

Expert Opinion

1. Introduction
2. Mechanisms of the immune response
3. Adjuvants targeting innate immunity
4. Adjuvants targeting adaptive immunity
5. Conclusion
6. Expert opinion

informa
healthcare

Vaccines & Antibodies

Adjuvants in vaccines and for immunisation: current trends

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Vaccines represent one of the most successful strategies in medical science. From the mechanistic perspective, vaccination works by manipulating the immune response through selecting, activating and expanding the memory of B and T cells. To determine the magnitude and quality of immune response, suitable vaccine adjuvants are required; therefore, much effort is going into finding new, effective and non-toxic adjuvant formulations focussed on the activation of key immune targets for inducing a long-term, potent and safe immune response. Significant research is being done in this area, to develop new classes of vaccines for use not only against infectious diseases, but also in the treatment of autoimmune disorders, allergies, chronic inflammatory diseases and cancer. Here the authors review and classify some of the main vaccine adjuvants on the basis of their immunomodulating properties on the immune system.

Keywords: adjuvant, immune response, vaccine

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1. Introduction

The development of vaccines against infectious diseases has been a triumph of medical science. Vaccines are capable of inducing long-lasting antibody responses, these being the principal agents of immune protection against most viruses and bacteria. Increased effort has gone into applying vaccination strategies for the treatment and immunomodulation of malignant diseases. In recent years, immunisation with naked plasmid DNA encoding tumour-associated antigens has revealed a number of advantages, and the improvement of vaccine efficacy has become a goal in the development of DNA vaccination as an antitumour therapy. Despite considerable success in the prevention field, vaccinations against intracellular organisms, which require a cell-mediated immunity, are not yet available, while infectious diseases such as tuberculosis and malaria remain a serious problem in the Third World. On the other hand, DNA vaccination against tumours offers a more promising prospect. That said, better strategies are needed in designing more effective vectors and combined protocols so as to induce a strong immune response to weakly immunogenic antigens and to overcome cancer self-tolerisation. One of these strategies to induce more effective immune responses relies on the discovery and use of new and effective adjuvants as essential components of vaccines.

1.1 Adjuvants: general considerations

Adjuvants are helper substances able to strengthen the immune response against antigens when used in combination with vaccines. They have been defined by Janeway [1] as the immunologist's 'dirty little secret', meaning that such substances radically improve the vaccine efficacy through mechanisms that are not yet fully elucidated. The main function of an adjuvant is to create a depot of antigen at the injection site, resulting in a gradual release of small quantities of antigen over a long period of time. The adjuvant also serves as a vehicle for delivering the antigen to

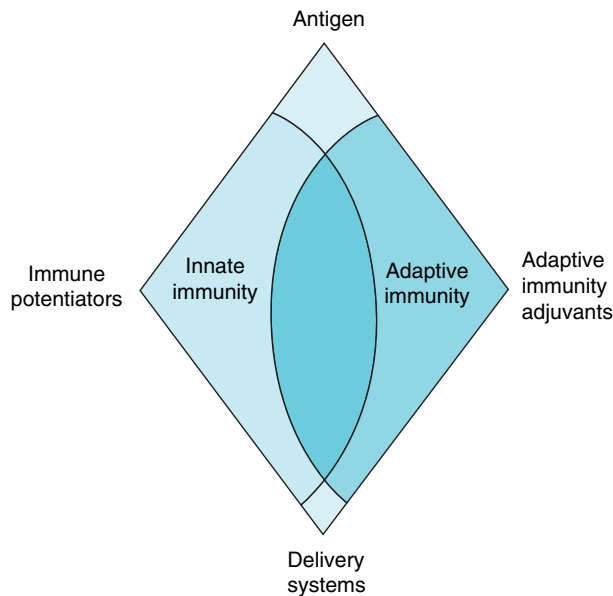


Figure 1. Essential components of a vaccine. Immune potentiators acting on the innate compartment of the immune system and adjuvants specific for the adaptive immune response are co-administered with antigen through appropriate delivery systems in order to elicit a strong immune response to the vaccine.

the lymphoid organs, where antigenic epitopes can be presented to T cells by 'professional' antigen-presenting cells (APCs). Furthermore, due to advances in the immunological research of the last decades, adjuvants are known to activate many immune cell types acting on mechanisms of the innate and adaptive immune response. In conventional procedures used for protein and DNA vaccination, adjuvants are co-injected together with the antigen, and they stimulate the immune system by triggering either the innate or the adaptive immunity (Figure 1). However, to develop innovative adjuvants, many key elements have to be considered and especially for human vaccines, many stringent requirements have to be fulfilled. These are safety, immunogenicity, clinical efficacy and patient compliance.

