Published in final edited form as:

Expert Rev Vaccines. 2011 April ; 10(4): 523–537. doi:10.1586/erv.11.30.

Carbohydrate-based immune adjuvants

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Abstract

The role for adjuvants in human vaccines has been a matter of vigorous scientific debate, with the field hindered by the fact that for over 80 years, aluminum salts were the only adjuvants approved for human use. To this day, alum-based adjuvants, alone or combined with additional immune activators, remain the only adjuvants approved for use in the USA. This situation has not been helped by the fact that the mechanism of action of most adjuvants has been poorly understood. A relative lack of resources and funding for adjuvant development has only helped to maintain alum's relative monopoly. To seriously challenge alum's supremacy a new adjuvant has many major hurdles to overcome, not least being alum's simplicity, tolerability, safety record and minimal cost. Carbohydrate structures play critical roles in immune system function and carbohydrates also have the virtue of a strong safety and tolerability record. A number of carbohydrate compounds from plant, bacterial, yeast and synthetic sources have emerged as promising vaccine adjuvant candidates. Carbohydrates are readily biodegradable and therefore unlikely to cause problems of long-term tissue deposits seen with alum adjuvants. Above all, the Holy Grail of human adjuvant development is to identify a compound that combines potent vaccine enhancement with maximum tolerability and safety. This has proved to be a tough challenge for many adjuvant contenders. Nevertheless, carbohydrate-based compounds have many favorable properties that could place them in a unique position to challenge alum's monopoly over human vaccine usage.

Keywords

adjuvants; carbohydrate; glucan; glycomics; immunity; inulin; vaccines

The goal of vaccination is to generate a protective immune response of sufficient strength and duration to prevent or attenuate the virulence of pathogenic organisms. This is currently achieved by parenteral or nasal immunization with live-attenuated, inactivated or

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Financial & competing interests disclosure

The authors are both affiliated with Vaxine Pty Ltd, a company holding intellectual property in the area of polysaccharide adjuvant technology. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

recombinant antigens to induce a neutralizing response against the relevant pathogen. In the early days of vaccinology, antigens, being impure and containing immunologically active contaminants, generated strong immunity often without the need for an adjuvant. On the negative side, owing to the same contaminants, early vaccines suffered from major injection site and systemic reactogenicity. Indeed, a use of aluminum adjuvants was to absorb and slow the systemic delivery of immunologically active contaminants such as bacterial lipopolysaccharide (LPS) and thereby reduce vaccine reactogenicity. Attempts over the years to improve the quality and purity of vaccine antigens to reduce their reactogenicity have paradoxically resulted in inadequate vaccine immunogenicity, necessitating corrective action by way of addition of an adjuvant.

An adjuvant is defined as any compound that enhances the immune response against a vaccine antigen; the word adjuvant comes from *adjuvare*, Latin for ‘help’ or ‘to enhance’. Adjuvants can be used for multiple purposes: to enhance immunogenicity, provide antigen-dose sparing, to accelerate the immune response, reduce the need for booster immunizations, increase the duration of protection, or improve efficacy in poor responder populations including neonates and the elderly [1]. To this day, alum-based adjuvants hold a monopoly over human vaccines in the USA, although oil emulsion adjuvants, such as MF59, have seen limited use in Europe and other jurisdictions. The reason for alum’s market dominance is not so much its potency, which can be bettered by many other adjuvants, but its long record of usage that predates founding of the US FDA. Regulatory requirements require any new adjuvant to prove that its benefits outweigh any safety or tolerability issues, making it difficult for new adjuvants to challenge alum’s supremacy. Alum’s long-term safety is taken for granted meaning that any new vaccine containing it is not forced to jump over the same hurdles as a vaccine containing any newer adjuvant.

The regulatory risk–benefit hurdle for any new adjuvant would be significantly reduced if it were made from a compound with known human safety and tolerability. Carbohydrates play major signaling roles within the immune system and have the advantage with rare exception of high biocompatibility and low toxicity. Carbohydrates are easily metabolized or excreted, with little risk of generating toxic metabolites or long-term tissue deposits such as may occur with aluminum salts in the condition known as macrophagic myofasciitis [2]. This means that adjuvant accumulation and excessive or chronic immune activation are less likely when using carbohydrate compounds. By contrast, aluminum salts have been shown to form long-term deposits at injection sites, potentially lasting for decades after vaccine administration [3]. A depot effect was initially thought important to alum’s adjuvant activity but was long ago disproved by showing adjuvant action even after excision of the alum depot, findings since largely forgotten [4,5]. While any detrimental pathophysiological effects of long-term aluminum tissue deposits and associated macrophagic myofasciitis remains a matter of debate [2], carbohydrate adjuvants present no such concern, a major factor in their favor. As discussed later, carbohydrate adjuvants come in many different forms (Table 1), each with their own unique physicochemical and immunological attributes and behaviors, providing a wide range of options in vaccine design.

A lack of animal studies or trials comparing multiple adjuvants with the same antigens is often noted as an impediment to adjuvant selection. However, even if they were available, extensive head-to-head comparisons of adjuvants in animals could be extremely misleading as many adjuvant effects including potency vary from species to species and thus comparisons of adjuvant potency performed, for example, in mice are often not predictive of relative adjuvant potency in humans. This is well demonstrated by studies showing that aluminum adjuvants are very effective at enhancing influenza antibody responses in mice but have no effect when the same influenza vaccine formulation is tested in humans [6]. Thus, there is a strong argument not to perform adjuvant selection for human trials based on

adjuvant ranking in animal potency studies, particularly if only a single animal species has been used. Instead, adjuvant selection for human trials may be better performed based on an assessment of all the merits and disadvantages of each candidate adjuvant for the intended vaccine application, with assessment of adjuvant potency a feature left to human testing. This suggests we are not yet at the point where trial and error has been completely excluded from adjuvant selection for human vaccine development.

Glucans

Glucans are plant- or microorganism-derived polysaccharides made up of repeating D-glucose units joined by glycosidic bonds in various alternative conformations. α -glucans include dextran (α -1,6-glucan), glycogen (α -1,4- and α -1,6-glucan), pullulan (α -1,4- and α -1,6-glucan) and starch (α -1,4- and α -1,6-glucan). β -glucans include cellulose (β -1,4-glucan), curdlan (β -1,3-glucan), laminarin (β -1,3- and β -1,6-glucan), chrysolaminarin (β -1,3-glucan), lentinan (purified β -1,6; β -1,3-glucan from *Lentinus edodes*), lichenin (β -1,3- and β -1,4-glucan), pleuran (β -1,3- and β -1,6-glucan isolated from *Pleurotus ostreatus*) and zymosan (β -1,3-glucan from *Saccharomyces*). The glucan field is confusing as each type and source of glucan can be of widely varying quality and purity, and may contain mixtures of different polymer chain structures with differing amounts of polymer branching and variation in polymer chain length. Because these polymer variables are often influenced by the source of the glucan, many glucans are named according to their plant or microbial source. Hence, while zymosan is a yeast cell wall extract that contains β -1,3-glucan, it is relatively impure and contains variable amounts of yeast cell wall proteins. Unfortunately, it is not always clear to what extent such variation in structure, or the presence of contaminants in glucan formulations, may influence their adjuvant activity, and this variability and lack of easy characterization is likely to have been a contributing factor holding back the development of glucan-based adjuvants, as strong proof-of-product characterization and consistency is required for regulatory approval. As shown in Table 1, glucans enhance both humoral and cellular immunity and exert their adjuvant activities by binding to specific carbohydrate receptors (lectins) such as the mannan, β -glucan and Dectin-1 receptors expressed on monocytes and other antigen-presenting cells (APCs), resulting in activation of NF- κ B, monocyte maturation and production of proinflammatory cytokines.

α -glucans

Dextran—Dextran is a branched microbial polysaccharide made up of α -1,6-glucan with α -1,3-branches. Along with its sulfated derivative, dextran sulfate, it has multiple human therapeutic applications, including use as a plasma volume expander and anti-thrombotic agent. Dextran sulfate has broad proinflammatory effects as exemplified by its use to induce inflammatory colitis in mice. These immune effects have led to examination of dextran's ability as a vaccine adjuvant. A study of *Streptococcus bovis* vaccine in sheep found dextran sulfate to be less effective at inducing protective antibody titers than either complete Freund's adjuvant (CFA), incomplete Freund's adjuvant, Quil A, alum, or Gerbu adjuvant [7]. Diethylaminoethyl (DEAE)-dextran is a polycationic derivative of dextran that has been tested as a veterinary adjuvant. DEAE dextran enhanced the primary immune response of rhesus monkeys to formalin-inactivated Venezuelan equine encephalomyelitis virus vaccine with a reduced time required for initiation of IgG production [8]. DEAE-dextran similarly enhanced antibody responses in mice to whole-cell cholera vaccine [9]. In an alternative approach, acetylated dextran (Ac-DEX) microparticles have been used to deliver the TLR-7 agonist, imiquimod, to immune cells. Ac-DEX-encapsulated versus free imiquimod was shown to significantly increase IL-1 β , IL-6, TNF- α , inducible nitric oxide synthase, PD1-L1 and nitric oxide expression by macrophages, suggesting that Ac-DEX micro particle-

encapsulation may enhance the potency of Toll-like receptor (TLR) ligands in vaccine formulations [10]. Dextran derivatives have yet to achieve success as human adjuvants.

β-glucans

Zymosan—Zymosan is prepared from yeast cell wall and consists of protein– carbohydrate complexes with the major constituent being β-1,3-glucan. Zymosan activates the alternative complement pathway and also binds to TLR-2 and Dectin-1 on monocytes, activating NF-κB and thereby inducing inflammatory cytokine production, arachidonate mobilization, protein phosphorylation and inositol phosphate formation [11–13]. Zymosan induced nonspecific resistance against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Serratia marcescens*, *Staphylococcus aureus* and *Candida* infection, with the effect being most pronounced 2–5 days after zymosan treatment [14–16]. Zymosan has also been shown to induce tumoricidal activity by polymorphonuclear cells [16]. Zymosan enhanced the mucosal adjuvant activity of polyriboinosinic polyribocytidylic acid (poly I:C) in a nasal inactivated influenza vaccine in mice, with the mechanism postulated to be a synergistic increase in cytokine production (TNF-α, IL-6, IL-10 and IFN-β) and increase in costimulatory molecules expression (CD86 and CD40) with poly I:C working via TLR-3 and zymosan through TLR-2 [17]. Zymosan has also been shown to enhance the humoral and cellular immune response to DNA vaccination in mice [18]. This effect was blocked by anticomplement C3 antibody and was not seen in complement (C5)-deficient DDD and AKR mice suggesting that the adjuvant effect on DNA vaccines was dependent on activation of complement by zymosan [18]. Zymosan's high reactogenicity may reflect its ability to directly activate the inflammasome through ASC and cryopyrin resulting in caspase-1 activation and IL-1β secretion, a feature that it shares with mannan [19]. β-glucan derived from zymosan and subjected to brief oxidation was able to substitute for CFA as an adjuvant for collagen-induced arthritis [20], consistent with zymosan's proinflammatory effects being sufficient to break immune tolerance; a major concern from a human regulatory standpoint. Zymosan, through TLR-2 activates the master proinflammatory transcription factor, NF-κB, and this action may be the key factor shared by adjuvants possessing autoimmune potential in animal models, such as CFA and TLR agonists. This could explain why carbohydrate adjuvants that do not activate NF-κB, for example, δ-inulin, are unable to induce autoimmunity when used as a replacement for CFA in autoimmune animal models such as the experimental allergic encephalitis (EAE) model of multiple sclerosis [Cooper P and Petrovsky N, Unpublished Data].

