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Old and new adjuvants

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Adjuvants have been deliberately added to vaccines since the 1920's when alum was discovered to boost antibody responses, leading to better protection. The first adjuvants were discovered by accident and were used in the safer but less immunogenic subunit vaccines, supposedly by providing an antigen depot to extend antigen presentation. Since that time, much has been discovered about how these adjuvants impact cells at the tissue site to activate innate immune responses, mobilize dendritic cells and drive adaptive immunity. New approaches to vaccine construction for infectious diseases that have so far not been well addressed by conventional vaccines often attempt to induce antibodies, polyfunctional $CD4^+$ T cells and $CD8^+$ CTLs. The discovery of pattern recognition receptors and ligands that drive desired T cell responses has led to development of novel adjuvant strategies using immunomodulatory agents to direct appropriate immune responses.

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Introduction

Unknowingly, as an accident of the material used, or deliberately, adjuvants have been used to improve vaccines for as long as vaccines have been in use. For example, for some centuries, small doses of live smallpox viruses were used in China, Turkey, Persia and elsewhere to immunize healthy people against the disease [1]. The live viruses in this case expressed intrinsic adjuvants in the shape of the viral nucleic acids and components of the virus coat. Later, Jenner's famous cowpox vaccine [2] again brought with it adjuvants derived from the live virus, in a context that was less risky than smallpox itself, since the cowpox virus reproduced less well and caused less attendant pathology than smallpox infection in people. So, we could view the cowpox vaccine as the first successful use of what was essentially an attenuated live virus vaccine in humans. Use of what are now presumed to have been live attenuated viruses in vaccines continued as the viruses responsible for various diseases were discovered. For example, rabies virus that had been passaged in rabbits' brains and then dried constituted the first rabies vaccine, produced by Pasteur and Roux [3,4] and attenuated yellow fever virus became the standard vaccine for that disease (all reviewed in Nunnally *et al.* [5]).

Meanwhile soluble toxins produced by various infections were discovered. Among these were the diphtheria and tetanus toxins, discovered respectively by Roux and Yersin in 1888 [6] and by Faber and Tizzoni and Cattani [7,8]. Von Behring and others found that immunization with these (at the time rather impure) proteins protected animals against the disease they induced and that antisera produced against them in, for example, horses, was also protective upon transfer into intoxicated hosts [9]. To protect against intoxication by these proteins, they were sometimes given with the antisera but later Glenny and Sudmersen and Ramon [10,11] realized that the toxins could be inactivated with formaldehyde and were still sufficient to induce antibody production without toxic sequelae [12]. The consequent demand for antitoxin antibodies led to large scale immunization of horses for production of such antisera and memorable events such as the transport of life saving diphtheria antitoxin to Nome in 1925. How did these toxin and toxoid preparations raise antisera even though they are given without obvious adjuvants? Perhaps the preparations contained contaminating bacterial products, or perhaps their partial denaturation during preparation created some adjuvant properties in the proteins themselves [13]. In attempts to improve the titers of immunized horses, Ramon co-injected the antigens with various materials including tapioca and breadcrumbs [10,14], which did actually work so we can think of these common kitchen commodities as being among the first officially added vaccine adjuvants. At about the same time Pope had discovered that salt precipitates brought down toxins from solution, and Pope and Glenny together showed that tetanus toxoid adsorbed to insoluble aluminum salts (called alum for simplicity, to refer to the several insoluble aluminum salts used in vaccines, for the rest of this article) would improve antibody responses in guinea pigs [11]. Only a few years later, trials in human populations showed that diphtheria

vaccines formulated with alum protected against diphtheria intoxication much better than the previous vaccines.

Since the 1920s many new adjuvants have been added to vaccines. Here we will discuss both one of the oldest adjuvants (alum) and new adjuvants below and in other chapters in this edition.

