polysaccharide β-glucan in the form β-1,3/1,6 is characterized by its appropriate immunogenicity because of its epigenetic properties due to the PAMP structure (β-linked chains), which is recognized by pattern recognition receptors. The most important sources of β-glucan are yeasts *Saccharomyces cerevisiae* and the mushroom *Grifola frondosa*.

Conclusions. Immunomodulation through targeting Toll-like receptors is fundamental in the creation of antigen-agnostic vaccines with epigenetic adjuvants of microbial origin. The polysaccharide β-glucan is a candidate for the status of a new type of immune adjuvant for new vaccines against a large number of diseases due to a wide range of immune responses regardless of the administration form. Such "universal vaccines" could have a significant impact during a pandemic when conventional vaccines that provide an antigen-specific immune response are not available.

Abreviations

APC – Antigen presenting cells
ASC – Apoptosis-associated speck-like protein containing a CARD
BVA – Bovine serum albumin
BMV – Bacterial membrane vesicle
CD – Cluster of differentiation
CpG – DNA section
DAMPs – Danger-associated molecular patterns
DC – Dendritic cells
Dectin – Natural killer (NK)-cell-receptor-like C-type lectin
EV – Extracellular vesicles
GSC – Glioma stem cells
HSPCs – Hematopoietic stem and progenitor cells
IFA – Indirect fluorescent antibody
IFN – Interferon
Ig – Immunoglobulins
IL – Interleukin
LPS – Bacterial lipopolysaccharides
MHC – Major histocompatibility complex
NF-κB – An ancient protein transcription factor
NK – Natural killer
OVA – Ovalbumin
OMV – Outer membrane vesicles
PAMP – Pathogen-associated molecular patterns
PRRs – Pattern recognition receptors
Th –T-helper
TNF – Tumor necrosis factor
TNF-α – Tumor necrosis factor alpha
TLR – Toll like receptors

Introduction

Despite rapid technological progress and global development of medicine, infectious diseases continue to occupy first place (after cardiovascular diseases) among the leading causes of death (Farina et al., 2023). With the beginning of the development and use of effective means of immunoprophylaxis in the world, there has been a significant reduction in mortality rates caused by the most common pathogens (whooping cough, polio, measles, tetanus, and diphtheria) (Karch et al., 2016). These cause-and-effect relationships are confirmed by the analysis of the course of the recent global pandemic of coronavirus infection caused by the zoonotic infectious agent SARS-CoV-2, which was largely overcome thanks to global immunization.

The World Health Organization recently released the first global priority list of endemic pathogens and noted the urgent need to develop vaccines for them. In particular, it includes HIV, tuberculosis and malaria, which annually claim 2.5 million lives worldwide, as well as those well known to mankind - streptococcal infection (the causative agent is group A streptococcus), hepatitis C (the causative agent is an RNA-containing virus belonging to the *Flaviviridae* family) and severe diseases caused by the causative agent *Klebsiella pneumoniae*. According to available statistics, over the past three years alone, the number of

confirmed cases of tuberculosis in Ukraine has increased from 75 to 112 (per 100,000 people) in 2020 and 2023, respectively (Statistics of the World Health Organization, 2024). Therefore, the development of new, scientifically based approaches to the creation of new vaccines and increasing the immunogenicity of existing drugs is a pressing task today.

In modern vaccinology, an adjuvant is an integral component of vaccines, the functionality of which is to stimulate the development of an immune response to an antigen, more intensive formulation of specific antibodies, and activation of the effector functions of T cells (Shah et al., 2017). In general, an ideal adjuvant should have no unacceptable side effects, should be proven safe and stable, should be easy to manufacture, cost-effective, and compatible with a wide range of vaccine components. Potential toxicity has generally remained a major constraint on the development of traditional adjuvants (Mohan et al., 2023).

The problem of adjuvant selecting is due to the widespread use of vaccines created based on inactivated pathogens, purified and recombinant antigens, which have insufficient immunogenicity and require the addition of adjuvants (Utama et al., 2022). However, currently only a small number of immunostimulants are allowed to be used in medical practice, and the approval of new types of adjuvants and their sources is associated with significant restrictions and is long-term, which hinders the implementation of innovations (Facciolà et al., 2022).

The issue of considering and selecting new adjuvants with high efficiency and safety for vaccine production is important and relevant for two main reasons: (a) absolutely all existing vaccination programs in the world require improvement of technologies and development of a new generation of vaccines; (b) there are still no clear generalized data on the mechanisms of action of adjuvants and their systems.

The aim of the present review is to systematize the data of scientific literature sources on the issues of analysis of the composition, structural features, immunological mechanisms of action and stimulating effects caused by the immunoadjuvant properties of immunopotentiating agents and their systems.

Immunological characteristics of chemical adjuvants

The correct selection of adjuvant is critical for immunopotentiating agent-mediated induction of the immune response of different types of vaccines. After all, the mechanism of activation of the immune response, its cellular and humoral links, is more moderate for vaccines without adjuvant. At the same time, vaccines with an adjuvant in their composition contribute to the maturation of a larger number of antigen-presenting cells, polarizing cytokines, multifunctional T-cells, and antibodies.

The scheme of manifestation of immunogenic properties of vaccines with and without adjuvants in their composition is shown in Figure 1.

Even if the antigen and adjuvant components are well matched individually, their mixture may be suboptimal or incompatible, which affects the safety and efficacy of the developed anti-infective agents (Harandi, 2018).

Currently, the following adjuvants are common in immunobiotechnological production: TLR9 agonist CpG1018, TLR4 agonist AS01b and AS04 5, 6, 7 or squalene-based adjuvants such as AS03 (containing α-tocopherol) and MF59 (oil-in-water nanoemulsion containing squalene). The list of adjuvants approved for use in licensed vaccines also includes Tween 80 and Span 85 (both are surface-active substances). In general, the most commonly used immunopotentiating agents are oil-in-water emulsion-based adjuvants (Chegrynets et al., 2021) and aluminum salts (Galson et al., 2016).

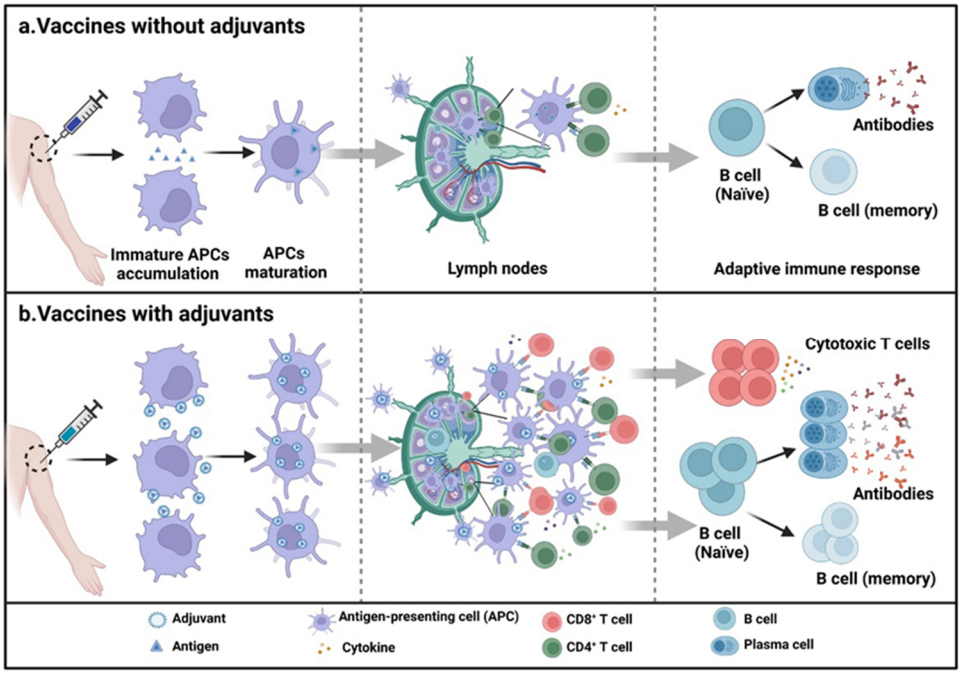

Figure 1. Generalized scheme of step-by-step manifestation of immunogenic properties of vaccines: a. vaccines without the use of adjuvants; b. vaccines with adjuvants in their composition (Adopted from Zhao et al., 2023)

Adjuvant based on an oil-water emulsion that has been widely used in animal experiments is Freund's adjuvant in two possible forms: incomplete (IFA) and complete (CFA). The latter also contains inactivated cells of *Mycobacterium tuberculosis* bacteria to enhance the immune effect by attracting macrophages and other immune cells to the injection site (usually used in primary immunization).

