REVIEW

nature medicine

Key roles of adjuvants in modern vaccines

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Vaccines containing novel adjuvant formulations are increasingly reaching advanced development and licensing stages, providing new tools to fill previously unmet clinical needs. However, many adjuvants fail during product development owing to factors such as manufacturability, stability, lack of effectiveness, unacceptable levels of tolerability or safety concerns. This Review outlines the potential benefits of adjuvants in current and future vaccines and describes the importance of formulation and mechanisms of action of adjuvants. Moreover, we emphasize safety considerations and other crucial aspects in the clinical development of effective adjuvants that will help facilitate effective next-generation vaccines against devastating infectious diseases.

Adjuvants have proven to be key components in vaccines that are today taken for granted. Indeed, many vaccines, comprised of whole or killed bacteria or viruses, have inherent immune-potentiating activity. However, attempts to develop a new generation of adjuvants, which will be essential for new vaccines, have been hindered somewhat by perceived, but most often undocumented, health risks and public misinformation, rather than by verified safety issues. Nonetheless, it is essential that vaccine and adjuvant developers fully utilize information on adjuvants' modes of action, avoid using undefined components in adjuvant formulations and develop comprehensive data packages on the safety, tolerability and efficacy of adjuvanted vaccines. Crucially, the inclusion of an adjuvant in a vaccine product or therapeutic regimen must be justified—that is, it should fill an unmet need. The degree of enthusiasm with which vaccine developers and regulators approach new vaccine adjuvants will depend largely on the contribution of the adjuvant and the importance of the vaccine. This Review addresses the contribution of adjuvants in current and future vaccines, their formulation aspects and safety considerations, and progress in understanding their mechanisms of action. We do not discuss other roles of adjuvant formulations as therapeutics, for example, in treating cancer or allergy.

Adjuvants, in the context of vaccines, are defined as components capable of enhancing and/or shaping antigen-specific immune responses. Biotechnology advances have enabled modern vaccines to be based on rationally designed recombinant antigens containing highly purified components with excellent safety profiles. Conversely, the immunogenicity of such well-defined vaccine antigens may be low compared to vaccines comprised of live attenuated or inactivated pathogen preparations. Live attenuated or inactivated vaccines may inherently contain natural adjuvants as they have heterogeneous compositions, which may include particulate forms of proteins, lipids and oligonucleotides, albeit in an undefined context[1] (**Fig. 1**).

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Received 19 December 2012; accepted 27 September 2013; published online 5 December 2013; doi:10.1038/nm.3409

Modern adjuvant development, which in spite of many hurdles is progressing, is based on enhancing and shaping vaccine-induced responses without compromising safety by selectively adding well-defined molecules, formulations or both. Because vaccines are often employed prophylactically in populations of very young people, it is important that medical risks to the subject (that is, safety) and other adverse effects (that is, tolerability) are addressed. Vaccine adjuvants designed for therapeutic uses, such as in cancer, may have a different risk-benefit profile. Adjuvants currently employed in human vaccines licensed for use in the US and/or Europe include aluminum salts, oil-in-water emulsions (MF59, AS03 and AF03), virosomes and AS04 (monophosphoryl lipid A preparation (MPL) with aluminum salt).

Adjuvant and formulation selection may be based on several parameters, including the physical and chemical natures of the vaccine antigen, type of immune response desired, age of the target population and route of vaccine administration. The desired qualities of each particular vaccine may necessitate adjuvants with specific properties. Indeed, the selection of the wrong adjuvant may render a particular vaccine antigen inadequate. Thus, vaccine antigen selection must take into account adjuvant selection to avoid discarding potentially effective vaccine antigen candidates.

Essential roles of adjuvants

Immunization with purified protein antigens typically results in the induction of a modest antibody response with little or no T cell response. Additionally, multiple immunizations may be required to elicit sufficient antibody responses. Developers may seek to include adjuvants in vaccine candidates to enhance the efficacy of weak antigens, to induce appropriate immune responses not sufficiently induced in the absence of adjuvant or both. For example, although there has been considerable investment in the development of recombinant influenza vaccines to better prepare for a pandemic, the candidates developed thus far require relatively high doses owing to their weak immunogenicity, which has a negative impact on the potential for a global supply. Adjuvants enable the use of lower vaccine doses, greatly expanding supply. This use and other practical applications of adjuvants are described below (**Fig. 2**).

Year	Vaccine	Adjuvant and mechanism	Scientific findings
1885	Rabies	ssRNA TLRs 7 and 8	
1886			Briegen describes endotoxin
1889			Coley shows tumor necrosis with bacterial extracts
1911	Typhoid	LPS, DNA TLRs 1, 2, 4, 5, 6 and 9	
1916		Lipovaccine	More durable immune response to typhoid vaccine
1921	BCG for TB	DNA, lipoprotein TLRs 1, 2, 6 and 9	
1926		Aluminum salts	Enhanced antibody responses to diphtheria vaccine
1937		Incomplete Freund's adjuvant (IFA) (water-in-oil emulsion)	Enhanced cellular and antibody responses to TB
1942	Diphtheria, pertussis and tetanus	LPS, DNA TLRs 1, 2, 4, 5, 6 and 9	
1949	Whole-cell influenza	ssRNA TLRs 7 and 8	
1955	Inactivated polio vaccine	ssRNA TLRs 7 and 8	
1966			LPS structure determined
1979			Ribi makes detoxified endotoxin MPL
1991	Hepatitis A		MPL tested in clinic
1996			TLRs discovered
1997	Fluad	MF59 (oil-in-water emulsion)	
1997	Epaxal (for hepatitis A) Inflexal (for influenza)	Virosome	
1998			LPS shown to be TLR ligand
2004	Invivac (for influenza; Europe)	Virosome	
2005	Fendrix (for hepatitis B; Europe)	MPL Defined TLR4	
2007–2009	Pandemic influenza vaccines (Europe)	MF59, AS03 (oil-in-water emulsion)	
2009	Cervarix (for HPV16 and HPV18; USA)	MPL Defined TLR4	

Debbie Maizels

Figure 1 A timeline of adjuvant development. The history of vaccines containing adjuvants is shown, indicating the development from natural adjuvants to defined adjuvants. BCG, bacillus Calmette-Guérin; LPS, lipopolysaccharide; ssRNA, single-stranded RNA; TB, tuberculosis.

Dose sparing. A recently issued report[2] specifically addressed solutions to increase the global supply of an influenza vaccine in the event of a pandemic. It was estimated that approximately 1 billion doses of the vaccine could be produced, which is insufficient to cover the worldwide population. Recommendations included the expansion of vaccine technologies beyond egg-based production (which itself could be compromised in the event of a pandemic involving bird flu) to include recombinant vaccines, as well as the use of adjuvants to increase global vaccine supply. Recombinant vaccines can have considerable manufacturing advantages, but they are weakly immunogenic on their own. The pairing of adjuvants with recombinant pandemic influenza protein can substantially reduce the amount of antigen needed to induce target antibody titers, a result with an obvious effect on manufacturing capacity. For example, inclusion of the adjuvant glucopyranosyl lipid adjuvant–stable emulsion (GLA-SE) reduced the amount of recombinant influenza H5 protein needed to reach 40% seroconversion after one immunization by greater than 30-fold compared with the antigen alone[3].