Insoluble salts and other particulate adjuvants

Besides alum, several other particulate adjuvants, calcium phosphate, chitin and other insoluble carbohydrates and nanoparticles have been used in vaccines for humans. Viral-like nanoparticles will not be considered here although the particulate nature of the virus products may in itself have some adjuvant-like qualities [15]. The viral-like particles are, however, often administered with added adjuvants such as alum (for example in the Gardisil vaccine) and, in some formulations, AS04 (alum plus monophosphoryl lipid A (MPL) in Cervarix). In the USA, alum is added to a number of vaccines including those for diphtheria and tetanus toxins and pertussis (DTaP), for hepatitis A and B and for the various polysaccharide conjugate vaccines (see Box 1). In Europe

Box 1

Adjuvant	Vaccines
Mineral salts	
Aluminum salts AS04 (alum + MPL)	DT, DTaP, HVA, HBV, HPV (AS04 — see below), HIB, Meningococcus, Pneumococcus, IPV, HAV, HPV
Calcium phosphate	DT, DTaP, IPV
Delivery systems	
Viral-like particles	HBV, HPV, in clinical trials for HAV, HCV, malaria, HIV, HPV, malaria, norovirus
Liposomes	HBV, HPV, in clinical trials for Hepatitis A, C, malaria, HIV, HPV, malaria, Norovirus
Microparticles (PLA/PLGA)	Malaria HPV, HBV
Emulsions	
IFA (water-in oil emulsion)	Influenza (1950s)
AS02 (MPL + QS21 in oil-in water emulsion)	Malaria
Squalene	
MF59	Influenza
AS03	AS03 — in clinical trial for HPV and malaria
TLR agonists	
MPL-SE	Influenza (MPL)
AS04 (MPL + alum)	HPV, HBV
AS01 (MPL + QS21 in liposomes)	
AS02 (MPL + QS21 in oil-in water emulsion)	Malaria
GLA, GLA-SE	In clinical development for MTb, Influenza, Leishmaniasis

Abbreviations: DT, diphtheria/pertussis; DTaP, DT with acellular pertussis; HAV, hepatitis A virus; HBV, hepatitis B virus; HPV, human papilloma virus; HIB, haemophilus influenza type B virus; IPV, inactivated polio vaccine; MPL, monophosporyl lipid a; GLA, glucopyrosyl lipid adjuvant.

Adjuvant	Innate responses	Effects on DC	Type of immune response
Aluminum salts	NALP3/P2X7R-dependent neutrophil recruitment, DAMP release: (chromatin, histones, IL-1α, NETs, uric acid), TBK1/Irf3/STING dependent effects on IgE	↑ migration to LN (i.p.) ↑ T cell interactions ↑ antigen presentation ↓ IL-12 secretion Reorganization of membrane lipids	TH2 TFH ↑ IgG1/IgE
Emulsions	Increases delivery to APC ↑ phagocytosis ↑ infiltration of monocytes ↑ cytokine production	↑ antigen presentation	TH1/TH2 ↑ IgG1/IgG2a
MF59	Monocyte recruitment, NALP3 activation (not required for adjuvant effects), DAMP release: (ATP), MyD88-dependent effects on cellular immunity	↑ migration to LN (i.p.) ↑ expression of costimulatory molecules	Polyfunctional TH1 TFH ↑ IgG2a, IgG1 ↑ antibody diversity/switching
MPL	TLR4/TRIF and type I IFNs Migration of monocytes to injection site	↑ expression costimulatory molecules and cytokines ↑ antigen presentation ↑ phagocytosis and endosomal activity	TH1 TFH CTL and ↑ antibody diversity and affinity
GLA	TLR4/MyD88+TRIF IL-18 from subcapsular macrophages IL-12 and type I IFNs		

calcium phosphate, another insoluble metal salt, is used in DT vaccines [16–19] and was suggested as a component of influenza vaccines by Salk and others [20••]. Of all these, alum is by far the most widely used and studied.

Here we will focus on what is known about the adjuvant properties of alum, because of all the particulate adjuvants, it is the most studied. However, it is likely that in some regards responses to alum are related to those of other particulate materials. Reports that injection of insoluble aluminum salts led to rapid accumulation of neutrophils and other cells and granulomas at the injection site have been in the literature for many years [21••,22,23]. The accumulation of these cells is accompanied by fibrinogen conversion to fibrin and accumulation of the tiny injected alum particles into large lumps [24••]. These lumps are composed of alum particles bound together by neutrophil extracellular traps (NETs) [25–27]. The NETs are made up of chromatin which the neutrophils expel after engagement of fibrin previously deposited on the alum [24••,28]. Do the neutrophils and other cells that are attracted to the alum and the NETs and their chromatin component have anything to do with the adjuvant properties of alum? It is difficult to evaluate the contributions of NETs since it is difficult to eliminate them entirely from injected alum. As for the accompanying chromatin, a couple of papers have suggested that host DNA may indeed increase alum's effects but host DNA is certainly not the only intrinsic adjuvant induced by alum ([29••,30,31] but see [32]). Alum may also act simply as a particle, lysing lysosomes and releasing lysosomal enzymes into the cytoplasm [33••,34,35••], by distorting the plasma membrane of antigen presenting cells [36••] and/or somehow by its ability to rapidly induce production if cytokines and chemokines [37,38].