A typical CFA compositions comprises 1 mg *Mycobacterium tuberculosis*, heat killed and dried, with 0.85 ml paraffin and 0.15 ml Arlacel® 83 (a mixture of oleic, palmitic, stearic and linoleic acids from 2-(3,4-dihydroniglyceride)) (Malik et al., 2018). The active component derived from *Mycobacterium tuberculosis* is a dipeptide, N-acetylmuramyl-L-alanine-D-isoglutamine (MDP). MDP is a molecule that activates macrophages and dendritic cells via an oligomerization domain, which binds nucleotides containing 2 (NOD2) and skeletal elements of the bacterial cell wall. In addition to stimulating inflammation, this immunopotentiation agent causes aggregation and precipitation of soluble protein antigens to form particles that facilitate their efficient uptake by antigen-presenting cells (APCs). CFA also positively influences the Th1 cell subpopulation, the synthesis of IgG immunoglobulins (as opposed to the synthesis of IgM class), suppresses the induction of tolerance and promotes delayed hypersensitivity reactions. However, due to toxicity and adverse reactions, the use of this adjuvant is unacceptable for vaccines used for human immunization (unlike IFA, which exhibits less toxicity and is therefore suitable for clinical use) (Tadepalli et al., 2017).

Another squalene-based immunological adjuvant, MF59, has been associated with increased helper T-cell activity and higher immunoglobulin concentrations, particularly IgG1 and IgG2a isotypes. MF59 has also been reported to have the ability to induce a Th1 response in CD8+ T-cells in healthy people of different age groups (Yang et al., 2020).

In studies (Clark et al., 2009) it was shown that MF59 induces seroconversion (production of primary antibodies in the blood serum) and seroprotection (titer of protective antibodies) 21 days after the administration of the first vaccine against influenza A/H1N1. Moreover, the same vaccine produced higher rates of seroprotection and seroconversion after 21 and 42 days with adjuvant than without adjuvant. As for safety, mild to moderate reactions have been reported, including headache, muscle pain, and bruising at the injection site. However, none of these adverse reactions lasted more than 72 hours.

Among the existing invasive immunobiological products, squalene-based vaccines (AS03) are the leaders, with more than 20 million doses administered worldwide and biosafety results ranging from "acceptable" to "excellent" (Reed et al., 2009).

Several adjuvant formulas containing squalene and other compounds have already been tested: GLA-SE (an oil-in-water emulsion with squalene) and GLA (includes a glucopyranosyl lipid adjuvant) (Desbien et al., 2015). The GLA-SE formulation has been shown to induce strong signaling through the TLR-4, caspase, IL-18 and IFN-γ pathways, resulting in a Th1 response. It is currently used as an adjuvant in tuberculosis vaccines and is characterized by strong antibody responses with peak levels after the second immunization, and mild side effects including headaches and fatigue (Coler et al., 2018).

Studies in humans who received influenza vaccine with the squalene-based AS03 adjuvant showed long-lasting histone modifications and epigenetic changes in monocytes for up to 21 days after vaccine administration (Wimmers et al., 2021). It was found that epigenetically changed monocytes are more effective because they retain increased levels of chromatin of antiviral interferon regulatory factors (IRF) loci, including key antiviral immune regulators – RIG-1 (DDX58), IRF1, IRF8 and a number of genes stimulated by interferon of dengue fever and Zika virus. This is evidenced by a decrease in viral load after vaccination and a correlation of viral titers with the expression levels of interferon regulatory factors.

Now, given the lack of unanimous scientific thought, the first aluminum-based adjuvants available to mankind (aluminum hydroxide or aluminum alum) do not lose their popularity The three most commonly used and approved salts in 146 vaccines for preventive diseases are aluminum hydroxide, aluminum phosphate, and potassium aluminum sulfate (Kooijman et al., 2022). However, despite its widespread use to effectively potentiate the immune response, special care should be taken to ensure that the type of aluminum adjuvant does not affect the stability of the antigen composition (Agarwal et al., 2020).

The mechanisms of action of aluminum hydroxide and aluminum-based adjuvants in general include formation of aggregates (ensures continuous release of antigens), formation of partial structures (promotes phagocytosis of APCs) and induction of local inflammation (via the NLRP3 inflammasome), which causes the attraction and activation of macrophages, major histocompatibility complex (MHC II) molecules and antigen presentation. Inflammasome activation induces the secretion of mature IL-1 and IL-18 by dendritic cells, differentiation of Th2 cells, promoting B cell activation and subsequent antibody production (primarily IgG). However, studies have reported NLRP3-independent antibody production pathways and a non-phagocytic mode of action of aluminum hydroxide (Ho et al., 2018).

The effectiveness of aluminum salts is due to the creation of a "depot" of antigen in the place of administration, slowing down its release (it has the ability to be absorbed, which extends the time of contact of the vaccine with the immune system), a strong humoral response with weak induction of a cell-mediated immune response, and strengthening of phagocytosis. To prevent the development of an abscess (possible with subcutaneous injection), the introduction of vaccines with aluminum salts should be carried out exclusively intramuscularly. In general, this type of adjuvant allows the body to increase the titer of antibodies against pathologies of viral or bacterial origin (Powell et al., 2015).

However, it should also be noted that aluminum hydroxide is considered a genotoxic substance, which, when present in excess in the body, can lead to irreversible changes, impair memory, lead to mental disorders and Parkinson and Alzheimer's diseases. At the same time, there is a possibility of increased development of atherosclerosis and epilepsy (Pulendran et al., 2021).

Confirmation of possible negative consequences of using aluminum salts in vaccines was noted back in the early 2000s (Gherardi et al., 2001; Rivas et al., 2005). A number of studies have confirmed the formation of long-term deposits at the injection sites of vaccines containing aluminum salts, which can persist for decades after the vaccine, is administered (macrophagic myofasciitis). The depot effect was initially thought to be important for adjuvant activity, but this was later disproved by the demonstration of their adjuvant action even after depot withdrawal. At present, any harmful pathophysiological effect of long-term aluminum deposition in tissues and associated macrophage myofasciitis remain a matter of debate.

A new TLR7/8 agonist, a synthetic small molecule adjuvant 3M-052, has recently emerged, inducing potent antigen-specific immune responses by stimulating Th1 cells, antibodies and plasma cells (Arunachalam et al., 2020). Despite its effectiveness, the mechanism of the immunostimulating action of 3M-052 remains insufficiently studied. Current experimental data based on PLGA nanoparticle or gallium adjuvants primarily assess peripheral immune responses in the blood of non-human primates (NHPs), but have little coverage of responses occurring in the lymph nodes where immune responses are initiated. To date, there are no studies directly comparing the immune responses induced by 3M-052 vaccines with those elicited by live virus vaccines that provide long-lasting antibody production (Kasturi et al., 2020).

In support of the above, a molecular atlas of immunity to adjuvanted and live attenuated vaccines was studied in experimental mice using flow cytometry, single-stranded RNA (scRNA-seq) and single-cell sequencing ATAC (scATAC-seq) to create a comprehensive cellular, transcriptomic and epigenomic maps of the innate immune response in dLN at early and late stages after immunization with antigen with adjuvant YF-17D or 3M-052-Alum (Lee et al., 2020). Immunization with antigen plus 3M-052/Alum was found to induce activation and recruitment of myeloid cells into dLNs with a robust humoral response in mice at 14 and 28 days post-immunization, indicating high titers of IgG, IgG2c, and IgG. Immunization with 5 μg 3M-025-Alum significantly enhanced germinal center and T-follicular helper cell responses and increased antibody titers compared to alum alone as an adjuvant. Immunological response and possible effects of chemical adjuvants is given in Table 1.

Table 1

Immunological response and possible effects of chemically synthesized adjuvants

Immunological response	Possible effects	Reference
Chemically synthesized adjuvants		
Aluminum salts; Activation pathway: PRR		
Depot effect; increased presentation of antigen by antigen-presenting cells (APC) and production of IgG1, IgE; induction of danger-associated molecular patterns (DAMPs) production, activation of pattern-recognition receptors (PRRs) innate immune pathways, leading to production of IL-1β cytokines; activation of NLRP3 inflammasome, leading to production of IL-1, IL-18 with subsequent local inflammation and recruitment of APC.	Low protective efficacy; potential side effects; potential neurotoxicity; allergic reactions; may contribute to the development of ASIA (autoimmune/ inflammatory syndrome)	Moni et al., 2023
LNP (Lipid nanoparticles); Activation pathway: TLR 4		
Enhancement of humoral and cellular immunity to proteins and polysaccharide antigens; generation of Th 1, Th 2 or Th 1/Th 2 phenotypes, Th 1/Th 2 switch; modulation of generation of CD4+, CD8+ mediated immune responses is possible; transfer of PAMP-associated or specific immunomodulatory molecules, liposomes; can activate DU, macrophages and NK cells; greater influence of antigen on APC after vaccination; prolongation of the half-life of antigens in the blood.	Comprehensive immune protection; protection of antigen from degradation and increase of its stability in the body; potential side effects	Owen et al., 2021
Oil-in-water emulsion adjuvants		
MF59 (squalene-based oil-in-water emulsion); Activation pathway: MyD88, TLR 4,7,9		
Activation of myeloid cells (macrophages, DC), which respond by producing chemokines CC-chemokine ligand 2 (CCL2), CCL4, CCL5 and CXC-chemokine ligand 8 (CXCL8) also known as IL-8, which in turn recruit neutrophils, eosinophils, DC to the injection site; may work through NLRP3-independent activation pathways – ASC and TLR, MyD88; Th2-biased immune response and weak induction of Th1 response; induction of antibody response via MyD88-dependent mechanism; induction of transcriptional changes in muscle cells.	Dual function: antigen delivery and immune stimulation; side effects at the injection site; erythema and induration are possible	Zhao et al., 2023; Mancini et al., 2020