Enabling a more rapid immune response. For many applications, including biodefense vaccines for pandemic flu, anthrax and other potential bioterrorism weapons, a single-shot vaccine is the goal. This may be accomplished by the addition of adjuvants to the target antigens, as exemplified by the addition of the AS04 adjuvant to hepatitis B antigen in GlaxoSmithKline's (GSK's) Fendrix, which enabled a reduction of a three-dose regimen to two doses[4,5].

Antibody response broadening. Many pathogens, such as influenza viruses, HIV, human papilloma virus (HPV) and the malaria parasite, display substantial antigenic drift, strain variations or both. Thus, the ability of adjuvants to broaden an immune response profile could be crucial to the success of vaccines against such targets. Experimentally, massively parallel sequencing has shown that the broadening effect of adjuvants may be mediated via expansion of B cell diversity, not merely through increased titers[6]. Clinically, antibody response broadening by adjuvants has been demonstrated in influenza and HPV vaccines[7–9].

Antibody response magnitude and functionality. It is well accepted that widely used adjuvants such as aluminum salts or oil-in-water emulsions induce a greater magnitude of antibody responses to vaccine antigens. There is now an increased appreciation of the capacity of adjuvants to increase not just overall antibody titer but greater numbers of functional antibodies, antibodies with higher affinity for vaccine antigens or both[10,11].

Developing vaccines for effective T cell responses. Several vaccines in development are aimed at targeting T cell responses, which are not optimally induced by the most commonly used adjuvants in vaccines approved for human use, including alum and oil-in-water emulsion–based adjuvants. A more refined objective may be to elicit more effective engagement of T helper cells for optimizing the quality and durability of antibody responses or to induce effector $CD4^+$ or $CD8^+$ T cells to kill intracellular pathogens. Therefore, the new generation of vaccines often incorporates agonists for Toll-like receptors (TLRs) and other innate immune receptors that facilitate the generation of T helper cell responses. This has been particularly important in the development of vaccines against pathogens that are controlled by cellular immune responses, including those causing malaria, tuberculosis and leishmaniasis.

Classes of adjuvants

The term adjuvant may have different meanings depending on the application. For example, delivery systems composed of

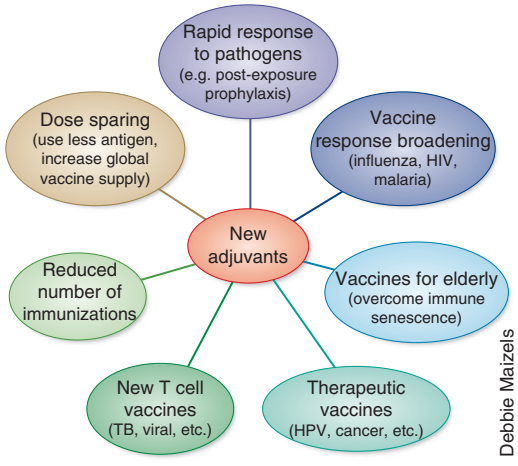

Figure 2 Potential benefits of adjuvants. Several crucial gaps in modern vaccine product development may be filled by appropriate adjuvant technologies.

Table 1 Classes of clinically used and tested adjuvants

Adjuvant name	Class	Mechanism or receptor	Type of immune response	Clinical phase or licensed product name
dsRNA analogues (for example, poly(I:C))	IM	TLR3	Ab, T_H1, $CD8^+$ T cells	Phase 1
Lipid A analogues (for example, MPL, RC529, GLA, E6020)	IM	TLR4	Ab, T_H1	Cervarix, Supervax, Pollinex Quattro, Melacine
Flagellin	IM	TLR5	Ab, T_H1,T_H2	Phase 1
Imidazoquinolines (for example, Imiquimod, R848)	IM	TLR7 and TLR8	Ab, T_H1	Aldara
CpG ODN	IM	TLR9	Ab, T_H1, $CD8^+$ T cells	Phase 3
Saponins (for example, QS21)	IM	Unknown	Ab, T_H1,T_H2, $CD8^+$ T cells	Phase 3
C-type lectin ligands (for example, TDB)	IM	Mincle, Nalp3	Ab, T_H1, T_H17	Phase 1
CD1d ligands (for example, α- galactosylceramide)	IM	CD1d	Ab, T_H1, T_H2, $CD8^+$ NKT cells	Phase 1
Aluminum salts (for example, aluminum oxyhydroxide, aluminum phosphate)	PF	Nalp3, ITAM, Ag delivery	Ab, T_H2	Numerous licensed products
Emulsions (for example, MF59, AS03, AF03, SE)	PF	Immune cell recruitment, ASC, Ag uptake	Ab, T_H1, T_H2	Fluad, Pandemrix
Virosomes	PF	Ag delivery	Ab, T_H1,T_H2	Epaxal, Inflexal V
AS01 (MPL,QS21, liposomes)	C	TLR4	Ab, T_H1, $CD8^+$ T cells	Phase 3
AS02 (MPL,QS21, emulsion)	C	TLR4	Ab, T_H1	Phase 3
AS04 (MPL, aluminum salt)	C	TLR4	Ab, T_H1	Cervarix
AS15 (MPL, QS21, CpG, liposomes)	C	TLR4 and TLR9	Ab, T_H1, $CD8^+$ T cells	Phase 3
GLA-SE (GLA, emulsion)	C	TLR4	Ab, T_H1	Phase 1
IC31 (CpG, cationic peptide)	C	TLR9	Ab, T_H1, T_H2, $CD8^+$ T cells	Phase 1
CAF01 (TDB, cationic liposomes)	C	Mincle, Ag delivery	Ab, T_H1, $CD8^+$ T cells	Phase 1
ISCOMs (saponin, phospholipid)	C	Unknown	Ab, T_H1,T_H2, $CD8^+$ T cells	Phase 2

Ab, antibody; Ag, antigen; ASC, apoptosis-associated speck-like protein containing caspase recruitment domain; C, combination of immunomodulatory molecule and particulate formulation; dsRNA, double-stranded RNA; IM, immunomodulatory molecule; ITAM, immunoreceptor tyrosine-based activation motif; PF, particulate formulation; TDB, trehalose dibehenate. Some particulate formulations (such as aluminum salts and emulsions) also generate immunomodulatory activity.

nonimmunostimulatory components may function as adjuvants by providing more effective antigen presentation to the immune system. In contrast, specific adjuvant molecules may directly activate innate immune receptors (for example, TLRs). Other formulation systems may include both delivery and immunostimulatory components. Thus, adjuvants may be broadly classified into three groups of delivery systems: immunomodulatory molecules, and combinations of the former two classes (combination systems) (**Table 1**). Moreover, the mechanisms of action of many adjuvants, including aluminum salts, the oldest adjuvant in use, are still being elucidated (**Box 1** and **Figs. 3** and **4**).

