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Vaccine adjuvants – understanding molecular mechanisms to improve vaccines

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Summary

Infectious pathogens are responsible for high utilisation of healthcare resources globally. Attributable morbidity and mortality remains exceptionally high. Vaccines offer the potential to prime a pathogen-specific immune response and subsequently reduce disease burden. Routine vaccination has fundamentally altered the natural history of many frequently observed and serious infections. Vaccination is also recommended for persons at increased risk of severe vaccine-preventable disease. Many current nonadjuvanted vaccines are poorly effective in the elderly and immunocompromised populations, resulting in nonprotective post-vaccine antibody titres, which serve as surrogate markers for protection. The vaccine-induced immune response is influenced by: (i.) vaccine factors i.e., type and composition of the antigen(s), (ii.) host factors i.e., genetic differences in immune-signalling or senescence, and (iii.) external factors such as immunosuppressive drugs or diseases. Adjuvanted vaccines offer the potential to compensate for a lack of stimulation and improve pathogen-specific protection. In this review we use influenza vaccine as a model in a discussion of the different mechanisms of action of the available adjuvants. In addition, we will appraise new approaches using "vaccine-omics" to discover novel types of adjuvants.

Key words: *vaccination; adjuvants; immune responses; alum; MF59; AS03; small molecules; in silico design; vaccine-ome*

A brief history of adjuvants

In 1796, Edward Jenner's first observations of protective effects after inoculation with inactivated cowpox, manifest by reduced disease burden following pathogen challenge, heralded the era of vaccination [1]. A century later, Emil von Behring and Shibasaburo Kitasato discovered that sera from animals immune to diphtheria contained an antitoxin activity, later called antibodies [2]. These key findings of an inducible immune response resulted in the development of vaccines at the beginning of the last century. Contemporaneously, the immunomodulatory potential of aluminium was discovered. In 1926, aluminium was the first commercially used adjuvant to improve the immunogenicity of diphtheria vaccine [3]. For several decades thereafter, oil-in-water emulsions were the only compounds added to the list of adjuvants (table 1). In recent years, many potential new classes of adjuvants have been discovered, most notably toll-like receptor (TLR) agonists, and these have undergone testing for efficacy and safety in humans.

Vaccine-induced immune responses

In this review article, we will address vaccine adjuvant themes using influenza as a model of acute viral infection. Influenza is a common acute viral infection and the currently available vaccines strive to generate neutralising antibodies. Influenza infection is associated with increased morbidity and mortality in elderly, obese and immunosuppressed patients (e.g., after transplantation or during chemotherapy) and newborns, and during pregnancy [4–7]. Influenza virus replication is controlled by a complex interaction of the innate and adaptive immune response. Neutralising antibodies prevent infection and CD8 cytotoxic T-cell responses (CTL) help to clear the infection [8–14]. Annual vaccination against influenza is recommended (http://www.who.int/influenza/vaccines/virus/en/).

The purpose of vaccination is to prime a naïve immune system and establish a pathogen-specific protective immunological memory. Essentially, vaccination induces two important immune phenotypes: (i.) a virus-specific B-cell response with production of neutralising antibodies [15]

with the help of a T helper cell type 2 (Th_2) memory and/or (ii.) a virus-specific CTL and T helper cell type 1 (Th_1) memory. Both are capable of providing protective memory, but one phenotype can predominate at the expense of the other. The relative importance for protection is likely dependent upon the particular pathogen. The type of vaccine-induced immune response is highly dependent on the antigen and adjuvant composition. Important factors to consider in vaccine design are: pathogen transmissibility, replication dynamics, tropism of the pathogen and the natural immune response. Currently, most commercially available vaccines target the induction of neutralising antibodies.

Key steps

Antigen-presenting cells (APCs: B cells, dendritic cells [DCs] or macrophages) play a central role in inducing vaccine responses. Antigens such as viral structural proteins are processed by the proteosome complex (CD8+ CTL) or lysosomal enzymes (CD4+ T helper cells), cleaved into peptides, and then presented in a human leucocyte antigen- (HLA-) dependent manner via the major histocompatibility complex class I (MHC-I) and MHC-II to CD8 and CD4 T cells, respectively [16] (signal 1). Simultaneously, APCs are activated via pattern recognition receptors (PRRs), such as TLRs [17] and cytokines such as interferons (IFNs). PRRs which sense ribonucleic acid (RNA) contained in inactivated whole influenza vaccines are endosomal TLR3 and TLR7/8, as well as cytoplasmic retinoic acid-inducible

Table 1: List of adjuvants.