Immunological response	Possible effects	Reference
AS03 ("Adjuvant System 03" containing DL-α-tocopherol and squalene in an oil-in-water emulsion); Activation pathway: My-D88, TLR4,7,9		
Activation of NF-κB, inducing the secretion of cytokines and chemokines in muscles and lymph nodes, promoting the migration of innate immune cells; increased magnitude breadth of antibody and CD4+ T cell responses, resulting in increased protection through antibody production and higher levels of memory B cells; increased serum levels of IL-6, IP10 (CXCL10), transcriptional signatures of interferon signaling, antigen processing and presentation in DCs, monocytes and neutrophils.	Proven antioxidant and immunostimulating properties of α-tocopherol; strong immune response (after 6 months of vaccination); subjective intolerance	Facciolà et al., 2022; Mancini et al., 2020
CFA (Compete Freund's adjuvant with heat-killed cells of *Mycobacterium*); Activation pathway: TLR 2,4,9		
May lead to potentiation of IgG and IgA production; induction of predominantly Th2-biased response due to the "depot" at the injection site; stimulation of plasma cells producing antibodies; auxiliary activity of IFA is based on continuous release of antigen at the injection site; increase in antigen lifespan and strong local stimulation of innate immunity with phagocytosis, recruitment and infiltration of leukocytes; production of cytokines.	Long-term local inflammation; severe side effects; possible ulceration at the injection site; toxicity	Mohan et al., 2023

Thus, despite the fact that chemical adjuvants are now widely used in vaccine preparations, they have a number of significant drawbacks. In particular, aluminum salts are recognized as genotoxic substances with potential mutagenic and carcinogenic effects and the ability to provoke mental disorders, impair memory, cause Parkinson's and Alzheimer's diseases, and increase the likelihood of developing epilepsy and atherosclerosis. Finally, their weak induction of mucosal immunity also has a limiting effect on the development of vaccination efficacy (Eldred et al., 2006; Exley et al., 2014). CFA (Freund's adjuvant), despite the proven stimulation of a powerful immune response, is characterized by toxicity, leading to the formation of granulomas, inflammation at the injection site, due to the high content of waste oils that are not subject to biodegradation (Burny et al., 2017). Emulsion adjuvants are considered toxic for conventional human prophylactic vaccines and have an incomplete immune response after immunization. Injections of MF59-adjuvanted vaccine stimulate extracellular ATP release, the inhibition of which by local injections of apyrase may not enhance, but, on the contrary, weaken the immune response (Kim et al., 2020). Thus, the search for adjuvants of biological origin is a promising alternative direction for the selection of immunopotentiating agents.

Immunological characteristics of microbial adjuvants

Adjuvants of microbial origin are currently being proposed for use in vaccinology (Abbasi et al., 2022; Chang et al., 2021; Durães et al., 2021; Verma et al., 2023). For example, the adjuvant effect of spherical nanometric outer membrane vesicles (OMV) of Gram-negative bacteria *Escherichia coli* on antigen-specific priming of T cells was studied by Lee with co-authors (2021). Parmaksız et al. (2023) proposed an adjuvant system based on a combination of *Salmonella typhi* nanoparticles porins in the form of nanoparticles with liposomes (cationic and neutral) using the model antigen, ovalbumin. Taleghani and colleagues (2019) in one of the latest studies considered the possibility of using monophosphoryl lipid A (MPL-A), isolated mainly from the genetically engineered *Salmonella minnesota* strain R595, as the historically first TLR ligand approved for use as an adjuvant in the creation of human vaccines.

Flagellin, a structural protein of the flagella of gram-negative bacteria, is considered to be an efficient adjuvant. When entering the body, most bacterial flagellin molecules remain in the flagella, but some are released into the environment, allowing them to be recognized by the immune system through Toll-like receptors on the surface of immune cells (Hajam et al., 2017).

Confirmation of the presence of epigenetic characteristics of flagellin is given in the work (Murthy et al., 2014), according to the results of which, the hyperconserved N and C ends of flagellin can be recognized by molecules of the Toll-like receptor of immunocompetent cells, in particular TLR-5 (expressed on macrophages, lymphocytes, and dendritic cells). This recognition of TLR-5 by flagellin results in signaling through MyD88-dependent and MyD88-independent pathways, resulting in the induction of transcription factors AP-1, NF-κB, and IRF3, which induce the production of cytokines and chemokines. These proteins, in turn, recruit (direct) dendritic cells, as well as T and B-lymphocytes to the lymph nodes. In addition, flagellin can promote a strong antigen-specific CD4+ T cell response by interacting with TLR-5 on CD11c+ cells. The result of this interaction is a high titer of antibody formation, demonstrating the potential of flagellin as a protein adjuvant (Bates et al., 2019).

As an adjuvant, flagellin is used by co-administration with the main antigen and through fusion proteins obtained by adding epitopes associated with the flagellin molecule. It has been proven that physical binding of vaccine antigens to flagellin creates a significantly more powerful vaccine than simple mixing with the antigen. It has already been determined into which specific areas of the flagellin molecule epitopes should be inserted in order to maximize antibody titers, but conclusions are not yet available (Huleatt et al., 2018).

For example, optimal antibody titers were obtained by introducing a hemagglutinin (HA) epitope into the hypervariable region of flagellin (Song et al., 2009). To achieve the set goals, an approach was developed to bind agonists of the Toll-like receptor (TLR-5) flagellin to the self-sufficient protective subunit (globular head) of HA. The globular head domain includes most of the neutralizing epitopes of HA in the context of the flagellin fusion protein and stably refolds to precisely form these conformationally sensitive epitopes. It was found that the R3 vaccine (configuration – both ends of the globular head of HA are linked to flagellin) of several H5 subtype vaccines (based on the influenza virus A/Vietnam/1203/2004 (VN04)), in which the D3 domain of the flagellar is replaced by the HA head, is the most effective in eliciting protective HAI titers and protecting mice from disease and death.

However, in a study (Delaney et al., 2010), insertion of the L1R epitope into the hypervariable region not only does not cause the production of antibodies, but also, if used

specifically at the N-terminus of flagellin, can lead to the formation of antibodies capable of interacting with native L1R.

Lin et al. (2016) showed that insertion of epitopes into the C-terminus of flagellin can induce signaling through NLRC4 and NAIP5, leading to CD8+ T cell responses against tumor cells. The demonstrated diversity of immune system responses experimentally confirms the versatility of flagellin as an adjuvant with different target orientations depending on the area of antigen administration. However, further fundamental and applied scientific research is required to reveal the full potential of flagellin.

It should be noted that from a practical point of view, flagellin as an immunopotentiating agent has a number of safety advantages: only low doses are required to achieve effectiveness; no toxicity when administered intranasally in animal experiments. Thus, results have been obtained confirming the safety of using fused flagellin proteins as an adjuvant in vaccines against influenza A H1N1, since all existing systemic side effects in some research groups of people stabilize after 4 days of rest, and in others after taking non-steroidal anti-inflammatory drugs (Treanor et al., 2010).

Among the adjuvants of microbial origin, outer membrane vesicles (OMV) of gram-negative bacteria, in particular *E. coli*, stand out due to their powerful inflammatory potential and ability to stimulate the innate immune response. The immune response induced by spherical nanometric vesicles (proteoliposomes) is provided by lipopolysaccharides (LPS), peptide glycans, phospholipids, proteins, DNA, RNA, as well as virulence factors such as exotoxins.

The rationale for using bacterial membrane vesicles (BMVs) in vaccines is due to the presence of pathogen-associated molecular patterns (PAMPs) to induce strong immune responses, as well as the ability to present proteins of various sources ranging in size from 50 to 200 nm for efficient processing and delivery of antigens (they can be easily captured by immune cells). There are already a number of experimental BMV-based vaccines against a wide range of pathogens (Krishnan et al., 2022). Thus, BMVs can accumulate and transport nutrients, virulence factors and toxins, while neutralizing antibacterial molecules produced by the host organism. BMVs express bacterial surface antigens, which can be an important vaccine target (Mehanny et al., 2022).