Immunomodulatory molecules include ligands of innate immune receptors such as TLRs, NOD-like receptors (NLRs), C-type lectins and RIG-I–like receptors (**Fig. 3**). The mechanisms of action of other immunostimulatory molecules, such as QS21 and other saponins, are not well understood. Among the most advanced compounds are the TLR4 ligand MPL, which comprises part of the adjuvant system in the Cervarix HPV vaccine (from GSK), and the TLR9 ligand CpG oligodeoxynucleotide (ODN), which is the adjuvant in the Hepislav vaccine candidate for hepatitis B from Dynavax that has completed a phase 3 clinical trial[12]. MPL and QS21 form part of the RTS,S malaria vaccine from GSK evaluated in a phase 3 clinical trial[13], although the adjuvant system in this case (AS01) and in the Cervarix vaccine (AS04) are classified as combination systems.

Another class of adjuvants includes delivery systems, meaning that their main function is to promote more effective delivery of vaccine antigens, immunomodulatory molecules or both. These adjuvants are perhaps best exemplified by conventional liposomes or virosomes. Liposomes are vesicles comprised of phospholipid bilayers. There are several related variations in development or in approved vaccines, such as virosomes (liposomes that include fusogenic viral proteins) and niosomes (vesicles composed of nonionic surfactants instead of phospholipids). Liposomes can range in size from <100 nm to several microns and are versatile delivery vehicles because antigens or immunomodulatory molecules can be encapsulated or associated with the vesicle surface. These lipid vesicle–based formulations are generally composed of nonimmunostimulatory components (for example, phosphatidylcholine) that provide delivery system capabilities, such as multimeric antigen presentation or fusogenic lipid activity, which enhance vaccine presentation to antigen-presenting cells (APCs). Approved virosome-based vaccines include the Inflexal V vaccine for influenza and the Epaxal vaccine for hepatitis A, both manufactured by Crucell. The RTS,S malaria vaccine mentioned above is also liposome based, wherein the liposomal formulation includes the immunostimulatory molecules QS21 and MPL.

Most adjuvants in advanced development provide delivery system and immunomodulatory properties. For instance, the Cervarix vaccine contains MPL and aluminum salt (AS04). Squalene-based

BOX 1 Recent findings on the mechanisms of action of alum

The adjuvant effects of alum were first discovered in the 1920s, and some billion doses of alum-adjuvanted vaccines have been administered to humans since then. Nevertheless, it seems that its multiple potential mechanisms of action are only now beginning to be elucidated. In 2008, De Gregorio *et al.*[21] concisely summarized the state of the field: alum enhances antigen uptake by DCs, cell recruitment to the injection site and stimulation of immune cells via the inflammasome, although there was some dispute regarding the specifics of the latter mechanism. For example, Kool *et al.*[119] proposed that administration of alum induces necrosis and uric acid production, a danger signal that activates the Nlrp3 inflammasome[120]. Eisenbarth *et al.*[121] confirmed a crucial role for the inflammasome in alum's actions; Franchi *et al.*[122] found that IgG antibody enhancement due to alum was uninhibited in Nlrp3 inflammasome-deficient mice. Since 2008, various other reports regarding alum have surfaced, some of which confirm or expand on previous conclusions regarding alum's impact on antigen uptake and immune cell recruitment[123–125], whereas others implicate a still greater range of diverse mechanisms of action[126]. Marichal *et al.*[127] proposed that alum adjuvant activity is related to the production of another danger signal, DNA released from necrotic cells exposed to alum. Flach *et al.*[23] employed atomic force microscopy to contend that aluminum salts interact with DCs but are not taken up by them; instead, they induce cell membrane lipid reordering, causing antigen uptake and upregulation of $CD4^+$ T cell adhesion molecules. Shah *et al.*[128] showed that type II natural killer T cells are involved in alum adjuvant activity in a CD1d-dependent manner, mediated by T_H2 cytokine production. IL-4–producing eosinophils are another recruited cell type that primes B cells in response to alum[129]. Recently, Wang *et al.*[130] confirmed the integral role of inflammasomes but suggested that this response is mediated by heat shock protein 70, indicating that alum acts as a stress-inducing agent. Interpreting the above reports is complicated by the lack of uniformity in the available reagents classified as aluminum salts; it is quite possible that each formulation elicits different effects. However, taken together, some common themes emerge: alum affects antigen uptake, induces danger signals, recruits various types of immune cells and elicits T_H2 responses. It is expected that further details will be elucidated and separate mechanisms will be identified in the future.

emulsions such as MF59 and AS03 have structure, and although the specific mechanisms of action of squalene and similar emulsions are incompletely understood, it is clear that they are not solely delivery systems because they significantly enhance the expression of various immune signatures depending on their oil composition[14–16]. By inducing a chemokine gradient, MF59 induces the recruitment of both monocytes and neutrophils to the site of immunization, where they take up the antigen[16–18]. Studies in mice indicate that this activity is dependent on the Myd88 and ASC signaling pathways, although it is probably independent of both the Nlrp3 inflammasome and TLR signaling[19,20]. Likewise, aluminum salts function as delivery systems in addition to their inherent adjuvant activity, although their mechanisms of action are still not completely understood[21–23] (**Box 1**).

Adjuvant formulation development

Most adjuvant formulation development focuses on micro- and nanoparticulate platforms, including aluminum salts, liposomes and emulsions. Aluminum salts have been employed as adjuvants in human vaccines for many decades, and they consist of crystalline nanoparticles that aggregate to form a heterogeneous dispersion of particles of several microns. They are highly charged and conducive to the adsorption of antigens or immunomodulatory molecules. Emulsions also have a long history of development, although until the 1990s they were not in approved vaccines. Modern emulsion adjuvants for human vaccines consist of oil-in-water, with nanosized oil droplets emulsified with biocompatible surfactants in an aqueous phase. Other formulations such as polymeric particles have undergone extensive research and development, but no approved vaccine products are on the horizon. The formulation platforms described above may have various effects on vaccine biological activity; they may have inherent adjuvant effects through modulating antigen delivery to APCs or through direct stimulation of immune cells.

There are many formulation parameters to consider, and each can have effects on shelf-life stability as well as biological activity: physicochemical characteristics (particle size and polydispersity, shape, surface charge, targeting moieties and component chemical structures (reviewed in refs. 24–26), association with antigen and immunomodulatory molecules and route of administration. Although a lack of standardization in comparative studies often complicates interpretation, formulation particle size and surface characteristics (including shape) may affect uptake by APCs[27–29], lymphatic trafficking[30–32], immune response quality and potency[33,34] and toxicity[35]. For example, Li *et al.*[34] showed that lipid-based nanoparticles of 230-nm diameter loaded with an ovalbumin antigen (OVA) were more efficiently internalized by dendritic cells (DCs) and macrophages, drained more efficiently to the lymph node and induced stronger IgG antibody and cytotoxic T lymphocyte responses than 708-nm-diameter particles, even though zeta potential and antigen loading parameters were constant. In order to more fully elucidate formulation effects on biological mechanisms, more thorough analytical characterization of adjuvant formulations will be essential. Thus, well-controlled sample preparation procedures and implementation of complementary analytical methods are required.

