Improvements in Adjuvants for New-Generation Vaccines

Lilly Ganju and Divya Singh

Abstract

Over the last decade, extensive research for development of new vaccine adjuvants is being carried out. Present generation vaccines, particularly those based on recombinant proteins and DNA, are not only less reactogenic and also less immunogenic. Therefore, there is an urgent need for the development of new and improved vaccine adjuvants. Many novel adjuvants have been cleared for license, and many are in late stages of clinical trials. Recent investigations in innate immunity have offered new insights into immunostimulatory actions of adjuvants and have facilitated a more rational selection of adjuvants. Despite the impressive response of approved adjuvants in generating immunity against pathogens, there remains a need for improved adjuvants that enhance strong T-cell immunity and protective antibody response. The discovery of more potent adjuvants will also allow engineering of vaccines against infections that do not naturally elicit protective immunity. A logical approach to the development of new and more effective vaccine adjuvants requires a better understanding of the action of adjuvant-antigen formulations. Here, we discuss these advances and the need for better adjuvant development.

Introduction

The invention of smallpox vaccine by Edward Jenner, in the year 1800 AD, brought a revolution in health-care techniques and engendered new fields of vaccinology and immunology. Today many vaccines are available in the market for diverse immune interventions. The fact is that many vaccines are developed in the laboratory, but unfortunately never see the light of the day as they have apparently no immunogenecity at all, when tested alone. This could virtually happen for any antigen, but it is particularly true for small peptides that serve as antigens [5]

In the process of recruiting the immune system to produce specific or nonspecific immunity, the antigen by itself may not be adequate as a stimulatory agent to trigger an immune response. This is the condition when immunologists look

L. Ganju (✉) • D. Singh
Immunomodulation Division, DIPAS, DRDO, Timarpur, New Delhi, India
e-mail: lganju@rediffmail.com

S.B. Singh et al. (eds.), *Translational Research in Environmental and Occupational Stress*,
DOI 10.1007/978-81-322-1928-6_11,

for an adjuvant, or an adjuvant strategy, in order to elicit a more pronounced immune response. The term adjuvant is derived from the Latin *adjuvāre*, meaning "to help" – first described by Ramon, while working with natural compounds, as "substances in combination with a specific antigen produced more robust and specific immune response than the antigen alone" [108]. An adjuvant can be defined as a substance or agent that serves as an immunological vehicle to enhance the innate immune response, or stimulate the adaptive immune response, to generate the desired levels of immunity with minimal toxicity or long-lasting immune effects on its own [129].

The Need for Adjuvants

There is a dearth of potent adjuvants for various vaccines. The demand for the development of new adjuvants and strategies that are safe and effective leads to continuing extensive research in this field, and as a result, different types of materials are being experimented with. This highly heterogeneous group of agents is categorized for rational selection of an adjuvant, with their classification based on chemical action, and mechanism of intervention.

Foreign invaders, e.g., bacteria, viruses, and parasites, contain antigens which a host immune system recognizes and mounts an immune response to. They also contain inbuilt "adjuvants" which trigger the immune system in a manner resulting in directing its response along an effective pathway. Thus, a purified antigen, derived from a bacterium, a virus, or a cancer cell, has a reduced ability to stimulate the immune system in the desired manner. Synthetic subunit vaccines are expensive to produce; but addition of an adjuvant will result in lesser quantity of antigen required, with fewer injections, thereby saving on the cost of vaccination. This will also help in developing better combination vaccines, the number of distinct vaccines, as well as the amount of vaccination antigen required will be lesser. This in turn may also reduce the competition of antigens and carrier-specific epitope suppression.

Table 1 Types of adjuvants, based on their composition

Type	Description
Mineral salt based	
Alum	Aluminum hydroxide; aluminum phosphate
Calcium phosphate	$CaHPO_4$, commercially available adjuvant
Bacteria derived	
CpG Oligodeoxynucleotides (ODNs)	Synthetic ODN containing unmethylated CpG motifs [12, 70]
Monophosphoryl Lipid A (MPL A)	Derived from LPS
AS04	TLR4-agonist MPL, with aluminum salt [23]
Emulsions	
MF59	Oil-in-water (o/w) emulsion [86]
AS03	α-tocopherol and squalene in an (o/w) emulsion[87]
Freund's incomplete adjuvant (FIA)	Water-in-oil emulsion
Montanide	Water-in-oil emulsion
Adjuvant 65, Lipovant	Water-in-oil emulsion
Carrier adjuvants	
Liposomes	Synthetic spheres of lipid layers encapsulating antigens [98]
Immunostimulatory complexes (ISCOMS)	Virus-like particles of 30–40 nm and dodecahedric structure, composed by Quil A, lipids, and cholesterol
Cytokines	
GM-CSF, IL-2, IL-12, Type 1 interferon, IFN-γ	Potentiate the immune response to vaccination
Carbohydrate adjuvants	
QS21	Saponin derived from a mixture of soluble triterpene glycosides purified from the soap bark tree (*Quillaja saponaria*)
Advax™ adjuvant	A crystallized natural plant-derived polysaccharide [94]
Acemannan	A natural polysaccharide extracted as a mucilaginous gel of the *Aloe barbadensis* [98]

For purified antigens, the importance of addition of effective adjuvants to optimize the immune response, is thus emphasized.

On the basis of quantum of adjuvanticity, a huge number of candidate adjuvants have been categorized (Table 1). However, such

classifications have inherent limitations though, as a consequence of highly diverse individual variation. Till date, there is no reliable algorithm available which can reliably predict the interaction of an adjuvant, permitting the selection of an antigen based on its physicochemical or immunological properties [109]. Very often, commercial aspects, viz. ease of production, cost, toxicity, availability, etc., become difficult deciding factors. The modal factors in the choice of an adjuvant are the ability to cause additive effects, or lower the quantity of antigens needed, or qualitatively reduce the side effects and enhance the safety of the adjuvant. A balance is struck on the principles of "risk benefit analysis." For routine childhood vaccines, mandatory safety is the biggest concern, while adjuvanticity may be restricted because these vaccines are needed to develop lifelong immunity against specific diseases in a normal healthy body where any risk of side effects is unacceptable; whereas for a person with an infection like HIV or with a disease like cancer, therapeutic vaccines with a certain level of toxicity are acceptable, based upon the accruing benefits.

Importance of Adjuvant Mediation in Vaccines

Vaccines confer protection mainly through humoral immunity [101]. But new-generation vaccines, including recombinant, conjugated or attenuated, polysaccharide subunit vaccines, etc., are developed with the intention to provide a stable and sustained protection. Despite the enhanced effect of such vaccines, substantial groups of people are not able to achieve adequate protection. Addition of an effective adjuvant or a switch from aluminum hydroxide (alum) – the most widely used adjuvant – helps enormously in boosting the immune response in such groups. Adjuvants have been used with a variety of vaccines to elicit safe, early, high, and long-lasting immune response. This has been clearly observed in aluminum adjuvants as compared to un-adjuvanted preparations [6, 52].

For the last decade, adjuvants have received much attention due to their ability to selectively improve the humoral immune response and stimulate the cellular immune response [7, 17, 47, 118]. Reports suggest this second role of adjuvants become increasingly important: guiding to the most effective adaptive response against each specific pathogen [68, 105]. Recent findings suggest that characterizing the immunostimulatory properties of an adjuvant will help in understanding and predicting the translational potential of a constrained or non-immunogenic vaccine into an efficacious one [104]. Besides generating strong antibody response (humoral response), they also act as immunomodulators by influencing the type and character of the antibody generated and help in seroconversion rates in the general population (including nonresponders, elderly, infants, and diseased) and generation of memory response [36, 76, 127] and rapid initial response, which is crucial during pandemic outbreaks of infections [37, 61, 69].

The choice of the adjuvant is of critical importance for the isotype and subclass of IgG, pattern of the cytokine production and recruitment of T cells. At times, it also modifies the antigen moieties in a controlled manner. Humoral response leads to the generation of IgG antibody subtypes, and increase their avidity and affinity. T cells recognize the antigen which is presented to it by antigen-presenting cells (APCs), through major histocompatibility complex (MHC) [89]. At this juncture where antigen recognition by T cell may get affected, adjuvants play an important role in modulating the response [47, 80], leading to elicit both T-helper cells and cytotoxic T lymphocytes (CTLs).

