REVIEW

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Toward precision adjuvants: optimizing science and safety

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Purpose of review
The gradual replacement of inactivated whole cell and live attenuated vaccines with subunit vaccines has generally reduced reactogenicity but in many cases also immunogenicity. Although only used when necessary, adjuvants can be key to vaccine dose/antigen-sparing, broadening immune responses to variable antigens, and enhancing immunogenicity in vulnerable populations with distinct immunity. Licensed vaccines contain an increasing variety of adjuvants, with a growing pipeline of adjuvanted vaccines under development.

Recent findings
Most adjuvants, including Alum, Toll-like receptor agonists and oil-in-water emulsions, activate innate immunity thereby altering the quantity and quality of an adaptive immune response. Adjuvants activate leukocytes, and induce mediators (e.g., cytokines, chemokines, and prostaglandin-E_2) some of which are biomarkers for reactogenicity, that is, induction of local/systemic side effects. Although there have been safety concerns regarding a hypothetical risk of adjuvants inducing auto-immunity, such associations have not been established. As immune responses vary by population (e.g., age and sex), adjuvant research now incorporates principles of precision medicine. Innovations in adjuvant research include use of human *in vitro* models, immuno-engineering, novel delivery systems, and systems biology to identify biomarkers of safety and adjuvanticity.

Summary
Adjuvants enhance vaccine immunogenicity and can be associated with reactogenicity. Novel multidisciplinary approaches hold promise to accelerate and de-risk targeted adjuvant discovery and development.

Video abstract
http://links.lww.com/MOP/A53.

Keywords
adjuvant, biomarker, reactogenicity, safety, vaccine

INTRODUCTION

While vaccines were originally made from live attenuated microorganisms, modern biotechnology, through the use of genetic engineering, contributed to the development of vaccines containing highly purified recombinant antigens, which has been gradually replacing self-adjuvanted live attenuated and killed vaccine formulations. This approach has successfully lowered reactogenicity rates as compared with live attenuated or killed vaccines. However, recombinant-antigen-based vaccines are often insufficiently immunogenic, especially in populations with distinct immunity, such as the very young, elderly, and chronically ill, highlighting the need for approaches to amplify protective vaccine responses.

Originally named on the Latin root *adjuvare*, to help or aid, adjuvants are defined as components which can enhance antigen-specific vaccine immunogenicity. Used only when needed, as vaccine formulations should be kept as simple as possible for development and regulatory purposes, adjuvants can

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KEY POINTS

- Established and novel adjuvants with increasingly elucidated mechanisms of action can greatly enhance immune responses in vulnerable populations such as young infants and the elderly.
- New approaches to discovery and development are ushering in a new era of personalized adjuvants tailored for optimal safety and efficacy in populations in need of protection.
- Continued comprehensive assessment of undesirable reactogenicity and other potential safety concerns associated with vaccine formulations including adjuvanted vaccines is warranted.
- Multidisciplinary approaches including standardized human in-vitro models, systems vaccinology, and innovations of formulation and delivery systems will enhance identification of mechanisms of action and biomarkers of safety and efficacy of adjuvants and adjuvanted vaccines thereby accelerating and derisking adjuvanted vaccine development.

be key for antigen/dose-sparing, broadening immunity to variable antigens and enhancing responses from vulnerable populations with weak immune responses. Characterizing adjuvant mechanism of action (MOA) is important for effective translation and increasingly desirable from a regulatory and licensing perspective. Aluminum salts (alum) have been widely used to enhance humoral immunity, but their MOA is still under study [1–3]. Over the past 2 decades, a greater understanding of innate immunity, including identification of pattern recognition receptors (PRRs), has informed development of modern adjuvants [4,5].

Because the immunostimulatory effects of adjuvants can potentially induce undesirable reactogenicity, discovery and development must focus on yielding adjuvanted vaccines that are not only immunogenic, but also highly tolerable. Herein, we comprehensively review current available adjuvants incorporated into modern vaccines, as well highlight several new classes under investigation, with a focus on MOA for enhancing immunogenicity as well as mechanisms that may impact safety, such as reactogenicity. To inform this review, we performed literature searches in PubMed with terms such as 'adjuvant' 'vaccines' AND/OR 'safety' AND/OR 'reactogenicity' in November 2019. Titles and abstracts of articles were screened by two authors (E.N. and O.L.), and those deemed most relevant, comprehensive and timely were reviewed.

OVERVIEW OF VACCINE ADJUVANTS

Alum is the most widely used vaccine adjuvant in history. Depot mechanisms were initially hypothesized to be the main MOA for alum's adjuvanticity. However, alum injection sites can be excised shortly after injection with no impact on immunogenicity [6]. Several MOAs, both direct and indirect, are thought to contribute to alum-mediated enhancement of antibody production [7,8], including first, enhancement of antigen delivery to antigen presenting cells (APCs) such as macrophages and dendritic cells [9,10], partially through preferential binding of lipids on the surface of dendritic cells without alum itself being internalized by the cells [11]; second, triggering of innate immunity via inflammasome complexes inducing production of IL-1β, independent of Toll-like receptor (TLR) signaling [3]; and third, induction of cell death with consequent release of host cell DNA that can act as an endogenous adjuvant [2]. Alum has been also used as a component of combination adjuvantation systems wherein it is codelivered with other adjuvants such as TLR agonists (TLRAs) [7]. While alum has been the main adjuvant in vaccine formulations licensed for pediatric use in the United States, other adjuvants have been or are currently employed in licensed pediatric vaccines including live attenuated vaccines containing endogenous adjuvants (i.e., are 'self-adjuvanted') such as Bacille Calmette-Guérin (BCG), given at birth or early infancy in tuberculosis-endemic countries, that activates multiple PRRs including TLRs 2, 4, and 8 as well as the C-type lectin receptor Mincle [12,13], or exogenously added adjuvants such as the formerly used outer membrane protein-adjuvanted Hib vaccine that activates TLR2 [14] as well as human papilloma vaccine (*Cervarix*) containing monophosphoryl lipid a (MPLA), available to in the European Union and China for girls (staring at age 9 years) and adolescents and young adults (from 16 to 25 years of age) (Table 1).