Although it is not possible to provide a comprehensive overview of the most commonly adjuvants used in vaccination, the authors briefly describe some of the well known immunostimulatory compounds on the basis of their immunomodulating properties on the innate and adaptive immune system.

2. Mechanisms of the immune response

In mammals, the crucial event responsible for the initiation of an adequate immune response is antigen recognition, uptake and presentation to naive lymphocytes by dendritic cells (DCs), the so-called professional APCs. These DCs, being resident in organs, act as sentinels in the periphery and are specialised in capturing antigens by receptor-mediated

endocytosis and pinocytosis mechanisms in order to undergo a complex multistep maturation process.

DC maturation occurs after DCs encounter the antigen and with microbial and pathogen-derived signals which increase their capacity to migrate towards the draining lymph node. On their journey to lymphoid organs, DCs interact with several ligands, in particular chemokines which can further enhance the DC maturation process. Once they reach lymph nodes, DCs shift from an antigen-capturing cell to a T-cell sensitising cell, which presents the antigen in the context of the MHC molecule to T cells and activates T lymphocyte clonal expansion and memory development via expression of co-stimulatory molecules (CD80, CD86 and CD40) and cytokine production.

To briefly summarise the mechanism of the immune response, activation of the immune system starts in the periphery, continues in lymphoid organs and culminates with DC-induced naive lymphocyte proliferation, differentiation and generation of effector and memory T/B cells. The adaptive immune response is a cascade of events where, despite the involvement of many cells, DCs and T cells act as main players, and the host defence against foreign antigens relies on the cross-talk between these two cell types. Therefore, the resulting immune response takes place because a contact site, namely the immunological synapse (IS), is formed between the APC and the T lymphocyte. The immunological synapse is characterised by four signals. The first signal (signal 0), is based on the stimulation of pattern recognition receptors (PRRs), which are expressed on macrophages and DCs. These cells are activated by pathogen-associated molecular patterns (PAMPs). As a result of this activation, DCs mature and are licensed to present antigens. The lymphocyte T-cell receptor (TCR) sensitisation takes place following interaction with the MHC-peptide complex (signal 1). It is followed by T-cell activation against the antigen via co-stimulatory molecules interaction (signal 2), and by a cytokine expression pattern that orientates T-cell differentiation towards the T_H1 or T_H2 specific functional pathway (signal 3).

The characterisation of the immunological synapse and the discoveries made in the immune research field in the last decades, allowed a better understanding of the principles of immune induction and immune regulation by vaccine adjuvants. Nowadays, most innovative adjuvants are being designed to selectively enhance the four signals of the immunological synapse.

3. Adjuvants targeting innate immunity

3.1 Toll-like receptor and pattern recognition receptor binding adjuvants

As described above, APCs on their surface express PRRs which bind highly conserved PAMPs of microbial derivation. PRRs are non-phagocytic receptors, such as toll-like receptors (TLRs) and nucleotide-binding oligomerisation

domain (NOD) proteins. These are distinguishable from receptors that induce phagocytosis such as mannose, scavenger and β -glucans receptors. Certain TLRs recognise PAMPs at an extracellular level, whereas proteins belonging to the NOD receptor family recognise PAMPs intracellularly. Following interaction with PAMPs, both TLRs and NOD transduce signals through a cascade leading to activation of transcription factors in the nucleus.

PAMPs include many components of the microbial cell wall, such as bacterial lipopolysaccharide, peptidoglycans, lipotechoic acids, mannans and glucans, as well as the nucleic acid of microbes and viruses such as bacterial DNA, double-strand RNAs and CpG DNA motifs [2]. All these PAMPs are known to have immunostimulatory effects on receptors belonging to the TLR family. They upregulate pro-inflammatory cytokine and chemokine expression, leading to maturation, differentiation and proliferation of DCs, macrophages, NK cells and B and T lymphocytes.

3.1.1 Water-in-oil formulations

Many adjuvants are water-in-oil formulations containing PAMPs of microbial derivation. Freund's complete and incomplete adjuvant is recognised as being as one of the most effective water-in-oil adjuvants. The complete formulation (FCA) has been developed as a water-in-oil emulsion containing heat-killed *Mycobacteria* [3]. The potent effect of FCA on the immune response is due to the ability of inactivated bacteria to stimulate TLRs, although the adverse effects on the host caused by *Mycobacteria* have discouraged scientists from using Freund's in clinical trials. The efficacy of killed or inactivated bacteria on the immune system, resides in some components of the cell wall that are known to stimulate PRRs.