Adjuvants help in modulating the immune response, with regard to MHC class I or class II [16, 112]. MHC class I response is usually generated by an intracellular pathogen, e.g., virus, leading to cytotoxicity [97]. This type of response is not usually obscured by proteins or peptide antigens which elicit MHC class II response. Adjuvants like ISCOMS and QS-21 can elicit CTL with protein [97, 112] and peptides [7, 51].

Adjuvants also contribute in modulating the immune response to different T-helper cells as Th1 or Th2. This is of prime importance for generating a response against different types of microbes or organisms. For intracellular organisms, e.g., protozoa, viruses, invasive bacteria, and parasitic infections, Th1 type of immune response is accompanied by secretion of IL-2 and IFN-γ leading to cell-mediated immune response and production of relatively high levels of IgG2a antibodies. The Th1 type response activates CTLs which induce death of cells infected with intracellular pathogens and natural killer cells (NK), playing a major role in apoptosis in tumors and virus-infected cells. Th2 type immune response is modulated by IL-4 and IL-10 and is stimulated by extracellular organisms, e.g., helminthes or toxins, protein antigens, and inactivated pathogens. Th2 response leads to antibody generation like IgG1 and IgE [47]. Aluminum adjuvants are known to stimulate a Th2-type response [7, 17, 52, 53]. Recent reports indicate that the combination of one or more adjuvants helps in a dramatic shift from Th2-type to Th1-type response [92, 129]. It has also been suggested that the addition of one adjuvant to another may modulate the adaptive response of the immune system [88].

Safety Issues

The triggering of the immune response is required only up to a level of immunogenicity. Certain adjuvants have complex immunomodulatory activities [73, 74], and one of them is the enhancement of IgE production [10] which could turn out to be an unfavorable adjuvant effect. At times, triggering of immune response in the host can result in an unintended attack on the host itself to which the host may react to save itself, failing which it may end up in developing adverse side effects or deterioration. Therefore balancing the required and desirable effects of an adjuvant becomes the most important safety issue. The incorporation of an adjuvant in a vaccine has to be critically reviewed and observed so as to guide the immune response in the most desirable direction.

Since the safety of adjuvants is the biggest concern, particularly in neonatal and pediatric vaccines, criteria have been set out to ensure the safety of adjuvanted vaccines [26]. Guidelines have been laid down by European Societies for development of adjuvants defining the criteria of an ideal adjuvant [29]. It is important to observe the initiation, quality and magnitude of the immune response which is influenced by many nonspecific factors: important ones being the type and dose of the antigen, route of immunization, number of boosters, and shelf life of the vaccine as well as of the adjuvant. The type of the vaccine immensely affects the strategy of adjuvant administration, e.g., oral delivery, transcutaneous, or intranasal route [35, 43, 48, 84, 111]. In addition to safety with regard to local reactions, systemic reactions (general toxicity and pyrogenicity), hypersensitivity, autoimmune reactions, carcinogenicity, teratogenicity, etc. need to be evaluated at the time of development and production. Adjuvant should be chosen based on the type of immune response desired and formulated with the antigen in such a way that both are optimally distributed and presented to the relevant lymphatic tissue. The most commonly used adjuvant, alum, has apparently been reported to have adverse reactions, and some of these events could be because of apparent antigenicity of alum itself [30].

Properties for Safety

Adjuvants should be chemically defined so as to get the consistent reproducibility while manufacturing. The preparation should elicit a protective immune response with weak and conjugated antigens, with a minimum of antigen dosage and a reduced number of boosters. It should be effective in neonatal infants, in elderly and immunocompromised (diseased) individuals. Adjuvants should be stable, with a comparatively long shelf life and be biodegradable and nonimmunogenic by itself. It should be able to work in a synergistic manner when mixed with other adjuvants in a formulation. These are the factors that go in the making of an effective adjuvant – eliciting an immune response which is also safe.

Mechanisms of Action

Research during the past two decades has offered new insight into the mechanisms of action of vaccine-adjuvant formulations. Evidence suggests that adjuvants employ different mechanisms to elicit different immune responses: (1) sustained release of antigen at the site of injection, which is also called the depot effect; (2) recruitment of immune cells at the site of injection; (3) increased antigen uptake and presentation to APC; (4) activation and maturation of APCs, which leads to increased MHC class II and co-stimulatory molecules expression and migration to the draining lymph nodes; (5) upregulation of cytokines and chemokines; and (6) activation of inflammasomes [18, 33, 58].

Till now, it was understood that vaccine adjuvants stimulate only adaptive immune response, but recent advances have identified the basic role of the innate immune response programming the protective immune response in a better way [104]. It is now clear how the innate immune response stimulates the adaptive immune response.

Decades of research have been spent in search of good adjuvants that can enhance the immune response to the desired level without any side effect, but very few adjuvants have been licensed for human use. These include alum (aluminum salts), ASO4 (combination of monophosphoryl lipid A, i.e., TLR4 ligand, adsorbed to alum), and water-in-oil emulsion (MF59) [15, 83]. The dearth of adjuvants for human use probably can be fulfilled by understanding their in-depth mechanism of action.

The design of an adjuvant is the key that stimulates the class of immune response, such as antigen-specific helper T-cell subset, cytotoxic T cells, or long-term sustenance and memory T cells/B cells. These pathways of action depend on how successfully antigens mediate their immunogenicity through dendritic cells (DC) and pattern recognition receptors (PRRs). The major role of these PRRs is to recognize a large group of microorganisms through their molecular structures.

In the last decade, various new families of PRRs have been identified, including TLRs, nucleotide oligomerization domain (NOD)-like receptors (NLRs), C-type lectin-like receptors (CLRs), and Retinoic acid-inducible gene 1 (RIG-1)-like receptors (RLRs). Sometimes particulate adjuvants are not recognized by specific PRRs, but they still induce adaptive immune responses [9]. The "Danger Hypothesis" [85] proposed that danger signals from damaged cells can also trigger activation of the immune system, apart from self-/nonself-discrimination against infection. Damaged cells at the injection site release molecules associated such as uric acid, nucleotides, adenosine triphosphate (ATP), reactive oxygen intermediates, and cytokines [117]. These noninfectious damage signals are named as damage-associated molecular patterns (DAMPs) to distinguish them from pathogen-associated molecular patterns (PAMPs) [9].

Alum – composed of precipitates of aluminum hydroxide and aluminum phosphate, to which the antigen gets adsorbed – has been widely used in most of the human vaccines for the last 90 years. Despite the fact that it has many undesirable side effects, it is used as a benchmark [51, 125, 126]. The mechanism of action – how on emulsification with the antigen, alum enhances the Th2-biased response – remained a "dirty little secret" until recent past [64]. It was believed that alum generates a depot effect where the emulsion retains the antigen at the site of injection and releases it slowly, which leads to sustained antigen presentation. A little bit of the dirty secret was revealed when it was reported that alum induces antibody responses independent of TLR signaling [40]. Very recently, it was also reported that removal of the injection site two hours after antigen and alum administration had no effect on humoral- or cell-mediated immunity [62]. Furthermore, it also acts on cells essential for clonal expansion and production [65]. It is also reported that alum works through NLRP-3 inflammasomes [27, 71, 77] though it is still a matter of controversy (Fig. 1). MF-59, an oil-in-water emulsion, has been licensed for the influenza vaccine: whereas other adjuvants work by promoting the recruitment of APCs, MF-59 triggers CD11

b + cells, induces cytokines, cytokine receptor, and adhesion molecules. The microarray analysis has revealed that adjuvants work at gene level as MF-59, CpG DNA, and alum do induce a set of 168 genes at the site of immunization. Though the role of the inflammasome in adjuvant activity of MF-59 has also been evaluated [28, 115], yet the PRR activation remains unknown [88]. Similarly, another novel adjuvant, poly-[di-(sodium-carboxylato-ethyl-phenoxy)-phosphazene] (PCEP), induced stronger expression of adjuvant core response genes compared to CpG at the site of injection. Locally, PCEP-triggered production of proinflammatory cytokines and chemokines, like CCL2 [8].

Though innate immunity plays an important role in regulation of T-cell responses, evidence also emphasizes its importance in regulating the quality, function, magnitude, and persistence of the antibody response [100]. The quality of the antibody which protects against infection matters most despite high antibody titers. Therefore, again, an understanding of the mechanisms of innate immunity regulation for the most appropriate quality of antibody response will help in designing better adjuvants.

