

Mechanisms and Performances of Adjuvants in Vaccine Immunogenicity

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Abstract

An adjuvant is a substance that is added to a vaccine to increase the body's immune response to the vaccine. Vaccines containing adjuvants are tested for safety in clinical trials before they are licensed for use. The basic action of adjuvants is stimulating adaptive immune responses. Adjuvants recently licensed for human utilization involve alum squalane oil or water emulsion, influenza virosomes, and few cytokines as IFN- γ and IL-2. Some adjuvants are currently under investigation such as DNA motifs, monophosphoryl lipid A, Cholera Toxin, *E. coli* heat Labile Toxin, Saponins, Immunostimulating complexes, liposomes, Flt3 ligand as a pleotropic glycoprotein, non-ionic block copolymers. This paper is an overview of most commonly used adjuvants, adjuvant mechanisms, adjuvant formulations and adjuvant limitations.

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Introduction

Adjuvants (originated from adjuvare as a Latin term, means assist or aid) are a cluster of diverse blends which increase or regulate the immunogenicity of the weakly proteins or poorly peptides which are used for vaccines [1, 2]. The basic action of adjuvants is stimulating adaptive immune responses. Choosing an adjuvant in vaccine development is often as notable as selecting the vaccine antigens which is enough to mimic natural infections or traditional vaccines. In the 1920s, the notion of adjuvant was conceptualized from observations such as those of Ramon et al who mentioned that horses with abscess at the inoculation site of diphtheria toxoid, produced higher distinct antibody titers. Then, they discovered that an abscess caused by the injection of unrelated substances accompanied with the diphtheria toxoid, enhanced the immunity against the toxoid [3]. The most suitable adjuvant for a determined vaccine antigen depends significantly on the kind of immune response which is indispensable for protective immunity. Although, some adjuvants are ostensibly potent, but they are too harmful to the host. Thus, their potency often conflicts with host safety and tolerability. They can be utilized for various aims: a) increasing immunogenicity of recombinant antigens, b) reducing the quantity of antigens or the multiplicity of immunizations required for safeguarding immunity, c) improving the efficiency of vaccine in newborn babies, the old persons or immunocompromised individuals, or d) up taking antigens by the mucosa just as systems for antigen delivery [4-6].

Adjuvant roles

A well practice in experiments of immunology is the insertion of adjuvant accompanied by antigens in production of vaccine which indicates one of the best approaches for developing the next generation of peptide subdivision vaccines. The potentiality to produce immunological 'incidents' that is essential to cause the wanted immune response is a factor to group adjuvants (Fig. 1). Alternative grouping through physical and chemical characteristics may be more profitable because few adjuvants act as stimuli more than one immunological track. Adjuvants may show their immunostimulatory impacts via the succeeding mechanisms: (1) supplying antigen depot; (2) activating the intrinsic immunity by Pathogen Recognition Receptors (PRR); (3) co-stimulating the immune cells; (4) immunomodulating, e.g., making mature Antigen Presenting Cells (APC) [7, 8].

Total procedures consist of direct or indirect inducement of APC, specifically Dendritic Cells (DCs). DCs play a pivotal role to bridge the intrinsic immune system by not specified internalization of the antigens which consequently are represented to the sequenced T cells. The confronted antigens may continuously be taken up by DCs through pinocytosis and/or phagocytosis and at the same time, their PRR play role as an "identifier" for infectious agents. DCs, upon inducement of PRR, deliver diverse soluble mediators like cytokines and interferon (IFN) type 1 as a part of the intrinsic immune response [10]. The activated DCs commence a response via processing the antigens and giving them to naive CD4⁺ T cells. Meanwhile, DCs by up