It is well known that alum rapidly activates the NALP3 inflammasome and consequent production of the potent T cell stimulating cytokine, IL-1β [37,39•,40,41]. Some have suggested that IL-1β is at the root of alum's adjuvant activity [39•,43] but others have failed to confirm this notion [37,41,44,46]. Alum also, in an inflammasome-independent manner, induces the release by macrophages of biologically active IL-1α. However, while IL-1α is required for neutrophil infiltration at the injection site, its absence does not impact T or B cell responses after i.p. injection of antigen with the adjuvant [47]. Interestingly, the proteins involved in IL-1 signaling, MyD88 and IL-1Rs, are dispensable for the effects of alum on antibody responses after i.p. or i.m. injection [44,48,49•] but are required for adjuvant effects of alum on IgE responses when instilled into the lung as a model particulate [50,51••]. These effects are independent of the NALP3 inflammasome and appear to be related to the fact that alveolar macrophages undergo necrosis and thus release IL-1α [51••] when exposed to alum, whereas peritoneal macrophages do not. Thus, the confusion about the mode of action of alum may be partly due to the properties of the resident macrophages (or other cell types) located at the site of injection. For example, resident alveolar macrophages are relatively anti-inflammatory [52,53], whereas the macrophages that migrate into challenged lungs are more inflammatory [54••]. Thus the various conflicting data about the mode(s) of action of alum may be occasioned by use of different sites of injection or different states of the resident cells in the various experiments.

Other insoluble metal salts such as beryllium hydroxide, have adjuvant properties that enhance sensitization and disease after pulmonary exposure [55]. Beryllium hydroxide induces release of caspase-1-independent IL-1α from necrotic alveolar macrophages and mediates its adjuvant effects on Th1 cells via MyD88-dependent receptors that enhance DC migration and function [56].

The fact that alum induces TH2-type responses, TFH cells and the antibody isotypes, IgG1 and IgE, that are induced by such T cells in mice is not in dispute [57••,58–62]. However, the bias in antibody isotypes induced by alum may be less pronounced in humans [63]. In contrast to other particulate vaccine adjuvants, alum has been reported to suppress secretion of IL-12, a critical third signal for TH1 cell differentiation [64]. Perhaps this is one of the ways in which alum biases responses toward T cell differentiation into TH2 type cells.

Emulsion based adjuvants

In 1930, Jules Freund developed the first deliberately added adjuvant that enhanced cellular immunity, a water-in-mineral oil emulsion containing heat killed *Mycobacterium tuberculosis* (MTb), Freund's Complete Adjuvant (CFA) [65••]. It induces potent antibody responses and cellular immunity [66], but the necrotic abscesses and granulomas induced by CFA at the injection site preclude its use in human vaccines. Muramyl dipeptide expressed by MTb in CFA activates NOD2 receptors [67,68••]. Incomplete Freund's adjuvant (IFA) composed of just the mineral oil component of CFA, was used in influenza vaccines in the 1950s to enhance antibody responses [69], and due to significant side effects [70] has only been studied in clinical trials of HIV and some cancer vaccines [71]. It enhances accumulation of APCs, antigen uptake and cytokine production and a non-polarized TH1/TH2 response with enhanced IgG1/IgG2a antibody responses [72–75,76••].