Another key factor is the persistence of antibody response. Some vaccines, such as carbohydrate or weak protein vaccines for children and elderly people, induce short-lived immunity. This has especially been demonstrated in some viral vaccines [110]. It is critical to enhance the persistence of such responses by internally stimulating memory cells, which mediate and facilitate in antibody-dependent cell-mediated cytotoxicity and induce an antibody response that lasts for several decades [16, 123]. A combination of adjuvants and vectors may be useful sometimes – in developing a synergistic specific response. This became clearly noticeable when a combination of antigen plus a TLR4 ligand, along with TLR7 ligand, induced synergistic antigen-specific neutralizing antibodies [67]. This mechanism could be possible due to the triggering of TLRs on a B cell and signaling via MyD88 and TRIF in DCs. These findings indicate that innate immunity regulates the magnitude, quality, and persistence of antibody response and generate memory B-cell development and persistence of plasma cells. Besides, there are certain molecules, such as CD40, CD95, BCL-6, IL-21, ICOS, BAFF, etc., known to enhance the number and survival of plasma cells [78]. Studies are in progress to use a combination of these molecules for enhanced results of memory response. There are certain adjuvants which work on this newly discovered trend in the mechanism of action via TLRs, the best example is ASO4 which works as a ligand through TLR4 because of monophosphoryl lipid A (MPL) and signals through TRIF [81]. Interestingly, LPS – from where MPL is derived – signals via both MyD88 and TRIF, which results in enhanced proinflammatory cytokines and toxicity. It is somewhat unanticipated to know that alum induces Th2 type of immune response, whereas ASO4, which has part of alum, induces Th1 type of immune response [23]. TLRs generally induce DCs to boost Th1 response, although it is also seen that mild TLR ligands induce Th2 or T-regulatory cell responses [105] and other Th17 responses [75]. Thus the design and selection of an effective and appropriate adjuvant also depends on and is influenced by the type of CD4 cells generated or required for protection.

We can infer that the precise mechanism of enhancing the protective response with adjuvant and at the same time antibodies protecting against pathogens, seems to prefer working via the innate immune system. An understanding of the basic levels and pathways of action of particular DC or specific PRRs will be helpful in designing adjuvants for the appropriate class of immune response. At higher levels, the immune response is also orchestrated by cell-to-cell interaction, e.g., between basophils and other immune cells and DCs [55, 93, 113, 124], confirming the action of adjuvants with multiple cell types; whereas at the highest level, finally cytokines and chemokines instruct the DCs to start functioning. It is also important to understand the mechanisms of different routes through which adjuvant may be administered – which must be able to reprogram the tissue-specific responses of DCs. The insight into the process of decision-making by DCs depends upon the various transcription factors, which remain to be fully understood.

Types of Adjuvants

Adjuvants represent different classes of compounds across the spectrum of products derived from microbes, oil emulsions, liposomes, microparticles, natural, and synthetic substances. These products function diversely and characterize different mechanisms of action [33, 54, 96]. Studies demonstrate two types of adjuvants based on their functional group – TLR-dependent and TLR-independent [40, 90]. TLR-dependent adjuvants act directly through DCs, causing them to migrate to the T-cell area of lymph nodes, in the process inducing the upregulation of MHC II, cytokines, chemokines, and other co-stimulatory molecules [33, 58], whereas TLRs independently induce inflammatory response, increase T-cell activation, and generate B-cell abundancy [40, 130].

Alum, a conventional adjuvant, provokes a strong Th2 response but ineffective against pathogens that require cell-mediated immune response. Furthermore, intraperitoneal administration of alum recruits monocytes at the site which uptakes the antigen and migrates to the draining lymph nodes and then differentiate into inflammatory DCs [71]. There is increase in Nalp3 inflammasome activation and production of IL1ß and IL-33 [77] which eventually leads to increased activation of immune cells (Fig. 1).

Jules Freund in the 1930s proved that an antigen contained in a water-in-oil emulsion markedly enhanced immune response [34, 57]. Known as Complete Freund's adjuvant (CFA), it is unusable for humans – with whole-killed mycobacteria in it. However, incomplete Freund's adjuvant (IFA), lacking mycobacteria, has been used as a potent adjuvant for a diverse number of vaccines [2, 19, 121]. Both Freund's adjuvants induce strong Th1 and Th17 responses, depending upon the mycobacterial components requiring host MyD88 or TRIF.

Dependent signaling pathways, independent of inflammasome [116, 122], vary with the experimental model [27, 40]. Emulsification of antigens with surfactant or paraffin oil alone without mycobacterium substantially boosts the antibody response. Although the mechanism of action is still unknown, one study suggested the requirement of NOD2 receptor [86]. It is a

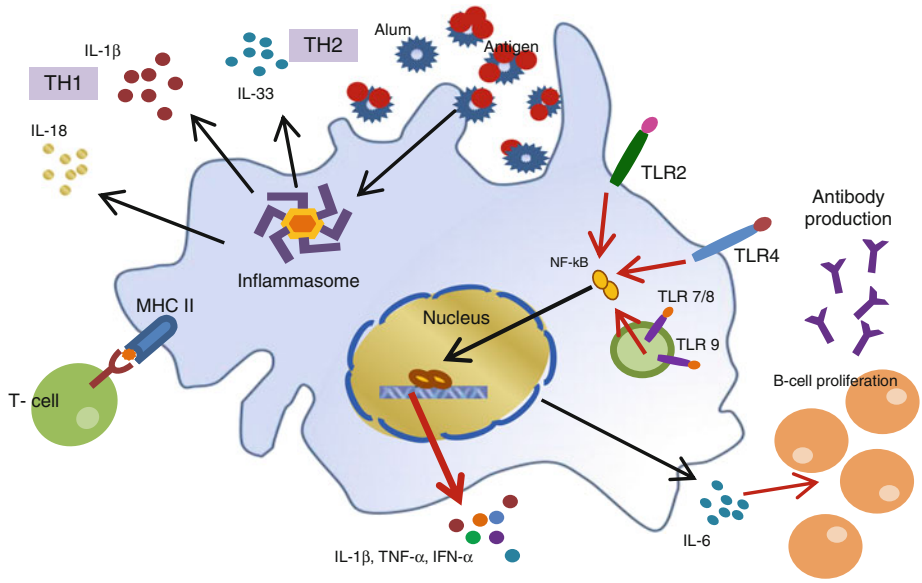

Fig. 1 Mechanism of induction of immune response by adjuvants

well-known fact that these emulsions upon injection cause cellular damage so it is speculated that necrotic cell death may be contributing to their adjuvant activity. Therefore, it becomes very important to select an appropriate adjuvant required for the protection.

MF59 a squalene-based oil-in-water emulsion has got approval for use with influenza vaccine. Squalene is an intermediate compound in the human steroid biosynthetic pathway and is precursor to cholesterol. Unlike alum, it induces increased CD8 response rather than CD4 T-cell response. Because of its ability to induce higher levels of hemagglutinin-inhibiting antibodies, it shows its potential to be used for influenza vaccines during pandemics [102, 107] along with similar ASO3 (GSK Biologicals), a 10% water-in-oil emulsion-based adjuvant, and both have been licensed for vaccines against pandemic influenza and widely used for 2009 influenza pandemic [88]. On comparison with other adjuvants like CPG and alum, MF59 showed the most efficient adjuvant activity by inducing maximum number of genes and rapid influx of CD11b + cells. Furthermore, it is a potent inducer of gene encoding, cytokine receptor, and cytokine adhesion molecules involved in leukocyte migration. Post-administration, it is internalized by APCs which migrate to the lymph nodes, eliciting efficient response [25].

Another oil/water emulsion adjuvant in use is MPL, a nontoxic derivative of LPS and a potent stimulator of Th1 response. It activates T-cell effector responses as part of HBV and HPV vaccines [13, 99]. In this category, another adjuvant giving rise to higher levels of specific antibody response with efficacy and fewer injections is ASO4, a combination of aqueous formulation of MPL and alum. Other forms of MPL-based adjuvant including ASO1B and ASO2A have undergone clinical trials for malaria [14].

Liposomes, the lipid membrane particles, LPS, lipid A, etc. can serve as vehicles or delivery systems for antigens [1, 3, 4]. Liposomes are usually made of biodegradable materials, e.g., phospholipids, and originally developed as carriers for drugs and biologically active substances. However, they have some limitations of causing sensitivity and instability. In addition, their high cost of manufacturing and scale up production limits their use. Instead, a superior liposome adjuvant referred as virosomes are prepared by inserting virus fusion protein into a liposome bilayer [45, 103]. These virosomes have proved to be potent adjuvants without any inflammatory reaction. They are membrane bound and serve to amplify fusogenic activity which facilitate the uptake by APC and induce a natural antigen-processing pathway. And this may be the reason why virosome-based vaccines stand out due to their excellent safety profile [44].