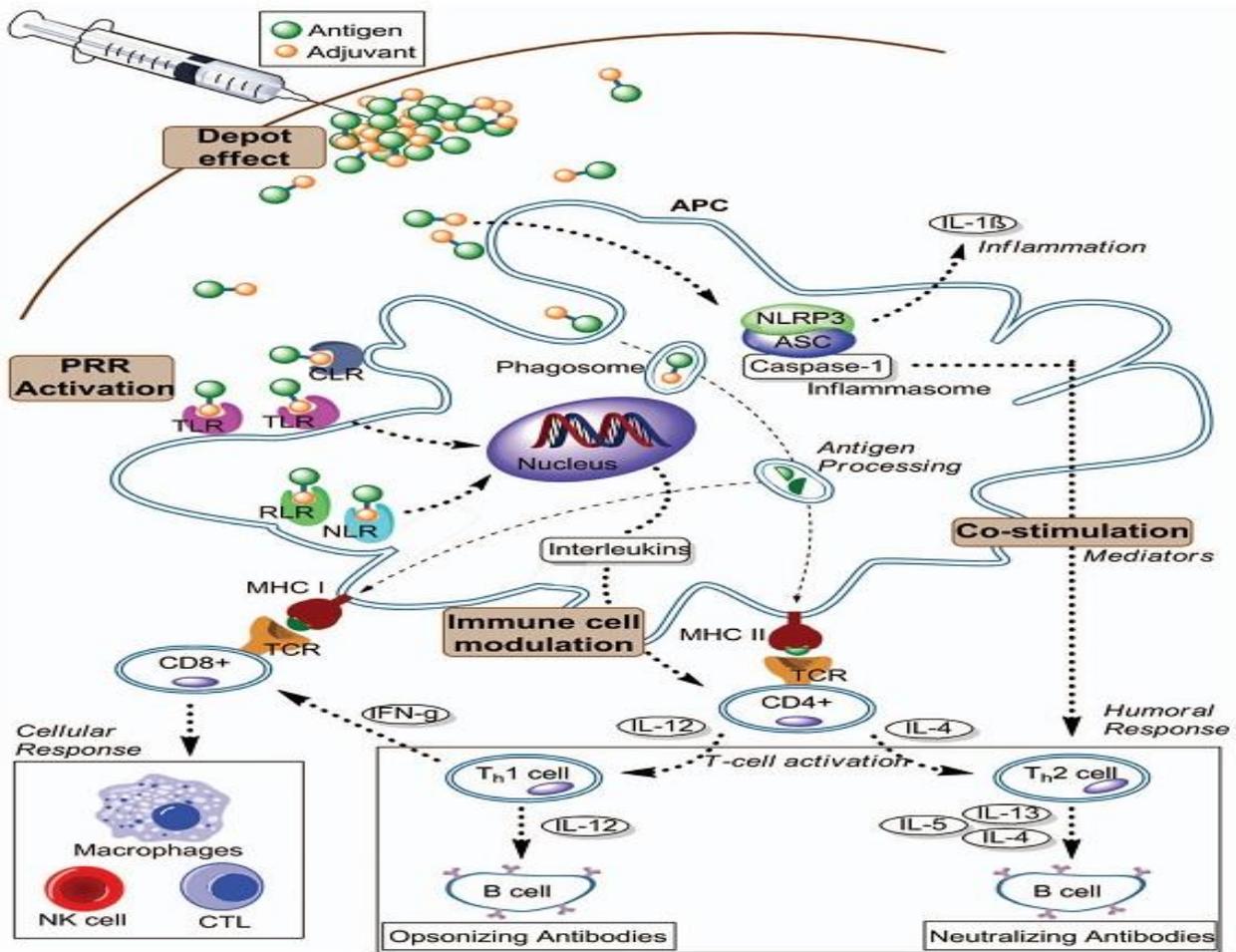


Figure 1. Cascade in schematic view caused by adjuvants. These events are necessary for increasing and adopting immune response opposed to vaccine antigens [9].

regulating MHC class II as well as co-stimulating molecules induce interactions between DC and CD4⁺ T cells. This immunological cascade leads to the promotion of optimal CD4⁺ stimulation. Anyway, this cascade is inadequate to prime CD8⁺ T cell which is vital for vaccine efficacy opposing to cancer or intracellular pathogens. For DCs, Cross-presentation is indispensable to exhibit MHC class I peptide complexes to CD8⁺ T cells to make a CTL response. This fact which the detailed immunological actions are needed to produce this response has been comprehensively appraised by Joffre and workmates [11]. Toll-Like receptors (TLR) among the PRRs are the most studied receptors that are well-known as the main interest of modern adjuvant targeting. TLR are transmembrane proteins type 1 along with an extracellular domain of interspersed leucine-rich repeat (LRR) motifs which are involved in the identification of pathogen associated with molecular patterns (PAMP), like lipopolysaccharide (LPS) [12]. Engagement of PAMP with TLR leads to the activation of mitogen-activated protein kinase pathway and NFκβ which ends in regulation of pro-inflammatory cytokines (interleukin-1β, TNF-α and chemokines). In initiating adaptive immune response, TLR activation also plays a main role as directing the immune system toward Th1- and Th2-biased responses [12]. Th1 responses

