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Review

Vaccine adjuvants revisited

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Abstract

The development of new adjuvants for human vaccines has become an expanding field of research in the last thirty years, for generating stronger vaccines capable of inducing protective and long-lasting immunity in humans. Instead of such efforts, with several adjuvant strategies approaching to requirements for their clinical application, limitations like adjuvant toxicity remain to be fully surpassed. Here we summarize the current status of adjuvant development, including regulatory recommendations, adjuvant requirements, and adjuvant categories like mineral salts, tensoactive compounds, microorganism-derived adjuvants, emulsions, cytokines, polysaccharides, nucleic acid-based adjuvants, and a section dedicated to particulate antigen delivery systems. The mechanisms of adjuvanticity are also discussed in the light of recent findings on Toll-like receptors' biology and their involvement on immune activation.

Keywords: Vaccine; Adjuvants; Toll-like receptors

Contents

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1. Introduction

The goal of vaccination is to generate a strong immune response providing long term protection against infection. Unlike attenuated live vaccines, killed whole organism or subunit vaccines generally require the addition of an adjuvant to be effective [1]. Adjuvants are compounds that enhance the immune response against co-inoculated antigens with the word adjuvant coming from the latin word *adjuvare*, which means to help or to enhance [2]. Adjuvants can be used for various purposes: (1) to enhance the immunogenicity of highly purified or recombinant antigens; (2) to reduce the amount of antigen or the number of immunizations needed for protective immunity; (3) to improve the efficacy of vaccines in newborns, the elderly or immuno-compromised persons; or (4) as antigen delivery systems for the uptake of antigens by the mucosa. The concept of adjuvants arose from observations that an abscess at the innoculation site assisted the generation of higher specific antibody titers [3,4]. Even an abscess generated by the injection of unrelated substances increased the antigen specific antibody response [3,4].

The adjuvant activity of aluminium compounds was first demonstrated in 1926 with diphtheria toxoid absorbed to alum [5]. So far, aluminium-based compounds (principally aluminium hydroxide or phosphate) continue to monopolize human vaccines [6]. Why this is the case? Despite the subsequent discovery of many other considerably more potent adjuvants, e.g. Freund's complete adjuvant (FCA) [7,8] or lipolysaccharide (LPS) [9], they are judged to be unsuitable for human use due to local and systemic toxicity. This has largely left the human adjuvant field to alum for the last 80 years. Will this stay the case for the next 80 years? Certainly, regulatory requirements have been raised since alum development and, arguably, its toxicity and side effects may preclude its registration for human use by regulatory bodies if it were only newly discovered as an adjuvant today. What chance for new and potentially more toxic adjuvants to obtain approval in a regulatory environment where even relatively mild or rare side effects may prevent drug registration? Furthermore, the scale of late phase human trials required to prove adjuvant safety makes adjuvant validation and registration increasingly expensive. Whereas once it may have been sufficient to demonstrate efficacy and safety in a study size of 200–500, now study populations of 5000–25,000 may be required before a product can be registered. For these reasons, relatively few adjuvants of the numerous under preclinical development are likely to make it through to human registration during the next 10–20 years.

2. The ideal adjuvant

Some of the features involved in adjuvant selection are: the antigen, the animal species to be vaccinated, the route of administration and the likelihood of side effects [10,11]. Ideally, adjuvants should be stable with long shelf life, biodegradable, cheap to produce, immunologically inert and promote an appropriate immune response, namely cellular or antibody immunity depending on requirements for protection [12]. There are marked differences in the efficacy of adjuvants depending on the administration route, *e.g.* between mucosal and parenteral routes. Hence, new vectors, antigen delivery systems or adjuvant compounds need to take into account the characteristics of the proposed administration route [13]. Although intradermal or subcutaneous immunization is far more effective in stimulating immunity than the intramuscular route, due to local toxicity alum is generally only used intramuscularly.

The benefits of adjuvant incorporation into a vaccine need to be balanced against the risk of adverse side effects [14,15]. Local reactions include pain, local inflammation, swelling, injection site necrosis, lymphadenopathy, granulomas, ulcers and the generation of sterile abscesses. Systemic reactions include nausea, fever, adjuvant arthritis, uveitis, eosinophilia, allergy, anaphylaxis, organ specific toxicity and immunotoxicity, *i.e.* cytokines release, immunosuppression or autoimmune diseases [16,17]. Unfortunately, potent adjuvant action is often correlated with increased toxicity, as exemplified by the case of FCA. Thus, minimizing toxicity remains as one of the major challenges in adjuvant research. The supremacy of alum application reflects current limitations for achieving this goal.

3. Regulatory barriers to adjuvant development

Adjuvants' regulations for human use are far more rigorous than those applied to veterinary vaccines. In addition to preclinical studies on the adjuvant itself, the combined antigen-adjuvant formulation also requires toxicological evaluation for entering phase 1 clinical trials [18]. Pre-clinical toxicology is normally tested in small animal species such as mice, rats or rabbits by the same administration route proposed for human use, with dose and frequency of vaccination similar to or higher than the proposed human dose to rule out potential safety problems [19,20]. Amongst the biggest regulatory hurdles is the required size of population that needs to be tested to prove efficacy and particularly safety of a new adjuvant or vaccine. These numbers have dramatically

increased in recent years, largely in reaction to the recognition that some approved drugs had rare serious and fatal side effects that were not identified because of inadequate sample sizes during the clinical drug development program.

The association of important adversed reactions with new vaccines has resulted in their market withdrawal. This is the case of the nasal inactivated influenza vaccine, associated to an increase in the number of cases of Bell's Palsy [21] and also the higher probability to develop intussusception related to oral vaccination with a rotavirus vaccine [22]. These kind of adverse events have challenged the public perception of safety associated to mucosal administration, and have an impact on the regulatory field.

4. Adjuvant categories

4.1. Mineral salts

Since the days of Glenny et al. [5], aluminium salts, principally aluminium hydroxide or phosphate, have been the most widely used adjuvants in humans [16]. Unfortunately, alum salts are relatively poor adjuvants in many situations, particularly at inducing cellular immune responses [23–25]. The mechanism whereby aluminium salts work remains unknown although one suggestion is that they work by the formation of an antigen depot at the inoculation site [26]. Other possible mechanisms of action may involve complement activation, or eosinophil or macrophage activation [27]. Granulomas are common when alum is administered via the subcutaneous or intradermal rather than intramuscular route [28–30]. Other side effects of alum are increased IgE production, allergenicity [25,28,29,31–33] and potential neurotoxicity. Normally, aluminium is excreted by the kidneys, although under certain conditions such as reduced renal function aluminium is accumulated in the body and can become toxic. High aluminium levels in the body predominately affect the brain and bone tissues causing fatal neurological syndrome and dialysis-associated dementia. Aluminium intoxication is also potentially linked to amyotrophic lateral sclerosis and Alzheimer's disease. Alternatively, the salts of calcium, iron and zirconium have also been used to adsorb antigens [29]. In particular, calcium phosphate has been used for diphtheria-tetanus-pertussis vaccines [34–36].