ISCOMS are particulate adjuvants composed of saponins purified from bark of the South American tree, *Quillaja saponaria*, formulated with cholesterol and phospholipids. This adjuvant doesn't act through PRRs, rather enhance antigen uptake and prolong retention by DCs in the draining lymph nodes, where DC activation leads to strong antibody and T-cell response [79]. They do not get biased about Th1 or Th2 cell response; at the same time, they induce CD8+ and CD4+ T-cell responses, both in human and animal vaccines [21].

Certain microspheres have also been evaluated as adjuvant with better targeting of antigen to APCs on mucosal surface or by reducing the number of doses by controlling the release of antigen. But there are still some issues related to antigen stability during encapsulation, hydration of microspheres, and their storage.

The discovery of novel plant compounds with immune system-modulating activities has become an increasingly important area of research, particularly in the search for new-generation vaccine adjuvants. Saponins are natural glycosides of steroid or triterpene, able to activate the mammalian immune system, which has led to significant interest in their potential as vaccine adjuvants. Their unique capacity to stimulate both the Th1 immune response and the production of CTLs against exogenous antigens makes them ideal for use in subunit vaccines and vaccines directed against intracellular pathogens as well as for therapeutic cancer vaccines. The most widely

used saponin-based adjuvants are Quil A and its derivatives QS-21. In some human vaccine designs, improving immunogenicity of a synthetic malaria peptide vaccine SPf66 was achieved by using SPf66/QS-21 formulation [66]. Adjuvants derived from *Ginseng saponins* (ginsenosides) – the active substances in the root of *Panax ginseng* – on bacterial antigens have shown a marked enhancing effect on vaccinating pigs against *Erysipelothrix rhusiopathiae* infection. The saponins from the root of *P. grandiflorum* increased a specific antibody and cellular response against ovalbumin in mice and could lead to the development of promising balanced Th1- and Th2- directing immunological adjuvants [131]. Advax™ adjuvant derived from inulin was evaluated with influenza vaccine to enhance immunogenicity and protection in mice [94].

The development of many other additional adjuvants is the result of the shortcomings of alum. Often, combinations of adjuvants in one formulation have yielded better results in a synergistic manner. Adjuvants like Montanides, MDP, etc. have been tried with various vaccines. An increasing number of experimental adjuvants are in developmental stages, such as squalene and phosphate adjuvants, QS21 [42, 128].

Realizing the need for a novel adjuvant, Defense Institute of Physiology and Allied Sciences (DIPAS), DRDO, India, has developed an adjuvant, DIP-HIP, derived from a high altitude medicinal plant, Seabuckthorn (*Hippophae rhamnoides* L. (SBT)). SBT, a unique and valuable plant from the family Elaeagnaceae, has recently gained worldwide attention, mainly for its medicinal and nutritional potentials [11, 38, 63, 95, 132]. In a comparative study, animals administered with DIP-HIP in formulation with different types of antigens – recombinant, conjugated, or native proteins, etc., evaluating an extensive range of different adjuvant systems – significantly and consistently enhanced the safe, stable, and efficacious antigen-specific response with minimum amount of antigen and reduced boosters with a long-term antibody sustenance. DIP-HIP was found to be consistently superior to alternative adjuvants when adjudged collectively by bioactive qualities, manufacturability, syringeability, stability, safety, and immunopotency criteria. This adjuvant can be produced as "point-of-use" by simple physical mixing procedure to generate an emulsion that is stable and homogenous. Alternatively, DIP-HIP has been produced in different batches stable at 2–8 °C for at least 3 years. The shelf life of DIP-HIP in the extract form and as formulation with antigens at 4 °C is quite reasonable.

A relative contribution of Th1/Th2 type of immune response was indicated by substantially enhanced titers of IgG1 and IgG2a antibody subtypes. The cytokine profile of IFN-γ and IL-4 correlated well with the Th1 and Th2 types of immune responses which are also supported by higher DTH response, indicating thereby the overall magnitude of humoral- and cell-mediated immune response generated by DIP-HIP and its ability to amplify both the arms of immune system. Interestingly, using different strains and species of animals, DIP-HIP responded equally well. Local tolerance evaluated by immunization through different routes like intramuscular or intraperitoneal using scoring systems, both macroscopically and histologically, did not show any variation nor caused any muscular damage, granulomatous reaction, or dystrophy. There was no hemolysis caused on treatment of both humans and animal erythrocytes with DIP-HIP. The herbal adjuvant developed by DIPAS is safe, effective, and comparable with commercially available adjuvants like CFA and alum. The product is being tested for commercial antigens to qualify for the license. The extract is in crude form and is being fractionated into various components using supercritical CO_2 extraction procedure followed by HPTLC and HPLC analyses. The bioactive fractions are being analyzed for their adjuvant activity.

Model Development

The most important part of adjuvant development is selection of a good model that can predict immune responses in humans. There are many limitations in developing a model relevant to

adjuvant development and evaluation. The most commonly used tools are activation of APCs *in vitro* and immunity in experimental animal models which could be inbred strains of mice, including knockout and transgenic mice. Despite many similarities, the immune responses of mice and humans differ in many aspects [41, 105] which lead to a failure of promising formulations in clinical confirmation trials. Different animal strains and species even behave differently to various adjuvants [56, 82]. In mouse studies, the route of immunization used is intraperitoneal or intravenous injections, whereas in humans, it is subcutaneous or intramuscular. It is understood that different routes affect activation of specific dendritic subsets. Sometimes, the choice of antigen itself can also affect the outcome of the study. Similarly, *in vitro* studies using blood cells have limitations in evaluating the adjuvant. Cell cultures are not reliable for studying the vaccine-adjuvant formulations that rely on inflammatory cytokine responses from noncirculating tissue cells. An adjuvant acts in part by altering the distribution of antigen or its presentation at the injection site, which may not give confirmatory results in cell culture. These differences pose an obstacle in rapid translation of findings from mice to humans or cell culture to humans. Some time ago, guinea pigs and rabbits were suggested to be better models for evaluating the adjuvanticity and toxicity of adjuvant formulations [46, 119].

To avoid these shortcomings, the possible approaches could be to use humanized mice or develop mice that have similar cell-specific expressions or finally use nonhuman primates. But all these approaches are very costly for maintaining such animals and cell culture conditions, which often limit the interest of development of a good adjuvant. Therefore, if one has to follow the traditional way of evaluating the adjuvanticity, then it is recommended that the evaluation may be performed in at least two strains of mice with different haplotypes or guinea pigs and/or rats for studying the immunological effect of adjuvant and its effective adjuvanticity.

Therefore, the present approach of systems biology has been considered of utmost importance to focus on the study of vaccine adjuvant formulations in humans [39, 106]. A consortium has been set up to analyze the human immune responses to vaccine formulations and infections by high-throughput approaches. This approach will benefit in systemically understanding and characterizing the immune response in humans to accelerate the studies in the field of human biology and vaccinology.

The age group distribution of the world population gives a deep insight into the vaccine adjuvant-induced immunity problems in humans, e.g., in the very young and very old, the immunocompromised, or the diseased. Therefore, whenever a new adjuvant is developed, there is a need to reexamine and reevaluate the clinical trials in which multiple parameters of both innate and adaptive immune responses can be evaluated by cutting-edge technologies [39, 105, 106]. Thus, systems biology is a needed and powerful modeling approach.

Novel Adjuvants and Approaches

Three-dimensional virtual screening, whereby a large number of small molecules are docked into the three-dimensional model of a protein receptor, is an important tool in the field of drug discovery and optimization. The identification of potential lead compounds from databases of small molecules significantly reduces the time spent on experimental screening and is therefore now an integral part of drug design. Within vaccinology, structure-based virtual screening is an approach of unprecedented power and scope.

Using virtual screening, CCR4 antagonists have been identified that act as adjuvants for both cellular and humoral immune responses. These molecules were tested *in vivo* with vaccines in mice; enhanced immunogenicity was observed with SP50. The enhancing effects observed in these experiments are particularly striking given that the vaccine vectors employed are known to be intrinsically immunogenic [20].