Lentianan—Lentianan is made up of β-1,3-glucosamine with β-1,6-branches. Intranasal lentianan induced nonspecific resistance against lethal influenza virus infection, in association with induction of an enhanced respiratory burst and nitric oxide and IL-6 production by bronchoalveolar macrophages [21]. As seen with other immunologically-active polysaccharides, lentianan also exhibited anti-tumor activity [22,23]. Shiitake mushroom (*Lentinus edodes*)-derived lentianan administered intranasally in guinea pigs at a dose of 1 mg/kg followed by administration of Bacillus Calmette–Guérin (BCG) vaccine enhanced the killing ability of alveolar macrophages against *Mycobacterium tuberculosis* and *Staphylococcus aureus*, while reducing systemic side effects [24]. Lentianan increased the efficacy of a dendritic cell (DC) vaccine prepared by transfection of adenovirus-mediated melanoma-associated antigen gene (*gp100*) into bone marrow-derived DCs for treatment of B16 melanoma-bearing mice, with enhancement of cytotoxic activity of T cells and natural killer cells, elevation of IL-2 and IFN-γ in splenocytes, and increased tumor inflammation and necrosis [25]. Lentianan enhanced by up to ninefold the phagocytosis of albumin, C3b- or IgG2b-coated fluorescent microspheres by resident or thioglycollate-elicited mouse macrophages in a dose-dependent manner, both by direct stimulation of macrophage phagocytosis as well as by serving as a supplementary opsonin for C3b-coated

beads, suggesting a role for the β -glucan receptor in initiating phagocytosis [26]. Sulfated lentinan was shown to be a more potent adjuvant than unmodified lentinan as measured by ability to enhance serum antibody titer, promote lymphocyte proliferation and reduce mortality of chickens immunized with Newcastle disease vaccine [27]. A synthetic lentinan analogue (β -glu6), when administered intraperitoneally with hepatitis B surface antigen (HBsAg) in BALB/c mice, enhanced macrophage chemotaxis and increased the B- and T-cell response to HBsAg [28]. β -glu6 enhanced the number of HBsAg-specific IL-4- but not IFN- γ -producing lymphocytes, consistent with a Th2 immune bias [28]. This Th2 bias is consistent with other studies showing that lentinan suppresses nonspecific macrophage tumoricidal activity whereas this activity is increased by proinflammatory adjuvants such as BCG extracts or LPS [29]. β -glu6 was also shown to enhance the antigen-specific CD8⁺ T-cell response induced by a DNA vaccine encoding hepatitis B core antigen in mice, with promotion of recruitment and maturation of DCs, enhanced anti-hepatitis B core IgG and IgG2a antibody titers and enhanced numbers and activation of antigen-specific CD4⁺ and CD8⁺ T cells [30].

Algal glucan—Algal glucan, a β -glucan extracted from an adapted strain of *Euglena gracilis* (SRI strain D86-G) grown heterotrophically in the dark, enhanced humoral and cellular immunity to coadministered peptide antigens [31]. Mice immunized with herpes virus glyco protein D (gD2) plus 100 μ g particulate algal glucan produced anti-gD2 antibodies and T-cell responses that were significantly higher and persisted longer than control animals injected with gD2 alone. Covalent conjugation of the algal β -glucan directly to the gD2 antigen resulted in even higher antibody titers than those achieved with a simple mixture of gD2 antigen and β -glucan [31]. Algal β -glucan was not toxic when administered intravenously at doses up to 25 mg/kg bodyweight.

β -glucan particles—Glucan particles (GPs) are purified cell walls of *Saccharomyces cerevisiae* treated so as to remove mannans and yeast cell wall proteins, leaving a skeleton primarily made of β 1,3- β -glucans [32]. GPs are phagocytosed by DCs via the Dectin-1 receptor, inducing inflammatory cytokine production. The hollow porous structure of GPs allows them to be loaded with antigens. Ovalbumin (OVA) electrostatically complexed inside hollow GP shells (GP-OVA) enhanced phagocytosis, upregulation of maturation markers and OVA proteolysis by DCs and these DC were 100-fold more potent at stimulating the proliferation of OVA-reactive transgenic CD8⁺ OT-I and CD4⁺ OT-II T cells [33]. Subcutaneous immunization of C57BL/6J mice with GP-OVA resulted in induction of anti-OVA IgG1 and IgG2c antibodies and higher OVA-specific T-cell responses with a Th1 bias.

Fructans

One of the first polysaccharides recognized to have immunological effects was β -D-(2-1) poly(fructo-furanosyl) β -D-glucose, more commonly known as inulin, a natural plant-derived storage carbohydrate of various plants, that is also produced by *Aspergillus* family microorganisms and *Streptococcus mutatis*. Inulin is a polymer comprising linear chains of fructosyl groups linked by β (2-1) glycosidic bonds terminated at the reducing end by an α -D-(1-2)-glucopyranoside ring group. Inulin's immune activity was first identified when inulin solutions were found to activate complement through a nonclassical pathway, which led to the discovery of the alternative complement pathway. Complement activation was found to be due to minute inulin particles contaminating inulin solutions, with these insoluble particles given the name γ -inulin to represent a completely new polymorphic form of inulin [34]. γ -inulin, like other complement-activating compounds, was subsequently found to have adjuvant activity of at least equivalent potency to alum adjuvants, a property not shared by α - and β -inulin [35]. Several subsequent studies demonstrated that γ -inulin also had anti-

tumor activity [36,37]. In head-to-head adjuvant comparison studies in mice, γ -inulin alone or complexed with aluminum hydroxide (algammaulin) was shown to be more potent than aluminum adjuvants and in several cases achieved equipotency to CFA, which to this day remains the gold standard for all adjuvant potency comparisons performed in animals [35,38–43].

More recently, a more temperature-stable inulin isoform, named δ -inulin (Deltin™, Vaxine Pty Ltd, Australia), has been identified and characterized with superior adjuvant potency to the earlier γ -inulin isoform [44]. Deltin adjuvant (also known by the tradename, Advax™, Vaxine Pty Ltd) has been shown to be effective in animal models of Japanese encephalitis [45] and HIV [46] vaccines, among others, and is currently in human clinical development in vaccines against influenza and hepatitis B [Petrovsky N, Unpublished Data]. A notable feature of Deltin adjuvant is its low reactogenicity as demonstrated in vaccine trials, which probably reflects its carbohydrate origins [40,47]. Deltin adjuvant enhances both humoral and cellular immunity to coadministered antigens, with induction of strong antigen-specific CD4⁺ and CD8⁺ memory T-cell responses, in addition to enhanced production of antigen-specific IgM, IgG and IgA [45]. The mechanism of action of Deltin is yet to be fully elucidated, but its action appears to be predominantly driven through interaction with APCs, including monocytes, macrophages and DCs, to which it binds and induces phenotypic changes associated with enhanced antigen presentation and antigen-specific T- and B-cell stimulation. Surprisingly, Advax adjuvant, unlike other adjuvants, does not induce macrophage production of the proinflammatory cytokines TNF- α and IL-1, consistent with its failure to induce NF- κ B activation [Petrovsky N, Unpublished Data]. This inability to induce NF- κ B activation could potentially explain the low reactogenicity of Advax adjuvant and its inability to substitute for CFA for the induction of autoimmune diseases such as murine EAE.

Mannans

Mannan is a storage polysaccharide produced by yeast, bacteria and plants that is a polymer of the sugar mannose in a 1,4 linkage. Binding of mannan by mannan-binding lectin and other C-type lectins of the mannose receptor family leads to complement activation, opsonization and phagocytosis [48,49]. Mannan also activates the inflammasome resulting in caspase 1 activation and production of IL-1 [19]. As C-type lectin receptors mediate endocytosis, mannan and derivatives, including oxidized and reduced forms, have been used as vaccine adjuvants to target antigens to APCs. Mannan, oxidized mannan and reduced mannan were all shown to stimulate mouse bone marrow-derived DCs, *in vitro* enhancing allogeneic T-cell proliferation and OT-I/OT-II peptide-specific T-cell responses and production of inflammatory cytokines including TNF- α and IL-1 β . *In vivo*, a mature phenotype of lymph node and splenic DCs were induced through a TLR-4-dependent mechanism [50]. In mice, mannose- or reduced mannan-coupled antigens stimulated Th2 type responses with IL-4 production whereas oxidized mannan-coupled antigens stimulated Th1 responses with IFN- γ , IL-2 and IL-12 production [51]. Mannan, oxidatively coupled to recombinant protein antigen and given intranasally was shown to be superior to cholera toxin in enhancing the production of antigen-specific IgA, IgG1 and IgG2a in serum, and secretory IgA in lung, tear duct, vaginal and salivary secretions [52]. Oxidative coupling was critical to its action, since neither mannan mixed with protein, nor mannan-protein conjugates that had been reduced by treatment with sodium borohydride, were effective [52].

Conjugation of myelin basic protein to reduced mannan was able to switch the anti-myelin basic protein immune response from Th1 to Th2 and thereby protect against EAE in an animal model [53]. Mannan conjugated to A β 28 enhanced anti-A β antibody production in

transgenic mice otherwise hyporesponsive to immunization with A β antigen alone [54]. Polymannose purified from the *Aloe barbadensis* Miller plant enhanced antibody titers against capsid protein epitopes in coxsackievirus B3-immunized mice [55]. Mannose has also been used to better target and enhance the immunogenicity of antigen-containing liposomes. A novel mannosylated cholesterol derivative enabled mannose receptor-mediated gene transfer with liposomes, with plasmid DNA complexed with mannosylated liposomes exhibiting high transfection of macrophages [56]. Coating of cationic liposomes with mannan significantly enhanced the ability of a DNA vaccine to induce HIV-specific Th1 cellular immunity, with similar results obtained with a DNA vaccine against melanoma [57,58].