Formulation components, even in the absence of TLR agonists or other immunomodulatory molecules, may have intrinsic adjuvant activity. For instance, the oil chemical structure in vaccine emulsion formulations seems to be a crucial factor in determining the resulting immune responses following immunization of mice; a squalene-based emulsion induced greater titers of IgG antibodies in response to a recombinant malaria antigen, as well as enhanced hemagglutination inhibition titers, numbers of long-lived antibody-secreting plasma cells and titers of IgG antibodies in response to an inactivated influenza antigen, compared to emulsions based on long-chain triglycerides, medium-chain triglycerides or perfluorocarbons[14]. Other formulation-intrinsic adjuvanticity may include the induction of complement or other danger signals[32]. Moreover, the intrinsic adjuvant activity of some formulation platforms may entirely be due to their more effective delivery of antigen components. For instance, aluminum salt adjuvant activity is generally thought to be improved when antigens are adsorbed to the aluminum particles, although this is not the case for all antigens[36]. Similarly, the association of some antigens to the surface of liposomal delivery vehicles has been shown to enhance their immunogenicity in some (but not all) cases; in turn, the particular association method may affect the type and/or extent

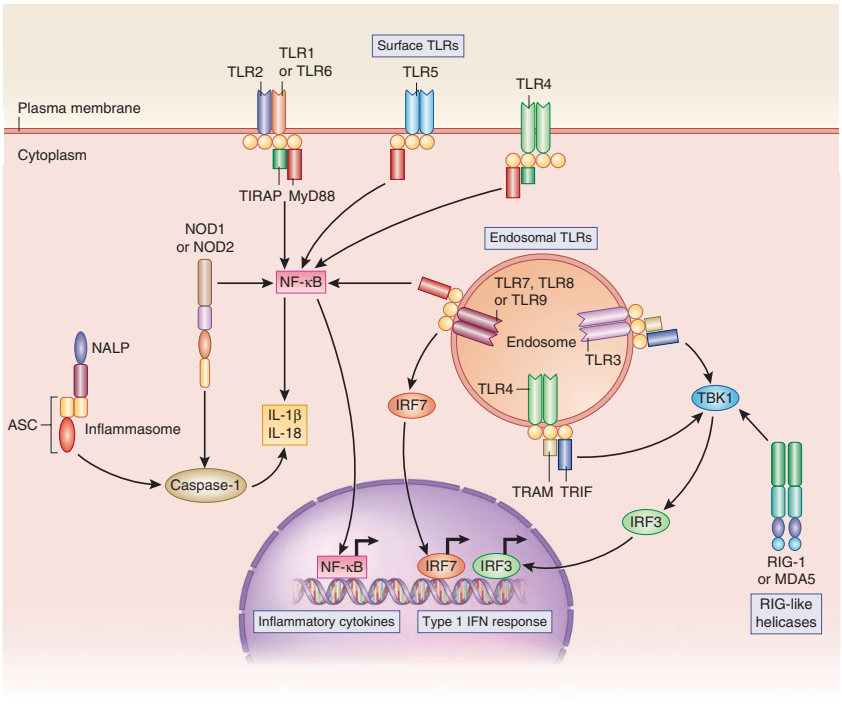

Figure 3 Target receptors on APCs for adjuvants. Several pattern recognition receptors (PRRs) that activate an innate immune response can be targeted by adjuvants, and details of their downstream signaling pathways are shown. TLRs, located at the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR11) or the endosome (TLR3, TLR7, TLR8 and TLR9) are targets for adjuvants, and when activated they stimulate signaling that leads to the activation of key transcription factors, such as nuclear factor-κB (NF-κB). These transcription factors then stimulate gene expression programs that lead to the production of chemokines and cytokines that help orient particular immune responses. Adjuvants can also target cytosolic PRRs such as NLRs and RIG-like helicases. The NLR NALP3 is part of a macromolecular assembly, the inflammasome, that leads to caspase 1 activation and the production of the proinflammatory cytokines IL-1β and IL-18. ASC, apoptosis-associated speck-like protein containing CARD; IRF3, interferon regulatory factor; MDA5, melanoma differentiation-associated protein 5; MyD88, myeloid differentiation factor 88; TBK1, TANK-binding kinase 1; TIRAP, Toll-interleukin 1 receptor domain–containing adaptor protein; TRAM, Trif-related adaptor molecule; TRIF, TIR-domain-containing adapter-inducing interferon-β.

of response[36–41]. The question of formulation association is important not only for antigens but also for TLR agonists or other immunomodulatory molecules. Thus, co-encapsulation of CpG and antigen in polymeric microparticles significantly increased cytotoxic T lymphocyte activity compared to the same particles with unencapsulated CpG[42]. Associating immunostimulants with particulate formulations may also promote localized immune activation and reduce systemic exposure and inflammation and thus improve the safety profile of an adjuvant. For instance, development of the new TLR7 and TLR8 ligand 3M-052 was designed to maintain the adjuvant activity but reduce the systemic exposure profile of the small molecule R-848, a similar TLR7 and TLR8 ligand, via the addition of an acyl chain[43].

Finally, the anatomical disparity in the various immunization routes and the surface modification of particle-based formulations by adsorbed host proteins (that is, the 'protein corona effect', wherein particles are surrounded by adsorbed proteins from the interstitial milieu) are essential factors in considering how to optimize formulations[44,45]. Formulations of a specific size or composition may be suitable for some routes but ineffective or even reactogenic when administered by another route[46–49]. For instance, Mohanan *et al.*[46] demonstrated that intralymphatic administration of different particle-based adjuvant formulations with OVA elicited strong IgG2a responses in mice compared to subcutaneous administration (with the exception of a chitosan-lipopolysaccharide nanoparticle formulation), whereas intramuscular and intradermal routes produced intermediate responses. However, some formulations at certain doses may not

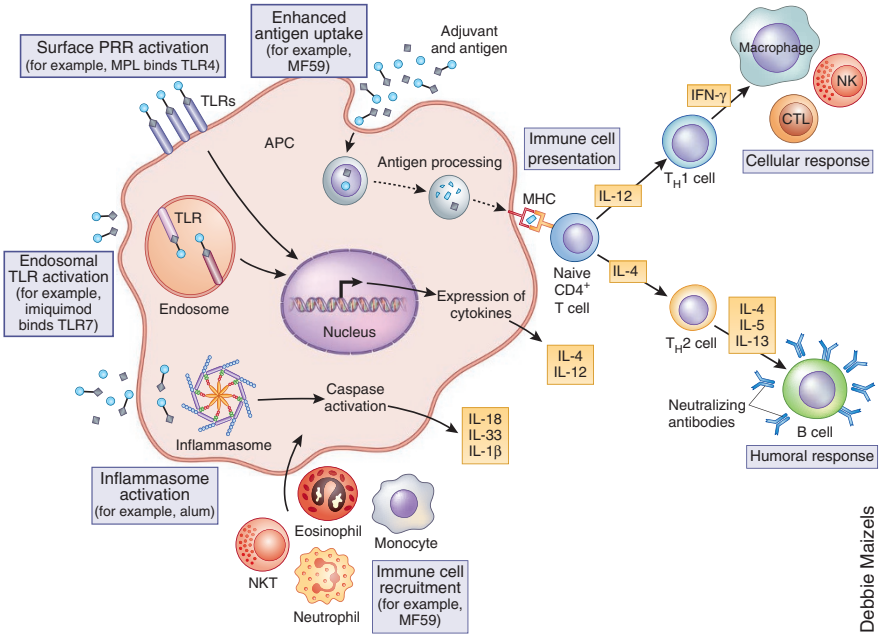

Figure 4 Putative mechanisms of action of adjuvants. A number of mechanisms have been postulated through which adjuvants mediate their activity. Many adjuvants can act as ligands for PRRs that activate an innate immune response. Receptor signaling can then activate transcription factors that induce the production of cytokines and chemokines that help direct a particular immune response, such as a T_H1 or T_H2 type response, as well as influence the immune cells that are recruited to the site of injection. Inflammasome activation has also been implicated as a mechanism for some adjuvants. Activation of the inflammasome leads to the production of the proinflammatory cytokines IL-1β and IL-18. Some adjuvants also influence antigen presentation by MHC. It is possible that some adjuvants can act through multiple mechanisms; for example, it has been suggested that alum can affect antigen uptake, PRR signaling, inflammasome activation and recruitment of immune cells. NK, natural killer cell.