Overall, bacterial vesicles have attracted increased attention as a powerful and versatile vaccine platform, as they are essentially immunostimulants capable of activating both innate and adaptive immune responses (CD4+ T-cell and B-cell responses) simultaneously. This mechanism is due to the recognition of LPS and other molecules on membranes by TLR molecules and the complement system, which leads to the recruitment of APC cells. Activated macrophages produce proinflammatory cytokines such as TNF-α and IL-6 and induce adaptive immune responses by enhancing the expression of the major histocompatibility complex class II (MHC-II) molecule HLADR, the costimulatory molecules CD80 and CD86, and intercellular adhesion molecules. Co-stimulation with molecules present in OMVs results in a more powerful response than that elicited by LPS alone. In this case, OMVs have the ability not only to induce the maturation of dendritic cells, which leads to enhanced capture of the antigen molecule and its presentation for the development of an adaptive immune response, but also to activate B cells to produce IgA, IgM, IgG and other antibodies (Zingl et al., 2020).

The studies (Buschmann et al., 2021; Elsharkasy et al., 2020; Vader et al., 2016) confirm the ability of BMV to induce long-term humoral and cellular immune responses when used in vaccines. These properties may be explained by the high density of pathogen-associated molecular patterns (PAMPs) on bacterial vesicles, which serve as potent immune stimulants.

Because BMVs carry a large number of antigens that can induce an immune response in vivo, a variety of BMVs including OMVs (outer membrane vesicles) and EVs (extracellular vesicles) are produced using wild-type or bioengineered bacteria (Treanor et al., 2010). Thus, a cloning system based on *Escherichia coli* and OMV derivatives fused with the outer membrane lipoprotein I (OprI) of *Pseudomonas aeruginosa* is currently in widespread use. This system has shown immunogenicity by stimulating dendritic cells/macrophages, demonstrating potential adjuvant properties.

Later, Gan with co-authors (2021) proposed the use of bacterial membrane vesicles OMV produced by gram-negative bacteria *Escherichia coli* and EV obtained from gram-positive bacteria (*Enterococcus faecium, Streptococcus pneumoniae, Staphylococcus aureus,* and *Bacillus anthracis*) as delivery systems for antigens in the fight against infections caused by bacterial pathogens. However, this use of OMVs and EVs as an adjuvant obtained using a bacterial producer also has its drawbacks. The problem is caused by the difficulty of maintaining the bacterial membrane due to the relative fragility of the structure, which can affect the uptake by antigen-presenting cells. Therefore, to solve this problem, it was proposed to coat MVs on nanoparticles to increase in vivo stability and circulation time (Gan et al., 2021).

In study of Afrough et al. (2020), the authors developed a vaccine candidate based on the fusion of PorA from serogroups A and B (*Neisseria meningitidis*) in combination with OMV, testing it in laboratory animals and comparing the efficacy of OMV with commonly used Freund's adjuvant. The elicited immune response in mice was assessed by enzyme-linked immunosorbent assay (ELISA) and serum bactericidal activity (SBA) assay. A significantly higher titer of bactericidal antibodies was observed in mice groups in response to injections with PorA+OMV compared to the groups receiving PorA+Freund's adjuvant. In this case, the recombinant PorA protein with OMV adjuvant additionally induces antibacterial antibody responses against capsular polysaccharides of serogroups A and B.

There is currently insufficient data on the specific effects of BMV and OMV adjuvants present in a number of vaccines against bacterial pathogens on the human immune system. The only licensed and widely used multicomponent meningococcal vaccine against *Neisseria meningitidis* is 4CMenB ("Bexsero") with OMV adjuvant in its composition (Masforrol et al., 2017).

Studies in recent years have shown the protective ability of the meningococcal vaccine containing OMVs against *Neisseria gonorrhoeae*, causative agent of gonorrhea, species close related to *Neisseria meningitidis*, a Gram-negative bacterium that can cause meningitis (Semchenko et al., 2019). Using enzyme immunoassay, it was shown that, as response to Bexsero (with OMV adjuvant) vaccination, human anti-gonococcal NHBA antibodies were generated providing additional cross-protection against gonorrhoea (Semchenko et al., 2019).

The potential impact of the outer membrane vesicle (OMV) meningococcal B vaccine, MeNZB, on specific immunogenicity and cross-protection against gonorrhoea were confirmed in study of Petousis-Harris et al. (2019).

Newly developed vaccines with OMV adjuvant in their composition have already begun to move to the clinical trial stage due to their preliminary demonstrated safety and efficacy. Thus, vaccines against allergens and shigellosis (the causative agent is *Shigella flexneri*) are in phase I clinical trials, and vaccines against influenza are in phase II. When administered intranasally, these immunobiological preparations induced the production of antibodies with very minor side effects, which emphasizes the potential of OMVs as immunopotentiating agents (Daleke-Schermerhorn et al., 2014; Martiñón et al., 2019).

However, the use of OMVs as adjuvants poses several additional challenges regarding their mass production, as OMV production is a complex procedure due to the need to control

the endotoxin content, which may be responsible for stimulating excessive inflammation. In particular, the lipopolysaccharide (LPS) dose needs to be carefully controlled, as OMVs with low LPS are less effective adjuvants, while excessive amounts of LPS may cause toxic effects (Martiñón et al., 2019).

Bacterial LPS and TLR4 ligands in vaccines are able to induce an immune response in the form of persistent epigenetic modification in bone marrow and myeloid progenitor cells with an increase in myeloid enhancers. This result is explained by the recognition of the TLR4 receptor by lipopolysaccharides and its transmission of immunological signals through the MyD88- and TRIF-dependent pathway (de Laval et al., 2020).

LPS-mediated resistance to infections is induced by increased leukocyte recruitment and attenuation of inflammatory processes. The manifestation of a stable immune response to bacterial antigens in the test mice after their vaccination using LPS is also shown (Rusek et al., 2018). It was found that transplanted hematopoietic stem and progenitor cells (HSPCs) from the bone marrow of LPS-exposed mice exhibit the ability to protect recipient mice from infectious diseases caused by *Pseudomonas aeruginosa*, demonstrating a reduced bacterial load in the spleen and liver (de Laval et al., 2020). However, despite existing studies on the immunostimulatory effects of LPS as an adjuvant, its use in clinical practice remains limited due to potential toxicity in humans (Hernandez et al., 2019).

Toll-like receptors as new generation adjuvants

According to previously established scientific ideas, immunological memory is a limited antigen-specific memory and is exclusively a privilege of the adaptive immune system, due to the work of lymphocytes. However, the description of specific memory in invertebrates, the system of acquired resistance in plants, antigen-specific memory in natural killer (NK) cells, as well as heterologous protection (provided by vaccines against smallpox, measles, and BCG) are evidence of the existence of an innate form of immune memory (Sánchez-Ramón et al., 2018).

Now, to reflect the possibility of the innate link of immunity to "remember"/adapter the first contact with a pathogen in order to create a robust immune response upon repeated exposure to a similar or dissimilar microbial stimulus, the term “trained immunity” has been proposed. It is noted that the molecular mechanisms that can form the basis of trained immunity include epigenetic, mechanical and functional reprogramming of progenitor cells of the myeloid line of hematopoiesis and the cell line of the innate link of immunity (Palgen et al., 2021).

According to evidence (Pulendran et al., 2021), the main functional task of adjuvants and their systems was previously attributed to enhancing the immunogenicity of vaccines by specifically targeting cells of the innate immune system. However, currently a number of other scientific works (Biering-Sørensen et al., 2017; Bruxvoort et al., 2022; Walk et al., 2019) show results indicating the actual possibility of pre-training the innate immune system, which can provide heterologous protection against secondary infection.

In addition, the ability of some immunopotentiating agents to induce persistent epigenetic reprogramming of the innate immune system in order to create improved resistance against pathogens is also emphasized. Such apotheosis testifies to the promising development of epigenetic adjuvants for the creation of new generation vaccine preparations.

Thus, it was confirmed the possibility of using transmembrane receptors of innate immunity (TLR) to provide longer-term protection against the next infectious agents due to the strengthening of innate immunity (Mifsud et al., 2014). At the same time, the FDA (Food and Drug Administration) in the USA has already approved several agonists of Toll-like

receptors for use as part of immunobiological drugs and recognized them as safe on a global scale.

Confirmation of heterologous protection (antigen-specific way) against a number of unrelated infectious agents is the results of epidemiological studies of immunization with measles vaccines (Aaby et al., 2010), BCG and OPG (Biering-Sørensen et al., 2017). The validity of these claims as a preventive measure against the disease of COVID-19 is described in the work (Bruxvoort et al., 2022), but is currently still at the stage of clinical trials.

Recent studies using Toll-like receptor (TLR) ligands have shown that antigens associated with their ligands can produce extremely high levels of antibodies and a rapid immune response. From the point of view of influencing the immune system cells, epigenetic adjuvants are able not only to stimulate persistent T- and B-cell responses, but also to provide additional protection against a wide range of pathogens by training innate immunity, which until recently was considered impossible. Therefore, when choosing an immunopotentiating agent for the creation of new vaccines, it is most appropriate to take into account its agonistic properties of Toll-like receptors and prospects for pharmaceutical use.