Another approach has been to use mannosylated niosomes as oral DNA vaccine carriers for the induction of humoral, cellular or mucosal immunity. Niosomes carrying recombinant HBsAg DNA and composed of span 60, cholesterol and stearylamine (all coated with the modified polysaccharide O-palmitoyl mannan to protect against degradation in the gut) were able to induce protective anti-HBsAg serum titers, cellular immune responses and secretory anti-HBsAg IgA in mucosal secretions [59]. A similar approach was used for topical vaccine delivery using O-palmitoyl mannan coating to target niosomes to Langerhan's cells in the skin [60]. Acemannan, a major acetylated mannan fraction of Aloe vera gel, enhances immune responses and has antiviral and anti-tumor activities. Acemannan induced maturation of immature DCs as detected through upregulation of MHC class II molecules and major costimulatory molecules CD80, CD86, CD40 and CD54, and increased the allogeneic mixed lymphocyte reaction and IL-12 production; these effects were almost completely abolished by chemical sulfation of the acemannan [61]. Acemannan adjuvant, however, performed poorly when compared with five other adjuvants including aluminum salts and incomplete Freund's adjuvant in immunizing mice against feline leukemia virus, feline immunodeficiency virus and heartworm antigen, only enhancing the antibody response against heartworm but not the other two antigens [62]. Similarly, acemannan adjuvant had mixed results when added to an oil-based vaccine (Breedervac III[®], Intervet Inc, USA) for immunization of chickens [63], suggesting insufficient potency to justify human vaccine development.

Chitin/chitosan

Chitin, a linear β -1–4-linked polymer of D-glucosamine and N-acetyl-D-glucosamine extracted from shrimp, and chitosan, which is obtained by partial deacetylation of chitin, exhibit a range of immunological effects, including macrophage activation and production of inflammatory cytokines including IL-12, IL-18, TNF- α and IFN- γ and enhanced antibody production when coadministered with antigens [64]. These effects are mediated by binding of chitin particles to cell surface receptors, including Dectin-1, macrophage mannose receptor and TLR-2, followed by internalization and degradation of the particles [64]. Phagocytosis of chitin particles by murine RAW cells resulted in phosphorylation of MAPK and release of high levels of TNF- α , COX-2 and prostaglandin E2, consistent with a strong proinflammatory signal [65]. The addition of 0.5% of chitosan derivative to inactivated influenza vaccines resulted in a four- to tenfold increase in antibody titers after intramuscular immunization of mice when compared with vaccine alone [66]. Mice injected intraperitoneally with a chitosan-adjuvanted H5N1 vaccine exhibited enhanced protection against lethal H5N1 challenge, which was equivalent to the enhanced protection seen with use of an alum adjuvant [67]. Chitosan particles produced by cross-linking the polysaccharide with a counter ion were shown to entrap HBsAg antigen and enhance its immunogenicity ninefold in mice compared with standard alum-adjuvanted HBsAg [68].

By virtue of their mucoadhesive qualities, chitin and its derivatives have been extensively tested as nasal adjuvants. Chitin derivatives such as *N*-trimethyl chitosan chloride have been shown to enhance the absorption of proteins at mucosal surfaces by inducing transient opening of tight junctions [69]. The concomitant use of chitosan microparticles and the mucosal adjuvant LTK63 significantly enhanced immunogenicity and protective efficacy of a group C meningococcal polysaccharide vaccine given intranasally [70]. Similarly, alginate-coated chitosan nanoparticles loaded with HBsAg and given to mice intranasally induced a measurable antibody response to HBsAg and a cellular response to HBsAg was obtained by administering chitosan nanoparticles together with CpG adjuvant [71]. Chitosan and glycol-chitosan were evaluated as nasal adjuvants to improve the immunogenicity in cattle of a replication-defective adenovirus type 5 vaccine expressing bovine herpesvirus 1 glycoprotein D. Here, the best virological protection was obtained with vaccine vector adjuvanted with glycol chitosan [72]. Plasmid DNA vaccine-loaded chitosan nanoparticles, when given intranasally, successfully induced humoral, cellular and mucosal immunization against hepatitis B [73]. Nanoparticles were developed by complexing the oppositely charged chitosan derivatives *N*-trimethyl chitosan (polycationic) with mono-*N*-carboxymethyl chitosan (polyampholytic) without using any cross-linker. Such particles loaded with tetanus toxoid antigen, when phagocytosed by mouse macrophages, were shown to induce both mucosal and systemic immune responses to tetanus toxoid, when administered intranasally [74]. BALB/c mice immunized with a *Helicobacter pylori* vaccine with chitosan adjuvant exhibited enhanced protection against *H. pylori* infection with reversal of *H. pylori*-induced inhibition of Th2 responses and a reduced rate of gastritis when compared with vaccine adjuvanted with the mucosal adjuvant, cholera toxin [75]. Addition of chitin particles upregulated Th1 and downregulated Th2 responses to a mycobacterial (MPB-59) vaccine in mice, with the magnitude of these responses being increased in IL-10 knockout mice [76]. The Th1 deviation induced by chitin has also been shown to be useful for the suppression of allergic responses. Chitin, when added to an oral ragweed desensitization regimen, downregulated the Th2 cytokines IL-4, IL-5 and IL-10, and reduced IgE and lung eosinophilia in a ragweed-sensitized murine model [77].

Mycobacterial carbohydrate adjuvant compounds

Mycobacteria are the major ingredient in CFA, which remains the gold standard in terms of adjuvant potency but also reactogenicity. Over recent decades, many of the chemical entities underlying the adjuvant activities of CFA and other microbial extracts have been elucidated. Among the adjuvant entities discovered in these extracts, many are carbohydrate-containing structures, including oligosaccharides, glycoproteins and glycolipids, as discussed later. Mycobacteria are recognized by APCs through various pathogen-associated molecular pattern receptors, including the TLRs and C-type lectins (e.g., the mannose receptor and DC-SIGN), with many of these interactions being dependent on carbohydrate structures within the mycobacterial cell wall [78]. These carbohydrate structures also contribute to CFA's adjuvant activity.

Lipoarabinomannan

Lipoarabinomannans (LAMs) are major mycobacterial structural cell surface components. LAMs have varying immunomodulatory activities, depending upon their structure. LAMs from nonpathogenic mycobacteria bind TLR-2 and activate macrophages through NF- κ B activation and induction of antimycobacterial inflammatory cytokines including TNF- α and IL-12 [79]. However, mannosylated LAM, for example, from pathogenic *M. tuberculosis*, binds to the mannose receptor and DC-SIGN resulting in inhibition of TNF- α and IL-12 and stimulation of the anti-inflammatory cytokine, IL-10 [79]. Hence, LAMs can be either pro- or anti-inflammatory, depending on their origin, and which innate receptors, for example, mannose receptor, TLR-1/TLR-2, TLR-4 or DC-SIGN, they signal through [80]. This

highlights the subtleties of carbohydrate signaling, with different adjuvant effects being potentially available by subtle modifications of carbohydrate structures. LAM-derived arabinomannan oligosaccharides from *M. tuberculosis* covalently conjugated to mycobacterial antigen Ag85B protected animals against mycobacterial challenge when administered with Eurocine L3® (Eurocine, Norway), a monoglyceride adjuvant [81].

Muramyl dipeptide

Muramyl dipeptide (MDP; *N*-acetyl muramyl-*L*-alanine-*D*-isoglutamine) was first identified from a mycobacterial peptido-glycan fraction known to have potent adjuvant activity [82]. MDP has been tested on its own and as a component of more complex adjuvant formulations. MDP binds and activates innate immune receptors, including the NOD2 [83] and TLR receptors providing synergistic coactivation [84]. This leads to potent activation of NF- κ B, the master immune regulator, and induction of inflammatory cytokine production. The carbohydrate moiety of MDP is critical to its adjuvant activity as shown by studies in which carbohydrate analogues of MDP were tested in the induction of delayed-type hypersensitivity in guinea pigs [85]. This confirmed a structural requirement of the carbohydrate moiety of MDP, as only *D*-mannosamine, *D*-galactosamine and *D*-glucose analogues of MDP were active as vaccine adjuvants [85]. Many analogues of MDP have been tested as vaccine adjuvants. MTP-PE (*N*-acetyl-*L*-alanyl-*D*-isoglutaminyl-*L*-alanine-2-[1,2-dipalmitoyl-*sn*-glycero-3-(hydroxy-phosphoryloxy)] ethylamide) was included as an immunostimulator in the original MF59 squalene oil emulsion adjuvant [86]. However, the MTP-PE component was discontinued from MF59 because of its propensity to cause excessive reactogenicity [87]. MDP analogues can be made that are either lipophilic or hydrophilic, with the lipophilic variants being more immunologically active. When formulated in saline, MDP analogues predominantly stimulate humoral immunity, whereas when incorporated into emulsions or liposomes they are potent inducers of cellular immunity. For example, *N*-acetylglucosaminyl-*N*-acetylmuramyl-*L*-Ala-*D*-isoGlu-*L*-Ala-glyceroldipalmitate (DTP-GDP) is a potent macrophage activator, and when formulated as adjuvant in liposomes, induced remission in human metastatic colorectal cancer and enhanced both humoral and cellular immunity. Similar to MDP, it induced high secretion of TNF- α , IL-1 and IL-6, and shared MDP's toxicity, producing fever, chills and hypotension at high doses (>0.8 mg/m²) [88,89].

D-Murapalmitine

D-Murapalmitine (Nac-Mur-*D*-Ala-*D*-isoGln-*sn*-glycerol dipalmitoyl) has been proposed to be distinct from other MDPs in that it did not induce EAE when administered with myelin basic protein, leading to the hypothesis that, when given with antigenic preparations containing heterologous and autologous epitopes, it favors the immune response to the heterologous determinants; how it might have such selectivity is not clear [90]. While the utility of MDP analogues as human adjuvants is restricted by their excess reactogenicity, they are still found in some veterinary adjuvants. For example, Gerbu adjuvants are a group of veterinary adjuvants all based on the use of the immunomodulator *N*-acetyl-glucosaminyl-*N*-acetylmuramyl-*L*-alanyl-*D*-isoglutamin (GMDP), a glycopeptide from the cell wall of *Lactobacillus bulgaricus* [91]. Gerbu adjuvants are complex mixtures that may also contain cimetidine, saponin, paraffin, dimethyldi(stearoylhydroxyethyl) ammonium chloride, mannide monooleate, glycerol, *L*-proline and ciprofloxacin. Although many attempts have been made to create modified analogues of MDP that are better tolerated and less reactogenic, the overall experience is that the potency and reactogenicity of MDP analogues are inseparable, making them unsuitable candidates for human adjuvants.