Cyclic-diguanylate (c-di-GMP), a bacterial signaling molecule, possesses protective immunostimulatory activity. In a study, c-di-GMP was evaluated as a vaccine adjuvant and compared to LPS, CpG oligonucleotides, and a conventional aluminum salt-based adjuvant. It elicited a more potent activator of both humoral- and Th1-like immune responses as evidenced by the robust IgG2a antibody response and the strong IFN-γ, TNF-α, and IP-10 responses in mice and *in vitro* in nonhuman primate peripheral blood mononuclear cells. Further, compared to LPS or CpG, c-di-GMP demonstrated a more pronounced ability to induce germinal center formation, a hallmark of long-term memory, in immunized mice. Together, these data add to the growing body of evidence supporting the utility of c-di-GMP as an adjuvant in vaccination for sustained and robust immune responses and provide a rationale for further evaluation in appropriate models of immunization [24].

Receptor-targeted small molecule adjuvants (SMA) are among the most under-explored type of immunomodulatory adjuvants. Examples include imidazoquinolines (Imiquimod and Resiquimod) which target toll-like receptors (TLRs) (specifically TLR-7 and TLR-8) and were developed as nucleoside analogues for antiviral or antitumor therapy; Bestatin, a tumor adjuvant acting as an inhibitor of aminopeptidase N [CD13]; and Levamisole and Bupivacaine (DNA vaccine adjuvants). Other examples of non-macromolecular adjuvants include monophosphoryl-lipid A, muramyl dipeptide, QS21, PLG, Seppic ISA-51, and CpG oligonucleotides. Optimized CpG oligonucleotides, which target TLR-9, are now entering late phase trials as adjuvants for the poorly immunogenic hepatitis B vaccine [22].

Evaluation of Novel Adjuvants and Delivery Systems

The use of repository adjuvants like mineral salts is accompanied by the formation of an inflammatory focus at the site of injection which may lead to the synthesis of proinflammatory cytokines and stimulation of innate immunity important for the initial steps of the immune response.

Quality evaluation of a vaccine-adjuvant formulation therefore covers aspects such as demonstration of the compatibility of the adjuvant(s) with the antigenic component(s) present in the vaccine, proof of an adequate and consistent association of the antigen with the adjuvant, demonstration that no significant de-association takes place in the course of the shelf life, degree of association throughout the shelf life, and effect of the adjuvant on the ability to assay components, biochemical purity, and pyrogenicity. As an example of association, adsorption is specific for aluminum hydroxide gels, aluminum phosphate gels, calcium phosphate gels, and ISCOMS, while ionic interaction occurs with charged dimethyldioctadecylammonium (DDA) micelles. For emulsions or liposomes, the mechanism is encapsulation. With saponin derivatives or other extracts, interactions with antigens are lipophilic/hydrophilic or ionic [29].

Adjuvants alone are evaluated for their local tolerance (inflammation, consideration of route of administration), induction of hypersensitivity and anaphylaxis (IgE antibody), antigen species (rabbit), pyrogenicity, validated *in vitro* models, systemic toxicity to tissues/organs (histopathology), pharmacodynamic studies (adsorption/elimination from tissues), and reproductive toxicity (reflecting intended schedule of use).

Regulatory Issues

The response generated by antigen-adjuvant formulations is vaccine specific and multifactorial. Thus, data cannot even be interpolated to another distinct antigen, a similar antigen with the same adjuvant or even the same antigen-adjuvant formulation administered via a different route. Therefore, the regulatory guidelines for issuing a license for one formulation would be inapplicable for other formulations [114]. This makes the task for the license-issuing authorities more difficult and vulnerable to wrong application of guidelines. However, the Center for Biologics Evaluation and Research (CBER)

of the US FDA has developed a new regulatory approach to vaccine adjuvants and adjuvanted preventive vaccines for infectious disease indications. The Committee for Medicinal Products for Human Use, of the European Medicines Evaluation Agency (EMEA), has also published a guide on similar issues [29].

Safety and tolerability are two critical considerations impacting early introduction of new adjuvants for human use and pose the greatest barrier to regulatory approvals for new adjuvants. Use of novel adjuvants demands extensive preclinical studies, including local reactogenicity and systemic toxicity testing, because vaccines are administered to healthy individuals including infants and children, and there are potential safety concerns. WHO has also suggested nonclinical evaluations to help in proceeding with the clinical development of new adjuvant-antigen formulations (WHO Guidelines on Nonclinical Evaluation of Vaccines, 2003). Applying for a license approval for any new adjuvant or vaccine development falls in the category of "Investigational New Drug Application," and it becomes mandatory to furnish a full report on the parameters evaluated – including its toxicology, pharmacology, biochemistry, and comparison with other similar adjuvanted and non-adjuvanted vaccines – and any incidental or clinical data obtained on human use with full justification – which will add value to the existing adjuvant, etc. All the clinical data should be fully justified.

Sometimes, the pressure of a looming pandemic also helps in streamlining the licensure mechanisms, which happened in the case of avian flu H5N1 influenza vaccine [32]. Sometimes, separate guidelines are set for frequent pandemic vaccines such as pandemic influenza vaccines, which advice clinical development approaches to help in expediting the licensing procedures. For more benefit of the society, for value addition of these pandemic vaccines, certain approaches have been demonstrated and briefly described in two FDA guidance documents [49, 50].

Presently, studies on specific projects on new adjuvant and their dose optimization are given much attention. Many funding agencies are releasing substantial amount of funds in developing the new adjuvants. The matter is being taken seriously as a global issue, more so for basic research on pandemic diseases and early product development of novel adjuvants, including both immunopotentiators and delivery systems. Research should be focused on the rational design of an adjuvant, founded upon a clear understanding of its mode of action on innate immune system, thus allowing for the enhancement of beneficial aspects and reduction of toxic side effects.

With the goal of new adjuvant discovery and development, multiple contracts for "Innate Immune Receptors and Adjuvant Discovery" have been awarded, including Development of Vaccines, Adjuvants, Immunotherapeutics, and Diagnostics for Biodefense, under "Adjuvant Development Program." This program also includes the designing of special units to assure availability of experienced individuals for rapid evaluation of new vaccines and novel adjuvant and use of high-throughput library screens. Other programs have encouraged both public and private sectors to participate in vaccine and adjuvant research in high-priority areas to help combat a wide variety of complex diseases. Such programs develop the strategies for dose reduction [31, 72, 91] using various appropriate technologies for enhancing the immunogenicity and effectiveness of vaccine-adjuvant formulations in collaboration with various industries and academia for use in the developing world and mass campaigns and in disseminating knowledge in this field to the scientific community [59, 60, 120].

Future

Advances in the newer technologies like recombinant DNA, genomics, proteomics, metabolomics, etc. have speeded up the synthesis of newer vaccines, but at the same time demand a need for improved and well-characterized adjuvants and formulations. There is more emphasis on vaccines for neonates and

immunocompromized individuals for a better understanding of mechanism of action of adjuvant and consequent immune response.

Several barriers have to overcome to meet the requirement of development of newer adjuvants. The first and foremost concern is the side effects and toxicity, particularly with the pediatric vaccines. In addition, the regulatory issues for approval of adjuvant have substantially raised the concerns. Adjuvants do not get FDA clearance as stand-alone compounds/products, but as a formulation of a registered vaccine adjuvant – antigen formulation. But this requires huge cost and effort for trying various combinations of untested antigens and adjuvants. Moreover, new antigen-adjuvant formulations become proprietary until the adjuvant evaluated is registered. All these regulations limit the development of new adjuvants and thus lead to slow development of novel adjuvants. The strategies to accelerate the development of adjuvants would be to create an organization for standardized evaluation of vaccine-adjuvant formulations capable of inducing safe, strong, stable, and long-lasting humoral and cellular immune responses, with a reduced number of protective boosters, for humans.

While investigating the mechanisms, contribution of TLR pathway has been significant in understanding how and why adjuvant used during vaccinations are important in augmenting adaptive immune responses to specific vaccine antigen. However, with the upcoming knowledge and the research findings, it is gathered that TLR activation is not necessarily required for enhancing the immunogenicity by adjuvant. Therefore, it can be concluded that there could be other receptors besides TLRs that have not yet been characterized, thus opening the door to future research.

Finally, development of candidate adjuvant should focus more on establishing easy, modular, reproducible, stable, and transferable standard operating procedures and fingerprint records for processing and production, which eventually will allow sustainable formulations with long shelf lives without major changes in large-scale manufacturing and sourcing.