For example, it was proposed a description of the concept of "learned" (trained) immunity, in terms of which the probability of acquiring new properties by the cells of the innate immunity increases due to metabolism and epigenetic reprogramming (Wimmers et al., 2021). Evidence for this description is provided in the paper (Lee et al., 2022), which confirms the ability of selected vaccine adjuvants to induce epigenetic imprinting of innate immune cells and, as a result, to obtain a long-lasting state of antiviral protection of vaccinated individuals against a wide range of other pathogens. Currently, any precise parameters, basic mechanisms of the innate reprogramming process and its impact on protection against a wide range of pathogens remain poorly understood. In this regard, the issue of a more in-depth study of the influence of previous vaccination on the secondary immune response during revaccination in the context of adjuvants is relevant.

The publication (Ciarlo et al., 2020) reports on the analysis of the effect of trained immunity against a wide range of bacterial infections caused by *Listeria monocytogenes*, *Escherichia coli*, *Staphylococcus aureus*, *Citrobacter rodentium* and *Pseudomonas aeruginosa*. Trained immunity in laboratory mice was obtained by intraperitoneal injection of 1 mg of adjuvant - zymosan (a yeast cell wall preparation rich in β-glucans) 3 and 7 days before infection. The degree of activity of trained immunity was determined by the number of monocytes formed in the bone marrow, cytokine production (enzyme immunoassay), bactericidal blood analysis, and chromatin immunoprecipitation. Mice with pre-trained immunity showed an increase in the number of cytokines, leukocytes and phagocytes in blood samples, a decrease in the severity of diseases and better survival rates, combined with an overall decrease in bacterial load. A group of mice with "trained immunity" survived after infection with *Listeria monocytogenes*, the causative agent of listeriosis, while the entire control group died within 5 days after infection. The bacterial pathogen was not detected in the blood of "trained mice" on the 2nd and 3rd days after infection, while up to 10^5 colony-forming units (CFU)/ml was determined in the blood circulation of the control group. Immunological protection of mice with "trained immunity" is due to increased production of cytokines (IL-1β) in combination with stimulation of myelopoiesis in the bone marrow.

The possibility of inducing trained immunity due to the simultaneous use of bacterial LPS and fungal β-glucan was shown (Owen et al., 2021). This confirms the potential of epigenetic adjuvants to influence the innate immune system.

From a chemical point of view, β-glucans, as carbohydrate polymers, are characterized by various structural variations, conformations, and physicochemical parameters that directly affect their physiological functions. Variations in the structure of β-glucan directly depend

on the source of the polymer (fungi, yeast, bacteria, plant, and grain crops), as well as on the type of glycosidic bond, the degree of its branching and polymerization (Wang et al., 2017).

Carbohydrate polymers of the β-glucan type were studied as auxiliary substances in the composition of anti-infective vaccines and immunomodulators in anti-cancer therapy. First, this interest is due to the possibility of regulating the immune response due to the stimulation of the innate and adaptive links of immunity, as well as the possibility of increasing the immunogenicity of vaccines (Colaço et al., 2023).

The effectiveness of β-glucans as epigenetic adjuvants is due to the presence of a PAMP structure (β-linked pathways) that can be recognized by PRRs – Dectin-1 and CR3. The mechanism of immunostimulating effect of polysaccharide (β-1,3/1,6 glucan) can be explained by its pronounced selectivity in relation to specific receptors on the surface of macrophages (Dectin-1, Complement 3, Lactosylceramide, etc.), which can bind only to unbranched section of the β-glucan molecule. The activation of macrophages caused by this leads to the implementation of a number of processes of immune protection of the body - activation of the phagocytic functions of macrophages, increased induction and release of cytokines, epidermal cell growth factors, and angiogenesis (De Marco Castro et al., 2021).

β-glucan-activated macrophages and monocytes induce a metabolic shift via the Dectin-1 receptor toward aerobic glycolysis. Such changes in cellular metabolism from oxidative phosphorylation to aerobic glycolysis (the «Warburg effect») are crucial for the induction of β-glucan-induced trained immunity. β-glucan training of monocytes induces a resetting of cellular metabolism that modulates epigenetic programming of metabolic genes (Elder et al., 2017). According to (Dos Santos et al., 2019; Novakovic et al., 2016), the ability of β-glucan to affect "trained immunity" is associated with metabolic rearrangement, induction of transcription factors associated with aerobic glycolysis, and activation of the cholesterol synthesis pathway.

The adjuvant properties of β-1,3-D-glucans isolated from yeast and fungi in experiments on laboratory rats were demonstrated in (Han et al., 2020). It is noted that the polysaccharide from the walls of *Saccharomyces cerevisiae* increases the production of TNF-α in alveolar macrophages, increases the oxidative burst of leukocytes, and increases their antimicrobial activity.

Simultaneous application of a mixture of β-glucan and LPS activates the induction of myeloid reprogramming at the level of progenitor cells was shown (Mitroulis et al., 2018; Saeed et al., 2014). β-Glucan (as a component of the cell wall of fungi) binds Dectin-1 and the membrane protein CLEC7A with further modulation of this myelopoiesis in mouse bone marrow at the level of hematopoietic stem cells and progenitor cells, which persists up to 28 days after immunization.

Currently, the use of yeast β-glucan as a platform for antigen delivery targeting TLRs of antigen-presenting cells is promising. In the work (De Smet et al., 2014), such a platform is highly purified empty and porous shells of S. cerevisiae cell walls obtained by stepwise extraction with alkaline and acid solvents. It is characterized by β-1,3-linked glucopyranosyl residues, a smaller number of β-1,6-linked branches, which after alkaline and acid treatments consist mainly of β-1,3-glucan (> 85%), chitin/ chitosan (2%) and are free of proteins, lipids (1%) and mannans. If the antigen enters with a delivery platform made of β-glucan, the interaction of immune cell receptors with membrane receptors Dectin-1 and CR3 is noted. Binding of β-glucan to Dectin-1 on macrophages and dendritic cells induces their activation, maturation, and increases the production of proinflammatory cytokines such as TNF-α, IL-6, IL-2, IL-10, and IL-23. The delivery platform additionally stimulates dendritic cells to produce cytokines IFN-γ and IL-17 (observed when interacting with TLR receptors) (Mentrup et al., 2022).

Yeast β-glucan antigen delivery platforms have been used in (Huang et al., 2012; Hurtgen et al., 2012; Soto et al., 2012) for oral and parenteral targeted delivery (peptide/protein antigens, siRNA/DNA, and nanoparticles) in studies on laboratory animals. Positive results from the use of such yeast cell wall particles for the delivery of pathogenic DNA are described in the work (Mirza et al., 2017). The authors designed a delivery system using electrostatic interactions and presented an encapsulation strategy that provides high capacity and delivery of nucleic acids in combination with the oral bioavailability of β-glucan pures with their inherent ability to affect APC cells. The disadvantage of this technology is cytotoxicity caused by a high concentration of polymers.

The solution to the above-mentioned problem is described in the work (Hwang et al., 2018) and is carried out due to alternating stages of hydration and lyophilization in the process of loading antigenic material into the middle of β-glucan shells. Using this approach, the delivery technology platform has been extended to enable multi-complex co-delivery of TLR adjuvants and/or siRNA (in separate layers around the antigen core) to improve vaccine efficacy and safety (Soto et al., 2023).

The principles described by Soto were used in the study by De Smet et al. (2013) to produce β-glucan particles (GP) for oral immunization with GP-OVA to further evaluate the biocompatibility and efficacy of this model. Studies of the interaction between GP-OVA and intestinal epithelial cells were carried out "in vitro" and "in vivo". It was established that oral immunization with GP-OVA causes persistent humoral responses in the epithelial cells of the small intestine (mucosal IgA and serum IgG responses), as well as the release of cytokines and chemokines due to close contact with the mucosal surface.

The above-described immunostimulatory effects from oral immunization with antigens in combination with β-glucan particles (antigen delivery platform) to promote an enhanced adaptive immune response are reported in (Berner et al., 2018; De Smet et al., 2023). Proliferation, differentiation, and activation of APC cells Th1 and Th17 were noted after sensitization of mice for 3 days (De Smet et al., 2023). Sensitization was carried out with 100 μg/300 μg of OVA-containing compositions with stimulation on the 14th and 28th day. Such oral administration of GP-OVA in mice promoted the generation of cellular and humoral immune responses and additionally activated an increase in the amount of IgA and the secretory component in the intestine. The above research results are in good agreement with the work of Huang and colleagues (Huang et al., 2013), where invasive subcutaneous administration of the GP composition led to the stimulation of antigen-specific antibodies and T-lymphocytes as a response, as well as the production of cytokines - IFN-γ and IL17a by CD4+ Th1 and Th17 cells.