4.2. Tensoactive compounds

Quil A is a saponin derived from an aqueous extract from the bark of *Quillaja saponaria*. Fractions purified from this extract by reverse phase chromatography, such as QS-21, have the ability to induce strong cellular responses against HIV-1 and other pathogen-derived antigens [16,37,38]. Quil A is a natural product composed of more than 23 different saponins and is too toxic for human use. In addition to severe local reactions and granulomas, toxicity includes severe haemolysis [14,39–42]. The Quil A-derived saponin QS-21, whilst less toxic than Quil A, has many of the same problems and is similarly unsuitable for most human uses other than cancer vaccines where higher toxicity may be accepted [43] or at relatively low doses.

4.3. Microorganism-derived adjuvants

Given their potent immunostimulatory capacity, bacterial or fungal substances constitute a productive source of potential adjuvants. Bacterial cell wall peptidoglycan or LPS enhances the immune response whilst themselves not being highly immunogenic. This adjuvant activity is mediated through activation of Toll-like receptors (TLRs) that mediate the danger signals activating the host immune defence system [31]. Different species of bacteria used as a source of adjuvants include *Mycobacterium* spp., *Corynebacterium parvum*, *C. granulosum*, *Bordetella pertussis* and *Neisseria meningitidis*. As whole killed microorganisms these are too toxic to be used as human adjuvants [25]. However, it appears the major adjuvant activity of these bacteria is mediated by *N*-acetyl muramyl-L-alanyl-D-isoglutamine, also called muramyl dipeptide (MDP) [44]. In saline, MDP mainly enhances humoral immunity [14,45,46], whilst when incorporated into liposomes or mixed with glycerol it induces strong cellular immunity [47]. Compounds with adjuvant activity derived from MDP include treonyl-MDP [17,48].

Another important group of compounds derived from the cell wall of Gram-negative bacteria is LPS. The major structural element of LPS responsible for their adjuvant effect is lipid A. In low acid conditions, lipid A can be hydrolysed to obtain monophosphoryl lipid A (MPL), a compound which retains the adjuvant activity of lipid A with reduced toxicity [49]. Another extract from bacterial walls is trehalose dimycolate (TDM), an adjuvant which simulates both humoral and cellular responses [50]. The demonstration that mycobacterial DNA had adjuvant activity, led to the discovery that the adjuvant activity correlated with a higher content of CpG motifs present in bacterial nucleic acids. DNA containing CpG motifs is one of the most potent cellular adjuvants and acts via activation of a Toll receptor pathway (see below) [51].

4.4. Emulsions

This class includes oil-in-water or water-in-oil emulsions such as the Freund's incomplete adjuvant (FIA), Montanide, Adjuvant 65 [52–54], or Lipovant [11]. The mechanism of action of adjuvant emulsions includes the formation of a depot at the injection site, enabling the slow release of antigen and the stimulation of antibody producing plasma cells [55]. In general, these adjuvants are too toxic for routine human prophylactic vaccine use, although they may be suitable for use in terminal conditions such as cancer where there is a greater tolerance of side effects. Frequent side effects of emulsions include inflammatory reactions, granulomas and ulcers at the injection site. Various types of emulsions have

been used, with different natural oils, in order to find more stable, potent and less toxic formulations. Different emulsions like oil in water [56] and water in oil in water [57] have shown been developed with the latter being as potent as FIA but more stable, less viscous and easier to administer with less resulting granulomas [58,59]. Montanide is a family of oil-based adjuvants that have been used in trial vaccines against HIV, malaria and breast cancer [60].

4.5. Particulate antigen delivery systems

Together with certain depot effect, it is the particulate nature that primarily decides whether the antigen-delivery system will be successful in inducing an immune response. If this first requirement is fulfilled, the chemical composition of the vaccine decides which type of immune response will develop, *e.g.* which isotype of antibodies the B cells will produce, and which cytokines the T cells will secrete, and can be controlled by combining the antigen with immunomodulatory or co-stimulatory molecules [61].

Several of the most studied adjuvants can be included in the category of "particulate antigen delivery systems": liposomes, polymeric microspheres, nano-beads, immunostimulating complexes (ISCOMs), virus-like particles (VLPs), among the most important antigen delivery systems. These adjuvants have been extensively used as carriers for protein subunit and DNA vaccines. There is an extensive focus on understanding their biological interactions and mechanisms of action related to their size and chemical nature [62].

4.5.1. Liposomes

Liposomes are synthetic spheres comprised by lipid bilayers that can encapsulate antigens and act as both a vaccine delivery vehicle and adjuvant. The potency of liposomes depends on the number of lipid layers [63], electric charge [64], composition [65] and method of preparation [65–67]. Recent results have suggested that, by choosing lipid components for liposomes, surface-coupled liposomal antigens might be applicable for the development of tumor vaccines to present tumor antigens to antigen-presenting cells (APC) and induce antitumor responses. The ability to induce cross-presentation of an Ag coupled to liposomes was higher in those consisting of unsaturated fatty acid. It was further confirmed by *in vivo* induction of CTL and tumor eradication in mice [68].

Although liposomes constitute one of most studied antigen delivery systems, they are still the subject of novel results on enhancing strategies. The synergistic effect of liposomally co-entrapped DNA and protein has been shown to exceed the well-known adjuvant effects of plasmid DNA and liposomes. This new approach to vaccination has been termed 'codelivery' and it may derive from the simultaneous presentation of antigen via MHC class-I (DNA) and MHC class-II (protein) pathways to CD8+ and CD4+ cells at the same antigen presenting cell -a mode of presentation that would commonly occur with live viral pathogens, opening new uses for this technology [69]. However, stability, manufacturing and quality assurance problems seem to have been major factors hampering the use of liposomes as adjuvants in humans.

4.5.2. Polymeric microspheres

Among particulated and polymeric systems, poly(DL-lactide-*co*-glycolide) microspheres have been extensively studied. These are biocompatible and biodegradable microspheres of nanometer-micrometer size able to incorporate different antigens. One of their advantages is the capacity to manipulate the degradation kinetics by varying the relative concentration of their components, thereby controlling the time of antigen release [70,71].

It has been recently shown that an alternative approach involving charged polylactide *co*-glycolide (PLG) microparticles with surface adsorbed antigen(s) can also be used to deliver antigen into APC. The preparation of cationic and anionic PLG microparticles which have been used to adsorb a variety of agents, including plasmid DNA, recombinant proteins and immunostimulatory oligonucleotides resulted in the induction of significantly enhanced immune responses in comparison to alum. The surface adsorbed microparticle formulation offers an alternative and novel way of delivering antigens in a vaccine formulation [72].

4.5.3. Nano-beads

Solid inert beads with a surface-adsorbed antigen have previously been used to stimulate CD8+ T cell responses, with an optimal bead diameter size of 1 μm, and <0.5 μm reported as inferior in targeting antigens for MHC class I-restricted presentation to T cells [73]. Recently, the use of solid inert beads of nanometric size (0.04–0.05 μm) was reported as a very promising strategy to achieve efficient antigen delivery to APC, generating potent and combined humoral and CD8+ T cell immunity [74]. In this sense, they have been considered similar to leading adjuvants for activating each arm of the immune response. The unusual potency of this novel nano-vaccine approach was demonstrated by the ability of the antigen-conjugated beads to protect from tumors in a tumor challenge model and also to clear large established tumor masses within 2 weeks after a single injection [74].

The nano-bead adjuvant, in contrast to alum, induced substantial cell mediated responses along with moderate humoral responses after large-scale testing in animals. Thus, nano-bead adjuvants are potentially useful for intracellular pathogens in humans as well as animals with immunogens in both therapeutic and prophylactic scenarios [75].