Conclusion

Learning how to enhance the persistence of immune responses with relevant adjuvants is critical. Recent in-depth research related to the possible mechanisms of action of few adjuvants has significantly progressed our understanding of the immunology and biochemistry of these adjuvants. However, does the new research explain the mechanism of action of clinically approved adjuvant? Probably the answer is "no," as the appropriate experiments performed particularly in humans do not give the clear indications, be it individual variation or behavior of adjuvant in humans. Moreover, advances in technologies have accelerated the development of newer vaccines which has increased the need for improved adjuvant and formulations beyond those currently available.

The foregoing considerations exigently lead to development of new adjuvants which can selectively modulate the immune response to the desired level and type. An adjuvant with potent properties is required for eliciting both – humoral and cell mediated – immune responses against all types of antigens, such as purified, subunit, combination, or synthetic vaccines. In addition, an adjuvant that helps in enhancing plasma cells, leading to generation of antigen-specific B-cell memory response, is a useful objective. This leads to the fact that the magnitude, quality, type, and persistence of antibody response are regulated by innate immunity. The intracellular signaling pathway, and transcription factors that control the innate immunity, are concepts currently under appreciation and investigation.

Incorporating the foregoing essential aspects, many new adjuvants are in the research pipeline. Further, based on preclinical and clinical observations, a number of adjuvants with some of the above properties are expected in the coming years.

References

1. Alving CR (1993) Lipopolysaccharide, lipid A and liposomes containing lipid A as immunologic adjuvants. Immunobiol 187:430–446

2. Alving CR (2002) Design and selection of vaccine adjuvants: animal models and human trials. Vaccine 20:S56–S64
3. Alving CR et al (1993) Novel adjuvant strategies for experimental malaria and AIDS vaccines. Ann N Y Acad Sci 690:265–275
4. Alving CR, Glass M, Detrick B (1992) Summary: adjuvants/clinical trials working group. AIDS Res Hum Retroviruses 8:1427–1430
5. Alving CR, Koulchin V, Glenn GM, Rao M (1995) Liposomes as carriers of peptide antigens: induction of antibodies and cytotoxic T lymphocytes to conjugated and unconjugated peptides. Immunol Rev 145:5–31
6. Aprile MA, Wardlaw AC (1966) Aluminum compounds as adjuvants for vaccines and toxoids in man: a review. Can J Public Health 57(8):343–360
7. Audibert FM, Lise LD (1993) Adjuvants: current status, clinical perspectives and future prospects. Trends Pharmacol Sci 14:174–178
8. Awate S et al (2012) Activation of adjuvant core response genes by the novel adjuvant PCEP. Mol Immunol 51:292–303
9. Awate S, Babiuk A, Mutwiri G (2013) Mechanisms of action of adjuvants. Front Immunol 4(114):1–10. doi:10.3389/fimmu.2013.00114
10. Baylor NW, Egan W, Richman P (2002) Aluminum salts in vaccines- US perspective. Vaccine 20: S18–S23
11. Beveridge T, Li TS, Oomah BD, Smith A (1999) Sea buckthorn products : manufacture and composition. J Agric Food Chem 47(9):3480–3488
12. Bode C, Zhao G, Steinhagen F et al (2011) CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 10(4):499–511
13. Bojang KA et al (2001) Efficacy of RTS, S/AS02 malaria vaccine against Plasmodium falciparum infection in semi-immune adult men in The Gambia: a randomised trial. The Lancet 358:1927–1934
14. Bojang KA et al (2005) Safety and immunogenicity of RTS, S/AS02A candidate malaria vaccine in Gambian children. Vaccine 23:4148–4157
15. Coffman RL, Sher A, Seder RA (2010) Vaccine adjuvants: putting innate immunity to work. Immunity 33:492–503
16. Cooper MA et al (2009) Cytokine induced memory-like natural killer cells. Proc Natl Acad Sci U S A 106:1915–1919
17. Cooper PD (1994) The selective induction of different immune responses by vaccine adjuvants. In: Ada GL (ed) Strategies in vaccine design. Landes, Austin, pp 125–158
18. Cox JC, Coulter AR (1997) Adjuvants-a classification and review of their modes of action. Vaccine 15:248–256
19. Davenport FM (1968) Seventeen years experience with mineral oil adjuvant influenza virus vaccines. Ann Allergy 26:288–292
20. Davies MN et al (2009) Toward the discovery of vaccine adjuvants: coupling *in silico* screening and *in vitro* analysis of antagonist binding to human and mouse CCR4 receptors. PLoS One 4(11):e8084: 1-12. PMCID-PMC2787246
21. Davis ID et al (2004) Recombinant NY-ESO-1protein with ISCOMATRIX adjuvant induces broad integrated antibody and CD4+ and CD8+ T cell responses in humans. Proc Natl Acad Sci U S A 101:10697–10702
22. De Gregorio E, Caproni E, Ulmer JB (2013) Vaccine adjuvant: mode of action. Front Immunol 4:214. doi:10.3389/fimmu.2013.00214. PMCID: PMC3728558
23. Didierlaurent AM et al (2009) AS04, an aluminum salt-and TLR4 agonist- based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. J Immunol 183:6186–6197
24. Dubensky TW, Kanne DB Jr, Leong ML (2013) Rational, progress and development of vaccine utilizing STING- activating cyclic dinucleotide adjuvants. Ther Adv Vaccines 1(4):131–143. doi:10.1177/2051013613501988
25. Dupuis M et al (2001) Immunization with the adjuvant MF59 induces macrophage trafficking and apoptosis. Eur J Immunol 31:2910–2918
26. Edelman R (1980) Vaccine adjuvants. Rev Infect Dis 2:370–383
27. Eisenbarth SC et al (2008) Crucial role for the Nalp3 inflammasome in the immunostimulatory properties of aluminum adjuvants. Nature 453:1122–1126
28. Ellebedy AH et al (2011) Inflammasome-independent role of the apoptosis-associated speck-like protein containing CARD (ASC) in the adjuvant effect of MF59. Proc Natl Acad Sci U S A 108:2927–2932
29. EMEA- Guidelines on adjuvants in vaccine for human use, 1/2005 and explanatory note on immunomodulators, 7/2006. Accessed at http://www.emea.europa.eu/pdfs/human/vwp/13471604en.pdf
30. Exley C et al (2009) A role for the body burden of aluminum in vaccine – associated macrophagic myofasciitis and chronic fatigue syndrome. Med Hypotheses 72:135–139
31. Fernandez J et al (2000) Randomized trial of the immunogenicity of fractional dose regimens of PRP-T Haemophilus influenzae type b conjugate vaccine. Am J Trop Med Hyg 62(4):485–490
32. Focetria®, the Novartis pandemic influenza vaccine, receives European union approval, 2007. Accessed 12th Oct 2007, at http://novartisvaccine.com/press-room/news/20070508focetria.shtml
33. Fraser CK et al (2007) Improving vaccines by incorporating immunological coadjuvants. Expert Rev Vaccines 6:559–578
34. Freund J (1956) The mode of action of immunologic adjuvants. Bibl Tuberc 10:130–148
35. Freytag LC, Clements JD (2005) Mucosal adjuvants. Vaccine 23:1804–1813
36. Galli G et al (2009) Adjuvanted H5N1 vaccine induces early CD4+ T cell response that predicts