Adjuvant	Class	Component	Company	Mechanism of action	Vaccines	References
Licensed adjuvants						
Alum	Aluminium mineral salts	- Potassium aluminium sulphate - Often wrongly classified		- Necrosis causing urate crystals - Induction of inflammasome - IL-1 secretion	Multiple	[162–166]
MF59	Oil-in-water emulsion	- Squalene - Polysorbate 80 - Sorbitan trileate	Novartis	- Slow release of antigen - Nonspecific immune stimulation	Fluad (seasonal influenza) Focetria (pandemic influenza) Aflunov (prepandemic influenza)	[98, 103, 167–171]
Virosomes	Liposomes	- Lipids - Haemagglutinin	Berna Biotech	- Slow release of antigen	Infexal (seasonal influenza) Epaxal (hepatitis A)	[172–174]
AS04	Alum-absorbed TLR4 agonist	- Aluminium hydroxide - MPL	Glaxo SmithKline	- induction of Th_1 response	Fendrix (hepatitis B) Cervarix (human papilloma virus)	[175–178]
AS03	Oil-in-water emulsion	- Squalene - Tween 80 - α-Tocopherol	Glaxo SmithKline	- Slow release of antigen - Nonspecific immune stimulation	Pandremix (pandemic influenza) Prepandrix (prepandemic influenza)	[179, 180]
Unlicensed adjuvants						
Pam3Cys	TLR2 agonist	- Lipopeptide	-	- Induction of Th_1 response	-	[181]
Poly I:C	TLR3 agonist	- ds-RNA analogues	Hemispherx Biopharma	- Induction of Th_1 response	-	[182, 183]
Flagellin	TLR5 agonist	- Bacterial protein linked to antigen	Moffitt Cancer Center	- Induction of Th_1 response	-	[184–186]
Imidazoquinolines	TLR7 and TLR8 agonist	- Small molecules	Cancer Research Technology	- Induction of Th_1 response - Direct activation of B cells	-	[187, 188]
CpG	TLR9 agonist	- CpG oligonucleotides ±alum/emulsion	Chiron	- Induction of Th_1 response - Direct activation of B cells	-	[189, 190]
AS01	Combination	- Liposome - MPL - Saponin	Glaxo SmithKline	- Slow release of antigen - Induction of Th_1 response	-	[191]
AS02	Combination	- Oil-in-water emulsion - MPL - Saponin	Glaxo SmithKline	- Slow release of antigen - Induction of Th_1 response	-	[192]
AF03	Oil-in-water emulsion	- Squalene - Montane 80 - Eumulgin B1PH	Sanofi Pasteur	- Slow release of antigen - Nonspecific immune induction	-	[193]
CAF01	Combination	- Liposome - DDA - TDB	Statens Serum Institute	- Slow release of antigen	-	[194]
IC31	Combination	- Oligonucleotide - Cationic peptides	Novartis	- Induction of Th_1 response	-	[195]
Iscomatrix	Combination	-Saponin - Cholesterol - Dipalmitoyl-phosphatidylcholine	CSL Behring	- Slow release of antigen	-	[196–198]

CAF = cationic adjuvant formulation; DDA = dimethyldioctadecylammonium; IL = interleukin; MPL = monophosphoryl lipid; poly I:C = polyinosinic:polycytidylic acid; RNA = ribonucleic acid; TDB = trehalose-6,6-dibehenate Th = T helper cell; TLR = toll-like receptor

gene-1- (RIG-I-) like receptors [18–20]. However, in subunit vaccines, only protein is present and stimulation of TLRs may need specific adjuvants. Activation of PRRs results in the up-regulation of costimulatory ligands on the cell surface of APCs. Costimulatory ligands on APCs modulate the activation and downstream signalling cascade of the T cell (signal 2). Important T-cell activating costimulatory ligands are CD80, CD86 and CD40; important T-cell inactivating costimulatory ligands are cytotoxic T-lymphocyte antigen-4 (CTLA-4) and programmed death ligand (PD-L)1/2 [21–23].

Successful induction of a CTL response involves the initial stimulation of DCs by influenza virus. DCs then preferentially secrete IFN-α [24] and interleukin-12 (IL-12), and up-regulate MHC-I and MHC-II [25]. Thus unprimed naïve T helper cell (Th_0) differentiation is skewed towards a Th_1 direction, with IFN-γ, IL-12 and IL-6 production further amplifying the response [26]. Th_1 cells, which produce a significant amount of IFN-γ, are essential for the induction of an optimal CTL killing response including release of perforin and granzyme from cytotoxic granules. In addition, IFN-γ generated from Th_1 CD4+ T cells can affect isotype switching in B cells [27]. IFN-γ can promote the induction of HA-specific neutralising antibodies and may in fact help with broadening responses to heterologous influenza viruses [28, 29]. This effect could be due to broadening the spectra of available epitopes after proteasome cleavage [30].

With respect to B-cell responses, adequate priming of a Th_2 immune response (IL-4, IL-5, IL-9 and IL-13) is important [31–33]. The Th_1 response is known to suppress Th_2 responses [34, 35] – via suppressor of cytokine signal (SOCS) proteins [36] – culminating in lower antibody titres [24]. In addition, a new class of T cells, T follicular helper cells (TFH), predominantly producing IL-21, also have a key role in B-cell maturation [37, 38]. For example, patients infected with human immunodeficiency virus (HIV) who had been successfully vaccinated showed significantly increased IL-21 serum levels and frequency and mean fluorescence intensity of IL-21R-expressing B cells, which correlated with H1N1 antibody titres [39, 40]. Other important growth factors for B cells, such as B-cell activating factor (BAFF) or a proliferation-inducing ligand (APRIL), are released by activated monocytes, DCs and macrophages in the lymph nodes [41]. Figure 1 highlights the most important interaction steps between monocytes/DCs, T cells and B cells during vaccination. The interaction between B cells and Th_2 cells leads to B-cell activation. The priming steps of a naïve B cell precede a maturation phase characterised by immunoglobulin class switching and affinity maturation. In turn this triggers the production of more specialised antibodies such as Immunoglobulin G (IgG) subclasses, IgA and IgE [37]. Seroconversion, which is commonly defined as a 4-fold antibody titre increase from baseline, serves as an important surrogate marker suggesting successful vaccination.

Adjuvants: mechanisms of action

Changing population demographics, in particular the increasing number of immunosuppressed hosts and elderly persons, significantly impacts vaccine-related outcomes. Overcoming the immunosuppressive effects of the aging immune system will become a key challenge for the vaccines of the 21st century [42–45]. Immunosenescence affects DCs, and T and B cells at various levels [46]. Nonadjuvanted influenza vaccines show low effectiveness, with seroconversion rates of only 30% in healthy adults above 65 years old [47–51], which is comparable to the weak response seen in transplant recipients taking immunosuppressive drugs [52]. Nonetheless, vaccination remains highly recommended for these groups in whom the prevalence of influenza-associated morbidity and mortality is high [53].

Adjuvants offer a strategy to improve vaccine outcomes via natural, synthetic or endogenous molecules that function to modulate and/or increase the immune effect. This may result in an enhanced, accelerated and prolonged pathogen-specific immune response. The immune response can be preferentially skewed in a certain direction, such as with respect to immunoglobulin classes or the induction of cytotoxic or T helper cell responses (Th_1 versus Th_2). Thereby, the immunogenicity of particular antigens can be improved and the nature of the immune response modified, whilst the amount of required antigen is also reduced. Adjuvants thus have the potential to boost immune responses in elderly and immunocompromised hosts. Table 1 provides a list of adjuvants and their proposed mechanisms of action.