Trehalose-6–6-dimycolate

Trehalose-6–6-dimycolate (TDM), previously known as *M. tuberculosis* cord factor, is a potent inducer of inflammatory cytokines, including TNF- α , and has biological effects including anti-tumor activity and stimulation of host resistance against infections [92,93]. A number of TDM analogues were synthesized for structure–activity studies, and some attenuation of TDM's toxicity was possible while still retaining adjuvant activity [94]. TDM augments both humoral and cell-mediated immune responses when combined with vaccine antigens, with a comparable efficacy to MDP [95–97]. TDM is a component of the commercial veterinary Ribi Aduvant System[®] (Sigma, USA) where it is formulated with squalene oil, monophosphoryl lipid A (MPL) and other components [96,97], but given its reactogenicity is unsuitable for inclusion in human vaccines.

Lipopolysaccharide

Bacterial lipopolysaccharide (LPS) is a potent inducer of macrophage activation and inflammatory cytokine production and, not surprisingly, has significant immune adjuvant activity. LPS itself is too toxic to be used as a human adjuvant, and this led to a search for less reactogenic LPS analogues, mostly focusing on the Lipid A component that consists of a β -(1,6)-linked α -glucosamine disaccharide phosphorylated at 1'-O and 4'-O-positions. While Lipid A is still too reactogenic for human use, in low acid conditions Lipid A can be hydrolyzed to remove the 1-phosphate group and subsequent mild alkaline treatment leads to specific removal of the fatty acid at position 3, resulting in the product MPL, which has a lower toxicity than Lipid A but retains immunostimulatory activity [98]. Lipid A, as with LPS, signals via TLR-4, but has been suggested to preferentially activate the TRIF over the MyD88 pathway, and this has been proposed as the reason why MPL may have a reduced toxicity compared with LPS [99]. MPL has been used in a variety of proprietary adjuvant formulations developed by GlaxoSmithKline, including their AS02 and AS04 adjuvants where MPL is used in combination with QS-21 in an oil-in-water emulsion, and aluminum hydroxide, respectively. The AS04 adjuvant is contained in licensed vaccines against hepatitis B and human papillomavirus (Fendrix[®] and Cervarix[®], respectively [GlaxoSmithKline]) with the head-to-head data from clinical trials of Fendrix showing the MPL component of the AS04 adjuvant increased antibody responses but at the same time significantly increased vaccine reactogenicity over alum adjuvant alone, consistent with MPL's proinflammatory actions [100,101].

Saponin compounds

A range of plant-derived compounds known as saponins have been identified to have immunological actions. Saponins from *Rhamnaceae*, *Araliaceae*, *Polygalaceae* and *Fabaceae* plant families have all been reported to have adjuvant activity [102]. For example, glycyrrhizina, a triterpenoid saponin glycoside of glycyrrhizic acid, which is the main sweet-tasting compound from liquorice root, has been shown to activate NF- κ B in murine macrophages resulting in production of IL-12 and other inflammatory mediators [103] and to enhance antigen-specific antibody production when coadministered with antigen [104]. Similarly, cycloartane type triterpene saponins from *Astragalus peregrinus* have been shown to stimulate proliferation of mouse splenocytes. However, the most extensively characterized plant saponin from an adjuvant perspective is QS-21. QS-21 is a saponin derived from Quil A, a mixture of triterpenoid glycosides derived from the bark of the South American soap bark tree, *Quillaja saponaria* [105]. QS-21 is an acylated saponin at the 4-hydroxyl position on fucose with two linked 3,5 dihydroxy-6-methyloctanoic acids [106]. *In vivo*, QS-21 induces inflammatory cytokines, IgG2a antibodies and CD8 T cells in mice, consistent with a Th1 bias [105,107]. Numerous clinical trials have been conducted using QS-21 alone as a vaccine adjuvant and it has also been used in more complex adjuvant

formulations such as complexed with cholesterol as immune-stimulating complexes (ISCOMs) [108] or mixed with MPL in an oil-in-water emulsion such as in the proprietary AS02 adjuvant. Because of its potent ability to lyse cell membranes, adverse reactions, including severe injection site pain and hemolysis, are major limiting factors in QS-21 use. Despite many decades of clinical development, no QS-21-based vaccine has yet to be approved [109]. In an attempt to overcome these problems of toxicity, a range of chemically modified variants of QS-21 have been created. GPI-0100 is a variant with an incorporation of a C-12 alkyl chain through a stable amide bond at the carboxyl group of the glucuronic acid residue of deacylated saponin [110–113]. GPI-0100 has been shown to be 20-times less lethal in mice than QS-21, and in association with this, stimulates a Th2-like immune response whereas QS-21 characteristically stimulates a more Th1-like response [114].

Mechanisms of action of carbohydrate adjuvants

Most of the aforementioned carbohydrate-based adjuvants, such as zymosan, mannan, MDP, TDM and LPS, work through binding to specific innate immune receptors designed to recognize pathogen-associated microbial patterns. These receptors include the TLRs, NOD2 and C-type lectins (e.g., mannan-binding lectin, β -glucan and Dectin-1 receptors), the binding of which results in activation of NF- κ B and production of inflammatory cytokines including TNF- α and IL-1 [32,65]. These cytokines then act as costimulatory molecules to enhance antigen-specific T- and B-cell responses. Activation by carbohydrate adjuvants of monocyte innate immune receptors also leads to upregulation of antigen presentation and costimulatory molecules including MHC class I and II, CD40, CD80 and CD86. This thereby induces mature, multifunctional immune cells including monocytes, macrophages and DCs, able to potently stimulate antigen-specific T- and B-lymphocytes [61]. Many carbohydrate adjuvants including MDP, LPS, zymosan, mannan, α - and β -glucan, γ -inulin and Deltin also activate the complement pathway, generating complement components such as C3a, C3b, C3d, C5a and C5b that act as opsonins and chemokines and provide adjuvant activity [115–118]. The potentially important role played by complement activation in the mechanism of action of carbohydrate adjuvants was demonstrated by the fact that, although zymosan activates multiple immune pathways including the inflammasome and NF- κ B pathways, its adjuvant activity for a DNA vaccine was completely lost in complement depleted or knockout mice [18].

Chemotaxis may also be important to the adjuvant action of carbohydrate compounds, with phosphomannosyl structures, for example, potently influencing lymphocyte migration [119]. Chemokines induced by carbohydrate adjuvants include IL-8, monocyte chemoattractant protein-1 (MCP-1), macrophage inflammatory protein-1 (MIP-1) α and MIP-1 β , as well as chemotactic complement components. The pattern of chemokines induced by a particular carbohydrate adjuvant would be expected to determine the type of immune cells attracted to the injection site and therefore the type of immune response generated [28].

Another way in which some carbohydrate compounds may have adjuvant action is via their ability to activate the inflammasome, with zymosan and mannan sharing the ability of alum adjuvants to bind NALP3 and thereby activate the inflammasome [19]. This results in the activation of caspase 1 and cleavage of pro-IL-1 into active IL-1. This synergizes with the increased *pro-IL-1* gene expression that results from zymosan and mannan activating proinflammatory TLR pathways [120].

Although QS-21 has been shown to result in activation of NF- κ B, the exact pathways through which it works remain unclear. Saponins integrate into cell membranes through interaction with cholesterol, resulting in pores through which antigens may enter into APCs [121]. The oligosaccharide chains in QS-21 may be important in allowing QS-21 to

selectively bind to APCs via cell surface selectins, thereby enabling it to insert itself into the membrane and trigger cell activation. QS-21 may also have a direct action on T cells, as the aldehyde group at C-4 of the aglycone unit of QS-21 may form a Schiff base with the amino groups of receptors on the surface of T cells, thereby providing them with direct costimulatory signals [106].

As excess inflammatory signals are responsible for the bulk of adjuvant toxicity, those adjuvants that primarily work through activation of NF- κ B and the inflammasome will probably share the dose-limiting excess reactogenicity of other noncarbohydrate inflammatory adjuvants that work through the same proinflammatory pathways [47]. Excessive adjuvant-induced inflammation can cause local reactogenicity, which manifests as pain, inflammation, swelling, redness, ulceration and sterile abscess, and systemic reactogenicity, which manifests as lymphadenopathy, splenomegaly, nausea and vomiting, diarrhea, fever, myalgia, fatigue, headaches, adjuvant arthritis, uveitis, eosinophilia, allergy, organ toxicity, autoimmunity and anaphylaxis. Many of these adverse effects can be reproduced in animal models by administering agents that activate NF- κ B, the inflammasome or both. In particular, the ability to activate NF- κ B appears to be common to adjuvants able to substitute for CFA in the induction of the autoimmune condition EAE. It is possible, therefore, that carbohydrate adjuvants, such as δ -inulin, that enhance vaccine immunogenicity via alternative as yet uncharacterized pathways that do not involve NF- κ B or inflammasome activation might avoid such toxicities.

Study of LAMs shows that carbohydrate compounds can also stimulate anti-inflammatory signals with the balance of pro-and anti-inflammatory signals mediated by a particular carbohydrate adjuvant determining differences in potency and/or toxicity [47]. As shown in Table 1, carbohydrate structures rarely bind exclusively to just one receptor, with examples of some binding four or more different immune receptors, and even this may be an underestimate of the total number of different receptors that a large multimeric carbohydrate structure may bind. The exact pattern of immune receptors triggered by a carbohydrate adjuvant is likely to be extremely important in determining the ultimate immune outcome, a situation unlike that of simpler compounds such as TLR agonists that may only bind one specific immune receptor. This immune receptor redundancy of carbohydrate adjuvants may also mean that they are effective even in individuals who have a genetic lack of a specific immune receptor or pathway providing an advantage over compounds dependent on just a single receptor for their action. Another advantage, particularly of multimeric semi-crystalline polysaccharide adjuvants such as δ -inulin, is that the low individual affinity of most carbohydrate-lectin bonds requires multimeric interactions for immune stimulation. This means that once a multimeric carbohydrate structure such as a δ -inulin particle solubilizes, it immediately ceases to have immune activity, with the soluble form being immunologically inert [44]. This should reduce the possibility of multimeric carbohydrate structures inducing toxicity due to chronic immune activation; a theoretical problem for long-lasting adjuvant depots such as formed by aluminum salts.