4.5.4. ISCOMs® and ISCOMATRIX® adjuvants

ISCOMs® are 40 nm large particles made up of saponins (Quil A), lipids, cholesterol and antigen, held together by hydrophobic interactions between the first three components. Cholesterol is the ligand that binds to saponin forming 12 nm rings. These rings are fixed together by lipids to form the

spherical nanoparticles. Hydrophobic or amphipathic antigens can be incorporated into this complex. They are versatile and flexible delivery systems with increased efficiency of antigen presentation to B cells and uptake by the APC (reviewed in [76]).

The ISCOMATRIX® adjuvant is identical to ISCOMs® except that it does not contain antigen. This adjuvant can be mixed with antigens and has some of the advantages of ISCOMS® such as the preferential targeting of antigen to APC. The ISCOMATRIX® has been analyzed in mucosal immunization in animals [77] and induced good mucosal IgA responses. However, the response obtained differed from that of ISCOMs® vaccination in that the ISCOMATRIX® induced a Th2-like response, whereas the ISCOMs®-based vaccine induced a mixed Th1/Th2 response.

The use of saponins in ISCOMs®-based vaccines retains the adjuvant activity of the saponin component but with a reduced toxicity. Saponin-adjuvanted particulate vaccines have significant potential as a novel strategy in vaccine development. Very recent reviews have addressed the practical aspects related to this antigen delivery system [78,79].

4.5.5. Virus-like particles

Virus-like particles are inert, empty capsids of viruses, which contain no DNA/RNA from the virus itself. However they retain the structure of a virus and they can be engineered to have antigens attached. Particles with similar size and shape to viruses and obtained by genetic engineering containing antigens from viral or non-viral sources are also regarded as VLPs. VLPs-displayed antigens are efficiently taken up by dendritic cells (DC) and induce potent immune responses after parenteral, mucosal and transcutaneous immunizations [80–82].

Several strategies have been directed to produce a given antigen with capacity to form VLPs, or to obtain it as part of a recombinant protein forming VLPs, for improving its immunogenicity. Antigens repeated on VLPs, like those naturally found in viral capsids, efficiently cross-link B-cell receptors and, therefore, induce strong IgG responses. Recent studies have shown the improvement in the immunogenicity of covalently coupled peptides from the allergen Der p 1 to a virus-like particle derived from the bacteriophage Qbeta (Qbeta-Der p 1), demonstrating that this strategy can be used to enhance the efficiency of allergen-specific immunotherapy and validating the approach in humans [83].

One of the most relevant examples of antigens in the history of vaccinology has been the recombinant hepatitis B surface antigen, produced as VLPs in *Saccharomyces cerevisiae* and *Pichia pastoris* yeasts. It has been used during more than 15 years as a very effective antigen in the preventive Hepatitis B vaccine. Recently, on June 8th, 2006, the U.S. Food and Drug Administration (FDA) approved an human papilloma virus (HPV) vaccine for clinical use, consisting in the recombinant VLPs of HPV 6, 11, 16, 18 mixed with an aluminum-containing adjuvant. In this sense, this is a very positive advancement for accepting the use of antigens as VLPs to develop new vaccines.

A new VLPs-based formulation has been developed since 1998 as a nasal therapeutic vaccine candidate for chronic hepatitis B therapy under the name of NASVAC, based on the surface and core hepatitis B virus (HBV) antigens (HBsAg and HBcAg, respectively). Pre-clinical experiments in mice induced higher immunogenicity and enhanced capacity to promote cellular and humoral (superior IgG2a) responses than the commercial vaccine. These results support therapeutic expectations as this Th1-prone immune response correlates with HBV control [84,85]. A double blinded, randomized and placebo controlled phase I clinical trial was recently concluded in healthy volunteers, evidencing safety and immunogenicity of this nasal vaccine candidate with a relatively low amount of antigen administered (50 μg each) [86]. This is the first nasal candidate for hepatitis B therapy, taking advantage of the cross-adjuvanting effect of the VLPs included in the formulation [84–86].

4.6. Cytokines

As a general rule, cytokines are included in the modern classification of adjuvants. For example, granulocyte-macrophage colony stimulating factor (GM-CSF) enhances the primary immune response by activating and recruiting APC [87]. However, the practical application of GM-CSF as an adjuvant has been limited by the requirement for multiple doses, toxicity and the immunogenicity of heterologous cytokines [11]. Cytokines may have particular potential in DNA vaccines where the cytokine can be co-expressed with the antigen by the same vector [88]. On the other hand, the direct application of IL-12 and other cytokines as soluble proteins has proven effective as mucosal adjuvants [89,90].

4.7. Polysaccharides

Inulin, a carbohydrate derived from plant roots of the Compositae family, when constituted into a micro-particulate form, is a potent humoral and cellular immune adjuvant. Micro-particulate inulin (MPI) is a potent activator of the alternate complement pathway and thereby activates the innate immune system [91]. MPI is particularly effective at boosting cellular immune responses without the toxicity normally exhibited by other adjuvants such as FCA, Montanide or QS21. MPI can be combined with a variety of other adjuvant components to produce a range of tailormade adjuvants with varying degrees of Th1 and Th2 activity. For example, algammulin is a combination of MPI and aluminium hydroxide, also considered as an adjuvant formulation. Algammulin exhibits a higher ratio of Th2 to Th1 activity than MPI alone, it's overall effect being equivalent to alum despite having a lower overall alum content [92,93]. MPI-based adjuvants have been successful in many animal models including diphtheria, tetanus toxoid, respiratory syncytial virus, E7 protein of HPV, herpes virus 2 glycoprotein D, HBsAg, influenza

haemagglutinin, *Haemophilus influenzae* and *Plasmodium falciparum* [91–93]. MPI advantageously induces both Th1 and Th2 immune responses, does not induce IgE, and is not associated with any significant local or systemic toxicity [94]. Inulin is metabolized into the simple sugars fructose and glucose in the body and, therefore, does not suffer from the safety concerns of alum-based adjuvants.

4.8. Nucleic acid-based adjuvants

The discovery of the immunostimulatory capacity of DNA [95] led to an impressive development of immunostimulating DNA-based molecules. CpG motifs are six deoxynucleotides-long DNA sequences with a central CpG dinucleotide, and normally occur in bacterial DNA twenty times more often than within mammalian DNA. They could be immunostimulatory [96] or immunosuppressive (certain motifs present on mammalian [97] or viral [98] DNA), depending on the CpG's cytosine methylation state [99] (unmethylated corresponding to immunostimulatory) or the flanking sequences. CpG motifs are recognized by the Toll-like receptor (TLR) 9 [100] in mammalian cells in an evolutionary fashion (see section *TLRs and adjuvant activity*), inducing the secretion of type I interferons (α and β) and IL-12 by cells of the innate immune system, promoting a Th1 cellular response and preventing allergic responses. Therefore, CpG-containing DNA-based molecules would be useful for therapeutic applications and also for adjuvanting other types of vaccines [101].