Diverse water-in-oil emulsions were originally evaluated in human trials during the mid-20th century [15]. Water-in-oil emulsions, wherein water droplets are held within a continuous mineral oil phase, either containing killed *Mycobacterium tuberculosis* (Freund complete adjuvant) or not (Freund incomplete adjuvant) provided potent immunogenicity and were used in early influenza vaccines [16,17]. However, they demonstrated intolerable reactogenicity such as abscess and cyst formation at the site of injection [18]. Furthermore, instability of the antigen and lack of formulation reproducibility led to the consensus to avoid the use of water-in-oil for human prophylactic vaccines for infectious diseases, although some water-in-oil emulsions are still used as therapeutic vaccines for cancer [19,20].

Table 1. US Food and Drug Administration-approved vaccines containing novel adjuvants

Adjuvant	Composition	Formulation	Vaccines	Year
(a) – Licensed adjuvanted pediatric vaccines				
Aluminum	One or more of the following: AAHS, aluminum hydroxide, aluminum phosphate, potassium Alum	Various/ Aqueous (PBS-based)	Anthrax (*BioThrax*), DT, DTaP (*Daptacel*), DTaP (*Infanrix*), DTaP-IPV (*Kinrix*), DTaP-IPV (*Quadracel*), DTaP-HepB-IPV (*Pediarix*), DTaP –IPV/Hib (*Pentacel*), Hep A (*Havrix*), Hep A (*Vaqta*), Hep B (*Engerix-B*), Hep B (*Recombivax*), HepA/Hep B (*Twinrix*), HIB (*PedvaxHIB*), HPV (*Gardasil* 9), Japanese encephalitis (*Ixiaro*), MenB (*Bexsero, Trumenba*), Pneumococcal (*Prevnar* 13), Td (*Tenivac*), Td (Mass Biologics), Tdap (*Adacel*), Tdap (*Boostrix*)	1930–Present
(b) – Licensed adjuvanted adult vaccines				
MF59	Oil in water emulsion composed of squalene	Emulsion-based	TIV (*Fluad*) (for adults aged 65 or older)	2015
AS01B	MPL and QS-21, a natural compound extracted from the Chilean soapbark tree	Liposome-based	RZV (*Shingrix*) (for adults aged 50 or older)	2017
CpG-1018	CpG, a synthetic form of DNA that mimics bacterial oligodeoxynucleotide and viral genetic material	PBS-based	Hep B (*Heplisav-B*) (for adults aged 18 or older)	2017
(c) – Approved, but not commercially available in the U.S.				
AS04	MPL + aluminum salt	VLPs + MPL adsorbed onto Alum, PBS based	HPV (*Cervarix*)	2009
AS03	α-Tocopherol, squalene, and polysorbate 80	Emulsion-based	Monovalent Pandemic H5N1 Swine Influenza A (Q-Pan H5N1) (for adults aged 18 or older)	2013

AAHS, aluminum hydroxyphosphate sulfate; Alum, aluminum sulfate; CpG, cytosine phosphoguanine; DT, diphtheria and tetanus toxoids; DTaP, diphtheria and tetanus toxoids with acellular pertussis; HPV, human papillomavirus; IPV, inactivated polio vaccine; MPL, Monophosphoryl lipid A; RZV, recombinant zoster vaccine; Td, tetanus and diphtheria toxoids; Tdap, tetanus and diphtheria toxoids with acellular pertussis; TIV, trivalent inactivated influenza vaccine; VLP, virus-like particle.

Oil-in-water emulsions were developed as an alternative to water-in-oil emulsions and demonstrated significantly better reactogenicity profiles. The most common oil phase contained in vaccine formulations is squalene, a natural organic compound originally obtained for commercial purposes from shark liver oil and some plant sources. Since the introduction of MF59, that enhances both humoral and cell mediated Th1 and Th2 responses, oil-in-water emulsions have been routinely used in many seasonal and pandemic influenza vaccines for adults [21–23]. The MOA of MF59, a squalene-containing oil-in-water emulsion, appears to include enhancing antigen uptake of monocytes and dendritic cells [24]; and secretion of chemokines to create an 'immunocompetent environment' for enhanced antigen transportation to the draining lymph nodes [25]. AS03, an oil-in-water emulsion containing squalene, polysorbate 80 and α-tocopherol (a form of vitamin E), is a component of multiple influenza vaccines (e.g., *Pandemrix*, *Arepanrix*). Studied in mice, α-tocopherol in AS03 modulated cytokine and chemokine expression, increased antigen loading in monocytes, enhanced recruitment of granulocytes in draining lymph nodes, and enhanced antibody responses [26].

Saponins are triterpenoid molecules extracted from a variety of plants. Quil-A, which is a heterogeneous product extracted from the Chilean soapbark tree *Quillaja saponaria*, has been used in veterinary vaccinology since the 1950s. Although Quil-A had excessive reactogenicity for human use, its affinity for cholesterol had prompted development of immune stimulating complexes (ISCOMs) [27,28]. ISCOMs are spherical cage-like nanoparticles formed via self-assembly of a mixture of Quil-A, cholesterol, phospholipids, and antigens [29,30]. ISCOMs in the absence of an antigen (called ISCOMATRIX) can be mixed with an antigen of interest. ISCOMs stimulate enhanced cellular responses with lower

antigen doses through enhanced antigen cross-presentation [31]. The saponin QS-21, a natural compound extracted from *Q. saponaria*, consists of a single saponin peak detectable by HPLC and is a component, together with MPLA, of the AS01B-adjuvanted zoster subunit vaccine (*Shingrix*) (Table 1) [32,33]. The combination adjuvant systems AS01 and AS02, components of the candidate RTS,S malaria vaccine, also contain both MPLA and QS21. Although the exact MOA of QS-21 is not fully elucidated, the nanoparticulate nature of saponin/ISCOM formulations may lead to their preferential interaction and pore formation within cholesterol-rich dendritic cell membranes [4]. Furthermore, QS-21 elicits synergistic NLRP3-Asc-caspase-1-dependent IL-1β and IL-18 release in APCs when costimulated with MPLA [34].

The discovery of PRRs has accelerated discovery and development of PRR agonists as adjuvants. A number of these are now in clinical use or late preclinical stages of development. At the forefront is the 3′-deacylated monophosphoryl lipid A (MPLA), a TLR4A. TLR4 recognizes several pathogen-associated molecular patterns, including lipopolysaccharides (LPS) from the outer membrane of Gram-negative bacteria. Although LPS have long been recognized as a potent adjuvant, its pyrogenic activity had limited its use in human [35]. MPLA is a detoxified form of the LPS from the bacterium *Salmonella minnesota*, with significantly lower reactogenicity (~1000-fold lower), but robust adjuvanticity via Th1 polarization [4,36].