Regulatory issues for carbohydrate adjuvants

As mentioned earlier, regulatory hurdles remain the biggest barrier to the introduction of new vaccine adjuvants. Beyond short-term issue of demonstrating efficacy and tolerability in preclinical animal models, adjuvants under development must surmount issues of production scale-up and manufacturing consistency under good manufacturing practice. Carbohydrate chemistry can be complex, making manufacturing scale-up difficult and also making it hard for carbohydrate-derived adjuvants to compete with existing simple and cheap adjuvant compounds such as aluminum hydroxide. Many carbohydrate compounds, for example, LPS, MPL or QS-21, are complex molecules with variable side chains and

modifications and, when purified from plant or microbial sources, may consist of many quasi-species making it difficult to purify them to homogeneity. In the absence of chemical entity homogeneity it may be hard to develop and validate release assays necessary to ensure there is no batch-to-batch variation that might affect biological potency or to detect the presence of trace contaminants that might cause excess reacto-genicity or toxicity. One approach taken to solve these purification and characterization problems has been to use chemical approaches to generate synthetic versions of the relevant native molecules. For example, tree bark-derived Quil A and QS-21 are complex and variable saponin fractions making the formulation of consistent batches very challenging. To overcome problems of instability and toxicity a semi-synthetic derivative of deacylated QS-21 modified at the glucuronic acid residue known as GPI-0100 was developed and shown to retain adjuvant potency [110]. A number of completely synthetic isomers of QS-21, called QS21-Xyl and QS-21-Api, have subsequently been developed [122]. Similarly, synthetic TLR-4 agonists have been created for use as vaccine adjuvants in place of bacteria-derived LPS and MPL [123,124]. A major virtue of polysaccharides, such as the glucans and fructans, is that they can usually be conveniently purified to a uniform product, thereby avoiding the problems of complex polyvalent mixtures. Nevertheless, variable branching and modifications of some polysaccharides could still present some challenges to the creation of a consistently good manufacturing practice product. As a final step for regulatory approval of a new adjuvant, there is also the need to generate long-term clinical safety data of the adjuvant when combined with a specific vaccine formulation, given that adjuvants cannot be approved as stand-alone products. These regulatory issues present major challenges for adjuvant developers and help explain the paucity of adjuvants that have successfully been approved for human use.

Expert commentary & five-year view

Carbohydrate structures play critical roles in immune function, and it is not surprising therefore that they should provide a fertile area for discovery of new immune adjuvants. Pure carbohydrate compounds are generally safe and well-tolerated; critical attributes for a general-purpose adjuvant. Among the many carbohydrate structures, the polysaccharides stand out as most promising as they are nontoxic and can be assembled into defined semi-crystalline structures able to multimerically engage monocyte receptors to enhance antigen presentation and costimulation. The key to avoiding excessive inflammation and toxicity will be to focus on those adjuvants that do not depend on activation of NF- κ B, as this can lead to excess inflammatory cytokine production and toxicity. Overall, the combination of strong immune enhancement and low reactogenicity makes polysaccharide particles and related carbohydrate-based structures strong candidates for future human adjuvant development. Several of these compounds have already been successful in reasonably large Phase II human trials, so providing that resources are available to take these compounds forward into definitive Phase III studies, it could only be a matter of years before one or more of these adjuvants may reach the licensure stage, thereby finally laying down a challenge to alum's supremacy.

Key issues

- Carbohydrate structures play important roles in the immune system and carbohydrate-derived compounds may make good vaccine adjuvant candidates.
- Carbohydrate compounds have a strong human safety and tolerability record; the biggest regulatory barriers to approval of new adjuvants.

- Among the carbohydrates with adjuvant activity, polysaccharide particles such as Deltin and β -glucan show particular promise and combine potency with lack of toxicity.
- Whilst no carbohydrate adjuvant classification system exists, a useful system is to separate them into those that activate NF- κ B or not, as this may help predict their toxicity profile.
- Whilst the exact mechanism of action of many adjuvants remains unclear, carbohydrate adjuvants have direct effects on monocytes and dendritic cells to upregulate costimulatory molecules and induce chemokine and cytokine secretion.

Acknowledgments

The authors are also supported by Contracts No. U01AI061142 and HHSN272200800039C administered by the National Institute of Allergy and Infectious Diseases, National Institutes of Health, Department of Health and Human Services.

References

Papers of special note have been highlighted as:

- of interest
- of considerable interest

1. Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. *Immunol. Cell. Biol.* 2004; 82(5):488–496. [PubMed: 15479434] • Provides a general overview of the adjuvant field.
2. Rivas E, Gomez-Arnaiz M, Ricoy JR, et al. Macrophagic myofasciitis in childhood: a controversial entity. *Pediatr. Neurol.* 2005; 33(5):350–356. [PubMed: 16243223]
3. Gherardi RK, Coquet M, Cherin P, et al. Macrophagic myofasciitis lesions assess long-term persistence of vaccine-derived aluminium hydroxide in muscle. *Brain.* 2001; 124(Pt 9):1821–1831. [PubMed: 11522584] •• While macrophagic myofasciitis is now accepted as a patho-histological abnormality induced by intramuscular injection of alum-containing vaccines, there is still no consensus on whether this muscle lesion is responsible for associated clinical symptoms of chronic inflammation and fatigue as described in the original report by Gherardi. Of course, the absence of definitive proof of an association cannot be taken as a proof of absence, leaving alum adjuvants on trial with a case to answer even if not convicted.
4. Holt LB. Quantitative studies in diphtheria prophylaxis; the primary response. *Brit. J. Exp. Pathol.* 1949; 30(4):289–297. [PubMed: 15393420] • One of the earliest studies disproving the ‘antigen depot’ explanation of action of adjuvants such as aluminum hydroxide.
5. Freund J. The effect of paraffin oil and mycobacteria on antibody formation and sensitization; a review. *Am. J. Clin. Pathol.* 1951; 21(7):645–656. [PubMed: 14846770]
6. Keitel WA, Campbell JD, Treanor JJ, et al. Safety and immunogenicity of an inactivated influenza A/H5N1 vaccine given with or without aluminum hydroxide to healthy adults: results of a Phase I-II randomized clinical trial. *J. Infect. Dis.* 2008; 198(9):1309–1316. [PubMed: 18808338]
7. Shu Q, Bir SH, Gill HS, et al. Antibody response in sheep following immunization with *Streptococcus bovis* in different adjuvants. *Vet. Res. Commun.* 2001; 25(1):43–54. [PubMed: 11214671]
8. Houston WE, Crabbs CL, Kremer RJ, Springer JW. Adjuvant effects of diethylaminoethyl-dextran. *Infect. Immun.* 1976; 13(6):1559–1562. [PubMed: 823105]
9. Kaistha J, Sokhey J, Singh S, Kumar S, John PC, Sharma NC. Adjuvant effect of DEAE-dextran and tetanus toxoid on whole cell heat inactivated phenol preserved typhoid vaccine. *Indian J. Pathol. Microbiol.* 1996; 39(4):287–292. [PubMed: 9009480]

10. Bachelder EM, Beaudette TT, Broaders KE, et al. *In vitro* analysis of acetalated dextran microparticles as a potent delivery platform for vaccine adjuvants. *Mol. Pharm.* 2010; 7(3):826–835. [PubMed: 20230025]
11. Schorlemmer HU, Bitter-Suermann D, Allison AC. Complement activation by the alternative pathway and macrophage enzyme secretion in the pathogenesis of chronic inflammation. *Immunology.* 1977; 32(6):929–940. [PubMed: 328387]
12. Sato M, Sano H, Iwaki D, et al. Direct binding of Toll-like receptor 2 to zymosan, and zymosan-induced NF- κ B activation and TNF- α secretion are down-regulated by lung collectin surfactant protein A. *J. Immunol.* 2003; 171(1):417–425. [PubMed: 12817025] • Important work elucidating the mechanism of action of polysaccharide adjuvant.
13. Dillon S, Agrawal S, Banerjee K, et al. Yeast zymosan, a stimulus for TLR2 and dectin-1, induces regulatory antigen-presenting cells and immunological tolerance. *J. Clin. Invest.* 2006; 116(4):916–928. [PubMed: 16543948]
14. Emod J, Joo I. Nonspecific resistance-enhancing activity of zymosan in experimental bacterial infections. *Acta. Microbiol. Hung.* 1990; 37(2):187–192. [PubMed: 2270737]
15. Morikawa K, Takeda R, Yamazaki M, Mizuno D. Induction of tumoricidal activity of polymorphonuclear leukocytes by a linear β -1,3-d-glucan and other immunomodulators in murine cells. *Cancer Res.* 1985; 45(4):1496–1501. [PubMed: 3156669]
16. Williams DL, Cook JA, Hoffmann EO, Di Luzio NR. Protective effect of glucan in experimentally induced candidiasis. *J. Reticuloendothel. Soc.* 1978; 23(6):479–490. [PubMed: 702473]
17. Ainai A, Ichinohe T, Tamura S, et al. Zymosan enhances the mucosal adjuvant activity of poly(I:C) in a nasal influenza vaccine. *J. Med. Virol.* 2010; 82(3):476–484. [PubMed: 20087927]
18. Ara Y, Saito T, Takagi T, et al. Zymosan enhances the immune response to DNA vaccine for human immunodeficiency virus type-1 through the activation of complement system. *Immunology.* 2001; 103(1):98–105. [PubMed: 11380697] • One of the first descriptions of the use of polysaccharide adjuvants for DNA vaccines along with the essential role of complement in the mechanism of action.
19. Lamkanfi M, Malireddi RK, Kanneganti TD. Fungal zymosan and mannan activate the cryopyrin inflammasome. *J. Biol. Chem.* 2009; 284(31):20574–20581. [PubMed: 19509280] • Important contribution to the understanding of the mechanism of immune action of polysaccharide compounds.
20. Hida S, Nagi-Miura N, Adachi Y, Ohno N. β -glucan derived from zymosan acts as an adjuvant for collagen-induced arthritis. *Microbiol. Immunol.* 2006; 50(6):453–461. [PubMed: 16785717] • Important work that serves as a warning that even relatively safe compounds such as carbohydrates may have adverse effects including induction of autoimmunity if they overactivate immune inflammatory pathways.
21. Irinoda K, Masihi KN, Chihara G, Kaneko Y, Katori T. Stimulation of microbicidal host defence mechanisms against aerosol influenza virus infection by lentinan. *Int. J. Immunopharmacol.* 1992; 14(6):971–977. [PubMed: 1428370]
22. Jeannin JF, Lagadec P, Pelletier H, et al. Regression induced by lentinan, of peritoneal carcinomas in a model of colon cancer in rat. *Int. J. Immunopharmacol.* 1988; 10(7):855–861. [PubMed: 3235240]
23. Chihara G, Hamuro J, Maeda YY, et al. Antitumor and metastasis-inhibitory activities of lentinan as an immunomodulator: an overview. *Cancer Detect. Prev. Suppl.* 1987; 1:423–443. [PubMed: 3319150]
24. Drandarska I, Kussovski V, Nikolaeva S, Markova N. Combined immunomodulating effects of BCG and Lentinan after intranasal application in guinea pigs. *Int. Immunopharmacol.* 2005; 5(4):795–803. [PubMed: 15710347]
25. Wang J, Zhou ZD, Xia DJ. Study on effect of lentinan in enhancing anti-tumor action of dendritic cytoplast vaccine and its mechanism. *Zhongguo Zhong Xi Yi Jie He Za Zhi.* 2007; 27(1):60–64. [PubMed: 17302067]
26. Abel G, Szollosi J, Chihara G, Facht J. Effect of lentinan and mannan on phagocytosis of fluorescent latex microbeads by mouse peritoneal macrophages: a flow cytometric study. *Int. J. Immunopharmacol.* 1989; 11(6):615–621. [PubMed: 2807634]