As antigens vary in immunogenic and biological characteristics, a particular adjuvant has to be optimised for a specific antigen. Adjuvants should be added on the basis of the type of immune response desired; for influenza vaccines, for example, cytotoxic and Th_1 responses against haemagglutinin might be of less clinical importance to prevent infection. More important is a robust Th_2 response to induce haemagglutinin-specific neutralising antibodies.

A large amount of data regarding adjuvanted vaccines concerns their safety and tolerability. Prevention of infection compared with nonadjuvanted vaccines is rarely examined and often compared with historical controls or literature. An important debate concerns the best immune biomarker indicative of protection and prevention of infection. Most research has considered antibody titres, which certainly

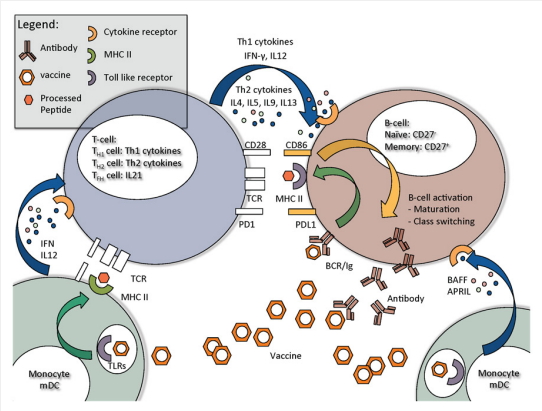

Figure 1

Key steps in B-cell activation and B-cell interaction with T-helper cells and monocytes, and monocyte derived macrophages and dendritic cells.

are easy to measure, as a surrogate marker for protection. However, T-cell responses, especially Th_2 cytokine release; and measuring B-cell activation directly might correlate better with protection [54–57]. An increase in antibody levels does not necessarily correlate with protection; nevertheless, as mentioned, seroconversion is the most commonly used surrogate marker of protection. It has been shown that seroprotection increases to a greater extent in patients with low or zero baseline titres, compared with patients with high baseline titres, and that the relative increase in vaccine recipients with prevaccination titres >1:40 is significantly lower [58, 59]. This could be due to a higher "activation threshold", which needs to be reached.

Differential cytokine responses are observed following administration of different adjuvants. In general, when used with pure proteins, oil-in-water (O/W) emulsions up-regulate Th_2 responses. Addition of TLR agonists to the emulsions skews the response to Th_1. Much of this work has been done with TLR4 agonists, including monophosphoryl lipid A (MPL1), and glucopyranosyl lipid A (GLA) oil-in-water formulations [60, 61].

Aluminium mineral salts

Mechanism of action

The adjuvant potential of aluminium salts was discovered in 1926 [62], but their mechanism of action is certainly complex and still not fully understood. Aluminium salts exist in various forms with various chemical specifications, however, and aluminium salts are often described as "alum". "True" alum should be reserved for hydrated potassium aluminium sulphate. The misleading term might explain the variability reported in the literature, as different aluminium salts induce distinct effects on the immune system. Aluminium is cytotoxic via the rupture of endolysosomes and the induced release of uric acid, which act as damage-associated molecular patterns (DAMP) [63]. This leads to an activation of the Nod-like receptor family protein-3 (NLRP3) inflammasome and caspase-1, and release of IL-1, IL-18 and IL-33 [64–67]. This promotes antigen uptake and presentation by human macrophages and their recruitment to sites of inflammation [64, 68]. In addition, HLA-DR, CD40 and CD86 are up-regulated on DCs [69] in a MyD88-dependent manner (MyD88 is a critical adaptor protein for most TLRs). Other studies have suggested that "alum" may not act through TLRs [70, 71]. The recruited DCs prime a naïve CD4+ T-cell response, in particular Th_2 [64, 72, 73]. Th_2 cytokines are crucial for the differentiation of B cells and the maturation processes leading to IgG1 production.

Clinical data

Although aluminium salts induce a favourable Th_2 response, not many licensed influenza vaccines are adjuvanted in this way – and no licensed influenza vaccine is available (table 1). In the last decade, new data on the mechanism of action have emerged, but most studies on vaccine efficacy have been performed in mice. The potential to reduce antigen amounts has been recently summarised in a meta-analysis [74]. Table 2 summarises the published studies with aluminium salts in influenza vaccine during the last 10 years.

Oil-in-water emulsions – MF59 and AS03

Mechanism of action

MF59 and AS03 are squalene-based oil-in-water emulsions. MF59 is composed of 0.5% Tween-80 as a water-soluble surfactant, 0.5% Span85 as an oil-soluble surfactant, 4.3% squalene oil, and water. The emulsion droplet size is approximately 130 nm. Experiments with nanoparticle adjuvants suggest that the particle size may be a key factor for the activity: microspheres with diameters of <10 nm seem to activate APCs, whereas particles with diameters of 30–100 nm show a slow release of antigen, known as "depot effect" [75]. MF59 squalenes are internalised by DCs [76] and act independently of the NLRP3 inflammasome [77], but are dependent on MyD88, which might have an adaptor protein function for tumour necrosis factor receptor superfamily member 13B (also known as TACI) or the IL-1 receptor [78]. Studies have shown that pretreatment with MF59 prior to vaccine application resulted in a maintained "immunocompetent environment" within the muscle [79]. Interestingly, this effect does not occur if MF59 is injected later. The number of leucocytes isolated in the muscle increased seven-fold within 2 days, and a slow decay was observed thereafter. MF59 function is dependent on CC chemokine receptor type 2 (CCR2), the receptor for monocyte chemoattractant proteins 1–5 (MCP-1–MCP-5), and on intracellular adhesion molecule-1 (ICAM-1) [80, 81]. MF59 additionally induces monocyte-to-DC differentiation [82]. Of note, the individual components of MF59 are not as effective as the entire formulation [83].