Table 2 Considerations for an ideal adjuvant

Category	Subcategory	Considerations
Biological activity	Safety	Formulation must be safe and effective in all age groups; metabolizable components preferred; adjuvant activity should be localized and transient; adjuvant should not have direct effect on lymphocytes: no nonspecific B or T cell responses
	Immunization route	Each immunization route may have different formulation requirements
	Antigen dose sparing	Adjuvant should enable reduction in required antigen dose or number of immunizations
	Response broadening	Adjuvant should broaden protective responses against heterologous pathogen strains
	Antibody responses	Neutralizing antibody responses should be enhanced or prolonged by adjuvant
	Cell-mediated immunity	Adjuvant should induce and/or prolong pathogen-specific $CD4^+$ and/or $CD8^+$ T cell responses
	Immune response quality	Adjuvant should enable shaping of immune response (for example T_H1 versus T_H2 balance)
	Improve responses in weak immune systems	Immune responses should be enhanced in very young, elderly or immunocompromised populations
Physicochemical aspects	Raw materials	Synthetic adjuvants are preferable for purity, sustainability and safety; plant-based adjuvants may be acceptable if synthetic ones are too costly or have low yield; animal sources should be avoided for sustainability and disease concerns; multiple sources should be available at low cost; metabolizable or excretable components preferred
	Manufacturability	Equipment and process should be scalable, transferable and able to produce consistent batches
	Particle morphology	<200 nm particles can be terminally filtered, avoiding requirement for aseptic manufacturing, and may enter lymph node more easily than large particles; orientation and shape of nonspherical particles affects cell uptake; charge and chemical structure of surface groups are crucial factors in resulting bioactivity; targeting molecules such as mannose may enhance delivery to APCs; some concern regarding potential toxicity of cationic particles
	Antigen compatibility, association	Effects of adjuvant formulation on antigen structure should be characterized; generally it is thought that some level of association of the antigen to the formulation is preferred, although direct association is not required for biological activity
	Stability	Excipients and active pharmaceutical ingredients (APIs) should maintain chemical structure and particle size, shape, polydispersity and visual appearance, and API localization should be constant for several years; packaging under inert gas guards against oxidative degradation

be suitable for intradermal use; for instance, aluminum hydroxide has been reported to cause persistent granulomatous and necrotic reactions at intradermal administration sites[49]. The considerations that should be taken into account in order to design an 'ideal' adjuvant', with a focus on formulation factors, are summarized in **Table 2**.

Adjuvant formulations for the development of new vaccines

Different formulations of the same immunomodulatory molecules may induce substantially different immune responses. This was illustrated in the malaria vaccine program wherein the RTS,S vaccine candidate formulated with AS02 (an oil-in-water emulsion containing MPL and QS21) protected six out of seven vaccine recipients from infection, whereas the same antigen with AS03 (emulsion without MPL or QS21) or AS04 (MPL and aluminum hydroxide) protected only two out of seven or one out of eight recipients, respectively[50]. Later, it was shown that switching from an oil-in-water emulsion formulation (AS02) to a liposome formulation (AS01) with the same antigen and immunostimulants increased efficacy, T helper type 1 (T_H1) cell–mediated immunity, and antigen-specific humoral immunity in both mice and humans[51–55]. This vaccine candidate retained almost 50% efficacy in children 5–17 months old, although efficacy waned in the very young (26% in infants aged 6–12 weeks)[56]. Pairing either AS01 or AS02 with the tuberculosis vaccine antigen M72 demonstrated that the liposomal formulation (AS01) with the same antigen and immunostimulants elicited greater frequencies of polyfunctional T_H1 cells in immunized volunteers than the oil-in-water emulsion[57]. Addition of MPL to aluminum hydroxide (AS04) significantly increased the titers of anti-HPV antibodies in both vaccinated mice and humans compared to a vaccine adjuvanted with aluminum hydroxide alone[58,59].

Another widely used adjuvant formulation, MF59, has been evaluated preclinically in the context of additional immunostimulants, systematically demonstrating the contribution of each component of the emulsion. Whereas MF59 boosts overall immune responses, addition of TLR ligands changes the quality of the immune response. For instance, inclusion of the TLR9 ligand CpG or the TLR4 ligand E6020 in an MF59-adjuvanted influenza vaccine did not further increase antibody titers in mice compared to treatment with an MF59-alone influenza vaccine, but it did induce a shift to a T_H1-type immune response[60]. In another influenza vaccine study in mice, addition of CpG to aluminum hydroxide or MF59 resulted in higher antibody titers as well as a T_H1 shift compared to CpG alone or either formulation alone[61]. Interestingly, an MF59-mimic formulation combined with CpG administered prophylactically with a recombinant antigen inhibited melanoma and prolonged survival in tumor-bearing mice, whereas the same composition administered in the absence of CpG actually promoted melanoma growth[62]. Finally, an MF59-E6020 formulation (oil-in-water emulsion with a TLR4 agonist) combined with recombinant meningococcus B antigens enhanced serum and bactericidal titers in mice compared to MF59 alone[63]. In contrast, clinical evaluation of the oil-in-water emulsion AS03 in the context of seasonal influenza vaccine for elderly people showed only a limited immunogenicity benefit from the addition of MPL[64]. Taken together, these two studies of oil-in-water emulsions combined with TLR4 ligands highlight an important point: the added benefit of a TLR ligand is dependent on the nature of the antigen. In other words, there may be less need for additional immunostimulants when the vaccine antigen is an inactivated virus that has inherent TLR ligands compared to a purified recombinant antigen where the addition of a TLR ligand will probably have more substantial immunogenic effects. We have found that the

MF59-like adjuvant SE enhances antibody responses to vaccine antigen[65] and induces interleukin-5 (IL-5)-producing T_H2 cells[66,67]. For intracellular pathogens such as *Leishmania* and *Mycobacterium tuberculosis* that probably require T_H1 responses for efficacy, this type of response may not be beneficial or may even be detrimental. Addition of the TLR4 agonist GLA to SE in the EM005 adjuvant induced interferon-γ (IFN-γ) production by $CD4^+$ T cells and provided significant protection against tuberculosis and leishmaniasis in mice and guinea pigs[66,67]. However, replacing squalene with triglyceride-based oils abrogated this adjuvant activity of GLA in a tuberculosis vaccine, even though other particulate formulations not containing an oil component (such as GLA-alum or GLA-liposomes) maintained protective efficacy[68]. Therefore, proper selection of both the immunostimulant and formulation components of an adjuvant is crucial for inducing an appropriate immune response tailored to control the target pathogen.