Synthetic single-stranded oligodeoxynucleotides (ODNs) containing unmethlyated cytosine phosphate guanine (CpG) motifs (CpG ODNs) found in bacterial DNA, have demonstrated adjuvant activity as a TLR9A [37]. CpG-ODN enhance antibody responses and enhance Th1-cell responses [38]. In humans, CpG motifs are recognized by TLR9 expressed on natural killer cells, B cells, and plasmacytoid dendritic cells but not myeloid dendritic cells and monocytes [39]. The licensed hepatitis B vaccine, *Heplisav-B*, indicated for use in adults at least 18 years, contains a TLR9A CpG adjuvant and, as compared with three doses of conventional alum adjuvanted hepatitis B vaccine, induces superior immunogenicity in older adults and the elderly (40–70 years of age) with only two doses [40,41].

Small molecule imidazoquinolines (IMQs) such as resiquimod (R848) are TLR7/8As [42]. These molecules activate human plasmacytoid dendritic cells and myeloid dendritic cells, enhancing expression of costimulatory molecules and production of type I IFN and IL-12 [43,44]. Although use of these small IMQ molecules is limited by their low molecular weight and rapid removal from the site of injection, several formulation methods, including covalent lipidation or incorporation into nanoparticles, enable use of these molecules as effective adjuvants *in vivo* [45▪]. Additional PRR agonists, including agonists of TLR3, TLR5, C-type lectin receptors, retinoic acid-inducible gene (RIG)-like receptors, and the stimulator of interferon genes are under evaluation as potential vaccine adjuvants [4,46,47].

Virosomes are enveloped virus-like particles that contain viral proteins in the liposomal membrane, and can act as adjuvants and carrier system for vaccinal antigens [48]. They are typically produced from reconstituted envelopes of influenza viruses, and enable robust and long-lasting immune responses with an excellent safety profile [49]. Virosomes have been licensed in vaccines against hepatitis A and pandemic influenza (Table 1), and also used in several clinical trials of malaria and hepatitis C vaccines.

Several additional types of adjuvants are under development. Proinflammatory cytokines such as granulocyte-macrophage colony-stimulating factor, IL-2, IL-12, and IL-15 were evaluated in vaccines against foot and mouth disease, hepatitis B, and HIV [50–53]. Diphtheria, tetanus, pertussis, and poliomyelitis vaccines containing calcium phosphate, in the form of hydroxyapatite nanoparticles with Th-polarizing cytokine inducing activity, have been licensed in France [54].

Combination adjuvantation systems can be a powerful approach to enhance immunogenicity [55]. As with any pharmacologic agents, adjuvant combinations can demonstrate additivity, antagonism or synergy [56]. Indeed, several of adjuvantation systems contained in licensed vaccines are comprised of combination systems such as AS01 (MPLA + QS21 in liposomes), AS02 (MPLA + QS21 in oil-in-water emulsion), AS03 (squalene + alpha-tocopherol in oil-in-water emulsion), and AS04 (MPL + alum) [57]. Of note, effects of at least some combination adjuvants may vary with age [58], though this has not been systematically studied.

DISCOVERY OF ADJUVANTS

The first adjuvant observation was recorded in 1893, reporting that administration of killed bacteria (Coley toxins) may be beneficial in treating some forms of cancer [59]. In 1925, Ramon observed that substances inducing sterile inflammation at the site of injection were able to increase antisera production of tetanus and diphtheria [60]. Since Glenny observed that alum enhanced antibody responses in 1926, alum was widely used as an adjuvant for numerous human vaccines. Although water-in-oil emulsions were withdrawn for their high reactogenicity in the 1960s, they were soon followed by development of oil-in-water emulsions. Liposomes

and virosomes that adsorb or encapsulate antigen were developed in the 1970s. Hepatitis A vaccine adjuvanted with virosome was licensed in the 1990s, as the first nonlive vaccine to use an adjuvant other than alum [60]. However, characterization of adjuvants and their MOA lagged for many years [61].

In 1990s and early 2000s, the discovery of PRRs and their agonists opened new opportunities in adjuvant discovery and development. Many adjuvants can activate PRRs directly or indirectly, to trigger different types of innate immune responses, and, if combined with an antigen, can initiate and enhance specific arms of the adaptive immune system [59,60]. In 2009, the US Food and Drug Administration (FDA) approved the first novel adjuvanted vaccine against human papillomavirus which contains AS04, comprised of alum and TLR4A MPLA. Thereafter, several vaccines with novel adjuvants were approved worldwide, such as hepatitis B vaccines containing TLR9 ligand CpG-ODN or AS04, and subunit zoster vaccine adjuvanted with AS01B, comprised of MPLA and saponin (Table 1).

EFFICACY OF VACCINE ADJUVANTS

Historically, vaccinologists sought to increase specific antibody responses with adjuvants such as alum. However, these classical adjuvants administered with subunit or recombinant antigens could not always induce sufficient immunity among naïve populations because some of the intrinsic immunogenic factor in the live, live attenuated or whole killed organisms are lost or inactivated via purification processes. Consequently, for children less than 2 years of age multiple booster doses of routine inactivated vaccines are typically needed to protect them from infectious diseases (Fig. 1). In addition, for multiple vaccines durability of humoral antibody responses is often insufficient due to the waning immunity, as is the case with acellular pertussis vaccines [62,63].

Modern adjuvants represent a powerful tool to overcome these challenges by enhancing a magnitude, durability, and/or quality of the immune response that in some instances mimics natural infection [5]. Adjuvants can therefore improve vaccine-specific immune responses in vulnerable populations such as young infants, elderly, immunocompromised, and chronically ill patients. For example, novel recombinant hepatitis B vaccines adjuvanted with AS04 (alum with the TLR4A MPLA) or with CpG-ODN (TLR9A) demonstrated enhanced immunogenicity in patients with chronic renal failure or elderly individuals with type 2 diabetes mellitus [64–66]. Adjuvants may be added to enhance responses to recombinant-antigen-based vaccines or be used to complement live attenuated vaccine formulations [60,67]. A key example is *Shingrix*, the recombinant subunit vaccine for varicella–zoster virus (VZV) adjuvanted with AS01B (MPLA and saponin) [32,33]. This adjuvanted vaccine was also immunogenic for recipients of autologous hemopoietic stem-cell transplants and solid organ transplant [68▪▪,69▪]. To our knowledge *Shingrix* has not yet been evaluated in pediatric cohorts wherein it may be promising for children with primary or secondary immunodeficiency, who are vulnerable to VZV but unable to receive live attenuated varicella vaccine.