The use of β-glucans in the form of microparticles for stimulating the innate link of immunity remains a relevant issue for the world scientific community. For example, the work (Berner et al., 2005) describes in-depth "in vitro" studies of the natural immunostimulating activity of yeast β-glucans obtained from *S. cerevisiae* in soluble form and as microparticles. Laboratory mice were vaccinated with 1 mg/kg preparation of β-glucan microparticles. As a result, increased expression of costimulatory molecules B7.1 and B7.2 (required as a concomitant signal during T-cell activation and proliferation) and increased phagocytic activity of macrophages were observed in mice. In addition, a rapid phagocytic reaction of macrophages with subsequent induction of pro-inflammatory cytokines TNF-α, IL-6 and IL-1β was noted; this response was additionally enhanced because of IFN-γ priming.

According to research (Yu et al., 2015), yeast β-glucan can induce the recruitment of macrophages, effector T cells, and DCs to the liver with additional enhancement of the Th1 immune response, which promotes the removal of viral DNA from it.

In global practice, the potential of β-glucan is also used in anticancer therapy. Solid β-glucan particles have proven to be physiologically active molecules in the microenvironment of tumors due to the stimulation of immunosuppressive macrophages classically activated through Dectin-1 (Liu et al., 2015). The immunostimulating effect of β-glucan with potential anticancer activity in lung cancer (Peymaeei et al., 2020), cervix (Chaichian et al., 2020), and ovarian cancer (Sadoughi et al., 2022) is currently noted. According to the study of Sadoughi et al. (2022), the positive effect of the polysaccharide is due to its binding to the CR3 receptor and cytotoxic degranulation of tumor cells (coated with iC3b). Due to these properties, the use of β-glucans in anticancer therapy is a topical topic presented in a number of studies in recent years (Caseiro et al., 2022; Geller et al., 2019; Sadoughi et al., 2022).

Adjuvant properties of β-glucans: immunological features in the composition of vaccine preparations

Immunostimulating properties of β-glucan and its chains for the delivery of vaccine antigen in oral immunization were confirmed in (Berner et al., 2015; Cheng et al., 2014; De Smet et al., 2013; 2014). The main functional features of β-glucans discussed are pattern receptor (PRR) recognition by immunocompetent cells and moderate enhancement of immunostimulatory properties without causing their excessive activity, which may cause undesirable consequences in the form of autoimmune diseases (Miyamoto et al., 2018).

β-glucan-induced changes in cellular metabolism lead to the induction of "trained immunity" in monocytes with profound changes in their metabolic processes. The three metabolic pathways involved in trained immunity are glycolysis, glutaminolysis, and cholesterol synthesis, which are associated with β-glucan H3K4me3 enrichment (required for the formation of trained immunity). There is already evidence of the principle of metabolic-epigenomic circuits in innate immune memory, demonstrating the essential role of fumarate in modulating HIF1α degradation, histone methylation and acetylation; taking into account the work of metabolic-epigenomic chains is important if β-glucans are used in the composition of the latest vaccines (Cheng et al., 2014).

Confirmation of the above-mentioned potential of the polysaccharide is described and is explained by the binding and stimulating properties of receptors in relation to APC in the form of a receptor-targeted antigen delivery system (Baert et al., 2015). This delivery system may be promising in the development of the latest vaccines.

The immunostimulating effects of separately prepared β-glucan-CpG complexes with protein antigens was investigated (Kobiyama et al., 2016; Miyamoto et al., 2018; Mochizuki et al., 2015). It was found that the proposed complexes contribute to the release of cytokines and the induction of a powerful cytotoxic T-lymphocyte response, because of which the antigen is released. It is worth noting that the tested drug in these studies overcame unwanted aggregation and initiated stronger antigen-specific reactions with the participation of CD8+ T-cell responses.

The results presented above testify to the potential of β-glucan, both independently and in combination, to manifest adjuvant properties in the composition of immunobiological drugs. Adjuvant properties of β-glucan in DNA vaccines are described by Wang et al. (2020). Such vaccine preparations are able to induce a CD8+ T-cell-specific immune response. Synthetic β-1,6- and β-1,3-glucohexaose administered together with HBcAg DNA (pB144) promotes the recruitment and maturation of dendritic cells along with enhancement of virus-specific lymphocyte cytotoxicity and antibody response.

The results of using β-glucan particles (GP) from yeast *S. cerevisiae*, which are purified cell walls mainly with β1,3D-glucans for OVA encapsulation, are described in the work

(Huang et al., 2010). The developed platform is made using a series of alkaline and acid extraction steps, combining the function of antigen delivery and adjuvant action. Mice were immunized 3 times with an interval of 3 weeks using 100 µl of sterile PBS (control) and 50 µg of OVA complexed with 5 × 10 7 GPs (GP-OVA) by subcutaneous injection above the abdomen. It was noted that GP-loaded OVA is able to stimulate sustained humoral and T-cell responses, whereby the cellular response is driven by Th1 and Th17. In addition, strong antibody reactions were observed, with the titer of antibodies increasing with each subsequent stimulation.

In the formulations of many immunobiological drugs, polysaccharide β-glucan from various sources of origin has already demonstrated significant adjuvant activity in experimental studies on animal models (Table 2).

As can be seen from Table 2, the polysaccharide β-glucan is a candidate for the status of a new type of immune adjuvant for the latest vaccines against diseases caused by infectious agents of both bacterial and viral nature, due to a wide range of potentiation of immune responses regardless of the form of administration. One of the most recent successful integration of polysaccharides into vaccine preparations is the attempt to use their adjuvant properties in influenza (H5N1) vaccines with sulfated (degree of sulfation 0.16) yeast (*S. cerevisiae*) β-glucan (GSC in its composition) (Wang et al., 2016). It has been shown that GSCs are able to significantly promote lymphocyte proliferation independently or synergistically with Con A and LPS, increase the ratio of CD4 + /CD8 + due to an increase in the percentage of T cells, and also stimulate cells to secrete cytokines (INF-γ, IL-2). These results support the adjuvant effect of sulfated glucan as an effective immune adjuvant for inactivated influenza (H5N1) vaccines.

Successful attempts to use sulfated yeast β-glucans from *S. cerevisiae* (GSC) are presented in (Wang et al., 2024), where GSCs were used to enhance the immunogenicity of Newcastle disease vaccines. The aim of the work was to study the effect of GSC on the proliferation of chicken spleen lymphocytes "in vitro". During the study, 7, 14, 21, 28, 35, 42 days after the first vaccination of chickens, the titer of antibodies in the blood serum, the amount of produced interleukins (IL-2), interferons (IFN-γ), and the proliferation of lymphocytes were measured.

A significant increase in the immune efficiency of the vaccine due to GSC is noted because of the increase in the titer of antibodies in chickens, the proliferation of lymphocytes, as well as an increase in the amount of IL-2 and IFN-γ in blood serum.

The status of β-glucan, as a new type of immune adjuvant, is confirmed by Berner et al. (2015), who studied the use of an immunobiological preparation with microparticles of β-glucan (MG) conjugated with a vaccine antigen. In experiments, bone marrow dendritic cells (BMDC) were treated with OVA-conjugated MG. and then interacted with splenocytes from DO11.10 transgenic mice expressing the OVA peptide-specific T-cell receptor. BMDCs treated with MG:OVA were found to induce significantly more activated (CD25+ CD69+) OVA-specific CD4+ T cells than BMDCs treated with OVA alone. However, BMDC treated with MG: OVA increased the expression of CD86 and CD40. Such obtained "in vitro" results once again demonstrate the adjuvant capabilities of MG in matters of enhancing DC antigen presentation to naïve antigen-specific lymphocytes (CD4, CD8).

Table 2

Action of β-glucans when used as adjuvants vaccines

Object of study	β-glucan (source form of administration	Antigens	Effect	Reference
Mice	β-glucan (barley); subcutaneous	Meningococcal group Y, subcutaneous	Increase in specific CPS titer of IgG and IgG1	Qiao et al., 2015
Dogs	β-1,3/1,6-glucan (yeasts *S. cerevisiae*), oral	Bivalent vaccine containing inactivated cells *Bordetella bronchiseptica* and parainfluenza virus type 2, subcutaneous	Increased production of serum immunoglobulins IgA, IgM. IgG level remains unchanged	Stuyven et al., 2010
Mice	β-1,6-branched β-1,3-glucohexaose (synthetic), intraperitoneal	HBcAg DNA (pB144), intramuscular	Increase in the number of recruited and maturing dendritic cells, virus-specific CTL antibodies. Strengthening of the Th2 immune response.	Wang et al., 2020
Mice	β-1,6-branched β-1,3-glucohexaose (synthetic), intraabdominal	HBsAg, intraabdominal	Increase in the number of recruited and maturing macrophages, dendritic cells; production of specific antibodies. Strengthening of the Th2 immune response.	Dong et al., 2007
Mice	β-1,3-glucan hexasaccharide (synthetic), intraperitoneal	Inactivated (detoxified) diphtheria toxin, intradermal	Increase in the titer of antigen-specific antibodies	Donadei et al., 2017
Chickens	Sulphated β-1,3-glucan (*Lentinus edodes*), intraperitoneal	Newcastle disease vaccine, intramuscular	Higher antibody titer and lower mortality than non-sulfated and non-adjuvanted	Guo et al., 2009
Mice	β-glucan microparticles (*Saccharomyces cerevisiae*), oral	Bovine serum albumin, oral	Increased antibody response to vaccine antigens. Activation of antigen-presenting cells	Berner et al., 2018
Chickens	Sulphated β-1,3–1,6-glucan, intraperitoneal	Newcastle disease vaccine, intramuscular	Increased serum antibody titer. Serum IL-2 and IFN-γ concentration	Wang et al., 2014
Ducks and chickens	β-glucan (*Aureobasidium pullulans*), oral	H5N1 and H5N2 influenza vaccine, subcutaneous	Increase in the titer of antigen-specific antibodies	Le et al., 2021

The argument for the feasibility of using yeast β-glucans as a "pathogen-like" adjuvant for hepatitis B antigen is given in study (Soares et al., 2018). It was proposed the use of chitosan biopolymers in combination with β-glucan and additional surface localization of its particles to imitate the cell wall of some pathogens. This combination will stimulate immune cells expressing the Dectin-1 receptor. β-glucan particles (ChiGluPs) were obtained using the method of chitosan precipitation in an alkaline solution followed by genipin cross-linking. This optimized method made it possible to create particles with an average diameter of 837 nm for ChiP and 1274 nm for ChiGluP.