As some water-in-oil formulations are used as delivery systems for antigen release, they will be also reported below, in Section 3.2.

3.1.2 Lipopolysaccharide and derivatives

Lipopolysaccharide (LPS) is a Gram-negative membrane molecule consisting of a hydrophilic polysaccharide and a lipophilic phospholipid (lipid A). The lipophilic portion of LPS is such a potent stimulus on the pro-inflammatory cytokine production that it can lead to septic shock [4] whereas monophosphoryl lipid A (MPL) is a lipid A derivative included in many adjuvant formulations because it retains good adjuvanticity and lower toxicity [5]. The adjuvanticity of these molecules depends on their capability to bind and activate the TLR4. As a result, many TLR agonists or lipid A mimetics displaying TLR4-dependent immunostimulating functions, have been synthesised and proposed as new adjuvants [6].

3.1.3 Bacterial CpG-DNA

CpG-DNA is an immunostimulatory oligodeoxynucleotide, consisting of unmethylated CpG motifs which are recognised

by TLR9, so leading to activation of innate immunity and cytokine-dependent promotion of the T_H -1 response. These motifs, present in bacterial DNA, consist of an unmethylated CpG dinucleotide flanked by two 5' purines and two 3' pyrimidines [7]. During an infection, the release of unmethylated CpG-DNA from bacteria serves as a danger signal stimulating the immune system of the host [8]. Bacterial DNA and synthetic unmethylated CpG oligodeoxynucleotides trigger an immunostimulatory cascade that culminates in maturation, differentiation and proliferation of several immune cells creating a pro-inflammatory and T_H -1-biased environment.

The immunostimulatory activity of this CpG-DNA is species-specific. As a result, sequences specific for the host are designed and optimised for selective TLR9 binding. In particular, *in vivo* CpG-DNA half-life has been improved by replacing phosphodiester CpG-DNA with a nuclease-resistant phosphothioate oligodeoxynucleotide [9], although it may induce an immune reaction leading to an anti-DNA immune response [10]. Addition of the immunostimulatory CpG-DNA to peptide vaccines, shifts the balance towards a T_H -1 immune response, which is often accompanied by a significant increase in IgG2a production in comparison to IgG1. The general effect of CpG-DNA addition is augmentation of the antibody serum titre against antigens and production of T_H -1 cytokines, such as IFN- γ [11]. By contrast, in DNA plasmid vaccination, the immunomodulating effect of bacterial CpG oligodeoxynucleotides on T_H cell balance, shows great variability. Such variability often depends on the route of immunisation [12,13].

3.1.4 Cochleate and proteoliposome

Cochleate and proteoliposome (PL), two bacterial derived compounds, induce the stimulation and activation of DCs as evidenced by increased expression of CD40, CD80, CD86, MHC-II and MHC-I molecules on these cells [14]. PL seems to be an efficient way to deliver antigens to DC for processing and presentation in association with MHC class I and MHC class II molecules [15]. Cochleate and PL are capable of inducing both systemic and mucosal antibody responses, as well as a T_H -1 type of immunity, as evident by high IFN- γ production and IgG2a antibody responses [16].

3.2 Delivery systems

The terms adjuvant and delivery system have often been used interchangeably although, to make a clear distinction, one might define the latter as a vaccine adjuvant when it enhances the immune response against the delivered antigen. The common feature shared by adjuvants and delivery systems is the maximisation of antigen concentration in lymph nodes in order to ensure efficient presentation by APCs. As highlighted previously, the antigen amount and availability to APCs at the site of injection is an important prerequisite for efficient antigen presentation to lymphocytes. Generally, adjuvants offering a slow delivery of the

antigen over time have been preferred, so that the antigen can be available to APCs in a controlled fashion. In fact, the rate of antigen delivery from a site seems to be a crucial factor in maintaining antigen-specific immune response during vaccination [17].

The principal mechanism of adjuvants such as water-in-oil emulsions is the triggering of an inflammatory response at the injection site with recruitment of APCs. These cells uptake the foreign antigen and migrate to draining lymph nodes, in order to present foreign epitopes to T lymphocytes (Figure 2). Water-in-oil emulsions and mineral salt adjuvants have the ability to retain the antigen at the injection site, thereby performing a depot effect [18].