Mechanistic insights from systems vaccinology

Recent reports have begun to address mechanisms of action of existing adjuvants, including recent reviews on widely used adjuvants such as MF59 and virosomes[69–72]. For instance, MF59 operates through multiple mechanisms, including the creation of a local immunocompetent environment that results in enhanced antigen uptake and immune cell recruitment[69]. Virosomes display influenza protein on their surface, which may help with antigen uptake and immune cell activation through their repetitive display of the antigen on particulates, and upregulate cytokines in peripheral blood mononuclear cells (PBMCs). Preexisting influenza immunity may enhance humoral and cellular responses to virosome-based vaccines, not just those against influenza[70]. Trehalose dibehenate, an ingredient of the cationic liposome formulation CAF01, binds a C-type lectin receptor and activates the inflammasome[73,74]. TLR ligands such as MPL have known receptors, but the specific structure of the adjuvant molecule may determine different signaling pathways through the same receptor[75,76]. However, additional research is needed to further investigate mechanisms of action of adjuvants. Below, we describe new approaches that may enable a more comprehensive understanding of the mechanisms underlying adjuvants' activities.

Candidate and licensed vaccines have historically been assessed using two metrics, immunogenicity and efficacy. The challenge with both these types of measurements is that they are temporally removed from the actual immunization. Recent efforts have been made to employ systems biology to describe the early events following immunization and identify proximal changes that can predict either immunogenicity or efficacy. Although systems biology can cover a wide variety of 'omics' fields, most systems biology approaches to vaccine development have focused on transcriptional profiles on account of the assay availability and expertise in this field. The goal of systems vaccinology is to identify unique immune signatures arising hours to days after immunization that can predict whether a recipient will develop the desired immune response (correlates of immunity) and/or will be protected from the targeted disease (correlates of protection). From a vaccine development standpoint, this approach holds the promise of quickly identifying effective and noneffective vaccines within days of immunization. These approaches may also predict immediate or long-term adverse effects stemming from the immunization.

Transcriptional profiling of human PBMCs acutely after immunization with the live attenuated yellow fever vaccine YF-17D revealed that expression of the stress response pathway protein eukaryotic translation initiation factor 2 alpha kinase 4 correlated with the magnitude of the virus-specific $CD8^+$ T cell response[77,78]. In the same study the amounts of tumor necrosis factor receptor superfamily member 17 (TNFRSF17), a receptor for the B cell growth factor BLyS (known to play a key part in B cell differentiation), correlated with the magnitude of neutralizing antibodies[79]. The amounts of TNFRSF17 following immunization with inactivated influenza virus were also found to predict hemagglutination inhibition (HAI) titers, whereas early expression of calcium/calmodulin-dependent protein kinase type IV inversely correlated with HAI titers[80]. Thus, it may be possible to identify universal predictors of particular immune responses.

Several licensed or candidate adjuvants have been studied for their immune signatures in humans and in animal models. Mosca *et al.*[16] analyzed the expression profile in the muscle tissue of mice immunized with alum, CpG or the oil-in-water adjuvant MF59. All three adjuvants induced changes in the levels of a core set of transcripts that probably indicate recruitment of neutrophils and APCs to the site of immunization, activation of a type I interferon response and inflammatory programs resulting from damage to the tissue arising from parenteral injection. Additionally, each adjuvant also independently regulated a number of transcripts. Two of the genes specifically activated by MF59, *Junb* and *Ptx3*, may indicate that MF59 acts directly on skeletal muscle tissue in addition to professional APCs. In a subsequent study the same group analyzed the effects of different adjuvants on the antibody responses to a subunit flu vaccine in mice[81]. Of the adjuvants tested, only MF59 and the TLR2 agonist Pam3CSK4 increased overall antibody and HAI titers. Transcriptional analysis of the injection site 6 h after intramuscular injection revealed an increase in the expression of the leukocyte transendothelial migration gene cluster, including *Itgam* (encoding CD11b). Analysis of cellular infiltrates into the muscle following immunization confirmed that only MF59 and Pam3CSK4 induced robust recruitment of $CD11b^+$ cells, primarily neutrophils. These data suggest that early $CD11b^+$ cell recruitment to the injection site after vaccination with an emulsion-based adjuvant may be predictive of a subsequent robust humoral immune response.

Molecular profiling of isotype-switched antigen-specific B cells from mice immunized with OVA adjuvanted with TLR7 and TLR4 agonists revealed several clusters of transcriptional changes that may be indicative of a productive antibody response[10]. These included *Bcl2*, *Bcl11a*, *Tank*, several type-I interferon (IFN)-related genes, *Plcg2* and *Cd38*, all of which are associated with memory B cell formation. Genes affecting B cell survival and proliferation were also induced by the combination of TLR7 and TLR4, including *Il17ra*, *Il18r1*, *Pax5*, *Ifngr2*, *Bcor* and *Ikzf1*. Importantly, the change in expression of most of these markers was enhanced by combining the two adjuvants, and this combination also enhanced the magnitude and quality of the antibody response. In another study by the same group, compared to MPL and R-848, only CpG increased the expression of TNFRSF17 in PBMCs following intradermal injection, in the absence of antigen, into rhesus macaques[82]. Thus, CpG may be a good adjuvant for intradermal immunization aimed at eliciting antibody responses.

A recent study analyzed transcriptional profiles of PBMCs from individuals immunized with the candidate malaria vaccine RTS,S/AS01B (ref. 83). Protection from parasitemia following challenge with malaria-infected mosquitoes correlated with increased expression of genes involved in the formation of the immunoproteasome 2 weeks after the third immunization, particularly expression of *PSME2*. The inducible immunoproteasome enhances major histocompatibility (MHC) antigen presentation by increasing the breadth of peptides presented. It is reasonable to hypothesize that increased MHC presentation of antigenic peptides enhances the development of both the

polyfunctional CD4+ T cells that make IFN-γ, TNF, IL-2 and CD40L, and, indirectly, the antibody response associated with the protective efficacy of RTS,S/AS01B.

The results of these studies highlight the potential for systems vaccinology to turn human clinical trials into hypothesis-generating exercises in addition to the traditional function of hypothesis testing. New clinical trials of candidate vaccines offer the opportunity to test hypotheses such as whether early TNFRSF17 expression is predictive of a robust humoral response or whether upregulation of the immunoproteasome predicts strong T cell responses to the vaccine. It will be important to test whether the signatures associated with the immunogenicity the YF-17D vaccine also predict the immune response magnitude and quality of other licensed and candidate vaccines[77–80]. Additionally, these studies should generate new hypotheses about the mechanistic nature of adjuvants that can be tested in a preclinical setting. Systems vaccinology approaches may also allow correlation between early gene expression changes and the occurrence of adverse events. Such correlations may provide more mechanistic insights into how adjuvant candidates elicit these adverse events. This would also allow for early identification and elimination of adjuvant candidates that have a high likelihood of producing unacceptable side effects, possibly even at the preclinical stage.