Use of appropriate adjuvants to enhance immunogenicity can decrease the amount of antigen contained in each vaccine (antigen sparing), reducing the quantity of costly vaccinal antigen and the number of vaccine doses required to achieve sufficient protection (dose sparing) (Fig. 1), thereby reducing the need for clinic visits and enhancing the effective vaccination rate for consequent herd immunity [70]. These adjuvant effects enhance sustainability of the global vaccine supply. Finally, adjuvants can broaden an immune response (Fig. 1) to variable antigens, and may thus be crucial for developing effective vaccines against key pathogens such as respiratory syncytial virus, influenza, and HIV, viruses that display substantial antigenic drift, strain variation or both [71–73].

REGULATORY PERSPECTIVE REGARDING THE SAFETY OF ADJUVANTED VACCINES

Clinical trials evaluating vaccines containing novel adjuvants have recently supported licensing by the FDA as described above. FDA defines adjuvants as one of the constituent materials of the vaccine, therefore, unless the adjuvant has a 'stand alone' indication, they do not usually evaluate and approve adjuvants in of themselves but rather as part of a vaccine formulation [74]. Key guidance from FDA states 'an adjuvant shall not be introduced into a product unless there is satisfactory evidence that it does not adversely affect the safety or potency of the product' [74]. While there is no requirement for demonstrating the safety of the adjuvant administered alone, the safety of an adjuvanted vaccine formulation has to be demonstrated with special considerations in adequate and well controlled studies. WHO published a guideline in 2013 and described the nonclinical, quality, pharmacological, toxicological, and other information needed to support initiation of clinical trials with a vaccine combined with a novel adjuvant [75]. In addition to appropriate safety studies, the guideline for supporting approval places emphasis on the importance of demonstrating the rationale for use of an adjuvant with defined MOA in human in-vitro and well defined animal models [76▪].

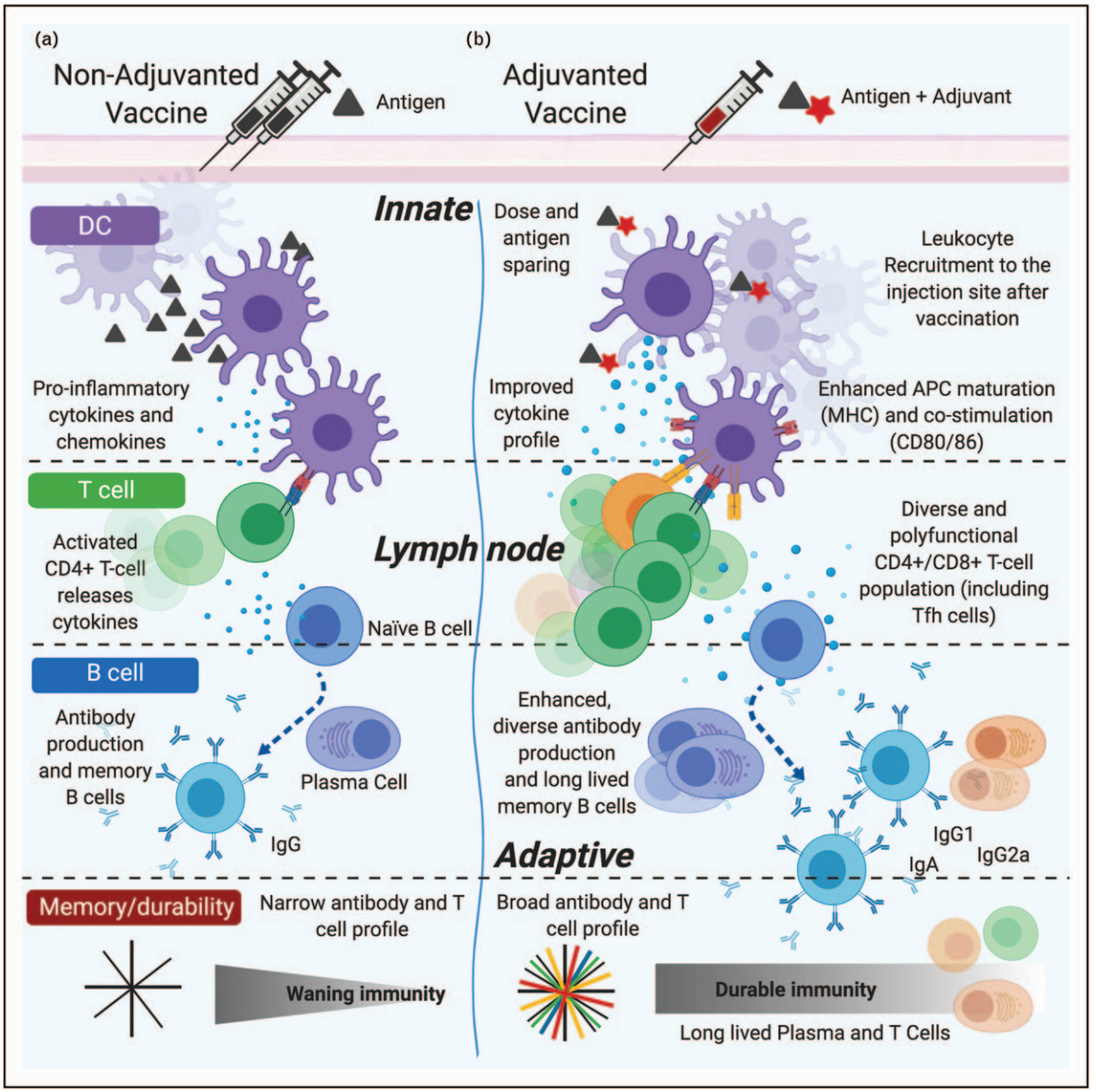

FIGURE 1. Adjuvants enhance vaccine immunogenicity. (a) Nonadjuvanted subunit vaccines that contain only purified recombinant antigens induce modest T helper-polarizing cytokines, T-cell activation, and antibody production typically require multiple booster doses and often result in narrow and/or waning immunity. (b) Adjuvanted vaccines, in turn, enhance the quality and quantity of antigen presenting cell maturation and costimulation, T helper-polarizing cytokine production, polyfunctional T cells, and antibody production, resulting in broad and durable immunity, as well as dose and antigen sparing. Multiple cell colors indicate broader protective immunity. APC, antigen presenting cell; DC, dendritic cell. This figure was created using BioRender (https://biorender.com/).