Three main approaches are under intense investigation: immunostimulatory deoxynucleotides (ODNs), ODN-antigen conjugates and DNA vaccine vectors with enhanced immunostimulatory sequences. All of them act through the specific binding of CpG motifs to the TLR9 molecule. ODNs are short synthetic single-stranded DNA molecules with a modified backbone (phosphorothyoate instead of phosphodiester linkage), bearing one or more CpG motifs and variable flanking sequences, with a preferential activation of APC subsets and patterns of cytokine secretion depending on the specific sequence combination [96,102], in a dose-dependent manner. Due to their lower molecular size, they show enhanced bioavailability and affinity for biological membranes (in part due to the phosphorothyoate modification), their main effect resulting from the interaction of the CpG sequence with the TLR9 molecule. Several vaccine strategies are under clinical evaluation with this kind of adjuvant, mainly in the field of cancer and viral infections [102]. However, toxicity may be related to the dose, treatment intervals, and the route of administration of CpG-ODN. Although no side effects have been reported to date in humans, results obtained in animal models of autoimmune diseases raise considerable concern about the safety of CpG-ODN therapy in systemic lupus erythematosus patients [103,104].

A related approach includes the chemical conjugation of an ODN to an antigen [105]. The adjuvant activity of this kind of compounds comes from targeting the antigen by the ODN to the TLR9-bearing APC, receiving at the same time the antigen and the co-stimulatory signals.

Another strategy relates to the DNA vaccine technology, based on engineering the CpG motifs into the plasmid DNA sequences. Since DNA vaccines have shown limited efficacy in large animals and humans, the insertion of these motifs would increase the immune activation of the administered DNA molecule, providing at the same time both the gene for antigen expression, processing and presentation to the immune system and the co-stimulation required. Engineering would also include the selective removal of immunosuppressive DNA sequences, contributing to the overall immunostimulatory capacity of the whole molecule [98]. This could additionally favor novel antigen strategies that use only protective or relevant fragments of natural antigens in a new molecular array, focusing the response in the context of a less structured molecule like a multiepitopic polypeptide [106]. Moreover, new vaccine approaches employing DNA vaccines in combination with subunit or viral immunogens would benefit from the immunoenhancing potential of plasmid DNA, like the prime-boost strategy [107]. Further analyses are required, with second generation DNA vaccines entering clinical trials.

4.9. Adjuvant formulations

New adjuvant formulations have resulted from the mixture of two or more adjuvants with different action mechanisms. The aim of this strategy is to further enhance and/or modulate the immune response against a given antigen compared to the adjuvants alone and in some cases, to combine delivery improvement and modulation.

A remarkable adjuvant formulation comprising MPL and alum has been recently included in the approved vaccine formulation Fendrix®, used for preventive immunization against hepatitis B in patients with renal diseases, including haemodialysis patients. This vaccine develops a more rapid, intense and long lasting immune response compared with the control vaccine in these high-risk groups, showing safety and clinically acceptable local reactions similar to other licensed hepatitis B vaccines. [108]. The AS04 adjuvant formulation has been tested also as part of a promising HPV vaccine development [109,110].

Additionally, some of the abovementioned adjuvant approaches were developed *per se* as adjuvant formulations, as in the case of ISCOMs® and Algammulin (see sections *ISCOMs®* and *ISCOMATRIX® adjuvants* and *Polysaccharides*, respectively). Several studies have shown the efficient stimulation of the immune responses using these adjuvant formulations [76,94,111,112].

Other formulations have been designed to take advantage of antigen delivery systems and the stimulating effect of bacterial compounds, such as CpG-ODN and MDP encapsulated into liposomes [113,114].

Table 1
Toll-like receptors (TLR), their ligands and related adjuvants

TLR	Natural ligand	Related adjuvant	References
TLR1	Bacterial/mycoplasmal lipopeptides (with TLR2)	*E. coli* type II heat-labile enterotoxins, triacylated lipopeptides	[117,118]
TLR2	Peptidoglycan, Lipoteichoic acid	MDP[a] derivatives, *E. coli* type II heat-labile enterotoxins, lipopentapeptide derivatives	[117,119,120]
TLR3	Bacterial and viral dsRNA[b]	Polyinosinic–polycytidylic acid	[121,122]
TLR4	Host hsp60/70[c], LPS[d], RSV[e] fusion protein, MMTV[f] envelope protein	MDP derivatives, synthetic MPL[g]	[123]
TLR5	Flagellin	Flagellin fusion proteins	[124]
TLR6	Bacterial/mycoplasmal lipopeptides (with TLR2)	Diacylated lipopeptides (macrophage-activating lipopeptide-2)	[125]
TLR7	Bacterial, viral and host ssRNA[h]	Imiquimod, R-848, ssRNA	[126–128]
TLR8	Bacterial, viral and host ssRNA	R-848, ssRNA	[127,128]
TLR9	Bacterial and viral CpG DNA, DNA-IgG complexes, malarial hemozoine	CpG ODNs	[96,127]
TLR10	NR[i]	NR	–

[a] MDP: muramyl dipeptide.
[b] dsRNA: double-stranded RNA.
[c] hsp60/70: heat shock proteins 60 and 70.
[d] LPS: lipopolysaccharide.
[e] RSV: respiratory sincitial virus.
[f] MMTV: mouse mammary tumor virus.
[g] MPL: monophosphoryl lipid A.
[h] ssRNA: single-stranded RNA.
[i] NR: not reported.

4.10. TLRs and adjuvanticity

As previously mentioned in this review, activators of TLRs have already been used as adjuvants to boost the immune responses of vaccines (see Table 1).

TLRs are transmembrane signaling proteins expressed by cells of the mammalian immune system, showing high specific binding to different ligands of varied molecular nature [115]. Those ligands are evolutionary signatures of invading pathogens, commonly known as pathogen-associated molecular patterns (PAMPs)(Table 1). TLRs derived from the *Drosophila* Toll, ten of them been described so far in humans. TLRs 1, 2 and 6 are triggered as homo or heterodimers by peptidoglicans and other bacterial products, TLR3 by dsRNA, TLR4 by LPS, TLR5 by flagellin, TLR7 and 8 by imidazoquinolines and ssDNA molecules, and TLR9 by unmethylated CpG DNA motifs [116] In the case of TLR3 recognition, it is not mediated by any specific RNA sequence motifs as characterized so far. Ligands for TLR10 still remain to be identified. After TLR engagement and depending on the specific ligand (interaction), a signaling pathway becomes activated, resulting in expression of genes associated with inducing host immune and inflammatory responses that abrogate or control the infection.

TLRs became fundamental for vaccine strategies due to their distribution mainly in monocyte/macrophage and dendritic cell populations. Instead of individual TLR stimulation, the success of the vaccination regime depends in part on proper activation of the appropriate TLR in the immunologically relevant subset of APC. It is also related to the route and delivery methods employed, according to the APC populations stimulated, either through TLRs or other immune receptors. Not all adjuvant families act through the same cell type and in the same manner depending on receptors and cellular processing. Therefore, the abovementioned considerations are mostly for adjuvants mimicking or sharing the structure and/or activating potential of the natural PAMPs, like microorganism-derived adjuvants and vaccine antigens with adjuvant capacity. They can be included in the formulation for triggering significant immune responses against a vaccine antigen if activating a co-stimulatory signal relevant for the model under study.

Special attention should be given to the stimulating capacity of PAMPs derived from the host cell employed for producing recombinant vaccine antigens, as in the case of the HBcAg. This antigen normally carries unrelated bacterial RNA molecules with immunomodulatory capacity [129]. This property of the HBcAg has been successfully exploited for modulating the immune response against other co-formulated antigens, such as HBsAg and the human immunodeficiency virus (HIV)-based CR3 protein [84,130].