Emulsion adjuvants approved for use in human vaccines are those that contain derivatives of squalene, a steroid precursor lipid. Squalene emulsified with surfactants serves as an adjuvant (MF59 and AS03) that drives robust antibody responses, polyfunctional TH1 cells and TFH cells, and enhanced immunity to heterotypic strains of influenza [77••,78–82]. Uniquely, antibody isotype

switching is induced by MF59 in the absence of $CD4^+$ T cells, however apparently the plasma cells making this antibody do not live for a long time in the absence of T cell help [83]. MF59 causes the production of a broader range of cytokine/chemokines than alum or CpG DNA, and more rapidly recruits $CD11b^+$ inflammatory cells to the injection site [38]. MF59 induced antibody responses are not affected by the absence of NALP3 or caspase-1 in mice, that is, the adjuvant properties of MF59 appear to be independent of the NALP3 inflammasome. However mice lacking ASC, a protein that is involved in activation of inflammasomes, have impaired antibody IgG responses suggesting an inflammasome-independent mechanism by which this adaptor promotes humoral immunity [84,85••]. MyD88 is involved in the adjuvant effects of MF59 on protective antibody production [85••] while type I IFNs are not required [86]. TLRs and innate receptors do not respond directly to squalene *in vitro* [85••]. Thus, MF59 may engage innate MyD88-dependent cell receptors via cell injury or stress and the release of DAMPS, such as ATP [87].

Innate pattern recognition receptor targeting adjuvants

Activation of innate pattern recognition receptors promotes DC function required for polyfunctional TH1 cells, T cell memory, and CTL responses [88,89••,90–92]. Targeting antigen and TLR agonists to DCs using monoclonal antibodies can improve the effects of TLR ligands [93–98] and addition of TLR ligand-targeting adjuvants to emulsions, insoluble metal salts, particles, and liposomes as delivery vehicles are more effective than soluble TLR-ligands alone [99–101].

Monophosphoryl lipid A (MPL), a diglucosamine derivative of lipopolysaccharide (LPS), has less toxic effects than LPS itself and is approved for use in human vaccines. MPL increases costimulatory molecule expression and IL-12 production by human DCs [102]. It mediates its adjuvant effects on TH1 cells via the TRIF-biased signaling pathway downstream of TLR4 [103], drives the production of TH1 cytokines [104] and enhances the production of antigen-specific CD8 cytotoxic T lymphocytes (CTLs) [105]. Glucopyranosyl lipid A adjuvant (GLA) is a synthetic hexaacylated lipid A derivative and TLR4 agonist. It is being studied in an emulsion based adjuvant (GLA-SE) for tuberculosis, leishmaniasis and influenza vaccines [106]. MPL is unable to initiate CD14 to augment dimerization of TLR4/MD2, which drives MyD88 activation at the plasma membrane and thus primarily mediates its effects on T cells via the TRIF pathway [103,107,108]. In contrast, GLA drives expression of MyD88-dependent genes expression at lower doses than MPL [109], and drives expansion of polyfunctional TH1 cells, $CD8^+$ T cells and TFH cells, via activation of both TRIF and MyD88 adaptors [110,111]. GLA-SE induces IL-12 production and TH1 cells [110]. When injected into the muscle GLA-SE accumulates in a network of subcapsular macrophages in the draining LNs, driving IL-18-production [112]. These macrophages are required for B cell expansion and differentiation, antibody secretion, and TH1 responses in vaccines using GLA-SE as adjuvant.

Agonists of TLR3 (polyI:C), TLR7/8 (remiquimod, imiquimod), TLR9 (CpG DNA) and NOD2 (muramyl dipeptide) are being studied in various formulations for clinical development. The yellow fever vaccine promotes lifelong immunity via multiple TLRs expressed on different DC subsets [113] and combination of different TLR agonists can lead to better protection than either TLR agonist alone [114]. The combination of TLR agonists is likely dependent on the targeted pathogen, as not all combinations offer the same protective effects [115].

Conclusion

Alum adjuvanted subunit vaccines have had a profound impact on early childhood mortality largely due to their ability to induce protective antibody responses. Alum does induce small $CD8^+$ T cell responses but does not drive CTL responses unless combined with a TLR agonist [116]. Diseases that contribute to mortality in children for which subunit vaccines have been more difficult to develop include intracellular pathogens (MTb), those with complex lifecycles (malaria), or ability to evade or disable the immune system (HIV, HPV, HSV, Influenza). Modern adjuvant strategies aim to improve vaccination to these more difficult targets by promoting polyfunctional TH1 and CTL memory cells and differentiation of TFH cells to drive long lived heterotypic antibody-mediated protection [92]. Formulations that use the benefits of both of older antigen adjuvants that promote cellular stress/DAMP release with newer adjuvants that target specific DCs and/or innate PRR, may lead to effective vaccines that offer safe protection against pathogens that have historically evaded successful vaccine strategies.

Conflict of interest

The authors declare no conflicts of interest.

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