are mediated by the secretion of IFNγ which are thought to be liable to kill intracellular pathogens. In contrast, the secretion of IL-4, IL-5, IL-6, and IL-10, which leads to the generation of antibodies, characterizes Th2 responses. It is shown that vaccine adjuvants that contain TLR ligands are able to induce T cell responses of higher avidity. To utilize the TLR ligand as a potential adjuvant, this immense research was prompted. As it is believed that for desired antigen presentation and later stimulation of antigen-specific T cell responses, adjuvants based on the TLR ligand should be co-delivered with the optimal antigen to target the same phagosome cargo at the APC. Therefore, a promising method of designing new vaccines is represented via the ability of TLR to connect innate and adaptive immune responses [13].

A delivery stand for the antigens of vaccine is executed by effects of diverse adjuvants. It is observed that, liposomes, emulsion and alum as particulate-based adjuvants increase antigen uptake by making prolong the contact of antigen to the DC [14]. Two discovered subpopulations of DC are Tissue-resident and migratory. Antigens at the site of administration are presented to resident DC placed in tissues close to the mucosal superficies. Tissue resident DC which subsequently named migratory DC, upon antigen exposure, undergoes a process of maturation and

commences to migrate to the lymph nodes [15]. Naïve T cells and B cells do not spread at the non-lymphoid places such as the most places of injection in the body and are often discovered at the lymphoid organs such as the lymph nodes. Thus, antigens in order to be given to the particular T cells require reaching the lymph nodes by migratory DC. These kinds of adjuvants supply a depot development that traps antigens at the site of injection, providing a release of antigens to be obtained by migratory DC. So, the vaccine antigen fate in time, area and concentration is affected by these adjuvants through the activation of antigen CD4⁺ T cells and promoting uptake of the antigen at site of injection. On this, particulate adjuvants can bind antigens and form aggregates. The aggregation complex, to some extent, shows same dimensions to pathogens that induces their uptake into APC by phagocytosis [16]. Hence, the maturation of APC may be contributed by these adjuvants. Other adjuvants act like immunopotentiators by having a more straight effect to induce an immune response. To do this, adjuvants need to interact with co-stimulatory molecules on APC which helps to prime T helper cells (CD4⁺) [17]. Through The activation of APC, it was exhibited for T cells that the given antigens are appropriate agents to induce an immune response. This eases the identification of antigen for more B cell increasing as well as antibody generation. The immunological endurance or energy may be resulted by the unavailability of this induction. Proteins, under physiological states, turn into a distinct fixed conformation as small peptides elicited from a protein are not usually able to keep their native conformation. Anyway, to activate B cell receptors to induce a humoral immune response, preserving this native conformation is indispensable [18]. For inducing the suitable secondary form of peptide epitopes, several strategies are accessible such as incorporating further flanking peptide and hydrophobic moieties like lipopeptide or polymers [19-21]. Unlike humoral immunity, for the peptide epitope, it is not needed to take a specific conformation to induce a CTL response. In order to produce defined length peptides, APC must make the antigenic peptides [22]. To induce CTL or antibody immunity, it is necessary to make T helper cells. It is typically attained through the blending of the peptide epitope to a carrier protein like tetanus toxoid which involves a T-helper epitopes. As an alternative, a universal t-helper such as PADRE (AKFVAAWTLKAAA) can be fused into the delivery system [23]. Adjuvants play a pivotal role to specify the quality and amplitude of immune response in opposition to the antigens. Different kinds of immune responses for different pathogens are required to aim the disease. Therefore, it is substantial to select a suitable kind of adjuvant for vaccines based on peptide, in order to induce the wanted immune response for efficient vaccination [23].

Most commonly used adjuvants

Adjuvants, recently licensed for human utilization, involve alum squalane oil or water emulsion (MF59), influenza virosomes, and few cytokines as IFN- γ and IL-2 [24]. Some adjuvants are currently under investigation such as DNA motifs [25], monophosphoryl lipid A [26], Cholera

Toxin (CT) [27], *E. coli* heat Labile Toxin (LT) [28-30], Saponins [31], Immunostimulating complexes (ISCOMs) [32], liposomes [33], Flt3 ligand as a pleotropic glycoprotein [34], non-ionic block copolymers [35].