AS03, containing an α-tocopherol (a form of vitamin E), modulates cytokine release and cell recruitment to regional lymph nodes, leading to enhanced antibody responses [84]. Slow release of antigen compounds seems not to be a primary mechanism of action. Squalene is rapidly degraded in tissues and studies with viral glycoproteins showed that antigens did not bind to the emulsion droplets, and that binding was not necessary to achieve a potent adjuvant effect [79, 85]. MF59 and AS03 showed higher immunostimulatory potential than aluminium salts in several clinical studies [74].

Clinical data

The immunogenicity of seasonal and pandemic MF59-adjuvanted influenza viruses has been widely evaluated in open and controlled studies involving a broad range of different patient groups – inclusive of elderly and immunosuppressed populations. To summarise, these studies showed that MF59-adjuvanted influenza vaccines induce a more potent immune response with higher rates of seroconversion compared with nonadjuvanted vaccines [86–91]. Local reactions – mainly mild reactions at the site of injection sites – were increased.

In addition, O/W emulsions carry the potential to generate cross-reactive antibody responses. MF59 increases the diversity of the epitope repertoire against haemagglutinin-1

[92]. Given the frequency with which antigenic drift occurs in influenza viruses, this seems to be the most important immune advantage arising from the use of MF59. In addition, the potency of vaccines against weakly antigenic pandemic vaccine strains could be significantly increased [93–95]. Reductions in the amount of antigen (lower than 15 µg of haemagglutinin) needed to generate a sufficient response is critical when the capacity to generate vaccines is limited, in particular during a pandemic [96–104]. Although the immunogenicity of MF59-adjuvanted vaccines has been demonstrated in terms of antibody responses, the increased protective effect has only been shown in two studies [105, 106].

The potency of AS03-adjuvanted influenza vaccines to increase antibody levels was shown for the avian A/H5N1 influenza virus [107–109] and the A/H1N1 pandemic virus [110–114]. Data from clinical trials involving the elderly, children of different ages and immunocompromised patients have shown enhanced antibody mediated immune responses to haemagglutinin. Again, only one study demonstrated the impact on infection prevention [115].

New types of adjuvants:

Peptide design

Protein interactions that modulate the immune response and have been identified in genome-wide association assays and gene hub analysis can also be used to design new adjuvants (see below). On the basis of these interactions, peptide libraries can be designed to block or stimulate the interaction. These peptides may be used as adjuvants by

Table 2: Studies of alum-adjuvanted influenza vaccines in humans.

Type of influenza	Study cohort	Treatment groups	Major outcomes	Type of aluminium salt	Reference
Whole virion inactivated Influenza A/California/7/2009 pandemic H1N1	Double-blind randomised Phase I (n = 50, 18–50 y) Phase II/III (n = 330, >3 y)	Phase I: 10 µg vs 15 µg, i.m. injection Phase II/III: 10 µg vs 15 µg Three age groups: 3–17 y, 18–49 y, >50 y	Phase I: In 20% mild side effects during 42-day follow-up. No adverse effects Phase II: same. Seroconversion in 18–49 y: 10 µg 90.4%, 15 µg 90.4% in >50 y: 10 µg 87%, 15 µg 76%	Aluminium hydroxide	Kulkarni et al. [199]
Whole virus inactivated Influenza A/Vietnam/1203/2004 H5N1	Randomised dose-escalation Phase I and II (n = 275)	18–45 y Adjuvanted: 3.75, 7.5, 15, and 30 µg Nonadjuvanted: 7.5, 15 µg.	Higher seroconversion in nonadjuvanted group Higher seroconversion in lower antigen group (7.5 vs 15 µg); Phase I; Phase II	"Alum" adjuvant	Ehrlich et al. [162]
Whole viron inactivated Influenza A/Vietnam/1194/2004–A/PR/8/34 H5N1	Randomised placebo-controlled double-blind study (n = 120)	18–60 y, i.m. injected 1.25, 2.5, 5, or 10 µg in two doses vs placebo	Seroconversion: 1.25 µg: 23.5%; 2.5 µg: 18.8% 5 µg: 78.6%; 10 µg: 80% 2nd booster dose increased seroconversion.	"Alum" adjuvant	Lin et al. [200]
Inactivated-split Influenza A/Vietnam/1194/2004	Prospective, randomised, observational, multicentre trial, n = 400 in each trial	Phase I, n = 400, 18–45 y Adjuvanted: 7.5, 15 µg Nonadjuvanted:7.5, 15 µg Phase II, n = 400, 18–64 y Adjuvanted: 30, 45 µg two doses	Phase I, d21, seroconversion: 7.5 µg: 21% 7.5 µg + Al: 14% 15 µg: 28% 15 µg + Al: 17% Phase II, d21, seroconversion: 30 µg + Al: 31% 45 µg + Al: 30% double seroconversion after 2nd dosage	Aluminium phosphate + thiomersal	Nolan et al. [201]
Inactivated Influenza A/Hong Kong/1073/99 H9N2	Randomised dose-comparison study, n = 353	1.7, 5, 15, 45 µg i.m. 5, 15 µg i.d. Whole virus vs virasomal >18 y, two doses	Seroprotection, d21 and d42, <40 y Dose-dependent increase Increase with alum Virosomal unit and intradermal injection minimal increased response	Aluminium phosphate	Nicholson et al. [202]
Inactivated-split Influenza A/California/7/2009, H1N1	Randomised double-blind, placebo-controlled study, n = 2,200	Age groups: 3–11, 12–17, 18–60, >61 y 7.5, 15, 30 µg 2nd dose vs placebo	18–60 y: 15 µg 97.1%; 30 µg 92.6%; Pl: 10.7% >61 y: 15 µg 79.1%; 30 µg 84.1%	"Alum" adjuvant	Zhu et al. [203]
Whole viron, inactivated Influenza A/Vietnam/1194/2004	Randomised study	Phase II/III trial Adult, n = 337; 20–59 y; i.m. vs s.c. 15 µg Children, n = 374; 3 m – 19 y; 3, 7.5 µg	Seroconversion Adult: i.m. 82.8%, s.c. 71.4% Children: no clear information	"Alum" adjuvant	Nakayama et al. [204]
Whole viron inactivated Influenza A/Vietnam/1194/2004, H5N1 and A/PR(8/34 H1N1	Randomised study (n = 120)	Phase I, healthy Japanese men 20–40 y s.c.: 1.7, 5, 15 µg i.m.: 1.7, 5, 15 µg 2nd dose	Seroconversion s.c. 15 µg 42.1%, vs i.m. 15 µg 65% Seroconversion after 2nd dose s.c. 15 µg. 68.4% vs 75%	Aluminium hydroxide	Ikeno et al. [205]