On the other hand, over-stimulation through several TLRs can also generate undesired toxic effects, as the case for the FCA. Thus, adjuvant dose and mechanism of action have to be carefully considered for preventing such drawbacks while not compromising the adjuvant effect.

5. Five years view

Despite an explosion of immunology knowledge over recent decades, there remains a surprising reliance on

aluminium-based compounds as the dominant adjuvants in human vaccines. This aspect will not change in the near future for already established vaccines with good efficacy and also in the scenery of prophylactic pediatric vaccines where safety issues are paramount. However, the introduction of new recombinant subunit and synthetic antigens in HIV, hepatitis C virus, Malaria and other "difficult" diseases as well as the development of therapeutic vaccines for chronic diseases and cancer will introduce new adjuvants to clinical trial pipelines.

New adjuvant formulations can be especially relevant for developing new vaccines against infectious agents causing pathological conditions characterized by immunodeficiency, low responders and high-risk groups. Further steps to generalize their applications seem to be affordable in a near future starting from these high-risk groups.

The study on new action mechanisms will further clarify molecular interactions behind adjuvant activity and this aspect, along with the development of bioinformatics, will improve the predictive capacity for scientists working on new adjuvant development.

References

[1] Petrovsky N, Aguilar JC. Vaccine adjuvants: current state and future trends. Immunol Cell Biol 2004;82(5):488–96.

[2] Vogel FR. Adjuvants in perspective. In: Brown F, Haaheim LR, editors. Modulation of the immune response to vaccine antigens. Dev. Biol. Stand. vol 92. Basel: Karger; 1998. p. 241–8.

[3] Ramon G. Sur l'augmentation anormale de l'antitoxine chez les chevaux producteurs de serum antidiphterique. Bull Soc Centr Med Vet 1925;101:227–34.

[4] Ramon G. Procedes pour accroïtre la production des antitoxins. Ann Inst Pasteur 1926;40:1–10.

[5] Glenny AT, Pope CG, Waddington H, Wallace V. The antigenic value of toxoid precipitated by potassium-alum. J Path Bact 1926;29:38–45.

[6] Vogel FR, Powell MF. A summary compendium of vaccine adjuvants and excipients. In: Powell MF, Newman MJ, editors. Vaccine design: the subunit and adjuvant approach. New York: Plenum Publishing Corp.; 1995. p. 234–50.

[7] Freund J, Casals J, Hosmer EP. Sensitization and antibody formation after injection of tubercle bacili and parafin oil. Proc Soc Exp Biol Med 1937;37:509–13.

[8] Stuart-Harris CH. Adjuvant influenza vaccines. Bull WHO 1969;41, 617–621.

[9] Johnson AG, Gaines S, Landy M. Studies on the O-antigen of Salmonella typhosa. V. Enhancement of antibody response to protein antigens by the purified lipopolysaccharide. J Exp Med 1956;103:225–46.

[10] Lindblad EB. Aluminium adjuvants. In: Stewart-Tull DES, editor. The theory and practical application of adjuvants. John Wiley & Sons Ltd.; 1995. p. 21–35.

[11] Byars NE, Allison AC. Immunologic adjuvants: general properties, advantages, and limitations. In: Zola H, editor. Laboratory Methods in Immunology. 1990. p. 39–51.

[12] Edelman R. Vaccine adjuvants. Rev Infect Dis 1980;2:370–83.

[13] Neutra MR, Pringault E, Kraehenbuhl JP. Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 1996;14:275–300.

[14] Warren HS, Chedid LA. Future prospects for vaccine adjuvants. CRC Crit Rev Immunol 1986;4:369–88.

[15] Edelman R. An update on vaccine adjuvants in clinical trials. AIDS Res Hum Retroviruses 1992;8:1409–11.

[16] Allison AC, Byars NE. Immunological adjuvants: desirable properties and side-effects. Mol Immunol 1991;28:279–84.

[17] Waters RV, Terrell TG, Jones GH. Uveitis induction in the rabbit by muramyl dipeptides. Infect Immunol 1986;51:816–25.

[18] Goldenthal KL, Cavagnaro JA, Alving CR, Vogel FR, National Cooperative Vaccine Development Working Group. Safety evaluation of vaccine adjuvants. AIDS Res Hum Retroviruses 1993;9:S45–9.

[19] Stewart-Tull DES. Recommendations for the assessment of adjuvants. In: Gregoriadis G, Allison AC, Poste G, editors. Immunological adjuvants and vaccines. New York: Plenun Press; 1989. p. 213–26.

[20] Edelman R, Tacket C. Adjuvants. Int Rev Immunol 1990;7:51–66.

[21] Couch RB. Nasal vaccination, *Escherichia coli* enterotoxin, and Bell's palsy. N Engl J Med 2004;350:860–1.

[22] Murphy TV, Gargiullo PM, Massoudi MS, Nelson DB, Jumaan AO, Okoro CA, et al. Intussusception among infants given an oral rotavirus vaccine. N Engl J Med 2001;344:564–72.

[23] Schirmbeck R, Melber K, Mertens T, Reimann J. Antibody and cytotoxic T-cell responses to soluble hepatitis B virus (HBV) S antigen in mice: implications for the pathogenesis of HBV-induced hepatitis. J Virol 1994;68:1418–25.

[24] Traquina P, Morandi M, Contorni M, Van Nest G. MF59 adjuvant enhances the antibody response to recombinant hepatitis B surface antigen vaccine in primates. J Infect Dis 1996;174:1168–75.

[25] Brewer JM, Conacher M, Satoskar A, Bluethmann H, Alexander J. In interleukin-4-deficient mice, alum not only generates T helper 1 responses equivalent to Freund's complete adjuvant, but continues to induce T helper 2 cytokine production. Eur J Immunol 1996;26:2062–6.

[26] Blagowechensky NN. Durée du séjour de l'antigène dans l'organisme et immunité. Rev Immunol París 1938;4:161.

[27] Walls RS. Eosinophil response to alum adjuvants: involvement of T cells in non-antigen-dependent mechanisms. Proc Soc Exp Biol Med 1977;156:431–5.

[28] Gupta RK, Rost BE, Relyveld E, Siber GR. Adjuvant properties of aluminium and calcium compounds. In: Powell MF, Newman MJ, editors. Vaccine design: the subunit and adjuvant approach. New York: Plenum Press; 1995. p. 229–48.

[29] Butler NR, Voyce MA, Burland WL, Hilton ML. Advantages of aluminum hydroxide adsorbed diphtheria, tetanus and pertussis vaccines for the immunization of infants. Br Med J 1969;1:663–6.

[30] Straw BE, MacLachlan NJ, Corbett WT, Carter PB, Schey HM. Comparison of tissue reactions produced by *Haemophilus pleuropneumoniae* vaccines made with six different adjuvants in swine. Can J Comp Med 1985;49:149.

[31] Audibert FM, Lise LD. Adjuvants: current status, clinical perspectives and future prospects. Immunol Today 1993;14:281–4.

[32] Bomford R. Aluminium salts: perspectives in their use as adjuvants. In: Gregoriadis G, Allison AC, Poste G, editors. Immunological adjuvants and vaccines. New York: Plenum Press; 1989. p. 35–41.