Freund's adjuvants

Jules Freund produced a powerful immunogenic adjuvant in 1940 which is known as Freund's complete adjuvant (FCA). It was consisted of a mixture of mineral oil, a surfactant (Aracel A), and heat killed *Mycobacterium tuberculosis* (MTB). This adjuvant prolongs antigen persistence. A muramyle dipeptide which is an element of the mycobacterial cell wall activates macrophages and makes FCA very potent. Considered as a gold standard for immunologists, FCA is so effective to enhance vaccine responses in animals. Since some problems are associated with its use as ulcerating tissue necrosis, it is not utilized for human vaccination. Freund's incomplete adjuvant (FIA) does not include the mycobacteria and was licensed to use in the influenza vaccine. Because of the toxic effect of the surfactant which causes tissue necrosis, it is no longer used for humans [36, 37].

ISCOMs

The notion of Immunostimulating complexes (ISCOMs) as a versatile delivery system was first defined in 1984 [38]. ISCOM is a 40nm cage like lipid carrier comprised of a glycoside, quillaja saponin and cholesterol. By addition of phospholipid, the assembly of the ISCOM structure and the incorporation of the antigen is facilitated and mediated mainly by hydrophobic interactions. ISCOMs which have a strong immunomodulatory capacity, increase the MHC class II expression on APCs [39], activate murine Th cells to secrete the Th1 type cytokines IL-2 and IFN- γ and up regulate IgG2a antibody responses [40-42]. It is able to deliver antigen to the MHC class I and to produce CTL responses after parenteral and mucosal operation [43, 44]. Immunization with gp120 ISCOMs has been exhibited to stimulate both IFN- γ and IL-4 production in primates and provide safety against HIV-1 infection [45]. Meanwhile, ISCOMs actuate a concomitant Th2 response [46] which ends in balanced Th1/Th2 response.

CpG (cytidine-phosphate-guanosine)

Unmethylated CpG dinucleotide motifs in bacterial DNA as uncommon in mammalian DNA are strong stimulators of immune responses for mammalian hosts. In the context of selected flanking sequences, CpGs are supposed to be identified by the cells of innate immune system to permit segregation of pathogen derived DNA from self-DNA [47]. The immune system is stimulated by these DNA sequences through a specific receptor, TLR-9, which is intracellularly showed in human and mouse B-cells and plasmacytoid DCs [25]. After some minutes, the activation of cell signaling pathways is resulted through the interaction of B cells or plasmacytoid DCs CpG motifs with TLR-9. Then, this terminates in the expression of MHC as well as co stimulatory molecules to elevate the secretion of Th1 polarizing cytokines as macrophage inflammatory protein-1, IFN inducible protein-10, TNF- α , IL-1, and IL-12 and IgG2a and IgG2b antibody production [48]. The immune impacts of CpG involve direct triggering of B cells which makes proliferation plus polyclonal

immunoglobulin synthesis, and low CpG concentrations which promote antigen specific immunoglobulin synthesis by synergistically acting in accompanied by the B cell antigen receptor [49]. The production of type I IFNs and IFN- γ is also induced by CpG which they activate NK cells for increased IFN- γ synthesis and enhanced lytic activity [50]. Various allergens and infectious agents are protected by CpG DNA all one via antigen-dependent mechanisms [51, 52] and the protective effects of antigen-specific immunity are enhanced immunity [53, 54]. The conjugation seems to maximize the adjuvant effect of CpG to plasmid protein antigens [55] or their formulation with delivery systems [56].

Bacterial toxins

Labile potent toxins from *E. coli* and CT from *Vibrio Cholerae* are able to induce both systemic and mucosal immune responses as administered through the parenteral, mucosal or intraperitoneal routes. CT treatment enhances the MHC class II expression on APCs and influences directly B-cell discrimination. Structural CT that is an AB₅-complex, is comprised of a pentamer of B-subunit (CTB). It is surrounded by a single A subdivision which involves a linker to the pentamer by the A₂ fragment or CTA₂ as well as enzymatically (ADP-ribosyltransferase) active A₁-fragment (CTA₁). Two mechanism of adjuvanticity have been proposed for CT. The first one is related to the structural binding characteristics of the AB₅-complex and the second one is dependent on the ADP-ribosylating role of the A₁-subunit. Unluckily, CT is so toxic to humans and just 5 mg of CT orally ended in overt diarrhea in human volunteers [Levine 1984]. The toxicity is associated without the binding of the B-subunit to the GM1-ganglioside receptor (present on all nucleated cells) and the ADP-ribosyltransferase activity of the A₁ subunit [28, 29, 57, 58].