The obtained particles demonstrated sufficiently high efficiency of antigen loading in combination with low cytotoxicity. Blood analysis of pre-vaccinated mice confirms that ChiPs and ChiGluPs exert an adjuvant effect on hepatitis B surface antigen (HBsAg): ChiGluPs induces a 16-fold higher increase in serum anti-HBsAg class G immunoglobulins than ChIPs when 1.5 μg HBsAg is administered per dose. In addition, an increase for induction of IgG1 (by 5 times) and IgG3 (by 4 times) with the use of ChiGluP as opposed to the use of ChIP is noted.

The above results from the use of β-glucan (from *Pleurotus ostreatus*) in the composition of the latest vaccines rationalize the possibility of their recommendation for use as a prophylaxis for competitive infections. This is especially true in conditions of primary and secondary immunodeficiency of various etiologies, as well as allergic diseases such as allergic rhinitis, bronchial asthma, and atopic eczema (Urbancikova et al., 2020).

However, in contrast to a number of studies on the positive immunological effect of β-glucan, the mechanisms of its adjuvant properties require additional research. Although the adjuvantity of β-glucans is now generally accepted, there are studies reporting opposite results and conclusions (Pence et al., 2022). For example, the absence of an immunological stimulus was noted with oral administration of β-1,3/1,6 glucan in relation to an increase in the production of antigen-specific antibodies to fibroblast antigen F4 in experiments on pigs (Kobiyama et al., 2016).

An identical situation was reproduced in analyzes of a group of male volunteers under the condition of oral administration of β-glucan, which also did not lead to the induction of cytokines and microbial activity of leukocytes (Leentjens et al., 2014).

Taking into account the lack of unified data on the adjuvant properties of β-glucan, the questions of a more detailed examination of the immunostimulating effects of the polysaccharide and the mechanism of interaction with the receptors of immune cells (DC, macrophages and granulocytes) remain important.

β-glucan and biotechnological methods for its production

The production of β-glucan for further use as an immunostimulant in immunobiological preparations is the subject of modern research. β-glucan is a non-starch soluble polysaccharide presented in the cell walls of cereals, yeasts, bacteria, and algae. β-D glucose units in glucan are linked by glycosidic bonds at (1,3), (1,4) or (1,6) positions, and they can be linear or branched. The sources of β-glucan are represented by cereal (mainly oats and barley), and non-cereal (fungi, algae, and yeast) groups (Hu et al., 2020; Murphy et al., 2020). β-glucans from cereal or grains have β-(1,3) and β-(1,4) glycosidic linkages between glucose units, while β-(1,3) and (1,6) are characteristics of non-cereal β-glucans (Figure 2).

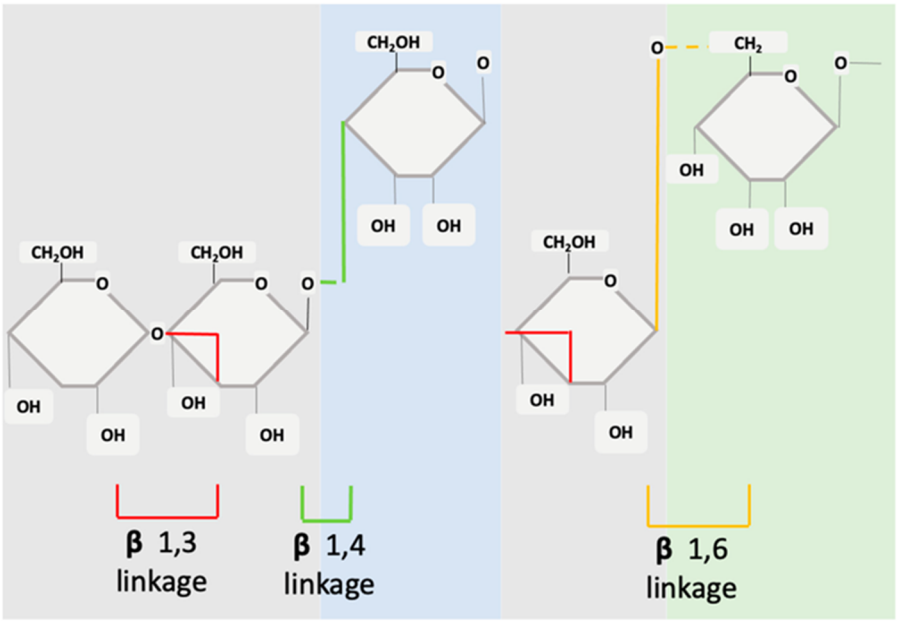

Figure 2. Structure of cereal β-glucans (1,3 1,4) and non-cereal β-glucans (1,3 1,6) (Adopted from Murphy et al., 2020)

β-glucan present in bacteria and algae is characterized by a linear structure, while β-glucan obtained from yeast, fungi, oats, and barley has a branched structure (Mudgil, 2017). The most biologically active form of β-glucans, known for their ability to optimise immune response, is β-1,3/1,6-glucan, having β(1-3) bonds between D-glucose units in the main chains and β(1-6) bonds in the side branches (De Marco Castro et al., 2021). Some prokaryotic (bacteria) and eukaryotic (yeasts, fungi, and algae) organisms proposed to be used for β-glucans production are shown in Table 3.

Table 3

Prokaryotic and eukaryotic organisms proposed to be used for β-glucans production

Glucan	Producer	Reference
β-1,3/1,6- glucan (branch-on-branch β-D-glucans structure)	Yeasts *Saccharomyces cerevisiae; Candida albicans*; *Candia utilis*	Philippini et al., 2012
β-1,3/1,6-glucan (highly branched) (extracellarly)	Yeast-like fungus *Aureobasidium pullulans*	Moriya et al., 2013
β-1,3/1,6-D-glucan (linear with 1,4-linked side branches)	*Pleurotus ostreatus* (white-rot fungus)	Dobsikova et al., 2012
β-1,3/1,6- glucan (linear with 1,6-linked β-glycoside side branches)	Algae *Laminaria* sp, *Eisenia bicyclis*; fungi *Schizophyllum commune*, *Aureobasidium pullulas, Grifola frondosa, Lentinula edodes*	Qu et al., 2021; Suzuki et al., 2021
β-1,3- glucan (linear and unbranched)	Bacteria *Alcaligenes faecalis;* algae *Euglena gracilis;* fungus *Poria cocos*	Suzuki et al., 2021

The attention is focused mostly on the following producers of β-1,3/1,6 glucans: yeasts *Saccharomyces cerevisiae* (Kim and Yun, 2006; Zhang et al., 2019), *Aureobasidium pullulans* (Chen et al., 2021; Moriya et al., 2013), actinomycetes *Streptomyces violaceus* and *S. lucensis* (Wu et al., 2018), microalgae (*Euglena gracilis*) (Qu et al., 2021), and Basidiomycetes fungi *Grifola frondosa* (hen-of-the-woods) and *Lentinula edodes* (shiitake) (Stabnikova et al., 2024).

One of the promising glucan producers is the mushroom *Grifola frondosa*, a Basidiomycete fungus, growing at the base of oaks or maples in Asia, Europe, and North America (Yang et al., 2023). This mushroom synthesizes a soluble homogeneous β-glucan with a β-D-(1-3)-linked glucan backbone with a single β-D-(1-6)-linked glucopyranosyl residue branched at C-6 on every third residue (Cui et al., 2020). The content of β-glucan in *G. frondosa* varied from 6.52% DM (Rodríguez-Seoane et al., 2018) to 25.99% DM (Sari et al., 2017).