themselves [116, 117] or be used as lead compounds for *in-silico* screening to design small molecules.

Small molecules

Small molecule adjuvants (SMAs) include both natural products such as muramyl dipeptide, byrostatin-1, monophosporyl Lipid A (MPL), QS-21 and QuilA (saponin based), and PAM2CSK4 [118–123]; and fully-synthetic drug-like molecules such as the group of imidazoquinolines and bestatine [124–126]. Best described is the so-called family of imidazoquinolines [127, 128), including imiquimod, resiquimod and gardiquimod [127–129], which act as TLR7/8 agonists. Other examples of SMAs are synthetic CpG oligodeoxynucleotides which act as TLR9 agonists [130]. For CpG oligonucleotides, structure-activity relationship data to design similar compounds [131] has been used; in addition, quantitative structure-activity relationship (QSAR) technology with optimised activity, selectivity and toxicity was used to generate novel variations of compounds such as, CPG-1826 [132] and CPG-7909 [133]. Polyinosinic:polycytidylic acid (or poly I:C) is another analogous immunostimulant, and acts as a TLR3 agonist [134].

The future of adjuvant development

Discovery of new adjuvants using "-omics"

During the last decade tremendous progress has been achieved in understanding the complex interaction between the various key components of the immune system. Complex data detailing messenger RNA (mRNA) and protein expression profiles using systems biology approaches has helped us to understand many key steps involved in immune activation [135–138]. The data-gathering techniques for modelling and simulation of immunological processes, and the required tools and techniques to question vaccine

Table 3: Summary of genome-wide association studies in vaccine cohorts.

Study	Vaccine	Sample size	Region	Mapped genes	SNP	p-value
Pajewski NM et al. 2012	Anthrax	726, European ancestry	18q21.2	SRSF10P1 – MEX3C	rs7230711–C	1×10^{-6}
			1p36.22	SPSB1	rs11121382–C	4×10^{-6}
			5q31.1	LOC100996485	rs634308–G	4×10^{-6}
			6p21.32	MTCO3P1 – HLA-DQA2	rs3104402–A	6×10^{-6}
			9q33.1	ASTN2	rs6478282–A	6×10^{-6}
			9p21.1	RPS11P4 – TMEM215	rs10758161–G	8×10^{-6}
			13q14.3	PCDH8 – OLFM4	rs732949–C	8×10^{-6}
			4q24	TET2 – PPA2	rs2647264–G	9×10^{-6}
Kennedy RB et al 2012	Smallpox	512, European ancestry 199, African American	18q21.2	MEX3C	rs8096445–A	9×10^{-9} (AA)
			5q11.2	PDE4D	rs17444059–G	2×10^{-8} (AA)
			2p22.3	LINC00486	rs6728021–G	4×10^{-8} (AA)
			3q28	PYDC2 – FGF12	rs1516489–C	8×10^{-8} (AA)
			9p21.1	NDUFB6	rs17290760–G	1×10^{-7} (AA)
			1p36.12	LINC00339 – CDC42	rs2501276–A	2×10^{-7} (AA)
			6q22.33	C6orf58 – THEMIS	rs17299841–C	2×10^{-7} (AA)
			8p23.1	BLK	rs2255327–A	3×10^{-7} (AA)
			5q34	TENM2	rs2973662–A	5×10^{-7} (AA)
Ovsyannikova IG et al 2012	Smallpox	217, African American ancestry 580, European ancestry 217, Hispanic ancestry	10p12.1	MKX	rs10508727–?	1×10^{-10} (AA)
			10q21.1	SNRPEP8 – PCDH15	rs12256830–?	2×10^{-10} (Hispanic)
			8p12	VENTXP5 – RPL6P22	rs10503951–?	3×10^{-9} (AA)
			10p12.1	GPR158 – GPN3P1	rs12775535–?	4×10^{-9} (AA)
			8q24.13	ZHX2	rs10108684–?	1×10^{-8} (AA)
			18p11.21	SPIRE1	rs9959145–?	3×10^{-8} (AA)
			10p14	PRKCQ	rs4748153–?	3×10^{-8} (Hispanic)
			1q31.1	RPS3AP9 – FAM5C	rs10489759–?	8×10^{-8} (EA)
			6p21.2	KIF6	rs9380880–?	1×10^{-7} (Hispanic)
			1q43	GREM2	rs10495471–?	2×10^{-7} (AA)
Png E et al. 2011	Hepatitis B	1683, Indonesian ancestry	6p21.32	BTNL2 – HLA-DRA	rs3135363–?	7×10^{-22}
			6p21.33	C2; ZBTB12	rs9267665–?	1×10^{-17}
			6p21.32	HLA-DPB1	rs9277535–?	3×10^{-12}
Fellay J et al. 2011	HIV	831, all male mixed ancestry	6p10	HLA B	rs4713462	1.9×10^{-6}
			6p10	HLA B	rs4713460	2.4×10^{-6}

AA = African American; EA = European ancestry; HIV = human immunodeficiency virus; SNP = single nucleotide polymorphism

responses have been reviewed recently [139]. Application of these approaches to vaccinology at the genome-wide, transcriptome, and proteome levels may identify new pathways and molecular targets for immune modulation.