ADJUVANT SAFETY

Although adjuvants are added to many vaccines for their immunostimulatory effects, they can potentially simultaneously induce undesirable reactogenicity, physical manifestations of the immunomodulatory and/or inflammatory response occurring within 72 h of vaccination [77▪▪,78▪]. Reactogenicity can be divided into local and systemic depending on the site of symptoms. Local reactogenicity includes erythema, swelling, pain, tenderness, or induration at the injection site, while systemic reactogenicity, often referred to as 'flu-like' symptoms, include chills, fever, fatigue, nausea, arthritis, myalgia, and headache.

Since the introduction of novel adjuvants, safety experience has been accumulated with their use in diverse vaccines as well as different target populations and settings. Although the safety profile of one adjuvanted vaccine in one target population cannot

be extrapolated to other vaccines or populations, novel adjuvants showed no increase in serious adverse events so far. However, some vaccines with novel adjuvants had higher rates of local reactogenicity compared with their controls (placebo, non-adjuvanted, or alum-adjuvanted vaccines) [57,79▪]. A systematic review evaluating safety data in vaccine trials in adults at least 50 years, focused on AS01 (liposomal MPLA and saponin QS21), AS02 (oil-in-water with MPLA and QS21), AS03 (squalene-based adjuvant), and MF59 [80▪▪]. Rates of local pain were as high as 45.7–91.1% for AS01/AS02, 41.0–68.6% for AS03, and 0.7–64.8% for MF59, respectively. Relative risk of fever was 5.51 (95% confidence interval = 3.49–8.71) for AS01/AS02, 1.51 (0.46–4.90) for AS03, and 1.41 (0.83–2.40) for MF59 [80▪▪]. A similar study evaluating safety data of novel adjuvants among children 10 years of age or less demonstrated that local pain was the most frequent adverse event, with rates reported as 8.0–91.4% for AS01/AS02, 31.7–84.6% for AS03, and 1.0–59.0% for MF59, respectively [81]. The rate of Grade 3 pain, defined as spontaneous pain or which induce crying when children move their limb, reported in phase 3 trials was 0.1–0.3% for AS01, less than 1% for MF59, and 4.3–12.4% for AS03-adjuvanted vaccines, respectively [82–85]. The incidence of Grade 3 fever defined as more than 39 °C was 2.5% for AS01 among children 5–17 months of age and less than 1% among infants 6–12 weeks of age (consistent with the generally lower reactogenicity of vaccines in early life [86]), 1.9–5.4% for AS03, and 1% or less for MF59-adjuvanted vaccines, respectively [82–85,87,88].

While reactogenicity is defined as acute inflammatory reactions after vaccination, the term 'safety' refers to all adverse events attributable to vaccination that could potentially be caused, triggered, or worsened after vaccine administration [77▪▪]. In addition to the symptoms described above as reactogenicity, safety would include adverse events such as anaphylactic reactions after administration of an adjuvanted vaccine. Although not proven via epidemiological research, and partially influenced by the immunomodulatory MOA of most adjuvants, concerns have been raised regarding the hypothetical potential of adjuvanted vaccine to increase autoimmune diseases [89–91]. Unexpected rare adverse events may also occur which are challenging to ascribe causality to. For example, an increased risk of narcolepsy was observed in several European countries after AS03-adjuvanted 2009 H1N1 influenza vaccine, and alum has been linked to macrophagic myofasciitis, although underlying mechanisms and causality are not fully elucidated [91–96]. Because the sample size of prelicensure clinical trials is not usually sufficient to detect rare adverse events, it is essential to monitor postlicensure data (i.e., phase 4) with well selected controls [79▪]. Reporting potential vaccine side-effects, including attributable adverse events, is crucial to the integrity of vaccine development and will inform development of vaccines optimized safer vaccines via transparency and public awareness of possible vaccine-associated adverse events [97]. If an adverse event is associated with a given vaccine, it is important to assess which of the vaccine components, potentially including the adjuvant, may contribute to that adverse event. The Vaccines Adverse Events Reporting Systems (www.vaers.hhs.gov) is important for reporting, assessing and addressing vaccine safety. WHO provides an e-learning course which is to establish a shared understanding among professionals whose work is linked to vaccine safety issues [98]. Prospectively designed vaccine studies in which systems biology assays are conducted on samples from rare events as compared with unaffected controls may represent an approach to get further insight into rare events.

MECHANISM OF REACTOGENICITY

After injection, adjuvants along with antigens rapidly stimulate the immune system. Adjuvants that are PRR agonists (e.g., MPLA, a TLR4A) directly activate innate immune cells, such as dendritic cells, and induce production of proinflammatory mediators including cytokines (e.g., IL-1β, IL-6, and tumor necrosis factor), chemokines (e.g., CCL2, CXCL1, and CXCL9), lipid mediators [e.g., prostaglandin-E_2 (PGE_2)], complement cascade components (e.g., C3a and C5a) and vasodilators (e.g., vasoactive amines and bradykinin) [77▪▪,99]. These soluble factors may sensitize peripheral nociceptive responses [100]. Adjuvants may drive injection site cell death/cytotoxicity and induce release of damage-associated molecular patterns (DAMPs) from injured/dead cells and tissue. DAMPs, such as chromatin-associated-protein-high-mobility group box 1, heat shock proteins, purine metabolites, and host cell DNA, can act as autologously derived endogenous adjuvants. Endogenous adjuvants may act in an additive or synergistic way with exogenous adjuvants [58,101].

Neutrophils, monocytes and lymphocytes accumulate at the site of immunization as early as 3–6 h after vaccine injection [26,99]. Vasodilators and chemokine promote cell recruitment from blood, but may also lead to the development of redness (erythema) and swelling. Most cytokines and chemokines in the injection site decrease within 24 h and reach baseline after 72 h [99,102,103]. For example, MF59 increases recruitment of immune cells into the injection site through secretion of chemokines, such as CCL2, CCL3, CCL4, and CXCL8 [76▪].