27. Guo Z, Hu Y, Wang D, et al. Sulfated modification can enhance the adjuvanticity of lentinan and improve the immune effect of ND vaccine. *Vaccine*. 2009; 27(5):660–665. [PubMed: 19056446]
28. Dong SF, Chen JM, Zhang W, et al. Specific immune response to HBsAg is enhanced by β -glucan oligosaccharide containing an α -(1 $\rightarrow$ 3)-linked bond and biased towards M2/Th2. *Int. Immunopharmacol*. 2007; 7(6):725–733. [PubMed: 17466906]
29. Bruley-Rosset M, Florentin I, Mathe G. In vivo and in vitro macrophage activation by systemic adjuvants. *Agents Actions*. 1976; 6(1–3):251–255. [PubMed: 782207]
30. Wang J, Dong S, Liu C, et al. β -Glucan oligosaccharide enhances CD8⁽⁺⁾ T cells immune response induced by a DNA vaccine encoding hepatitis B virus core antigen. *J. Biomed. Biotechnol.* (Epub ahead of print).
31. Mohagheghpour N, Dawson M, Hobbs P, et al. Glucans as immunological adjuvants. *Adv. Exp. Med. Biol.* 1995; 383:13–22. [PubMed: 8644497]
32. Huang H, Ostroff GR, Lee CK, Wang JP, Specht CA, Levitz SM. Distinct patterns of dendritic cell cytokine release stimulated by fungal β -glucans and Toll-like receptor agonists. *Infect. Immun.* 2009; 77(5):1774–1781. [PubMed: 19273561] •• Important work highlighting the potential combined use of polysaccharide particles as both adjuvant and antigen delivery systems.
33. Huang H, Ostroff GR, Lee CK, Specht CA, Levitz SM. Robust stimulation of humoral and cellular immune responses following vaccination with antigen-loaded β -glucan particles. *MBio*. 2010; 1(3):e00164–e00110, pii.
34. Cooper PD, Carter M. Anti-complementary action of polymorphic “solubility forms” of particulate inulin. *Mol. Immunol.* 1986; 23(8):895–901. [PubMed: 3796631]
35. Cooper PD, Steele EJ. The adjuvanticity of γ inulin. *Immunol. Cell. Biol.* 1988; 66(Pt 5–6):345–352. [PubMed: 3265692] •• The first paper describing how an inulin polysaccharide, when semi-crystallized, developed both complement activation and vaccine adjuvant activity.
36. Cooper PD, Carter M. The anti-melanoma activity of inulin in mice. *Mol. Immunol.* 1986; 23(8):903–908. [PubMed: 3540619]
37. Korbelik M, Cooper PD. Potentiation of photodynamic therapy of cancer by complement: the effect of γ -inulin. *Br. J. Cancer*. 2007; 96(1):67–72. [PubMed: 17146472]
38. Cooper PD, Steele EJ. Algamulin, a new vaccine adjuvant comprising γ inulin particles containing alum: preparation and in vitro properties. *Vaccine*. 1991; 9(5):351–357. [PubMed: 1872020]
39. Cooper PD. Vaccine adjuvants based on γ inulin. *Pharm. Biotechnol.* 1995; 6:559–580. [PubMed: 7551236]
40. Petrovsky N. Novel human polysaccharide adjuvants with dual Th1 and Th2 potentiating activity. *Vaccine*. 2006; 24 Suppl. 2:26–29.
41. Silva DG, Cooper PD, Petrovsky N. Inulin-derived adjuvants efficiently promote both Th1 and Th2 immune responses. *Immunol. Cell. Biol.* 2004; 82(6):611–616. [PubMed: 15550119]
42. Cooper PD, Turner R, McGovern J. Algamulin (γ inulin/alum hybrid adjuvant) has greater adjuvanticity than alum for hepatitis B surface antigen in mice. *Immunol. Lett.* 1991; 27(2):131–134. [PubMed: 1827427]
43. Fuentes P, Cooper PD, Barnadas R, Sabes M, Osterhoff C, Martinez P. Use of γ -inulin/liposomes/Vitamin E adjuvant combination in contraceptive vaccines. *Int. J. Pharm.* 2003; 257(1–2):85–95. [PubMed: 12711164]
44. Cooper PD, Petrovsky N. δ inulin: a novel, immunologically-active, stable packing structure comprising β -d-[2 $\rightarrow$ 1] poly(fructo-furanosyl) α -d glucose polymers. *Glycobiology*. 2010 (In Press). •• Latest work describing the unique δ -inulin polymorphic form which now forms the basis of the Advax™ family of human and veterinary adjuvants.
45. Lobigs M, Pavy M, Hall RA, et al. An inactivated Vero cell-grown Japanese encephalitis vaccine formulated with Advax, a novel inulin-based adjuvant, induces protective neutralizing antibody against homologous and heterologous flaviviruses. *J. Gen. Virol.* 2010; 91(Pt 6):1407–1417. [PubMed: 20130134]
46. Cristillo AD, Ferrari MG, Hudacik L, et al. Induction of mucosal and systemic antibody and T-cell responses following prime-boost immunization with novel adjuvanted human immunodeficiency virus-1-vaccine formulations. *J. Gen. Virol.* 2011; 92(Pt 1):128–140. [PubMed: 21169215]

47. Petrovsky N. Freeing vaccine adjuvants from dangerous immunological dogma. *Expert Rev. Vaccines*. 2008; 7(1):7–10. [PubMed: 18251687]
48. Thiel S, Gadjeva M. Humoral pattern recognition molecules: mannan-binding lectin and ficolins. *Adv. Exp. Med. Biol.* 2009; 653:58–73. [PubMed: 19799112]
49. Takahara K, Yashima Y, Omatsu Y, et al. Functional comparison of the mouse DC-SIGN, SIGNR1, SIGNR3 and Langerin, C-type lectins. *Int. Immunol.* 2004; 16(6):819–829. [PubMed: 15096474]
50. Sheng KC, Pouniotis DS, Wright MD, et al. Mannan derivatives induce phenotypic and functional maturation of mouse dendritic cells. *Immunology*. 2006; 118(3):372–383. [PubMed: 16827898] •• Describes the ability of polysaccharides to activate and mature dendritic cells therefore contributing to their immune adjuvant activity.
51. McKenzie IF, Apostolopoulos V, Lees C, et al. Oxidised mannan antigen conjugates preferentially stimulate T1 type immune responses. *Vet. Immunol. Immunopathol.* 1998; 63(1–2):185–190. [PubMed: 9656453]
52. Stambas J, Pietersz G, McKenzie I, Cheers C. Oxidised mannan as a novel adjuvant inducing mucosal IgA production. *Vaccine*. 2002; 20(7–8):1068–1078. [PubMed: 11803067]
53. Katsara M, Yuriev E, Ramsland PA, et al. Altered peptide ligands of myelin basic protein (MBP87–99) conjugated to reduced mannan modulate immune responses in mice. *Immunology*. 2009; 128(4):521–533. [PubMed: 19930042]
54. Petrushina I, Ghochikyan A, Mkrtichyan M, et al. Mannan-A β ₂₈ conjugate prevents A β -plaque deposition, but increases microhemorrhages in the brains of vaccinated Tg2576 (APPsw) mice. *J. Neuroinflammation*. 2008; 5:42. [PubMed: 18823564]
55. Gauntt CJ, Wood HJ, McDaniel HR, McAnalley BH. Aloe polymannose enhances anti-coxsackievirus antibody titres in mice. *Phytother. Res.* 2000; 14(4):261–266. [PubMed: 10861969]
56. Kawakami S, Sato A, Nishikawa M, Yamashita F, Hashida M. Mannose receptor-mediated gene transfer into macrophages using novel mannosylated cationic liposomes. *Gene Ther.* 2000; 7(4):292–299. [PubMed: 10694809]
57. Toda S, Ishii N, Okada E, et al. HIV-1-specific cell-mediated immune responses induced by DNA vaccination were enhanced by mannan-coated liposomes and inhibited by anti-interferon- γ antibody. *Immunology*. 1997; 92(1):111–117. [PubMed: 9370932]
58. Lu Y, Kawakami S, Yamashita F, Hashida M. Development of an antigen-presenting cell-targeted DNA vaccine against melanoma by mannosylated liposomes. *Biomaterials*. 2007; 28(21):3255–3262. [PubMed: 17449093]
59. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant-carrier system for oral genetic immunization against hepatitis B. *Immunol. Lett.* 2005; 101(1):41–49. [PubMed: 15869802]
60. Jain S, Vyas SP. Mannosylated niosomes as carrier adjuvant system for topical immunization. *J. Pharm. Pharmacol.* 2005; 57(9):1177–1184. [PubMed: 16105238]
61. Lee JK, Lee MK, Yun YP, et al. Acemannan purified from Aloe vera induces phenotypic and functional maturation of immature dendritic cells. *Int. Immunopharmacol.* 2001; 1(7):1275–1284. [PubMed: 11460308]
62. Usinger WR. A comparison of antibody responses to veterinary vaccine antigens potentiated by different adjuvants. *Vaccine*. 1997; 15(17–18):1902–1907. [PubMed: 9413100]
63. Chinnah AD, Baig MA, Tizard IR, Kemp MC. Antigen dependent adjuvant activity of a polydispersed β -(1,4)-linked acetylated mannan (acemannan). *Vaccine*. 1992; 10(8):551–557. [PubMed: 1320308]
64. Arca HC, Gunbeyaz M, Senel S. Chitosan-based systems for the delivery of vaccine antigens. *Expert Rev. Vaccines*. 2009; 8(7):937–953. [PubMed: 19538118]
65. Nishiyama A, Tsuji S, Yamashita M, Henriksen RA, Myrvik QN, Shibata Y. Phagocytosis of N-acetyl-d-glucosamine particles, a Th1 adjuvant, by RAW 264.7 cells results in MAPK activation and TNF- α , but not IL-10, production. *Cell Immunol.* 2006; 239(2):103–112. [PubMed: 16781693]