Animal models versus clinical experience with adjuvants

Evaluation of preclinical data regarding adjuvant activity is fraught with caveats. First, animal models clearly have different TLR expression patterns compared to humans[84]. Moreover, the TLR specificity for adjuvant molecules may also be dramatically different in different species. For example, mouse TLR4 is more promiscuous in its binding affinity for lipid A derivatives even with substantial variations in lipid A acyl chain number and length, whereas human TLR4 is highly specific regarding lipid A structure; in fact, lipid A molecules that are TLR4 agonists in mice may be TLR4 antagonists in humans[85–88]. Despite these species-specific differences, the TLR4 agonist monophosphoryl lipid A was the first TLR agonist to be approved for inclusion as a vaccine adjuvant. TLR8 was proposed to be expressed in humans, but not in mice, somewhat complicating immunological studies[89]. Many immunostimulatory molecules activate both TLR7 and TLR8, which may lead to unexpected activities of such agonists as they are translated from preclinical models to human testing[90]. As is the case with TLR4, human and rodent TLR9s recognize slightly different molecules, making the translation of an adjuvant that is effective in animals to testing in humans challenging. Additionally, the cellular expression pattern of TLR9 differs between humans and rodents, further complicating development of TLR9 agonist adjuvants (reviewed in ref. 91). TLR9 agonists face an additional challenge, as substantial safety concerns about their use were raised by a study showing that TLR9 agonists contribute to the production of autoreactive antibodies in mice[92]. Despite these challenges, Dynavax's Heplisav vaccine, which includes the TLR9 agonist 1018 ISS, demonstrated a robust immune response toward the vaccine antigen with no apparent induction of autoreactive antibodies in a recently completed phase 3 study[93]. Nevertheless, to date the vaccine has not been approved[94]. However, the success of MPL-containing vaccines confirms that the considerable challenges in translating TLR agonists from animal models to human usage are not insurmountable. Difficulties in translating results from animal systems to the development of human vaccines are not unique to the development of new adjuvants. For example, DNA vaccination is very efficient in mouse models but is much less immunogenic in humans[95]. Similarly, adenovirus-vectored vaccine candidates have shown great promise in animal models but have been less successful in humans, where preexisting immunity to the vector may limit efficacy[96].

Selection of appropriate preclinical animal models is essential for the efficient development of new vaccines. Nonhuman primates (NHPs) are likely to be the most predictive animal model for many vaccines, yet ethical and financial considerations limit the widespread use of these models. Furthermore, NHPs do not always respond to adjuvants in a manner predictive of responses in humans, particularly regarding adjuvant doses. Another limitation of animal models is that none of the commonly used models are ideal to study intradermal or transdermal immunization procedures, largely owing to differences in skin architecture. There is interest in developing more robust methods of intradermal immunization given the potential advantages of this route (more efficient antigen presentation and enhanced TLR repertoire of skin-resident APCs). Device makers have developed several products for intradermal vaccine delivery using hollow microneedles (from BD and NanoPass) or solid microneedles, and the recent approval of Intanza (Sanofi), an intradermal influenza vaccine, demonstrated some advantages of intradermal immunization by increasing positive responses in elderly populations, an area remaining to be explored in more depth.

Preclinical animal models are needed to establish a basic safety profile of adjuvants, but they may not be predictive of all potential safety issues. Even large phase 3 clinical trials may not be sufficiently powered to detect rare side effects. For example, oil-in-water emulsion adjuvants have recently undergone increased scrutiny as a result of adverse reactions observed during the 2009 H1N1 influenza pandemic, at which time millions of doses of vaccines adjuvanted with the oil-in-water emulsions MF59 or AS03 were administered. Some Nordic countries first noticed an increased risk of narcolepsy in children and adolescents immunized with the AS03-containing vaccine Pandemrix (GSK)[97,98], which led to a revised use recommendation by the European Medicines Agency, although the overall benefit-to-risk ratio was considered positive[99]. However, subsequently, additional cause for concern has arisen, as several other European countries, including the UK, have now published related findings showing an increased risk of narcolepsy in young people after vaccination with Pandemrix[100–103]. To date, preliminary assessments of a potential mechanism for the association between narcolepsy and Pandemrix vaccination involving an induced autoimmune response have been considered inconclusive by the European Medicines Agency[104]. Interestingly, although Canada and Brazil also employed an AS03-adjuvanted H1N1 vaccine (Arepanrix, also), these countries have not reported an increased risk of narcolepsy, nor has any link been established between MF59-adjuvanted vaccines and narcolepsy[105]. Although the mechanisms responsible for causing narcolepsy are unknown, it should be noted that major differences exist in the compositions of AS03 (squalene, α-tocopherol and polysorbate 80) and MF59 (squalene, polysorbate 80 and sorbitan trioleate); moreover, the antigens used in various H1N1 vaccines are also substantially different, with Pandemrix containing an inactivated split vaccine and Focetria (Novartis) containing a purified subunit vaccine. Furthermore, H1N1 infection independently of vaccination has been associated with increased incidence of narcolepsy in China[106]. More research is needed to investigate the specific causes of narcolepsy in vaccine recipients, the role of differences in vaccine composition and the genetic makeup of vaccinated populations and the corresponding implications to future vaccine development.

In the clinical development of adjuvanted vaccines, the chances for success are higher when there is a clear unmet need. Otherwise, the