MF59 also accelerates and enhances monocyte differentiation into mature dendritic cells, and facilitates migration of dendritic cells into tissue-draining lymph nodes to prime adaptive immune responses [76▪]. Because each adjuvant has a distinct MOA that may further vary with vaccine dose and demographics of the target populations, including age [58,104], their contribution to reactogenicity symptoms may vary. Although these phenomena may be crucial for strong immunogenicity, these same inflammatory events may also lead to the development of local reactogenicity. Systemic reactogenicity may be initiated when pyrogenic factors such as proinflammatory cytokines (e.g., IL-1β) and chemokines, and PGE_2 are produced in sufficient levels to enter the systemic circulation and the central nervous system (Fig. 2). Within the brain, the coupled induction of the inducible enzymes cyclooxygenase-2 and microsomal PGE synthase-1 enhances intracerebral PGE_2 concentrations, thereby causing an elevation of body temperature [105,106].

BIOMARKERS FOR REACTOGENICITY

Several new scientific approaches are proposed to strengthen the assessment of science and safety of vaccine adjuvants. Preclinical human in-vitro models that consider age-specific and sex-specific differences can generate hypotheses to be tested in appropriate animal models and eventually in targeted clinical trials [107]. Using in-vitro biomarkers as surrogate markers of in-vivo immunogenicity and reactogenicity would be highly desirable as well [4]. Indeed, given the substantial list of infectious disease pathogens and cancers for which new or improved prophylactic or therapeutic vaccines are urgently needed; the long list of candidate vaccinal antigens; the growing list of candidate adjuvants, combinations adjuvants, and formulations thereof; and the diversity and variability of target populations, it will not be possible to conduct multiple large-scale phase 3 clinical trials for each and every potential adjuvanted vaccine formulation. In this context, in-vitro systems that model innate and adaptive immune responses may be key to

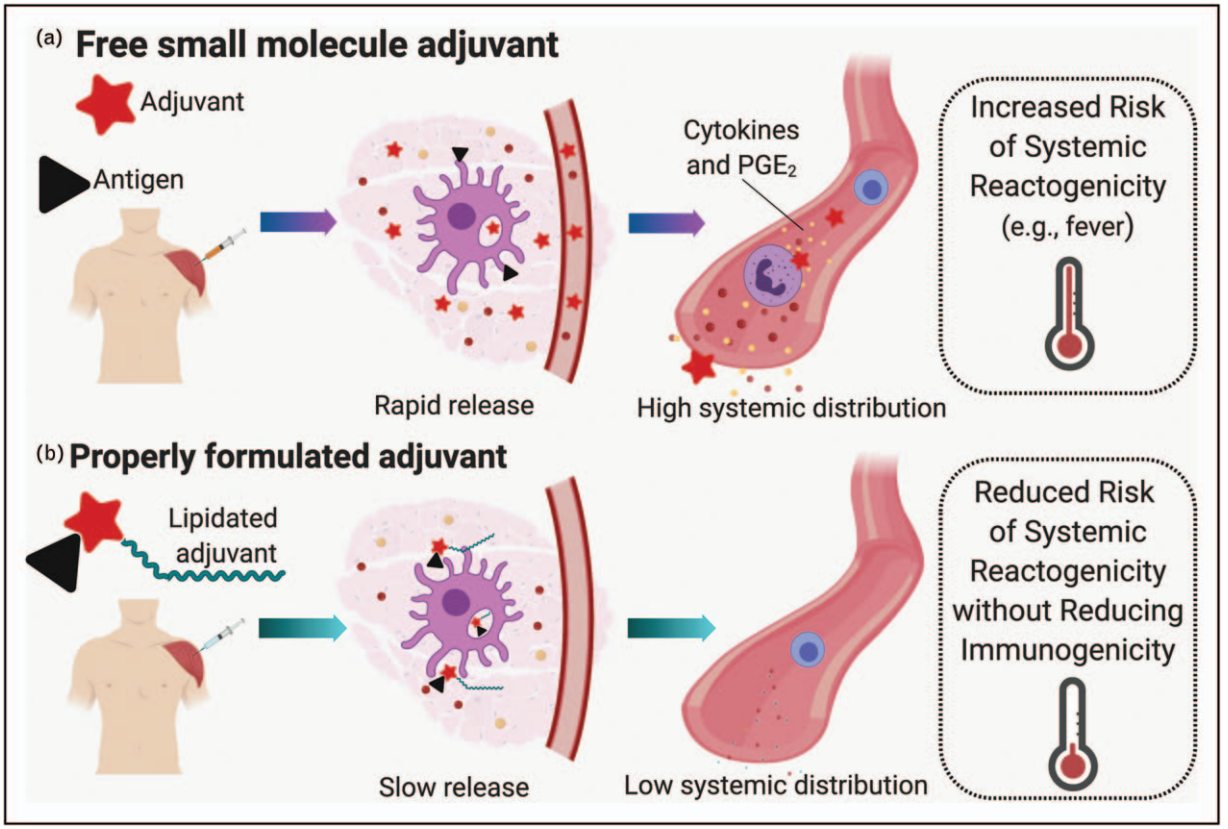

FIGURE 2. Medicinal chemistry and formulation can limit adjuvant-related systemic reactogenicity. (a) Free small molecule adjuvants are rapidly released and dissipated from the injection site. Entry of such adjuvants into the systemic circulation induces production of cytokines and prostaglandin-E_2 thereby increasing systemic reactogenicity, including fever and malaise. (b) Lipidation of small molecules adjuvants, is one approach to reduce the systemic reactogenicity without reducing immunogenicity through the slow release of the adjuvant with low systemic distribution. PGE_2, prostaglandin-E_2. This figure was created using BioRender (https://biorender.com/).