Another suitable producer of β-glucan is the yeast *Saccharomyces cerevisiae*, which synthesizes linear β-1,3-glucan with a small amount of β-1,6-linked branches that accumulates in the cell walls (Kim and Yun, 2006). However, it should be noted that, according to the results of Boutros et al. (2022), the structure of glucan obtained from the yeast biomass of *S. cerevisiae* is influenced by both the yeast strain and the method used for its isolation and purification, so there may be slight chemical deviations in the structure of the final products. Cell wall accounts for up to 30% of the cell dry weight and is composed up to 55–65% β-glucan. The highest β-glucan content in cell wall among 10 *S. cerevisiae* strains cultuvated in a nutrient medium with glucose as a carbon source was 41.69% (Pengkumsri et al., 2022). 0.13 g of β-glucan per g of dried biomass was obtain during fed-batch cultivation of *S. cerevisiae* in medium with glucose as substrate for yeast growth (Kim and Yun, 2006).

The biological activity of β-glucan (immunostimulating, antitumor and antibacterial) is manifested only under the condition of increasing the degree of its solubility. At the same time, "soluble β-glucan" (molecular weight 130 kDa) refers to its water-soluble fragments (obtained by partial fragmentation of molecules) and various derivatives of the composition. In this regard, methods of obtaining polysaccharides with an increased degree of solubility using biotechnological modification are relevant (Kaur et al., 2019).

The influence of cultivation conditions and isolation methods on the yield of polysaccharides has been described in a number of studies, which demonstrate the possibility of significantly increasing the amount of β-glucan synthesized (Amer et al., 2021; Bzducha-Wróbel et al., 2020; Fu et al, 2022; Khadam et al., 2023; Utama et al., 2021; Zhang et al., 2019). During the cultivation of the yeast *S. cerevisiae*, it was found that the content of soluble polysaccharide and resistance to β-glucanase are higher in the early stationary phase of growth (Avramia et al., 2021). Under the condition of continuous cultivation, the content of soluble β-glucan and resistance to the action of the enzyme were higher.

To evaluate the immunomodulatory effect "in vivo" of β-glucan from yeast *S. cerevisiae*, laboratory mice were given 50, 100, 150 mg of dried β-glucan per kilogram of body weight (Pengkumsri et al., 2016). It was found that even a small dose of β-glucan induces the expression of selected pro-inflammatory (IL-17, IFN-γ) and significantly anti-inflammatory cytokines (IL-10); a relatively high dose is required to alter IL-6 and TGF-β expression. In general, the research data demonstrate that β-glucan extracted from *S. cerevisiae* HII31 cells can change the expression of pro-inflammatory and anti-inflammatory cytokines.

Taking into account all the above, the most important producers of β-glucan yeasts *Saccharomyces cerevisiae* and the mushroom *Grifola frondosa* can be considered.

Immunological response and possible effects of adjuvants of microbial origin is shown inTable 4.

Table 4

Immunological response and possible effects of microbial adjuvants

Immunological response	Possible effects	Reference
Adjuvants of microbial origin		
β-glucan; Activation pathway: Metabolic reprogramming and epigenetic modifications, Dectin-1, TLR 2,4,5		
Induces trimethylated histone H3 at Lys4 (H3K4me3) and H3 acetylated at Lys27 (H3K27ac) in monocytes and macrophages. Using the Dectin-1 receptor activates the PI3K (phosphoinositol-3-kinase)/AKT/ mTOR (mammalian target of rapamycin)/HIF-1α (hypoxia-inducible factor-1α) pathway and as a result of an activation, a metabolic shift towards aerobic glycolysis is induced enhancing glutaminolysis, which replenishes the tricarboxylic acid cycle, activates the cholesterol synthesis pathway and blocks the itaconate pathway. Formed and accumulated metabolites (fumarate, succinate, mevalonate) act as cofactors of epigenetic modifiers and enhancers of trained immunity. Induces the production of 1β [IL-1β], IL-32y, interleukin 6 [IL-6]); TNF; increases the total titer of serum immunoglobulins IgA, IgM, and IgG.	Epigenetic action and metabolic reprogramming of cells; no side effects	Kumar et al., 2019; Shah et al., 2009
OMV (Outer membrane vesicles); Activation pathway: TLR4		
Increases antigen absorption and induces the expression of cell surface molecules, receptors and costimulatory molecules, which leads to increased production of T cells; stimulates the production of T-helpers (Th1 and Th2), as well as cellular and humoral immunity. Activates embryonic kidney cells through the TLR-2/TLR-1 complex, increasing the titer of IgG.	Technological difficulty of obtaining; provocation of inflammatory processes; endotoxicity	Tan et al., 2018

In summary, the ideal adjuvant should be safe, stable and not cause unwanted side effects. Its production must be simple, cost-effective and compatible with all components of the vaccine. However, the toxicity of traditional adjuvants remains a key limiting factor for the development of new and improvement of existing immunobiological drugs. Thus, the long-term strategy for choosing of adjuvants is to select the optimal platform and identify key targets of innate immunity that ensure the generation of potent yet safe immune responses according to the nature of the pathogen targeted by the vaccine (Belemets et al., 2024).

According to the data given in Table 4, OMV adjuvants in combination with some TLR agonists can cause uncontrolled immune reactions in the type of excessive inflammation factor in addition to the possible provocation of inflammatory processes due to the presence of a significant amount of endotoxins in the composition of OMV. Accordingly, there is a need to rationalize their design to elicit an optimal immune response (Tan et al., 2018; Van Der Pol et al., 2015).

The disadvantages of using LPS as an immunopotentiating agent include toxicity, which makes it quite difficult to translate the use of LPS adjuvant into a clinical scenario. Moreover, problems with stability, production and quality assurance are the main factors in the absence of any registration of a liposome-based adjuvant for use as part of immunobiological drugs for humans (Facchini et al., 2021).

The use of polysaccharide β-glucan as an epigenetic adjuvant is appropriate due to its ability to influence the immune phenotype. This makes it possible to form the most effective immune response for each particular causative agent of the disease. Due to its epigenetic characteristics, β-glucan can be used for imprinting an enhanced antiviral state (resistance against a wide range of viruses in humans). This property may be particularly advantageous during a pandemic (the COVID-19 pandemic) to confer enhanced antiviral resistance for a limited period (approximately a few weeks) in a susceptible population (Arts et al., 2018).

Research results (Biering-Sørensen et al., 2017; Lee et al., 2022) demonstrate the induction of epigenetic imprinting of innate immune cells under the influence of epigenetic adjuvants, which leads to a long-lasting state of antiviral immunity and non-specific protection against re-infection. The promise of these adjuvants is enhanced by stimulation of robust T- and B-cell responses and protection against a wide range of pathogens through training of the innate immune system (Arts et al., 2016).

The epigenetic status of β-glucan has been repeatedly confirmed in a number of studies. It was noted that PAMPs induce trimethylated histone H3 at Lys4 (H3K4me3) and H3 acetylated at Lys27 (H3K27ac) in monocytes and macrophages. These changes persist for several weeks after elimination of stimuli, resulting in improved epigenetic status. However, what is important is the improvement of the epigenetic status, which induces increased expression of genes (related to metabolic, immune and host defense pathways), obtained during the secondary stimulus by the same or other PAMP. Thus, trained monocytes/macrophages produce increased levels of cytokines (tumor necrosis factor (TNF), interleukin 1β [IL-1β], and interleukin 6 (IL-6) when exposed to microbial compounds. This raises the attractive possibility of targeting small molecules to relevant cell types as immunological modulators to reprogram the epigenetic landscape of innate immune memory (Netea et al., 2017).

Vaccines using β-glucans as adjuvants of the new generation can be used both orally and parenterally, which makes them widely applicable in medical practice. Therefore, immunostimulating polysaccharides with inherent biocompatibility and biodegradability are considered a rational strategy for the development of new adjuvants that mimic the properties of pathogens ("pathogen-like") (Stothers et al., 2021).

Conclusions

Therefore, immunomodulation through targeting of TLRs opens up new perspectives in the development of new generation vaccines that use epigenetic adjuvants to persistently reprogram the innate immune system. This makes it possible to achieve long-term protection

against a wide range of pathogens, which is especially relevant in pandemic conditions, when traditional antigen-specific vaccines may not be available.

The selection of safe and effective adjuvants or their systems remains a key area of research. The development of new pharmacological drugs, molecules or a combination of excipients plays an important role in the progress of modern immunobiotechnology. In this context, the creation of innovative biotechnological approaches aimed at obtaining safe, stable and compatible adjuvants capable of ensuring the effectiveness of vaccination is of particular importance.

The widespread use of chemical adjuvants, such as aluminum salts or emulsion preparations, has significant limitations, including toxicity, potential mutagenic and carcinogenic effects, a number of side effects, and insufficient effectiveness in stimulating immunity. This creates a need to find alternative approaches, in particular adjuvants of microbial origin.

The β-glucan polysaccharide shows significant potential as an epigenetic adjuvant due to its ability to modulate the immune phenotype, enabling adaptive immune responses optimized for a specific pathogen. The biotechnological production of β-glucan is very simple, which contributes to its prospects in the development of new, safe and effective immunotherapeutic drugs.

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