Currently, it has been presented that one toxic form of the CT could be attained by redirecting the thorough enzymatic activity of the CTA₁-subdivision to target B cells through the expression of CTA₁-encoding gene as a fusion protein along by a dimer (DD) of an Ig-binding particle of *Staphylococcus aureus* protein A [59, 60].

In this way, not only the enzymatic action of CTA₁ in CTA₁-DD fusion protein is maintained, but also the A₁ subdivision is prevented from binding to cells (epithelial and nerve cells) [61], where it could exert a more generalized toxic effect. It has been shown that CT and CTA₁-DD bind directly to B cells and escalate the expression of co stimulatory molecules strongly (CD80/86) in vivo as well as in vitro [59], through increased production of cytokines as IL-1 and IL-6 [62].

CTA₁-DD increases T-cell priming and germinal center reactions following administration, ending in elevated specific antibody responses. Recently, it has been noticed that CTB subunit can act as a carrier of antigens, and noticeably enhances and slightly directs the DC vaccine induced immune response with respect to Th1 and Th2 responses [63, 64].

Alum

Alum is aluminum-based mineral salt (generally called alum). For the first time in 1926, Glenny introduced it.

Aluminum salts are insoluble gel like which precipitate aluminum hydroxide or aluminum phosphate. Immunogen is bound by electrostatic interactions to pre-formed gel or during gel formation in situ. Alum has been extensively utilized in human and veterinary vaccines since 1930 and has a good safety record. Alum prompts strong Th2 type of responses, and latest work in vitro demonstrated that alum up regulated co stimulatory signals on human monocytes and induces the delivering of IL-4. Unfortunately, alum is a weak adjuvant for cell-mediated immunity and is able to induce IgE antibody responses which are related to allergic reactions in some subjects. The administration of alum which contains vaccines might be associated with the emergence of macrophagic myofasciitis (MMF). MMF is an inflammatory myopathy has been determined recently [65, 66].

Liposome adjuvants

Liposomes as synthetic spheres are formed of lipid layers which can encapsulate antigens and play role as a vaccine delivery vehicle as well as an adjuvant. In experimental vaccines, liposomes have been applied broadly. The effectiveness of liposomes relies on the quantities of lipid surfaces [67], electric charging [68], composition [69] and method of making ready [70, 71]. Humeral immunity and cellular immunity for protein antigens and polysaccharide antigens can be enhanced through liposomes [69-72]. Liposomes aid to grow the life of antigens in blood ensuring. It means a higher antigen contact to antigen giving cells at the back of vaccination [73]. Stability, producing and quality assuring problems look to have been main agents behind the matter that no adjuvant for human utilization related to the liposomes has been recorded yet.

Cytokines as adjuvants

In the modern categorization of adjuvants, Cytokines are taken into consideration. IFN- γ as a pleiotropic cytokine can increase cellular immune responses via a number of procedures [74]. By activating and recruiting antigen presenting cells, Granulocyte-macrophage colony stimulating factor (GM-CSF) boosts the primary immune response [75]. Mean while, in need of multiple doses, toxicity and the immunogenicity of heterologous cytokines, the practical application of GM-CSF as an adjuvant has been limited. Cytokines are specifically observed to have potentiality in DNA vaccines where the cytokine can be expressed by the same vector as an antigen [76].

Adjuvant formulations

From the mixture of various adjuvants in the same formulation, new adjuvants have been resulted. Two or more adjuvants, as a general rule, with different mechanisms of actions are mixed to enhance the potency and type of the immune response to the vaccine antigen [77]. For instance, in combination with other adjuvants such as Lipid A, alum salts can be formulated to increase immunogenicity. Similarly, algamulin which is the mixture of γ -inulin plus alum has enhanced absorptive capacity as well as increased ability to stimulate Th2 responses [78]. Saponins like Quil A have also been applied as an element of immunostimulatory complexes (ISCOMS) [79]. ISCOMS are virus like particles of 30–40 nm with dodecahedral structure, comprised of Quil A,

lipids and cholesterol. Antigens can be encapsulated or inserted in the membrane. A large number of proteins have been inserted in these cage-like structures [72, 80, 81]. ISCOMS can be utilized via the oral, respiratory and vaginal routes [82]. Although, they are particularly efficient to activate cellular immunity and cytotoxic T cells but often show problems with stability and toxicity [79].