Table 2. Biomarkers of adjuvant reactogenicity

Biomarker	Adjuvant tested (and vaccine containing similar adjuvant)	*In vivo*	*In vitro*	Comment	References
IL-1β	FSL-1, Pam3CSK4, flagellin, R848	–	Human	Strong correlation between in-vitro production of IL-1β and PGE_2	[111,113,116]
IL-6	AS01, AS03, AS04, FSL-1, Pam3CSK4, flagellin, R848	Human	Human	Associations with systemic or severe local symptoms	[111,112▪▪,117]
Tumor necrosis factor, IL-8	FSL-1, Pam3CSK4, flagellin, R848	–	Human	Associations with systemic or severe local symptoms	[111]
IFNγ, CXCL10	AS01, AS03, AS04	Human	–	Associations with systemic or severe local symptoms	[112▪▪,117]
CCL4, CCL8	AS01	Human	–	Associations with systemic or severe local symptoms	[112▪▪]
CRP	AS01, AS03, AS04, FSL-1, Pam3CSK4, flagellin, R848	Human, rabbits	–	Associations with systemic or severe local symptoms	[111,112▪▪,117,118]
Fibrinogen	AS01, AS03, AS15	Rabbits	–	Associations with systemic or severe local symptoms	[119]
PGE_2	FSL-1, Pam3CSK4, flagellin, R848	Rabbits	Human	Strong correlation between in-vitro production of PGE_2 and an increase in body temperature in rabbits	[115,118]
PTX3	R848, MPLA, alum (*Bexero*, *EasyFive*, *Cervarix*)	Mouse	Human, mouse	PTX3 is a glycoprotein produced in response to proinflammatory stimuli (e.g., tumor necrosis factor, and IL-1β) and TLR agonists. PTX can act as an endogenous adjuvant	[102,113,114,120]
Lactoferrin, matrix metalloproteinase-9	R848, MPLA, alum (*Bexero*, *EasyFive*, *Cervarix*)	–	Human	Correlated with PTX3 adjuvant-induced human monocytes secretome *in vitro*	[114]
Creatine kinase	N/A	Rabbits	–	A marker of muscle damage at the injection site. Appears to have poor concordance with the systemic CRP response	[118]
Transitional B-cell subset	AS03	Human	–	Healthy adults with a relatively high number of a transitional B-cell subset at baseline who received AS03-H1N1 flu vaccine demonstrated greater frequency of medium and high adverse events	[121]
Clustered biomarker genes	CpG-K3, virosome, alum, pam3CSK4, nanoSiO_2, DMXAA	Mouse, rats	–	Genes (*Cxcl11, Cxcl9, Zbp1, Mx2, Irf7, Lgals9, Ifi47, Tapbp, Csf1, Timp1, Trafd1, Lgals3bp, Psmb9, Ifrd1, C2, Tap2, Psme1, Ngfr*) expressed in the lungs were correlated with reactogenicity. Genes are functionally classified into 3 groups: IFN pathway; modification and presentation of antigens; and intracellular signaling of chemokines and cytokines	[122–125]

DMXAA, stimulator of interferon agonist; FSL, TLR2/6 agonist (also a putative TLR10 ligand); nanoSiO_2, single particles of silica dioxide; pam3CSK4, TLR1/2 agonist. CRP, C-reactive protein; PGE_2, prostaglandin-E_2; PTX3, pentraxin 3; TLR, Toll-like receptor.

accelerating and derisking vaccine development [101,108–110].

Although there is a growing evidence of general associations between local and systemic inflammatory mediators and systemic symptoms after vaccination, no single biomarker of systemic reactogenicity has been definitely identified (Table 2). However, several contenders, either analyzed alone, or in combination are under investigation. PGE_2 was reported as an early marker of febrile responses *in vivo*. PGE_2 upregulation in the plasma preceded elevation of body temperature (within 6–8 h), while upregulation of C-reactive protein (CRP) occurred only at 24 h [111]. A study of healthy adults vaccinated with AS01-adjuvanted hepatitis B vaccine reported that several cytokines, chemokines, and acute phase reactants such as IL-6, IFN-γ, CXCL10 (IP-10), CCL8 (MCP-2), and CRP were correlated with intensity of systemic symptoms, but not with local reactogenicity [112▪▪]. In-vitro production of PGE_2 correlated with tumor necrosis factor, IL-1β production in human primary monocytes and a human monocytic cell line *in vitro*, and with reactogenicity in rabbits *in vivo* [111,113,114]. PGE_2 may be a useful biomarker to

predict reactogenicity [115], but its utility in predicting reactogenicity in the pediatric area has yet to be defined [114,116]. Ultimately, benchmarking of novel adjuvants and adjuvanted vaccines to established adjuvants (e.g., Alum, MPLA, CpG), licensed adjuvanted vaccines [e.g., alum-adjuvanted pneumococcal conjugate vaccine (PCV), the group B meningococcus (MenB) vaccine *Bexsero* (containing TLR4A activity), *Heplisav* (containing CpG, a TLR9A)], and live attenuated vaccines (e.g., BCG), may provide fresh insight into their age-dependent activation of innate signatures important for reactogenicity [116]. These approaches will enable us to apply precision medicine principles to tailor vaccines for optimal safety and efficacy for vulnerable populations.

Systems vaccinology, employing integration of approaches such as genomics, transcriptomics, proteomics, metabolomics, and miRNomics with conventional immunological and clinical data, is another promising approach to characterizing adjuvant MOA and defining biomarkers of adjuvanticity and safety [115,126]. For example, a global proteomic approach to characterize adjuvant-induced protein release (i.e., secretome analysis) from human neonatal and adult monocytes, demonstrated adjuvant-specific and age-specific differences (Table 2) [114]. This comprehensive approach has begun to shed light on adjuvanted vaccine-induced molecular signatures that correlate with reactogenicity and immunogenicity [22,127▪]. Several studies have demonstrated promising underlying mechanisms possibly associated with vaccine efficacy and safety, but considerable effort is still required to comprehensively standardize and apply these approaches [127▪]. Thus, systemic and local reactions after vaccination may involve different innate immune response parameters, or a different kinetic of the same parameters. Among healthy adults who received the squalene-based AS03-adjuvanted H1N1 flu vaccine moderate or severe local or systemic reactogenicity (as calculated and categorized from self-reported participant parameters), correlated with a higher quantity of a transitional B-cell subset at baseline prior to vaccination. In contrast, neither basal numbers of memory T cells nor antibody titers correlated with adverse events [121].