66. Ghendon Y, Markushin S, Vasiliev Y, et al. Evaluation of properties of chitosan as an adjuvant for inactivated influenza vaccines administered parenterally. *J. Med. Virol.* 2009; 81(3):494–506. [PubMed: 19152418]
67. Chang H, Li X, Teng Y, et al. Comparison of adjuvant efficacy of chitosan and aluminum hydroxide for intraperitoneally administered inactivated influenza H5N1 vaccine. *DNA Cell. Biol.* 2010; 29(9):563–568. [PubMed: 20380570]
68. Prego C, Paolicelli P, Diaz B, et al. Chitosan-based nanoparticles for improving immunization against hepatitis B infection. *Vaccine.* 2010; 28(14):2607–2614. [PubMed: 20096389]
69. Kotze AF, Luessen HL, de Leeuw BJ, de Boer BG, Verhoef JC, Junginger HE. N-trimethyl chitosan chloride as a potential absorption enhancer across mucosal surfaces: *in vitro* evaluation in intestinal epithelial cells (Caco-2). *Pharm. Res.* 1997; 14(9):1197–1202. [PubMed: 9327448]
70. Baudner BC, Giuliani MM, Verhoef JC, Rappuoli R, Junginger HE, Giudice GD. The concomitant use of the LTK63 mucosal adjuvant and of chitosan-based delivery system enhances the immunogenicity and efficacy of intranasally administered vaccines. *Vaccine.* 2003; 21(25–26):3837–3844. [PubMed: 12922117]
71. Borges O, Cordeiro-da-Silva A, Tavares J, et al. Immune response by nasal delivery of hepatitis B surface antigen and codelivery of a CpG ODN in alginate coated chitosan nanoparticles. *Eur. J. Pharm. Biopharm.* 2008; 69(2):405–416. [PubMed: 18364251]
72. Gogev S, de Fays K, Versali MF, Gautier S, Thiry E. Glycol chitosan improves the efficacy of intranasally administrated replication defective human adenovirus type 5 expressing glycoprotein D of bovine herpesvirus 1. *Vaccine.* 2004; 22(15–16):1946–1953. [PubMed: 15121307]
73. Khatri K, Goyal AK, Gupta PN, Mishra N, Vyas SP. Plasmid DNA loaded chitosan nanoparticles for nasal mucosal immunization against hepatitis B. *Int. J. Pharm.* 2008; 354(1–2):235–241. [PubMed: 18182259]
74. Sayin B, Somavarapu S, Li XW, Sesardic D, Senel S, Alpar OH. TMC-MCC (N-trimethyl chitosan-mono-N-carboxymethyl chitosan) nanocomplexes for mucosal delivery of vaccines. *Eur. J. Pharm. Sci.* 2009; 38(4):362–369. [PubMed: 19733658]
75. Gong YF, Xie Y, Zhou NJ, et al. Cellular immunity induced by *H. pylori* vaccine with chitosan as adjuvant. *Xi Bao Yu Fen Zi Mian Yi Xue Za Zhi.* 2007; 23(7):595–599. [PubMed: 17618575]
76. Shibata Y, Honda I, Justice JP, Van Scott MR, Nakamura RM, Myrvik QN. Th1 adjuvant N-acetyl-d-glucosamine polymer up-regulates Th1 immunity but down-regulates Th2 immunity against a mycobacterial protein (MPB-59) in interleukin-10-knockout and wild-type mice. *Infect. Immun.* 2001; 69(10):6123–6130. [PubMed: 11553551]
77. Shibata Y, Foster LA, Bradfield JF, Myrvik QN. Oral administration of chitin down-regulates serum IgE levels and lung eosinophilia in the allergic mouse. *J. Immunol.* 2000; 164(3):1314–1321. [PubMed: 10640745]
78. Gringhuis SI, den Dunnen J, Litjens M, van der Vlist M, Geijtenbeek TB. Carbohydrate-specific signaling through the DC-SIGN signalosome tailors immunity to *Mycobacterium tuberculosis*, HIV-1 and *Helicobacter pylori*. *Nat. Immunol.* 2009; 10(10):1081–1088. [PubMed: 19718030]
79. Doz E, Rose S, Nigou J, et al. Acylation determines the Toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. *J. Biol. Chem.* 2007; 282(36):26014–26025. [PubMed: 17617634]
80. Doz E, Rose S, Nigou J, et al. Acylation determines the toll-like receptor (TLR)-dependent positive versus TLR2-, mannose receptor-, and SIGNR1-independent negative regulation of pro-inflammatory cytokines by mycobacterial lipomannan. *J. Biol. Chem.* 2007; 282(36):26014–26025. [PubMed: 17617634]
81. Hamasur B, Haile M, Pawlowski A, et al. Mycobacterium tuberculosis arabinomannan-protein conjugates protect against tuberculosis. *Vaccine.* 2003; 21(25–26):4081–4093. [PubMed: 12922145]
82. Yamamura Y, Azuma I, Sugimura K, Yamawaki M, Uemiya M. Adjuvant activity of 6-O-mycoloyl-N-acetylmuramoyl-l-alanyl-d-isoglutamine. *Gann.* 1976; 67(6):867–877. [PubMed: 828599]

83. Uehara A, Yang S, Fujimoto Y, et al. Muramyl dipeptide and diaminopimelic acid-containing desmuramyl peptides in combination with chemically synthesized Toll-like receptor agonists synergistically induced production of interleukin-8 in a NOD2- and NOD1-dependent manner, respectively, in human monocytic cells in culture. *Cell. Microbiol.* 2005; 7(1):53–61. [PubMed: 15617523]
84. Takada H, Uehara A. Enhancement of TLR-mediated innate immune responses by peptidoglycans through NOD signaling. *Curr. Pharm. Des.* 2006; 12(32):4163–4172. [PubMed: 17100619]
85. Azuma I, Okumura H, Saiki I, et al. Adjuvant activity of carbohydrate analogs of N-acetylmuramyl-l-alanyl-d-isoglutamine on the induction of delayed-type hypersensitivity to azobenzenearsonate-N-acetyl-l-tyrosine in guinea pigs. *Infect. Immun.* 1981; 33(3):834–839. [PubMed: 7287186]
86. Valensi JP, Carlson JR, Van Nest GA. Systemic cytokine profiles in BALB/c mice immunized with trivalent influenza vaccine containing MF59 oil emulsion and other advanced adjuvants. *J. Immunol.* 1994; 153(9):4029–4039. [PubMed: 7930610]
87. Ott G, Barchfeld GL, Van Nest G. Enhancement of humoral response against human influenza vaccine with the simple submicron oil/water emulsion adjuvant MF59. *Vaccine.* 1995; 13(16):1557–1562. [PubMed: 8578842]
88. Vosika GJ, Cornelius DA, Gilbert CW, et al. Phase I trial of ImmTher, a new liposome-incorporated lipophilic disaccharide tripeptide. *J. Immunother.* 1991; 10(4):256–266. [PubMed: 1931863]
89. Vosika GJ, Cornelius DA, Bennek JA, Sadlik JR, Gilbert CW. Immunologic and toxicologic study of disaccharide tripeptide glycerol dipalmitoyl: a new lipophilic immunomodulator. *Mol. Biother.* 1990; 2(1):50–56. [PubMed: 2185794]
90. Audibert, F.; Leclerc, C.; Chedid, L. Muramyl peptides as immunopharmacological response modifiers. In: Torrence, PF., editor. *Biological Response Modifiers*. Orlando, FL, USA: Academic Press, Inc.; 1985. p. 307–337.
91. Schwarzkopf C, Thiele B. Effectivity of alternative adjuvants in comparison to Freund's complete adjuvant. *ALTEX.* 1996; 13(5):22–25. [PubMed: 11178467]
92. Sueoka E, Nishiwaki S, Okabe S, et al. Activation of protein kinase C by mycobacterial cord factor, trehalose 6-monomycolate, resulting in tumor necrosis factor- α release in mouse lung tissues. *Jpn J. Cancer Res.* 1995; 86(8):749–755. [PubMed: 7559098]
93. Masihi KN, Brehmer W, Lange W, Ribi E. Effects of mycobacterial fractions and muramyl dipeptide on the resistance of mice to aerogenic influenza virus infection. *Int. J. Immunopharmacol.* 1983; 5(5):403–410. [PubMed: 6654538]
94. Fujita Y, Okamoto Y, Uenishi Y, Sunagawa M, Uchiyama T, Yano I. Molecular and supra-molecular structure related differences in toxicity and granulomatogenic activity of mycobacterial cord factor in mice. *Microb. Pathog.* 2007; 43(1):10–21. [PubMed: 17434713]
95. Koike Y, Yoo YC, Mitobe M, et al. Enhancing activity of mycobacterial cell-derived adjuvants on immunogenicity of recombinant human hepatitis B virus vaccine. *Vaccine.* 1998; 16(20):1982–1989. [PubMed: 9796054]
96. Ribi E, Meyer TJ, Azuma I, Parker R, Brehmer W. Biologically active components from mycobacterial cell walls. IV. Protection of mice against aerosol infection with virulent *Mycobacterium tuberculosis*. *Cell. Immunol.* 1975; 16(1):1–10. [PubMed: 803876]
97. Ribi E, Granger DL, Milner KC, et al. Induction of resistance to tuberculosis in mice with defined components of *Mycobacteria* and with some unrelated materials. *Zentralbl. Bakteriell. Mikrobiol. Hyg A.* 1982; 251(3):345–356. [PubMed: 6805154]
98. Masihi KN, Lange W, Brehmer W, Ribi E. Immunobiological activities of nontoxic lipid A: enhancement of nonspecific resistance in combination with trehalose dimycolate against viral infection and adjuvant effects. *Int. J. Immunopharmacol.* 1986; 8(3):339–345. [PubMed: 3733302]
99. Cekic C, Casella CR, Eaves CA, Matsuzawa A, Ichijo H, Mitchell TC. Selective activation of the p38 MAPK pathway by synthetic monophosphoryl lipid A. *J. Biol. Chem.* 2009; 284(46):31982–31991. [PubMed: 19759006]