Based on genome-wide association studies, several critical single nucleotide polymorphisms (SNPs) influencing vaccine-induced responses have been found. These genes reflect "gene-hubs"; critical interaction points in an inflammatory signalling cascade. SNPs with a high frequency are attractive targets for compensatory molecules specifically targeting the immune response of an individual (personalised vaccination). Figure 2 summarises such a screening approach and gives a hypothetical example using IL-4 as a key cytokine for B-cell activation.

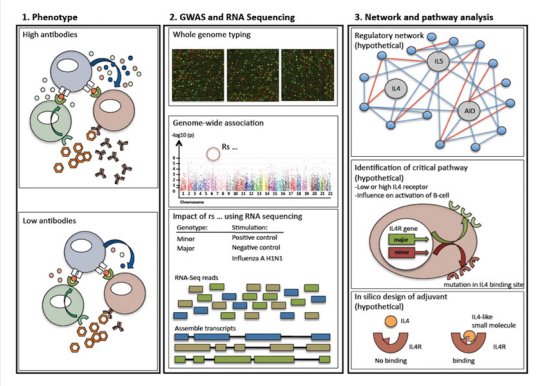

Figure 2

Possible work-flow to develop novel adjuvants. The following example is only used as an illustration and not based on "real" data – it should highlight critical steps, which could be used for different vaccines and pathways. First step: observation of distinguished phenotypes, such as antibody production different due to defective cytokine production. Second step: description of respective phenotype – identification of involved genes using a genome-wide association study. Next, RNA sequencing could be used to describe the pathophysiological impacts of the newly discovered SNP. Third step: generation of a genetic network to identify critical interaction points (so called gene hubs). In our "imaginary example" an important cytokine receptor would be affected – the interleukin 4 receptor. *In-silico* design of small molecules, which can modulate or compensate for the polymorphisms in the signalling cascade. In this example IL4 cannot bind sufficiently owing to a mutation within the IL4-receptor. A small molecule could be specifically designed to overcome this.

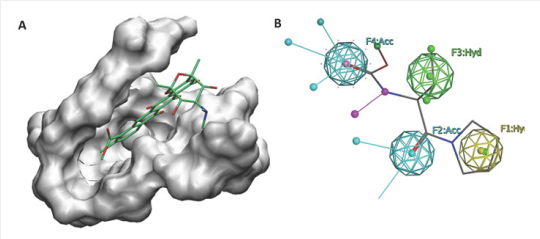

Figure 3

In-silico drug discovery strategies.

(A) Virtual screening campaigns usually identify small molecules that fit within a particular pocket in a protein.

(B) An illustration of a pharmacophore construction describing the distribution of important features for a drug that has three hydrophobic regions with two hydrogen bond acceptors. Based on these models virtual screens can be performed to focus only on a couple of interesting candidate compounds rather than screening a whole library.

At the transcriptome level, several high-frequency sampling studies of vaccine responses to yellow fever [148] and influenza [12, 149] vaccination have identified transcriptome signatures of the unadjuvanted vaccine response. Application of this knowledge to adjuvant design has yet to be published.

Studies continue to suggest the promise of this approach. Umlauf and colleagues associated the measles-mumps-rubella (MMR) induced vaccine response with 307 common candidate SNPs from 12 antiviral genes / immune signalling cascade such as RIG-I, interferon-induced GTP-binding protein Mx1 (Mx1) 2'-5'-oligoadenylate synthetase 1 (OAS1), etc. Genetic variants within the DDX58/RIG-I and OAS1 gene were associated with measles-specific antibody variations. DDX58 and ADAR polymorphisms were associated with variations in both measles-specific IFN-γ and IL-2 secretion. After correction for false discovery rate, 15 single-SNP associations (11 SNPs in Caucasians and 4 SNPs in African-Americans) still remained significant at the q-value (minimal false discovery rate) <0.20 [140]. Larger studies have been performed using genome-wide association of SNPs (GWAS) with vaccine-induced immune responses against smallpox [141–143], anthrax [144], HIV [145], and hepatitis B virus [146]. Table 3 summarises the findings of all mentioned studies. One GWAS explored the association of SNPs with side effects from vaccines [147].

One caveat to the "-omics" approach as applied to adjuvants is the need for better data at the site of the adjuvant effect (i.e., the site of injection and the lymph nodes), to better characterise *in-situ* molecular and cellular responses. Virtually all human studies have involved examining transcriptome and proteome profiles of peripheral blood. With current technology, muscle and lymph node sampling of inflammatory cells and parenchymal tissue is highly invasive and not an option for ethical reasons in human studies. Complicating matters is that murine models may differ in the fundamental biology of key immune responses. For example, there are significant differences in murine B-cell responses to some classes of TLR agonists [150].

In-silico screening

A successful adjuvant should be specific and selective for a target receptor either in an agonistic or antagonistic fashion to modulate the immune response. This could significantly reduce any possible side effects and lead to a robust and controlled modulation of the immune response. These side effects signify a major problem in adjuvant development and are not always predictable, as in the likely association of narcolepsy with the AS03 adjuvanted influenza vaccine [151, 152]. Nevertheless, computer-modelling (or *in-silico*) techniques could be the new path of hope to develop novel adjuvants that are potent, selective and safe (Figures 3A and B).

For the last two decades, molecular modelling approaches have been used to develop new drug candidates (small molecules or short peptides) that fit within a binding site in a particular target (usually protein). The objective is to complement a binding pocket that would regulate the activity of

the target in terms of shape, charge and other physiochemical properties. In order to reduce side effects and enhance the pharmacological properties, this regulator has to be potent for and selective toward the designated pocket, and to be suitable for further modifications and optimisations. In this regard, for an adjuvant to act as a modulator of the immune response, it must be specific for one of the receptors involved in this process and regulate its activity. Depending on the availability of a receptor structure or a number of predetermined potent regulators, one of many modelling strategies can be pursued.