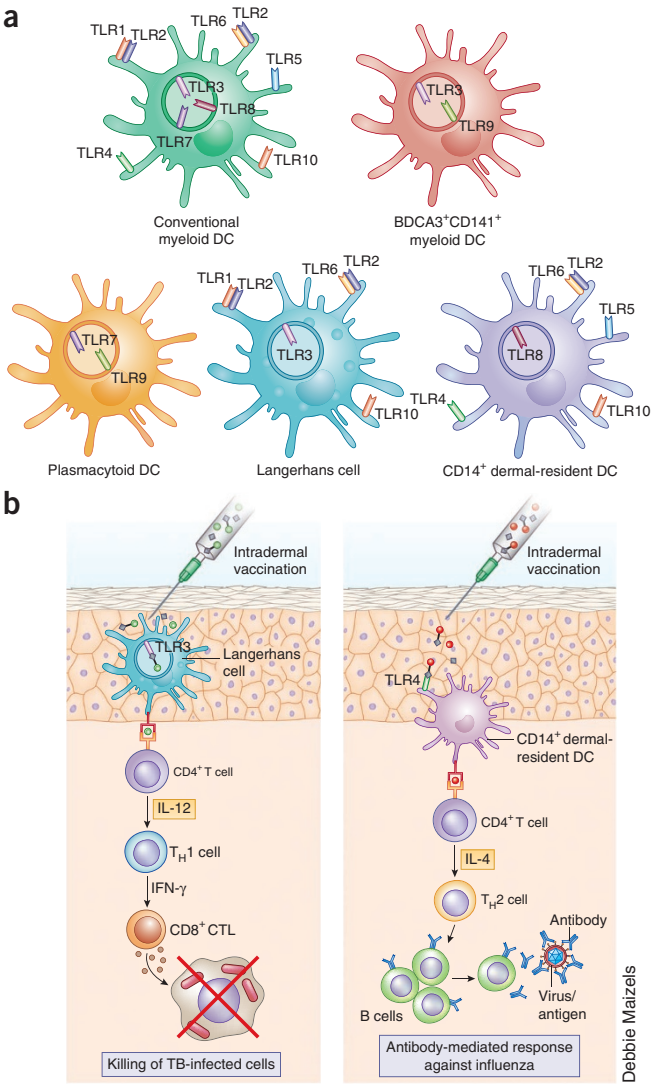

Figure 5 Central role of DCs in adjuvant activity. (**a**) Patterns of TLR expression by human DC subsets. (**b**) Theoretical modulation of immune responses by targeting adjuvant-DC interactions. For example, an optimal vaccine for tuberculosis, which is controlled by T_H1 $CD4^+$ T cells and cytotoxic $CD8^+$ T cells, would activate Langerhans cells via intradermal immunization with a TLR3-activating agonist (left). For a flu vaccine, the protective response is thought to be mainly mediated by humoral immunity, so use of a TLR4 agonist delivered intradermally to target $CD14^+$ dermal DCs might be beneficial (right).

perceived safety risks with the introduction of new adjuvants may not be considered as justifiable. For instance, a US Food and Drug Administration committee recently decided that a new hepatitis B vaccine containing the TLR9 agonist 1018 ISS demonstrated adequate immunogenicity but that there were insufficient data to support approval[94]. Notably, there are several other hepatitis B vaccines on the market. In contrast, the same committee unanimously recommended approval of GSK's H5N1 pandemic influenza vaccine containing the emulsion adjuvant AS03 for use in adults during a pandemic, despite the potential concerns discussed above[94].

Future directions

Recent work has shed considerable light on the mechanistic actions of both alum and oil-in-water emulsion adjuvants. Similar mechanistic insights are needed for next-generation adjuvants, particularly those that elicit a different type of immune response from the first-generation adjuvants (that is, cell-mediated versus humoral immunity). Of particular interest will be determining how different formulations of the same immunostimulant alter the molecular pathways activated by vaccination. For example, it will be important to understand why MPL and QS21 formulated in liposomes as AS01 elicit a different quality of immune response than when the same molecules are formulated in an oil-in-water emulsion. Similarly, it will be important to elucidate the molecular underpinnings that make squalene-based but not other oil-in-water–based emulsions effective adjuvants[68]. Finally, the potential benefits of alternative routes of delivery remain to be fully realized. Such findings will enable high-throughput, rational screening of potential adjuvant candidates and instruct the optimization and development of new adjuvants.

Another important new area of vaccine development is the targeting of DCs, the most potent professional APCs of the immune system[107]. Three populations of human blood DCs and two populations of skin-resident DCs have been described with varying expression of TLRs and capacities to induce different types of adaptive immune responses (reviewed in ref. 107) (**Fig. 5**). Conventional myeloid DCs are able to activate both $CD4^+$ and $CD8^+$ T cell responses as well as antibody responses[108]. The recently identified $BDCA3^+CD141^+$ myeloid DCs are proficient at cross-presenting exogenous antigens via MHC class I to induce robust $CD8^+$ T cell responses[109,110]. Plasmacytoid DCs are characterized by their ability to produce massive amounts of type I interferon upon stimulation, which may be important to their ability to prime $CD8^+$ T cell responses as well as the induction of plasma cell formation[111–113]. Skin-resident Langerhans cells are adept at inducing both $CD4^+$ and $CD8^+$ T cell responses, whereas their ability to induce B cell responses by driving follicular helper T cells is reduced compared to other DC populations[114,115]. Dermal-resident $CD14^+$ DCs are able to drive the formation of CXCL13-secreting follicular helper cells that enhance class switching but are inefficient at driving cytotoxic T lymphocyte formation[114,116]. Future studies are needed to assess the feasibility of rationally selecting an adjuvant and administration route that will optimally activate the DC subset best suited to inducing the desired immune response.

Another crucial avenue for future adjuvant development is in regards to induction of mucosal immunity. Many enteric diseases, which disproportionately affect disadvantaged populations, lack effective vaccines. In cases where effective injectable vaccines have been developed, such as with the inactivated polio vaccine, the induced mucosal immune responses are less than optimal. For these reasons, it will be important to devise ways to elicit stronger mucosal immune responses. This may involve alternative routes of delivery, such as intranasal or sublingual, although such routes come with their own challenges regarding vaccine stability and administration and have not proven consistently superior at inducing mucosal immunity. Although they are at a very early stage, intriguing new approaches based on vitamin metabolites offer potential alternatives. For example, the vitamin A metabolite retinoic acid has been shown to induce T cell homing to the gut and increased IgA responses after parenteral immunization[117], and a new report suggests that vitamin B metabolites may activate a specific mucosal-associated T cell population[118]. In the future, vaccine adjuvants that offer more controlled targeting in their delivery and biological effects should enhance vaccine efficacy while minimizing required antigen and adjuvant doses.

Conclusions

Adjuvant development for human vaccines has been a circuitous process. Adjuvants used in animal models, often with high oil content and complex bacterial extracts, in general did not meet adequate safety or quality standards, which may have contributed to negative perceptions regarding adjuvants for human vaccines. Breakthroughs in the design and use of safe and effective adjuvants came with the development of emulsions (for example, MF59) and the alum-MPL combination (AS04), both of which have been used in millions of individuals. These are examples of advances around which next-generation formulations can be and are being developed, including emulsions based on synthetic, yeast-derived or plant-derived oils (as opposed to fish-derived squalene) and adjuvants based on synthetic TLR4 ligands rather than those from bacterial extracts. Such advances are expanding the availability of defined adjuvants with better-understood mechanisms of action, which can be produced on a large scale. Application of systems biology in both animal and human studies will help in understanding adjuvant activity and selecting adjuvant components for further development and optimization.

In the development of new adjuvanted vaccines, it will be important to focus on clear unmet needs to establish a favorable benefit-to-risk ratio. Moreover, to engender positive public perception, rigorous clinical and post-marketing testing will be required to identify potential safety issues, as well as the mechanisms involved to guide subsequent vaccine development projects. Understanding the limitations of preclinical models will help avoid surprises in the clinic. Recognition of the impact of formulation factors and exploitation of systems vaccinology approaches will help ensure that the developed adjuvant systems are optimized for each particular vaccine. Furthermore, understanding of the proposed mechanisms of action of existing adjuvants must continue to be refined. All of these aspects must play vital parts in order to realize all of the potential benefits that adjuvants offer.

ACKNOWLEDGMENTS

We thank M. Friede for his helpful discussions and preparation of **Figure 1**. This project has been funded in part with funds from NIAID/US National Institutes of Health, the US Department of Health and Human Services, under Contract No. HHSN272200800045C. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government. This work was also supported with funding from the Bill & Melinda Gates Foundation, under grant 42387.

COMPETING FINANCIAL INTERESTS

The authors declare competing financial interests: details are available in the online version of the paper.

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