DRUG DELIVERY SYSTEM FOR TARGETING ADJUVANTED VACCINE FORMULATIONS AND REDUCING REACTOGENICITY

As described previously, several adjuvant platforms such as liposome, nanobeads, and VLPs are categorized as vaccine delivery systems, representing their inherent adjuvant effects through modulating antigen delivery to APCs [5]. Advances in immunoengineering and formulation science have enabled some adjuvants to be rationally engineered as 'pathogen-like particles' to mimic the size, shape, and surface chemistry of pathogens [4,10,109]. Furthermore, formulation technologies may not only increase adjuvant immunogenicity but also optimize safety [101,128]. Formulation strategies to increase vaccine delivery to draining lymph nodes can often simultaneously decrease the systemic distribution of adjuvants, thereby limiting systemic reactogenicity [67]. For example, we had previously reported some promising innovations in formulation and use of delivery systems for TLR7/8As including lipidation approaches, encapsulating nanoparticles, adsorption to alum adjuvants, and conjugation to polymers and/or protein antigens [45▪,101,109]. Lipidated IMQ TLR7/8As, such as 3M-052, maintain the adjuvant for an extended period within a localized tissue region to decrease metabolism and systemic reactogenicity (Fig. 2) [128].

TOWARD 'PRECISION ADJUVANTS'

Traditionally adjuvants have been developed on a 'one size fits all' basis. However, a growing body of evidence suggests that responses to adjuvants can markedly vary based on genetic and epigenetic factors. Ontogeny, the change in an individual across their life span, is a key factor in determine the quality and quantity (magnitude) of an immune response [129]. Early life immunity varies with age during fetal gestation, neonatal phase, and infancy [108]. For example, human neonatal APCs (e.g., monocytes, dendritic cells) demonstrate reduced Th1 but robust anti-inflammatory IL-10 responses to stimulation by most PRR agonists studied to date [130]. Human newborn cord blood includes soluble mediators such as maternal antibodies, high levels of immunosuppressive adenosine, and low levels of complement, important for triggering adaptive immune responses [131]. Thus, newborns present with distinct ontogeny resulting in decreased magnitude of immunogenicity and reduced persistence of functional antibodies, both limit early life immunization strategies [45▪].

The concept of precision medicine can be applied to vaccinology to generate precision vaccines, that is, those optimally tailored to a given target population. Indeed, with the growing realization that responses to adjuvants may vary by target population, the most recent National Institutes of Health strategic plan on adjuvants (2018) highlights the importance of discovery and development of adjuvants targeted for particular demographic groups such as age and sex [132]. Accordingly,

Table 3. Characteristics of ideal vaccine adjuvants

Efficacy
Initiate and enhance specific arms of the immune system
Optimally enhance immunogenicity in a target population
Improve immune response in vulnerable populations
Enhance inactivated vaccine immunogenicity to mimic that of a live vaccine
Induce potent and durable immunity
Broaden an immune response to variable antigens
Reduce number of vaccinations – dose sparing
Known mechanism of action and biomarkers for adjuvanticity
Safety
No or limited systemic reactogenicity
Low local reactogenicity
Broad safety spectrum
Known mechanism and biomarkers for reactogenicity/adverse effects
Material
Age-optimized formulation to increase immunogenicity and reduce reactogenicity
Ease of production, scalability, high stability, and ease of use
Enable dose – and antigen-sparing and reduced vaccine manufacturing costs

precision vaccines are defined as vaccines that take into account the target population; are formulated to selectively activate the immune system by targeting anatomic sites, cells, and pathways that generate a protective response; and may, as needed, contain adjuvantation systems that optimally enhance immunogenicity in a target population (Table 3) [107]. The use of optimized adjuvanted vaccine formulations targeted to a vulnerable population such as newborns or young infants may overcome barriers in vaccine development. The target of precision vaccines can also be applied to vulnerable populations such as the elderly, immunocompromised, and therapeutic vaccines for immunosuppressed opioid users [133,134].

TLR7/8As, as a key example for this context, have demonstrated unique utility. Unlike most TLRAs that elicit reduced Th1 cytokine production by newborn and infant leukocytes, TLR7/8 or TLR8 agonists induces robust Th1-polarizing responses from neonatal dendritic cells in human and nonhuman primates (NHPs) [135–137]. The robust activity of TLR7/8As, including IMQs and ssRNAs, likely reflects that these agents are refractory to mechanisms that limit Th1 polarization, including the adenosine/cAMP axis [137]. Indeed, as predicted by human and NHP in-vitro studies, newborn NHPs immunized with PCV resulted in accelerated and enhanced specific B cells, specific antibody titers, and antibody-mediated phagocytic killing when the vaccine was adjuvanted with TLR7/8A [101]. These data suggest that appropriately formulated TLR7/8A adjuvants could enhance responses to vaccines administered even very shortly after birth, though much remains to be learned regarding optimal formulation safety and efficacy.

LESSONS FROM LIVE VACCINES: ADJUVANTS FOR TRAINED IMMUNITY

Although they represent an older approach to vaccine development, we have much to learn from live attenuated vaccines. Indeed, live vaccines with inherent adjuvant activity (i.e., 'self-adjuvanted' vaccines), such as BCG, yellow fever vaccine, and varicella vaccine, are still routinely administered to newborns and infants [138]. Not only does such live vaccine tend to induce more durable immunity, but it is increasingly appreciated that they may have beneficial effects in preventing a range of infections that exceed those attributable to prevention of the infection they are targeted to [139,140]. This phenomenon of heterologous immunity is apparently due in part to immunometabolic and epigenetic enhancement of innate immunity, potentially via trained immunity, that is, an altered innate immune response to subsequent stimulation [141–143]. Certain adjuvants or combination adjuvantation systems may be able to reproduce the benefit of live vaccines in animal models [144] but whether these effects can be demonstrated in humans remains to be determined.

CONCLUSION

Currently employed and novel adjuvants with well defined MOA can greatly enhance immune responses in vulnerable populations such as young infants. Concurrently, a comprehensive understanding and assessment of undesirable reactogenicity associated with adjuvanted vaccine formulations is pivotal for developing optimal prophylactic and therapeutic vaccines. Recent advancements in adjuvant discovery and development including systems immunology, immunoengineering and population-specific human in-vitro modeling for identification of MOA and biomarkers of immunogenicity and reactogenicity promises to open a new era of personalized adjuvanted vaccines tailored to vulnerable populations.

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Conflicts of interest

D.D. and O.L. are named inventors on several patent applications related to vaccine adjuvants.

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