If the target three-dimensional structure is available and the binding site that would regulate the activity of the target is known, one could use receptor-based virtual screening [153]. In this technique, a set of small molecules is docked to the surface of the identified pocket (Figure 3A). The ones that best fit within the pocket are then retained and their binding affinities are further calculated and used to rank them for experimental testing [154–156]. Only a few success stories reported in the literature followed this path to discover immune-related adjuvants. This small number of computer-developed adjuvants could be attributed to the limited number of protein structures involved in the immune response process. Among the few examples of success is the work done by Goel et. al. to modulate the chemokine receptor-4 (CCR4). They designed a set of small molecules specific for CCR4, a protein that is expressed on Th_2 cells [157–161].

When only a set of potent regulators is available, ligand-based approaches are used. This may involve one of two strategies: pharmacophore construction or quantitative structure activity relationship (QSAR) modelling. In the former, the common chemical and physical features that exist in the training set are used to build a hypothetical structure that distributes these features in space (Figure 3B). These features include hydrogen-bond donors and acceptors, hydrophobic regions, aromatic rings and excluded volumes. QSAR usually uses two-dimensional properties of the ligands to construct a mathematical model that would predict the activity of a future ligand. These properties include, but are not limited to, the molecular weight, the hydrophobicity of the ligand, the number of hydrogen bonds and many other properties.

All of the above-mentioned methodologies hold the promise to develop novel classes of adjuvants. Combining information from "vaccine-ome" studies identifying crucial junctures in immune activation, together with powerful *in-silico* modelling tools will certainly become the future for adjuvant design.

Conclusion

Infectious diseases are a continuous threat. Without doubt the development of vaccination has saved millions of lives. However, immunosuppressed and elderly people remain vulnerable to vaccine preventable disease as a consequence of reduced vaccine responses. Despite profound advances in the understanding of the immunological processes involved in vaccine responses, we have failed to improve further vaccine responses. In the last 150 years only a few adjuvants have been discovered and applied clinically. However, the future is bright. New small molecules may help to significantly increase vaccine responses. Emerging technology such as *in-silico* modelling of adjuvant receptor interactions using super-computers; combined with RNA sequencing and microarray SNP discovery, uncovering critical steps in vaccine responses, will bring about the design of novel classes of adjuvants.

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Figures (large format)

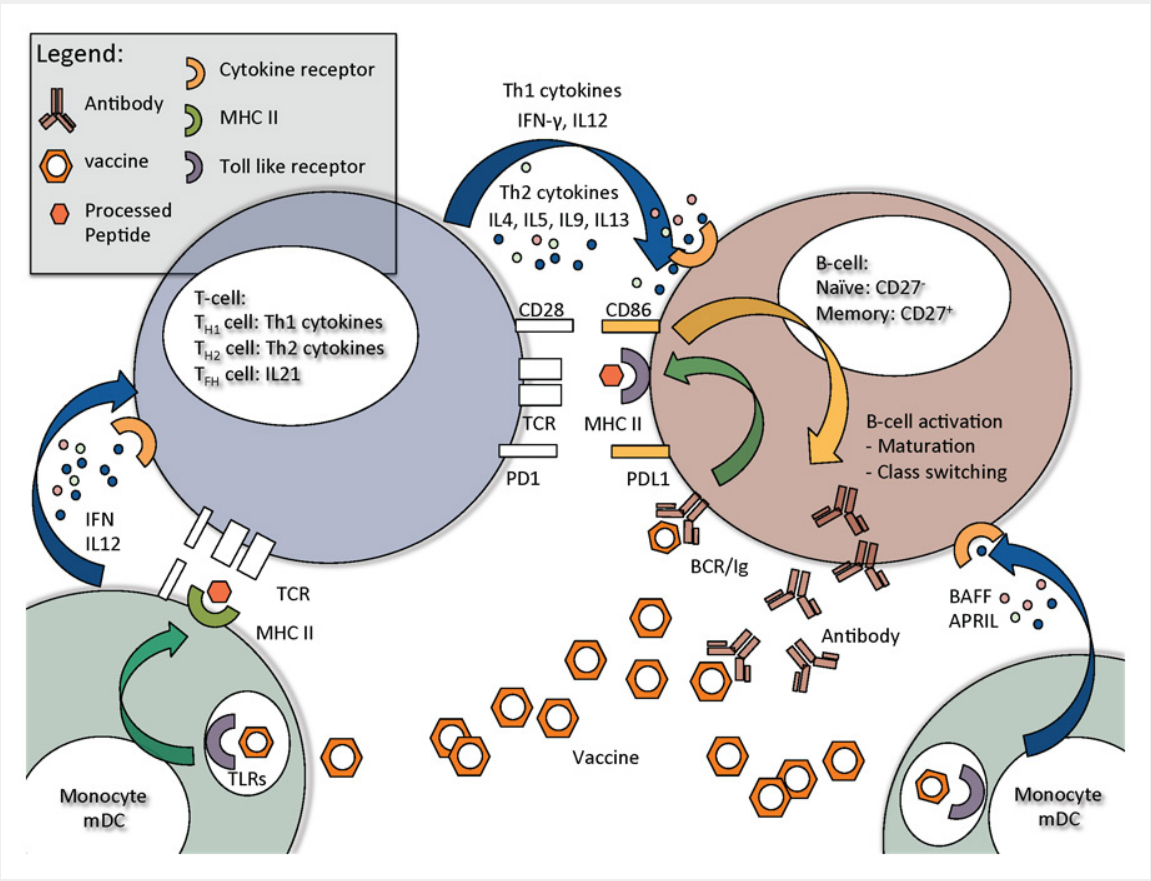

Figure 1

Key steps in B-cell activation and B-cell interaction with T-helper cells and monocytes, and monocyte derived macrophages and dendritic cells.