[33] Goto N, Kato H, Maeyama J-I, Eto K, Yoshihara S. Studies on the toxicities of aluminium hydroxide and calcium phosphate as immunological adjuvants for vaccines. Vaccine 1993;11:914–8.

[34] Relyveld EH, Hencoq E, Raynaud M. Etude de la vaccination antidiphterique de sujets alergiques avec une anatoxine pure adsorbee sur phosphate de calcium. Bull WHO 1964;30:321–5.

[35] Gupta RK, Siber GR. Adjuvants for human vaccines-current status, problems and future prospects. Vaccine 1995;13:1263–76.

[36] Relyveld EH. Preparation and use of calcium phosphate adsorbed vaccines. Dev Biol Stand 1986;65:131–6.

[37] Kensil CR. Saponins as vaccine adjuvants. Crit Rev Ther Drug Carrier Syst 1996;13:1–55.

[38] Takahashi H, Takeshita T, Morein B, Putney S, Germain RN, Berzofsky JA. Induction of CD8+ cytotoxic T cells by immunization with purified HIV-1 envelope protein in ISCOMs. Nature 1990;344: 873–5.

[39] Dalsgaard K. Adjuvants. Vet Immunol Immunopathol 1987;17: 145–53.
[40] Bomford RHR. The differential adjuvant activity of Al(OH)3 and saponin. In: Madje J, editor. Immunopharmacology of infectious diseases: vaccine adjuvants and modulators of non-specific resistance. New York: Alan R. Liss; 1987. p. 65–70.
[41] Rönnberg B, Fekadu M, Morein B. Adjuvant activity of non toxic *Quillaja saponaria* Molina components for use in iscom-matrix. Vaccine 1995;13:1375–82.
[42] Kensil CR, Patel U, Lennick M, Marciani D. Separation and characterization of saponins with adjuvant activity from *Quillaja saponaria* Molina cortex. J Immunol 1991;146:431–7.
[43] Kensil CR, Wu J-Y, Soltysik S. Structural and immunological characterization of the vaccine adjuvant QS-21. In: Powell MF, Newman MJ, editors. Vaccine design: the subunit and adjuvant approach. New York: Plenum Press; 1995. p. 525–41.
[44] Kotani S, Watanabe Y, Shimoto T, Narita T, Kato K, Stewart-Tull DES, et al. Immunoadjuvant activities of cells walls, their water soluble fractions and peptidoglycan subunits, prepared from various gram-positive bacteria, and of synthetic *N*-acetylmuramyl peptides. Z Immunitatsforsch 1975;149S:302–5.
[45] Audibert F, Leclerc C, Chedid L. Muramyl peptides as immunopharmacological response modifiers. In: Torrence PF, editor. Biological response moodifiers. New approaches to disease prevention. Orlando: Academic Press; 1985. p. 307.
[46] Audibert F, Chedid L, Lefrancier P, Choay J. Distinctive adjuvanticity of synthetic analogs of mycobacterial water-soluble components. Cell Immunol 1976;21:243–5.
[47] Parant MA, Audibert FM, Chedid LA, Level MR, Lefrancier PL, Choay JP, et al. Immunostimulant activities of a lipophilic muramyl dipeptide derivative and of a desmuramyl peptidolipid analogue. Infect Immun 1980;27:826–30.
[48] Leclerc C, Vogel F. Synthetic immunomodulators and synthetic vaccines. CRC Crit Rev Ther Drug Carrier Syst 1986;2:353–7.
[49] Tomai MA, Johnson AG. T cell and interferon-gamma involvement in the adjuvant action of a detoxified endotoxin. J Biol Resp Modifiers 1989;8:625–30.
[50] Lemaire G, Tenu JP, Petit JF, Lederer E. Natural and synthetic trehalose diesters as immunomodulators. Med Res Rev 1986;6: 243.
[51] Weiner GJ, Hsin-Ming L, Wooldridge JE, Dahle CE, Krieg AM. Immunostimulatory oligodeoxynucletides containing the CpG motif are effective as immune adjuvants in tumor antigen immunization. Proc Natl Acad Sci USA 1997;94:10833–7.
[52] Hilleman MR, Woodhour AF, Friedman A, Phelps AH. Studies for safety of adjuvant 65. Ann Allergy 1972;30:477–80.
[53] Smith JWG, Fletcher WB, Peters M, Westwood M, Perkins FT. Response to influenza vaccine in adjuvant 65-4. J Hyg (Camb) 1975; 74:251–5.
[54] Weibel RE, McLean A, Woodhour AF, Friedman A, Hilleman MR. Ten-year follow-up study for safety of Adjuvant 65 influenza vaccine in man. Proc Soc Exp Biol Med 1973;143:1053–6.
[55] Freund J. The mode of action immunological adjuvants. Adv Tuberc Res 1956;7:50–5.
[56] Woodard LF, Jasman RL. Stable oil-in-water emulsions: preparation and use as vaccine vehicles for lipophilic adjuvants. Vaccine 1985;3:57–61.
[57] Kimura J, Nariuchi H, Watanabe T, Matuhasi T, Okayasu I, Hatakeyama S. Studies on the adjuvant effect of water-in-oil-in-water emulsion in sesame oil. I. Enhanced and persistent antibody formation by antigen incorporated into the water-in-oil-in-water emulsion. Jpn J Exp Med 1978;48:149–52.
[58] Freestone DS, Hamilton-Smith S, Schild GC, Buckland R, Chinn S, Tyrrel DAJ. Antibody responses and resistance to challenge in volunteers vaccinated with live attenuaded detergent split and oil adjuvant A2/Hong Kong/68 (H3N2) influenza vaccines. J Hyg (Camb) 1972;70:351–5.
[59] Taylor PJ, Miller CL, Pollock TM, Perkins FT, Westwood MA. Antibody response and reactions to aqueous influenza vaccine, simple emulsion vaccine and multiple emulsion vaccine. J Hyg (Camb) 1969;67:485–90.
[60] Jones GL. Peptide vaccine derived from a malarial surface antigen: effect of dose and adjuvant on immunogenicity. Immunol Lett 1990;24:253–60.
[61] Storni T, Kundig TM, Senti G, Johansen P. Immunity in response to particulate antigen-delivery systems. Adv Drug Deliv Rev 2005;57:333–55.
[62] Bramwell VW, Perrie Y. Particulate delivery systems for vaccines. Crit Rev Ther Drug Carrier Syst 2005;22:151–214.
[63] Shek PN, Yung BYK, Stanacev NZ. Comparison between multilamellar and unilamellar liposomes in enhancing antibody formation. Immunology 1983;49:37–40.
[64] Allison AC, Gregoriadis G. Liposomes as immunological adjuvants. Nature 1974;252:252–8.
[65] Heath TD, Edwards DC, Ryman BE. The adjuvant properties of liposomes. Biochem Soc Trans 1976;4:49–52.
[66] Tyrrel DA, Heath TD, Colley CM, Ryman BE. New aspects of liposomes. Biochim Biophys Acta 1976;457:259–63.
[67] van Rooijen N, van Nieuwmegen R. Use of liposomes as biodegradable and harmless adjuvants. Methods Enzymol 1983;93:83–5.
[68] Taneichi M, Ishida H, Kajino K, Ogasawara K, Tanaka Y, Kasai M, et al. Antigen chemically coupled to the surface of liposomes are cross-presented to CD8+ T cells and induce potent antitumor immunity. J Immunol 2006;177:2324–30.
[69] Laing P, Bacon A, McCormack B, Gregoriadis G, Frisch B, Schuber F. The 'co-delivery' approach to liposomal vaccines: application to the development of influenza-A and hepatitis-B vaccine candidates. J Liposome Res 2006;16:229–35.
[70] Eldrige JH, Staas JK, Meulbroek JA, Tice TR, Gilley RM. Biodegradable microspheres as a vaccine delivery system. Mol Immunol 1991;28:287–90.
[71] Eldrige JH, Staas JK, Meulbroek JA, Tice TR, Gilley RM. Biodegradable and biocompatible poly (DL-lactide-*co*-glycolide) microspheres as an adjuvant for Staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies. Infect Immun 1991;59:2978–83.
[72] Singh M, Kazzaz J, Ugozzoli M, Malyala P, Chesko J, O'Hagan DT. Polylactide-*co*-glycolide microparticles with surface adsorbed antigens as vaccine delivery systems. Curr Drug Deliv 2006;3: 115–20.
[73] Falo Jr LD, Kovacsovics-Bankowski M, Thompson K, Rock KL. Targeting antigen into the phagocytic pathway in vivo induces protective tumour immunity. Nat Med 1995;1:649–53.
[74] Fifis T, Gamvrellis A, Crimeen-Irwin B, Pietersz GA, Li J, Mottram PL, et al. Size-dependent immunogenicity: therapeutic and protective properties of nano-vaccines against tumors. J Immunol 2004;173:3148–54.
[75] Scheerlinck JP, Gloster S, Gamvrellis A, Mottram PL, Plebanski M. Systemic immune responses in sheep, induced by a novel nano-bead adjuvant. Vaccine 2006;24:1124–31.
[76] Cox E, Verdonck F, Vanrompay D, Goddeeris B. Adjuvants modulating mucosal immune responses or directing systemic responses towards the mucosa. Vet Res 2006;37:511–39.
[77] Pearse MJ, Drane D. ISCOMATRIX adjuvant for antigen delivery. Adv Drug Deliv Rev 2005;57:465–74.
[78] Wikman M, Friedman M, Pinitkiatisakul S, Andersson C, Lovgren-Bengtsson K, Lunden A, et al. Achieving directed immunostimulating complexes incorporation. Expert Rev Vaccines 2006;5:395–403.
[79] Skene CD, Sutton P. Saponin-adjuvanted particulate vaccines for clinical use. Methods 2006;40:53–9.
[80] Antonis AF, Bruschke CJ, Rueda P, Maranga L, Casal JI, Vela C, et al. A novel recombinant virus-like particle vaccine for prevention of porcine parvovirus-induced reproductive failure. Vaccine 2006;24:5481–90.