long-term persistence of protective antibody levels. Proc Natl Acad Sci U S A 106:3877–3882
37. Galli G, Hancock K, Hoschler K, DeVos J et al (2009) Fast rise of broadly cross-reactive antibodies after boosting long-lived human memory B cells primed by an MF59 adjuvanted prepandemic vaccine. Proc Natl Acad Sci 106:7962–7967
38. Ganju L et al (2005) Anti-inflammatory activity of Seabuckthorn (*Hippophae rhamnoides*) leaves. Int Immunopharmacol 5:1675–1684
39. Gaucher D et al (2008) Yellow fever vaccine induces integrated multilineage and polyfunctional immune responses. J Exp Med 205:3119–3131
40. Gavin AL et al (2006) Adjuvant-enhanced antibody responses in the absence of toll-like receptor signaling. Science 314:1936–1938
41. Germain RN (2010) Vaccines and the future of human immunology. Immunity 33:441–450
42. Ghochikyan A et al (2006) Prototype Alzheimer's disease epitope vaccine induced strong Th2-type anti-Aβ antibody response with Alum to Quil A adjuvant switch. Vaccine 24(13):2275–2282. doi:10.1016/j.vaccine.2005.11.039.PMC 2081151. PMID: 16368167
43. Glenn GM, Taylor DN, Lix FS et al (2000) Transcutaneous immunization a human vaccine delivery strategy using a patch. Nat Med 6:1403–1406
44. Gluck R (1999) Adjuvant activity of immunopotentiating reconstituted influenza virosomes (IRIVs). Vaccine 17(13–14):1782–1787
45. Glück R, Mischler R, Brantschen S et al (1992) Immunopotentiating reconstituted influenza virus virosome vaccine delivery system for immunization against hepatitis A. J Clin Invest 90(6):2491–2495. doi:10.1172/JCI116141
46. Goldenthal K et al (1993) Safety evaluation of vaccine adjuvants. NCVDG meeting working group. AIDS Res Hum Retroviruses 9:S47–S51
47. Golding B (1991) Cytokine regulation of humoral immune responses. In: Sprigs DR, Koff WC (eds) Topics in vaccine adjuvant research. CRC press, Boca Raton, pp 25–37
48. Gonzalez AM, Nguyen TV, Azevedo MSP et al (2004) Antibody response to human rotavirus (HRV) in gnotobiotic pigs following a new prime/vaccine boost strategy using oral attenuated HRV priming and intranasal VP2/6 rotavirus like particle (VLP) boosting with ISCOM. Clin Exp Immunol 135:361–372
49. Guidance for Industry – Clinical Data Needed to Support the Licensure of Pandemic Influenza Vaccines (2007a). Accessed at http://www.fda.gov/cber/gdlns/panfluvac.pdf
50. Guidance for Industry – Clinical Data Needed to Support the Licensure of Trivalent Inactivated Influenza Vaccines (2007b). Accessed at http://www.fda.gov/cber/gdlns/trifluvac.pdf
51. Gupta RK et al (1993) Adjuvants- a balance between toxicity and adjuvanticity. Vaccine 11(3):293–306
52. Gupta RK, Rost BE, Relyveld E, Siber GR (1995) Adjuvant properties of aluminum and calcium compounds. In: Powell MF, Newman MJ (eds) Vaccine design: the subunit and adjuvant approach. Plenum Press, New York, pp 229–248
53. Gupta RK, Siber GR (1995) Adjuvants for human vaccines-current status, problems and future prospects. Vaccine 13:1263–1276
54. Guy B (2007) The perfect mix: recent progress in adjuvant research. Nat Rev Microbiol 5:505–517
55. Hammad H et al (2010) Inflammatory dendritic cells–not basophils–are necessary and sufficient for induction of Th2 immunity to inhaled house dust mite allergen. J Exp Med 207:2097–2111
56. Hardegree MC, Pittman M, Maloney CJ (1972) Influence of mouse strain on the assayed potency (Unitage) of tetanus toxoid. Appl Microbiol 24(1):120–126
57. Hilleman MR (1966) Critical appraisal of emulsified oil adjuvants applied to viral vaccine. Prog Med Virol 8:131–182
58. Hoebe K, Janssen E, Beutler BF (2004) The interface between innate and adaptive immunity. Nat Immunol 5:971–974
59. Hoelscher MA, Garg S, Bangari DS et al (2006) Development of adenoviral-vector-based pandemic influenza vaccine against antigenically distinct human H5N1 strains in mice. Lancet 367 (9509):475–481
60. Hoelscher MA, Jayashankar L, Garg S et al (2007) New pre-pandemic influenza vaccines: an egg- and adjuvant-independent human adenoviral vector strategy induces long-lasting protective immune responses in mice. Clin Pharmacol Ther 82(6):665–671
61. Huleatt JW, Jacobs AR, Tang J et al (2007) Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 25:763–775
62. Hutchison S et al (2012) Antigen depot is not required for alum adjuvanticity. The FASEB J 26:1272–1279
63. Jain M et al (2008) Effect of Hippophae rhamnoides leaf extract against Dengue virus infection in human blood–derived macrophages. Phytomedicine 15(10):793–799. doi:10.1016/J.phymed.2008.04.017, Epub 2008 June 30
64. Janeway CA Jr (1989) Approaching the asymptote? Evolution and revolution in immunology. Cold Spring Harb Symp Quant Biol 54:1–13
65. Jordan MB et al (2004) Promotion of B cell immune responses via an alum-induced myeloid cell population. Science 304:1808–1810
66. Kashala O et al (2002) Safety, tolerability and immunogenicity of new formulations of the Plasmodium falciparum malaria peptide vaccine SPf66 combined with the immunological adjuvant QS-21. Vaccine 20(17–18):2263–2277
67. Kasturi SP et al (2011) Programming the magnitude and persistence of antibody responses with innate immunity. Nature 470:543–547

68. Kenney RT, Cross AS (2010) Adjuvants for the future. In: Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R (eds) New generation vaccines. Informa Healthcare USA, Inc., New York, pp 250–262
69. Khurana S, Chearwae W, Castellino F et al (2010) Vaccines with MF59 adjuvant expand the antibody repertoire to target protective sites of pandemic avian H5N1 influenza virus. Sci Transl Med 2:15ra15–15ra15
70. Klinman DM (2003) CpG DNA as a vaccine adjuvant. Expert Rev Vaccines 2(2):305–315
71. Kool M, Soullie T, van Nimwegen M et al (2008) Alum adjuvant boosts adaptive immunity by inducing uric acid and activating inflammatory dendritic cells. J Exp Med 205:869–882
72. Lagos R, Valenzuela MT, Levine OS et al (1998) Economisation of vaccination against Haemophilus influenzae type b: a randomised trial of immunogenicity of fractional-dose and two-dose regimens. The Lancet 351(9114):1472–1476
73. Lavelle EC, Jarnicki A, McNeela E et al (2004) Effects of cholera toxin on innate and adaptive immunity and its application as an immunomodulatory agent. J Leukoc Biol 75:756–763
74. Lavelle EC, McGuipk P, Mills KHG (2004) Molecules of infectious agents as immunomodulatory drugs. Curr Top Med Chem 4:499–508
75. Leibundgut-Landmann S, Groβ O, Robinson MJ et al (2007) Syk-and CARD9-dependent coupling of innate immunity to the induction of T helper cells that produce IL17. Nat Immunol 8:630–638. Pubmed:17450144
76. Leroux-Roles I, Roman F, Forgus S et al (2010) Priming with AS03 A-adjuvanted H5N1 influenza vaccine improves the kinetics, magnitude and durability of the immune response after a heterologous booster vaccination: an open non-randomised extension of a double- blind randomised primary study. Vaccine 28:849–857
77. Li H, Willingham SB, Ting JP, Re F (2008) Cutting edge: inflammasome activation by alum and alum's adjuvant effect are mediated by NLRP3. J Immunol 181:17–21
78. Mackay F, Schneider P (2009) Cracking the BAFF code. Nat Rev Immunol 9:491–502
79. Maraskovsky E, Schnurr M, Wilson NS et al (2009) Development of prophylactic and therapeutic vaccines using the ISCOMATRIX adjuvant. Immunol & Cell Biol 87:371–376
80. Martin S, Daniel SL, Rouse BT (1991) Cytokines and regulation of cellular immune responses to viruses. In: Spriggs DR, Koff WC (eds) Topics in vaccine adjuvant research. CRC Press, Boca Raton, pp 39–50
81. Mata-Haro V, Cekic C, Martin M et al (2007) The vaccine adjuvant Monophosphoryl lipid A as a TRIF- biased agonist of TLR 4. Science 316:1628–1632
82. Matsuhasi T (1991) Influence of mouse strain on the results of potency test of diphtheria and tetanus toxoid components of D, T, DT and DPT vaccines. In: Manclark CR (ed) Proceeding of an informal consultation on the World Health Organization Requirements for diphtheria, tetanus, pertussis and combined vaccine. Department of Health and Human Services, United States Public Health Service, Bethesda. DHHS Publication No. (FDA) 91-1174, pp 55–58
83. Valiante NM et al. (2010) New adjuvants for human vaccines. Curr Opin Immunol 22:411–416
84. McGhe JR, Mestecky J, Dertzbaugh MT et al (1992) The mucosal immune system from fundamental concepts to vaccine development. Vaccine 10:75–88
85. Metzinger P (1994) Tolerance, danger and the extended family. Annu Rev Immunol 12:991–1045
86. Moreira LO, Smith AM, Defreitas AA et al (2008) Modulation of adaptive immunity by different adjuvant- antigen combinations in mice lacking Nod2. Vaccine 26:5808–5813
87. Morel S, Didierlaurent A, Bourguignon P et al (2011) Adjuvant system AS03 containing α-tocopherol modulates innate immune response and leads to improved adaptive immunity. Vaccine 29 (13):2461–2473
88. Mosca F et al (2008) Molecular and cellular signatures of human vaccine adjuvant. Proc Natl Acad Sci U S A 105:10501–10506
89. Neefjes JJ, Schumacher TNM, Ploegh HL (1991) Assembly and intracellular transport of major histocompatibility complex molecules. Curr Opin Cell Biol 3:601–609
90. Nemazee D, Gavin A, Hoebe K, Beutler B (2006) Immunology: toll like receptors and antibody responses. Nature 441:E4. doi:10.1038/nature04875
91. Neuzil KM, Jackson LA, Nelson J et al (2006) Immunogenicity and reactogenicity of 1 versus 2 doses of trivalent inactivated influenza vaccine in vaccine-naive 5-8-year-old children. J Infect Dis 194(8):1032–1039
92. O'Hagan DT, Ott GS, De Gregorio E, Seubert A (2012) The mechanism of action of MF59 – an innately attractive adjuvant formulation. Vaccine 30(29):4341–4348
93. Ohnmacht C et al (2010) Basophils orchestrate chronic allergic dermatitis and protective immunity against helminths. Immunity 33:364–374
94. Okubo HY, Saade F, Petrovsky N (2012) Advax™, a polysaccharide adjuvant derived from delta inulin, provides improved influenza vaccine protection through broad-based enhancement of adaptive immune responses. Vaccine 30(36):5373–5381
95. Padwad Y, Ganju L, Jain M et al (2006) Effect of leaf extract of Seabuckthorn on lipopolysaccharide induced inflammatory response in murine macrophages. Int Immunopharmacol 6(1):46–52