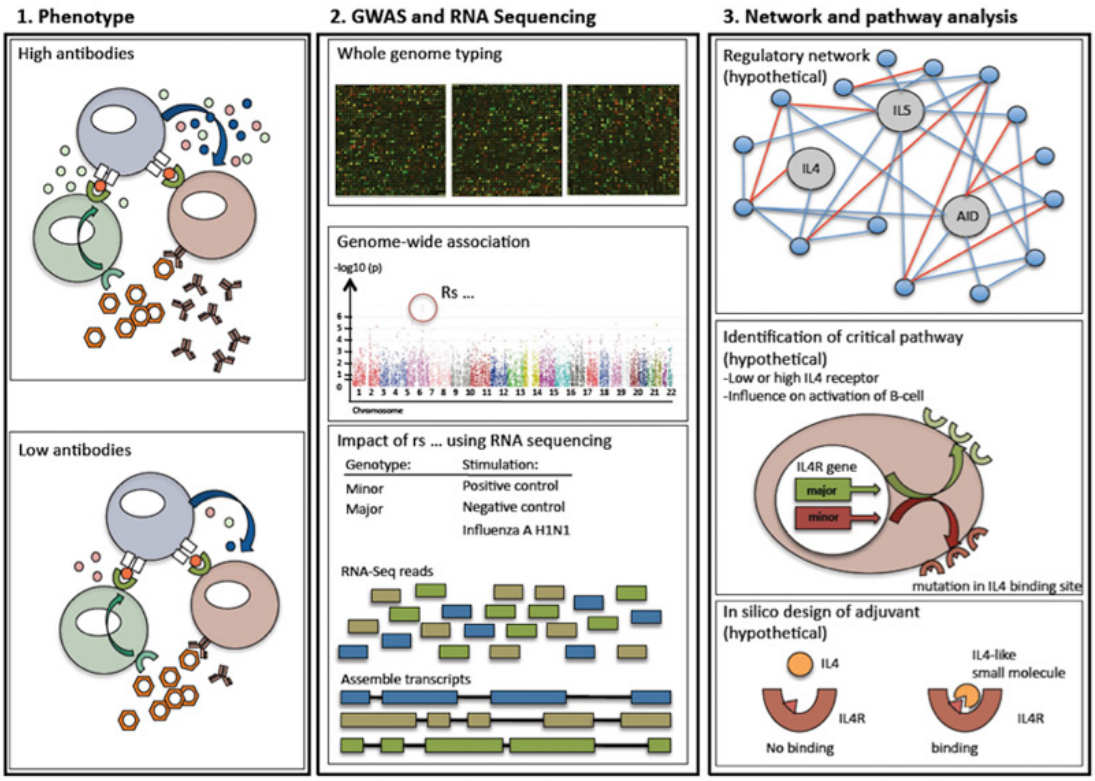

Figure 2

Possible work-flow to develop novel adjuvants. The following example is only used as an illustration and not based on "real" data – it should highlight critical steps, which could be used for different vaccines and pathways. First step: observation of distinguished phenotypes, such as antibody production different due to defective cytokine production. Second step: description of respective phenotype – identification of involved genes using a genome-wide association study. Next, RNA sequencing could be used to describe the pathophysiological impacts of the newly discovered SNP. Third step: generation of a genetic network to identify critical interaction points (so called gene hubs). In our "imaginary example" an important cytokine receptor would be affected – the interleukin 4 receptor. *In-silico* design of small molecules, which can modulate or compensate for the polymorphisms in the signalling cascade. In this example IL4 cannot bind sufficiently owing to a mutation within the IL4-receptor. A small molecule could be specifically designed to overcome this.

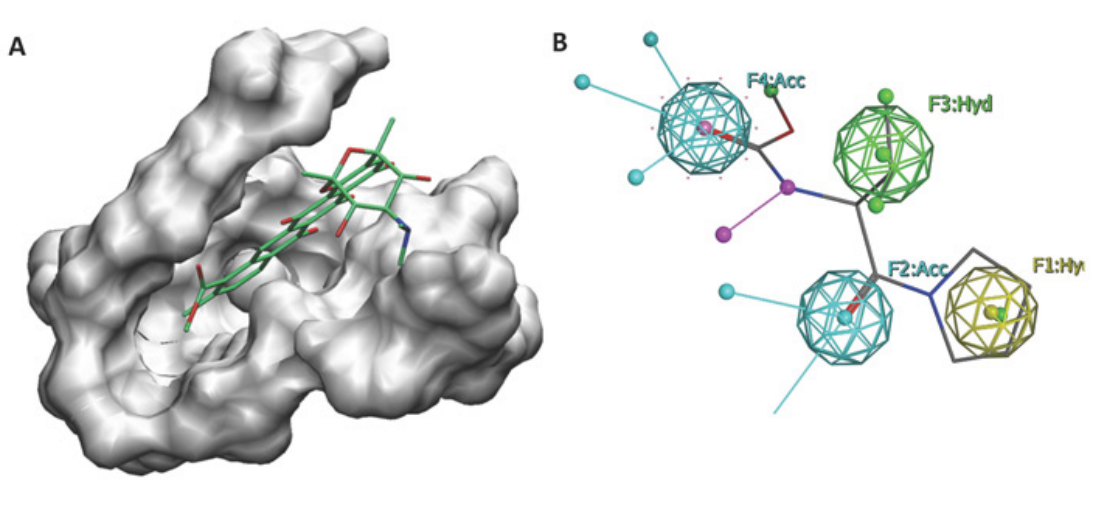

Figure 3

In-silico drug discovery strategies. (A) Virtual screening campaigns usually identify small molecules that fit within a particular pocket in a protein. (B) An illustration of a pharmacophore construction describing the distribution of important features for a drug that has three hydrophobic regions with two hydrogen bond acceptors. Based on these models virtual screens can be performed to focus only on a couple of interesting candidate compounds rather than screening a whole library.