[81] Young SL, Wilson M, Wilson S, Beagley KW, Ward V, Baird MA. Transcutaneous vaccination with virus-like particles. Vaccine 2006; 24:5406–12.
[82] Dell K, Koesters R, Linnebacher M, Klein C, Gissmann L. Intranasal immunization with human papillomavirus type 16 capsomeres in the presence of non-toxic cholera toxin-based adjuvants elicits increased vaginal immunoglobulin levels. Vaccine 2006;24:2238–47.
[83] Kundig TM, Senti G, Schnetzler G, Wolf C, Prinz Vavricka BM, Fulurija A, et al. Der p 1 peptide on virus-like particles is safe and highly immunogenic in healthy adults. J Allergy Clin Immunol 2006;117:1470–6.
[84] Aguilar JC, Lobaina Y, Muzio V, Garcia D, Penton E, Iglesias E, et al. Development of a nasal vaccine for chronic hepatitis B infection that uses the ability of hepatitis B core antigen to stimulate a strong Th1 response against hepatitis B surface antigen. Immunol Cell Biol 2004;82:539–46.
[85] Lobaina Y, Palenzuela D, Pichardo D, Muzio V, Guillen G, Aguilar JC. Immunological characterization of two hepatitis B core antigen variants and their immunoenhancing effect on co-delivered hepatitis B surface antigen. Mol Immunol 2005;42:289–94.
[86] Aguilar A, González C, Cinza Z, Martínez J, Véliz G, Aureoles-Roselló SR, et al. Phase I clinical trial in healthy adults of a nasal vaccine candidate containing recombinant Hepatitis B surface and core antigens. Int J Infect Dis, 2007, doi:10.1016/j.ijid.2006.09.010.
[87] Heufler C, Koch F, Schuler G. Granulocyte/macrophage colony-stimulating factor and interleukin 1 mediate the maturation of murine epidermal Langerhans cells into potent immunostimulatory dendritic cells. J Exp Med 1988;167:700–5.
[88] Egan MA, Israel ZR. The use of cytokines and chemokines as genetic adjuvants for plasmid DNA vaccines. Clin Appl Immunol Rev 2002;2:255–87.
[89] Lynch JM, Briles DE, Metzger DW. Increased protection against pneumococcal disease by mucosal administration of conjugate vaccine plus interleukin-12. Infect Immun 2003;71:4780–8.
[90] Bradney CP, Sempowski GD, Liao HX, Haynes BF, Staats HF. Cytokines as adjuvants for the induction of anti-human immunodeficiency virus peptide immunoglobulin G (IgG) and IgA antibodies in serum and mucosal secretions after nasal immunization. J Virol 2002;76:517–24.
[91] Cooper PD. Vaccine adjuvants based on gamma inulin. In: Powell MF, Newman MJ, editors. Vaccine design: the subunit and adjuvant approach. New York: Plenum Press; 1995. p. 559–80.
[92] Cooper PD, Steele EJ. Algammulin: a new vaccine adjuvant comprising gamma inulin particles containing alum, preparation and in vitro properties. Vaccine 1991;9:351–7.
[93] Cooper PD, McComb C, Steele EJ. The adjuvanticity of algammulin, a new vaccine adjuvant. Vaccine 1991;9:408–15.
[94] Silva DG, Cooper PD, Petrovsky N. Inulin-derived adjuvants efficiently promote both Th1 and Th2 immune responses. Immunol Cell Biol 2004;82:611–6.
[95] Tokunaga T, Yamamoto H, Shimada S, Abe H, Fukuda T, Fujisawa Y, et al. Antitumor activity of deoxyribonucleic acid fraction from Mycobacterium bovis BCG. I. Isolation, physicochemical characterization, and antitumor activity. J Natl Cancer Inst 1984;72:955–62.
[96] Krieg AM. CpG motifs in bacterial DNA and their immune effects. Annu Rev Immunol 2002;20:709–60.
[97] Gursel I, Gursel M, Yamada H, Ishii KJ, Takeshita F, Klinman DM. Repetitive elements in mammalian telomeres suppress bacterial DNA-induced immune activation. J Immunol 2003;171:1393–400.
[98] Krieg AM, Wu T, Weeratna R, Efler SM, Love-Homan L, Yang L, et al. Sequence motifs in adenoviral DNA block immune activation by stimulatory CpG motifs. Proc Natl Acad Sci USA 1998;95(21):12631–6.
[99] Cornelie S, Poulain-Godefroy O, Lund C, Vendeville C, Ban E, Capron M, et al. Methylated CpG-containing plasmid activates the immune system. Scand J Immunol 2004;59:143–51.
[100] Cornelie S, Hoebeke J, Schacht AM, Bertin B, Vicogne J, Capron M, et al. Direct evidence that toll-like receptor 9 (TLR9) functionally binds plasmid DNA by specific cytosine-phosphate-guanine motif recognition. J Biol Chem 2004;279:15124–9.
[101] Klinman DM. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat Rev Immunol 2004;4(4):249–58.
[102] Marshall JD, Fearon KL, Higgins D, Hessel EM, Kanzler H, Abbate C, et al. Superior activity of the type C class of ISS *in vitro* and *in vivo* across multiple species. DNA Cell Biol 2005;24:63–72.
[103] Anders HJ, Vielhauer V, Eis V, et al. Activation of toll-like receptor-9 induces progression of renal disease in MRL-Fas(lpr) mice. FASEB J 2004;18:534–6.
[104] Anders HJ. A toll for lupus. Lupus 2005;14:417–22.
[105] Datta SK, Cho HJ, Takabayashi K, Horner AA, Raz E. Antigen-immunostimulatory oligonucleotide conjugates: mechanisms and applications. Immunol Rev 2004;199:217–26.
[106] Rodríguez EG, Vazquez DM, Herrera AM. Duarte CA Enhanced cell-mediated IFN-g-secreting activity against the HIV-1IIIB V3 peptide of the TAB9 multiepitope after DNA vaccine backbone engineering. Biochem Biophys Res Commun 2003;308:713–8.
[107] Robinson HL. New hope for an AIDS vaccine. Nat Rev Immunol 2002;2:239–50.
[108] Tong NK, Beran J, Kee SA, Miguel JL, Sanchez C, Bayas JM, et al. Immunogenicity and safety of an adjuvanted hepatitis B vaccine in pre-hemodialysis and hemodialysis patients. Kidney Int 2005;68: 2298–303.
[109] Giannini SL, Hanon E, Moris P, Van Mechelen M, Morel S, Dessy F, et al. Enhanced humoral and memory B cellular immunity using HPV16/18 L1 VLP vaccine formulated with the MPL/aluminium salt combination (AS04) compared to aluminium salt only. Vaccine 2006;24:5937–49.
[110] Harper DM, Franco EL, Wheeler C, Ferris DG, Jenkins D, Schuind A, et al. Efficacy of a bivalent L1 virus-like particle vaccine in prevention of infection with human papillomavirus types 16 and 18 in young women: a randomised controlled trial. Lancet 2004;364: 1757–65.
[111] Petrovsky N. Novel human polysaccharide adjuvants with dual Th1 and Th2 potentiating activity. Vaccine 2006;24:S2-26–9.
[112] Cooper PD. Vaccine adjuvants based on gamma inulin. Pharm Biotechnol 1995;6:559–80.
[113] Turanek J, Ledvina M, Kasna A, Vacek A, Hribalova V, Krejci J, et al. Liposomal preparations of muramyl glycopeptides as immunomodulators and adjuvants. Vaccine 2006;24:S2-90–1.
[114] Tafaghodi M, Jaafari MR, Sajadi Tabassi SA. Nasal immunization studies using liposomes loaded with tetanus toxoid and CpG-ODN. Eur J Pharm Biopharm 2006;64:138–45.
[115] Iwasaki A, Medzhitov R. Toll-like receptor control of the adaptive immune responses. Nat Immunol 2004;5:987–95.
[116] Takeda K, Akira S. Toll-like receptors in innate immunity. Int Immunol 2005;17:1–14.
[117] Hajishengallis G, Tapping RI, Martin MH, Nawar H, Lyle EA, Russell MW, et al. Toll-like receptor 2 mediates cellular activation by the B subunits of type II heat-labile enterotoxins. Infect Immun 2005;73:1343–9.
[118] Schroder NW, Heine H, Alexander C, Manukyan M, Eckert J, Hamann L, et al. Lipopolysaccharide binding protein binds to triacylated and diacylated lipopeptides and mediates innate immune responses. J Immunol 2004;173:2683–91.
[119] Uehori J, Fukase K, Akazawa T, Uematsu S, Akira S, Funami K, et al. Dendritic cell maturation induced by muramyl dipeptide (MDP) derivatives: monoacylated MDP confers TLR2/TLR4 activation. J Immunol 2005;174:7096–103.
[120] Muller SD, Muller MR, Huber M, Esche Uv U, Kirschning CJ, Wagner H, et al. Triacyl-lipopentapeptide adjuvants: TLR2-dependent activation of macrophages and modulation of receptor-mediated cell activation by altering acyl-moieties. Int Immunopharmacol 2004;4:1287–300.
[121] Salem ML, Kadima AN, Cole DJ, Gillanders WE. Defining the antigen-specific T-cell response to vaccination and poly(I:C)/TLR3