Adjuvants for DNA immunization

When immunization of uncovered DNA introduced in the 1990s, it was imagined that this would not require adjuvants. It is now obvious that new methods require enhancing the power of candidates with vaccine based on DNA. Co-inoculating plasmids coding diverse cytokines or co-stimulatory agents has been utilized efficiently to increase the immune response caused by the plasmid of vaccine [83]. Co-inoculating the plasmid exhibiting B7-2 in accompany with a DNA-based vaccine candidate from HIV-1 escalated the cellular immune response particular to HIV-1. Moreover, when co-inoculation of plasmids coding for each protein were done, a plasmid showing GM-CSF promoted the humoral kind of immune response to protein G from rabies virus co-inoculation of IL-12 expressing plasmid in addition to the other plasmid coding for an HIV-1 protein increased the cell mediated immunity particular for VIH-1[74, 84].

Adjuvant limitations

Alum remained as the main adjuvant for human vaccines, as the discovering procedures of adjuvant actions were progressing. Although numerous adjuvants have been given over the years, they have not been successful to use for humans to a large extent because of being toxic, stable or not, and their bioavailability and costs. It is difficult to foresee an exact basis which adjuvant will be effective with a specific protein or peptide because of extent, electrical charge and being hydrophobic which regulate the incorporating of proteins into the adjuvant formulation. Also, epitope modifications may be resulted by formulation or conjugation. A pre-existing immunity is a main restriction for carrier proteins [85]. Additionally, each adjuvant produces an immune response profile. For instance, as alum-based adjuvants are unable to cause Th1 antibody isotypes or cellular immune responses and have poor impact on polysaccharide antigens to restrict their applicability to various vaccines [86].

Conclusion

In spite of the development of knowledge about immune function over current decades, we are almost completely dependent on compounds based on aluminum for human adjuvants whose actions was first discovered about 80 years ago. Recent progresses in vaccine forming and the enhancing employing of recombinant subdivision and synthetic vaccines induces the need for ameliorated adjuvants. Although there is a hope that new adjuvants may amend some of the deficits of adjuvants based on aluminum, there is a worry that many of these adjuvants won't be accepted for logistical or commercial use for human beings rather than scientific scopes. Obviously, not only there exists a lack of knowledge of adjuvants that are in the way of accessibility of new adjuvants, there are some leading

obstacles. Firstly, undesirable side-effects and being toxic it prevent the utilization of many candidate adjuvants. Also, this is peculiarly right for prophylactic pediatric vaccines where safety matters are of greatest importance. Secondly, when alum was first discovered as an adjuvant for human; the regulatory bar has been enhanced seriously. In fact, it is possible that if alum hadn't been utilized all these years and was first used to regulatory bodies for approbation, it would be rejected to register regarding safety concerns today. Thirdly, it is not feasible for adjuvants to be accepted as materials in their own right as they can just be recorded as an element of the vaccine combination. Many good adjuvant candidates have not been successful to be registered because of this fact that vaccine combination was not efficient or had been toxic, not for being any problems with them. Fourth, since so much money has spent to produce antigen, few firms are ready to endanger this investment in order to continue clinical trial plan of candidate antigens. Fifth, most companies tend to keep their adjuvant data secret. Therefore, as they themselves like to register their outcome based on their adjuvants, they will not give their data to others. Finally, it is now preventing to spend so much money to produce a new product like an adjuvant.

Although, it may be possible to give reasons to spend several hundred million dollars to make a new vaccine with regaining such money from selling vaccines, but it does not come true for adjuvant production costs because there does not exist any trouble-free origin of cost recapturing. There is, for all the mentioned logistical reasons and commercial causes for adjuvants which can boost strongly immune responses related to undue toxicity. This key objective, in spite of abundant valuable promotions of immunology, has remained vaccinology as a 'holy grail'.

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