100. Schiller JT, Castellsague X, Villa LL, Hildesheim A. An update of prophylactic human papillomavirus L1 virus-like particle vaccine clinical trial results. *Vaccine*. 2008; 26 Suppl. 10:K53–K61. [PubMed: 18847557]
101. Tong NK, Beran J, Kee SA, et al. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. *Kidney Int*. 2005; 68(5):2298–2303. [PubMed: 16221232]
102. Lacaille-Dubois, MA. Bioactive saponins with cancer related and immunomodulatory activity: recent developments. In: Atta ur, R., editor. *Studies in Natural Products Chemistry*. Amsterdam, The Netherlands: Elsevier; 2005. p. 209–246.
103. Dai JH, Iwatani Y, Ishida T, et al. Glycyrrhizin enhances interleukin-12 production in peritoneal macrophages. *Immunology*. 2001; 103(2):235–243. [PubMed: 11412311]
104. Raphael TJ, Kuttan G. Effect of naturally occurring triterpenoids glycyrrhizic acid, ursolic acid, oleanolic acid and nomilin on the immune system. *Phytomedicine*. 2003; 10(6–7):483–489. [PubMed: 13678231]
105. Kensil CR, Wu JY, Soltysik S. Structural and immunological characterization of the vaccine adjuvant QS-21. *Pharm. Biotechnol*. 1995; 6:525–541. [PubMed: 7551234]
106. Kensil CR, Soltysik S, Wheeler DA, Wu JY. Structure/function studies on QS-21, a unique immunological adjuvant from *Quillaja saponaria*. *Adv. Exp. Med. Biol*. 1996; 404:165–172. [PubMed: 8957293]
107. Meraldi V, Romero JF, Kensil C, Corradin G. A strong CD8⁺ T cell response is elicited using the synthetic polypeptide from the C-terminus of the circumsporozoite protein of *Plasmodium berghei* together with the adjuvant QS-21: quantitative and phenotypic comparison with the vaccine model of irradiated sporozoites. *Vaccine*. 2005; 23(21):2801–2812. [PubMed: 15780728]
108. Pham HL, Ross BP, McGeary RP, Shaw PN, Hewavitharana AK, Davies NM. Saponins from *Quillaja saponaria* Molina: isolation, characterization and ability to form immuno stimulatory complexes (ISCOMs). *Curr. Drug Deliv*. 2006; 3(4):389–397. [PubMed: 17076641]
109. Sun HX, Xie Y, Ye YP. Advances in saponin-based adjuvants. *Vaccine*. 2009; 27(12):1787–1796. [PubMed: 19208455]
110. Marciani DJ, Press JB, Reynolds RC, et al. Development of semisynthetic triterpenoid saponin derivatives with immune stimulating activity. *Vaccine*. 2000; 18(27):3141–3151. [PubMed: 10856794]
111. Marciani DJ, Reynolds RC, Pathak AK, Finley-Woodman K, May RD. Fractionation, structural studies, and immunological characterization of the semi-synthetic *Quillaja* saponins derivative GPI-0100. *Vaccine*. 2003; 21(25–26):3961–3971. [PubMed: 12922132]
112. Quenelle DC, Collins DJ, Marciani DJ, Kern ER. Effect of immunization with herpes simplex virus type-1 (HSV-1) glycoprotein D (gD) plus the immune enhancer GPI-0100 on infection with HSV-1 or HSV-2. *Vaccine*. 2006; 24(10):1515–1522. [PubMed: 16288820]
113. Quenelle DC, Collins DJ, Rice TL, Prichard MN, Marciani DJ, Kern ER. Effect of an immune enhancer, GPI-0100, on vaccination with live attenuated herpes simplex virus (HSV) type 2 or glycoprotein D on genital HSV-2 infections of guinea pigs. *Antiviral Res*. 2008; 80(2):223–224. [PubMed: 18573279]
114. Ragupathi G, Coltart DM, Williams LJ, et al. On the power of chemical synthesis: immunological evaluation of models for multiantigenic carbohydrate-based cancer vaccines. *Proc. Natl Acad. Sci. USA*. 2002; 99(21):13699–13704. [PubMed: 12359877]
115. Rawal N, Rajagopalan R, Salvi VP. Stringent regulation of complement lectin pathway C3/C5 convertase by C4b-binding protein (C4BP). *Mol. Immunol*. 2009; 46(15):2902–2910. [PubMed: 19660812]
116. Ray TL, Hanson A, Ray LF, Wuepper KD. Purification of a mannan from *Candida albicans* which activates serum complement. *J. Invest. Dermatol*. 1979; 73(4):269–274. [PubMed: 383852]
117. Kawasaki A, Takada H, Kotani S, et al. Activation of the human complement cascade by bacterial cell walls, peptidoglycans, water-soluble peptidoglycan components, and synthetic muramylpeptides-studies on active components and structural requirements. *Microbiol. Immunol*. 1987; 31(6):551–569. [PubMed: 3670125]

118. Bohana-Kashtan O, Ziporen L, Donin N, Kraus S, Fishelson Z. Cell signals transduced by complement. *Mol. Immunol.* 2004; 41(6–7):583–597. [PubMed: 15219997]
119. Weston SA, Parish CR. Different carbohydrate structures control entry of lymphocytes into spleen and lymph nodes. *J. Immunol.* 1991; 146(12):4180–4186. [PubMed: 2040795] •• Important early work on the role of carbohydrate structures in immune cell migration and chemotaxis.
120. Hawn TR, Ozinsky A, Underhill DM, Buckner FS, Akira S, Aderem A. Leishmania major activates IL-1 α expression in macrophages through a MyD88-dependent pathway. *Microbes Infect.* 2002; 4(8):763–771. [PubMed: 12270723]
121. Liu G, Anderson C, Scaltreto H, Barbon J, Kensil CR. QS-21 structure/function studies: effect of acylation on adjuvant activity. *Vaccine.* 2002; 20(21–22):2808–2815. [PubMed: 12034108]
122. Deng K, Adams MM, Damani P, Livingston PO, Ragupathi G, Gin DY. Synthesis of QS-21-xylose: establishment of the immunopotentiating activity of synthetic QS-21 adjuvant with a melanoma vaccine. *Angew. Chem. Int. Ed. Engl.* 2008; 47(34):6395–6398. [PubMed: 18624313]
123. Cluff CW, Baldrige JR, Stover AG, et al. Synthetic Toll-like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infect. Immun.* 2005; 73(5):3044–3052. [PubMed: 15845512]
124. Stover AG, Da Silva Correia J, Evans JT, et al. Structure-activity relationship of synthetic Toll-like receptor 4 agonists. *J. Biol. Chem.* 2004; 279(6):4440–4449. [PubMed: 14570885]

Table 1

Immunologically active carbohydrate-containing compounds tested as vaccine adjuvants.

Carbohydrate-containing compound	Chemical structure	Modified forms	Source	Delivery route(s)	Receptor(s)	Immune actions	Development stage (if known)	Ref.
Dextran	α -1,6-glucan with α -1,3-branches	Dextran sulfate, DEAE-dextran, acetylated dextran	Lactobacilli		GR	Proinflammatory, activates NF- κ B, enhances macrophage IL-1 β , IL-6, TNF- α expression	Used in veterinary adjuvants	[7–10]
Lentinan	β -1,3-glucohexaose with β -1,6-branches	Sulfated lentinan	Shiitake mushroom	im./in./iv.	GR	Enhanced respiratory burst, nitric oxide, IL-6, phagocytosis	Approved in Japan for iv. use for treatment of cancer	[21–30]
Zymosan	β -1,3-glucan		<i>Saccharomyces cerevisiae</i>	im.	GR, TLR-2, Dectin-1, complement and receptors	Proinflammatory through activation NF- κ B, complement and inflammasome	No known active human-development program	[11–20]
β -glucan	β -1,3-glucan	Algal glucan, glucan particles	<i>Eniglena gracilis</i> , <i>Saccharomyces cerevisiae</i>	im.	Dectin-1	Proinflammatory, activates NF- κ B, complement and inflammasome	Some versions of β -glucan adjuvants remain in preclinical development	[28–33]
Deltin	β -D-[2-1] poly (fructo-furanosyl) α -D-glucose	γ -inulin and algammulin	Compositae	im.	Not known	Does not activate NF- κ B, activates complement, induces chemokine secretion, upregulates costimulatory molecules	Has been successfully tested in multiple human Phase II clinical trials	[34–47]
Mannan	1,4-polymaltose	Oxidation, reduction, acylation, mannosylated miosomes	<i>Aloe barbadensis</i>	im./in./oral	MBL	Proinflammatory, activates NF- κ B, DC maturation	Tested in Phase I and II human cancer vaccine trials	[19,48–60]
Chitin	N-acetyl-D-glucosamine	Acetylation (chitosan), mono-N-carboxymethyl chitosan	Crustaceans	im./in.	Dectin-1, Macrophage mannose receptor and TLR-2	Phosphorylates MAPK, induces TNF- α , COX-2, prostaglandin E2, matures immature DCs, transient opening of tight junctions	Tested in early phase human vaccine trials	[64–77]
Muramyl dipeptide	N-acetyl muramyl-L-alanine-D-isoglutamine	D-murapalmitine, GMDP	<i>Mycobacteria</i> , <i>Lactobacillus bulgaricus</i>	im.	NOD-2	Proinflammatory, activates NF- κ B	MTP-PE was a component of human MF59 adjuvant, was removed because of excess toxicity, but remains a component of Gerbu veterinary adjuvants	[82–89]
Cord factor	TDM	Multiple	<i>Mycobacterium tuberculosis</i>	im.	Mincle	Activates monocyte Syk-CARD9 signaling	TDM is a component of the veterinary Ribi Adjuvant	[92–97]

Carbohydrate-containing compound	Chemical structure	Modified forms	Source	Delivery route(s)	Receptor(s)	Immune actions	Development stage (if known)	Ref.
LPS	LPS	MPL	Gram-negative bacteria	im.	TLR-4	resulting in NF-κB activation and inflammation Proinflammatory, activates, NF-κB	System® MPL is a component of GSK AS04 adjuvant in licensed human HBV and HPV vaccines	[98–101]
QS-21	Triterpenoid glycosides	GPI-0100	Bark of <i>Quillaja saponaria</i>	im./in.	Unknown	Proinflammatory, activates NF-κB	Has been used in multiple human Phase II trials	[102–114]

ASC: Apoptosis-associated speck-like protein containing a caspase recruitment domain; DC: Dendritic cell; DEAE: Diethylaminoethyl; GMDP: *N*-acetyl-glucosaminyl-*N*-acetyl-muramyl-L-alanyl-D-isoglutamin; GR: Glucan receptor; GSK: GlaxoSmithKline; HBV: Hepatitis B virus; HPV: Human papillomavirus; im.: Intramuscular; in.: Intranasal; iv.: Intravenous; LPS: Lipopolysaccharide; MBL: Mannose-binding lectin; MPL: Monophosphoryl lipid A; MTP-PE: *N*-acetyl-L-alanyl-D-isoglutaminyl-L-alanine-2-[1,2-dipalmitoyl-sn-glycero-3-(hydroxy-phosphoryloxy)] ethylamide; TDM: Trehalose-6-6-dimycolate.