96. Pashine A, Valiante NM, Ulmer JB (2005) Targeting the innate immune response with improved vaccine adjuvant adjuvants. Nat Med 11(4):S63–S68
97. Paul WE (2003) Antigen processing and presentation. In: Paul WE (ed) Fundamental immunology, 5th edn. Lippincott Williams & Wilkins Publishers, Philadelphia
98. Petrovsky N, Aguilar JC (2004) Vaccine adjuvants: current state and future trends. Immunol & Cell Biol 82:488–496
99. Pichyangkul S, Gettayacamin M, Miller RS et al (2004) Pre-clinical evaluation of the malaria vaccine candidate P. falciparum MSP1(42) formulated with novel adjuvants or with alum. Vaccine 22:3831–3840
100. Plotkin SA (2008) Vaccines: correlates of vaccine –induced immunity. Clin Infect Dis 47:401–409
101. Plotkin SA (2010) Correlates of protection induced by vaccination. Clin Vaccine Immunol 17:1055–1065
102. Podda A, Del Giudice G (2003) MF59-adjuvanted vaccines: increased immunogenicity with an optimal safety profile. Expert Rev Vaccines 2:197–203
103. Poovorawan Y, Theamboonlers A, Chumdermpadetsuk S et al (1995) Safety, immunogenicity, and kinetics of the immune response to a single dose of virosome-formulated hepatitis A vaccine in Thais. Vaccine 13(10):891–893
104. Pulendran B, Ahmed R (2011) Immunological mechanisms of vaccination. Nat Immunol 131 (6):509–517
105. Pulendran B, Powell J, Flavell RA (2010) Modulating vaccine responses with innate immunity. In: Levine MM, Dougan G, Good MF, Liu MA, Nabel GJ, Nataro JP, Rappuoli R (eds) New generation vaccines. Informa Healthcare USA, Inc., New York, pp 183–190
106. Querec TD, Akondy RS, Lee EK et al (2009) Systems biology approach predicts immunogenicity of the yellow fever vaccine in humans. Nat Immunol 10:116–125
107. Radošević K, Rodriguez A, Mintardjo R et al (2008) Antibody and T-cell responses to a virosomal adjuvanted H9N2 avian influenza vaccine: impact of distinct additional adjuvants. Vaccine 26:3640–3646
108. Ramon G (1924) Sur la toxine et sur I' anatoxine diphteriques. Ann Inst Pasteur 38:1–10
109. Reed SG, Bertholet S, Coler RN et al (2009) New horizon in adjuvants for vaccine development. Trends Immunol 30(1):23–32
110. Rerks-Ngarm S, Pitisuttithum P, Nitayaphan S et al (2009) Vaccination with ALVAC and AIDSVAX to prevent HIV infection in Thailand. N Engl J Med 361:2209–2220
111. Rimoldi M, Rescigno M (2005) Uptake and presentation of orally administered antigens. Vaccine 23:1793–1796
112. Rock KL (1996) A new foreign policy MHC class I molecules, monitor the outside world. Immunol Today 17:130–137
113. Saenz SA, Noti M, Artis D (2010) Innate immune cell populations function as initiators and effectors in Th2 cytokine responses. Trends Immunol 31:407–413
114. Sesardic D, Dobbelaer R (2004) European Union regulatory developments for new vaccine adjuvants and delivery systems. Vaccine 22(19):2452–2456
115. Seubert A et al (2011) Adjuvanticity of the oil-in-water emulsion MF59 is independent of Nlrp3 inflammasome but requires the adaptor protein MyD88. Proc Natl Acad Sci U S A 108:11169–11174
116. Shenderov K (2010) Inflammasome-dependent IL-1 {beta} production is critical for complete Freund's adjuvant-induced helper T cell polarization. J Immunol 184:136–144
117. Shi Y, Evans IE, Rock KL (2003) Molecular identification of danger signal that alerts the immune system to dying cells. Nature 425:516–521
118. Spriggs DR, Koff WC (1991) Topics in vaccine adjuvant research. CRC Press, pp 119–136
119. Stewart-Tull DES (1989) Recommendations for the assessment of adjuvants (immunopotentiators). In: Gregoriadis G, Allison AC, Poste G (eds) Immunological adjuvants and vaccines. Springer, pp 213–226
120. Stout R, Gutierrez M, Freeland P et al (2007) Needle-free injections using a spring-powered device for subcutaneous, intramuscular & intradermal injections. Drug Deliv Technol 7(2):40–43
121. Stuart Harris CH (1969) Adjuvant influenza vaccines. Bull WHO 41:617–621
122. Su SB, Silver PB, Grajewski RS et al (2005) Essential role of the MyD88 pathway, but nonessential roles of TLRs 2, 4, and 9, in the adjuvant effect promoting Th1-mediated autoimmunity. J Immunol 175:6303–6310
123. Sun JC, Beilke JN, Lanier LL (2009) Adaptive immune features of natural killer cells. Nature 457:557–561
124. Tang H, Cao W, Kasturi SP et al (2010) The T helper type 2 response to cysteine proteases requires dendritic cell-basophil cooperation via ROS-mediated signaling. Nat Immunol 11:608–617
125. Tomljenovic L, Shaw CA (2011) Aluminum vaccine adjuvants: are they safe? Curr Med Chem 18 (17):2630–2637
126. Tomljenovic L, Shaw CA (2012) Mechanisms of aluminum adjuvant toxicity and autoimmunity in pediatric populations. Lupus 21(2):223–230. doi:10.1177/0961203311430221
127. Vandepapeliere P, Horsmans Y, Moris P et al (2008) Vaccine adjuvant systems containing monophosphoryl lipid A and QS21 induce strong and persistent humoral and T cell responses against hepatitis B surface antigen in healthy adult volunteers. Vaccine 26:1375–1386
128. Vogel FR (1995) immunologic adjuvants for modern vaccine formulations. Annals of the New York Academy of Sciences 754:153–160

129. Wack A, Rappuoli R (2005) Vaccinology at the beginning of the 21st century. Curr Opin Immunol 17:411–418
130. Wickelgren I (2006) Mouse studies question importance of toll-like receptors to vaccines. Science 314 (5807):1859–1860. doi:10.1126/Science.314.5807.1859a. PMID 17185572
131. Xie Y, Pan H, Sun H et al (2008) A promising balanced Th1 and Th2 directing immunological adjuvant, saponins from the root of *Platycodon grandiflorum*. Vaccine 26(31):3937–45. doi:10.1016/J.Vaccine.2008.01.061. Epub 2008 May 20
132. Xu M, Sun X, Tong W (1994) Medical research and development of sea buckthorn. Hippophae 7:32–40