signaling: evidence of enhanced primary and memory CD8 T-cell responses and antitumor immunity. J Immunother 2005;28: 220–8.

[122] Gill N, Deacon PM, Lichty B, Mossman KL, Ashkar AA. Induction of innate immunity against herpes simplex virus type 2 infection via local delivery of Toll-like receptor ligands correlates with beta interferon production. J Virol 2006;80:9943–50.

[123] Baldridge JR, McGowan P, Evans JT, Cluff C, Mossman S, Johnson D, et al. Taking a Toll on human disease: Toll-like receptor 4 agonists as vaccine adjuvants and monotherapeutic agents. Expert Opin Biol Ther 2004;4:1129–38.

[124] Huleatt JW, Jacobs AR, Tang J, Desai P, Kopp EB, Huang Y, et al. Vaccination with recombinant fusion proteins incorporating Toll-like receptor ligands induces rapid cellular and humoral immunity. Vaccine 2007;25:763–75.

[125] Takeuchi O, Kawai T, Muhlradt PF, Morr M, Radolf JD, Zychlinsky A, et al. Discrimination of bacterial lipoproteins by Toll-like receptor 6. Int Immunol 2001;13:933–40.

[126] Stockfleth E, Trefzer U, Garcia-Bartels C, Wegner T, Schmook T, Sterry W. The use of Toll-like receptor-7 agonist in the treatment of basal cell carcinoma: an overview. Br J Dermatol 2003;149(Suppl 66):53–6.

[127] Weeratna RD, Makinen SR, McCluskie MJ, Davis HL. TLR agonists as vaccine adjuvants: comparison of CpG ODN and Resiquimod (R-848). Vaccine 2005;23:5263–70.

[128] Heil F, Hemmi H, Hochrein H, Ampenberger F, Kirschning C, Akira S, et al. Species-specific recognition of single-stranded RNA via toll-like receptor 7 and 8. Science 2004;303:1526–9.

[129] Riedl P, Stober D, Oehninger C, Melber K, Reimann J, Schirmbeck R. Priming Th1 immunity to viral core particles is facilitated by trace amounts of RNA bound to its arginine-rich domain. J Immunol 2002;168:4951–9.

[130] Iglesias E, Thompson R, Carrazana Y, Lobaina Y, Garcia D, Sanchez J, et al. Coinoculation with hepatitis B surface and core antigen promotes a Th1 immune response to a multiepitopic protein of HIV-1. Immunol Cell Biol 2006;84:174–83.