Among delivery systems, it is worth mentioning particulate delivery. Typical examples of particulate delivery vehicles are polymer microparticles, liposomes, virosomes and immunostimulatory complexes [19]. Several formulations, such as antigen-entrapping liposomes or microcapsules based on biodegradable polylactic acid-polyglycolic acid copolymer (PLGA), provide a depot effect. Possible advantages of particulate delivery are that antigen and adjuvant are delivered to the same cell and synthetic microparticles have excellent potential for targeting cells of the immune system and stimulating antigen uptake. An extensive description of particulate delivery systems is described elsewhere [19,20].

3.2.1 Aluminium salts

Aluminium salt adjuvants are used with protein antigens as alum-precipitated vaccines and as alum-adsorbed vaccines. An efficient, safe and well tolerated adjuvant in humans is aluminium hydroxide. Its use has been licensed in many clinical trials performed with peptide vaccination. This adjuvant normally induces a T_H2 -type immune response but it can also stimulate T_H1 cells when used in combination with specific molecules that bias towards T_H1 -type immunity. Recently, aluminium hydroxide has been also shown to work well in DNA vaccination in preclinical models [21]. However, the enhancing effect of aluminium hydroxide on the immune response elicited by DNA vaccines, is not related to the levels of antigen expression. Rather, it seems to affect antigens after *in vivo* expression, suggesting the adjuvanticity of this substance is strictly related to the antigen delivery mechanism [22].

3.2.2 Electroporation

Among delivery systems electroporation emerged and was classified as a new adjuvant in DNA vaccination. This technique was initially designed for organ-targeted gene therapy, then the applicability shifted towards genetic vaccination. In fact it was discovered that electroporation not only allowed the increase of antigen expression in the target organ, through augmentation of gene transfer efficiency but also improved the immune response to the injected antigen [23,24]. The idea of an adjuvant effect of electroporation is in accordance with the danger model of Matzinger, who postulated the immune system can be activated by

danger signals liberated after tissue damage and stress [25]. Tissue stress may result from infections or from trauma, such as heat shock or physical injury, all of which cause necrosis and release of harmless signals able to activate APCs of the immune system. Signals derived from stressed, necrotic or virally infected cells may act as natural adjuvants responsible for DC recruitment and activation [23]. In DNA vaccination, electroporation exploits the application of electric pulses on the skeletal muscle to allow DNA transfer within recipient cells. Application of electric fields leads to pore formation on the cell membrane. This forces the DNA molecule to enter muscle cells and causes local tissue damage, which has been shown to play a role in enhancing the antigen-specific immune response [26]. On the one hand, electroporation induces a substantial increase in antigen expression and availability to resident APCs in the muscle cells, that become a long-term antigen reservoir. On the other hand, the tissue damage induced by electroporation, triggers a site-specific inflammatory reaction which enhances the antigen presentation mechanisms through activation of cells and factors belonging to the innate compartment of the immune system [24,27,28].

4. Adjuvants targeting adaptive immunity

Adjuvants that are classified as those targeting the adaptive immune response comprise a class of different molecules and substances that can differ from each other in terms of their origin, chemical nature and effect on the target of the immune system. Adaptive immunity adjuvants can be natural and synthetic products that include water-soluble adjuvants, emulsions, as well as recombinant proteins such as cytokines, chemokines, co-stimulatory molecules and T_H peptides derived from some bacterial toxins or viral components (Figure 2).

4.1 Water-soluble adjuvants and oil-in-water emulsions

QS-21 and OM-174 are water-soluble adjuvants highly suitable for vaccine development because they show good solubility in aqueous buffers. QS-21 is an acylated triterpene saponin derived from the bark of the South American tree *Quillaja saponaria* Molina [29]. This adjuvant has been tested in several animal models and in human trials with different antigens (e.g., in vaccination against malaria, influenza, HIV-1, human hepatitis B virus [HBV] and cancer). QS-21 seems to increase both T_H1 and T_H2 -type immune response and to elicit *in vivo* priming of antigen-specific CD8⁺ cytotoxic cells [30]. A semisynthetic saponin compound (GPI-0100) has also been produced. It seems to be safe and stimulates T_H1 -type immunity with cytotoxic T lymphocyte (CTL) production [31].

OM-174 is a synthetic soluble derivative of monophosphoryl lipid A (MPL), which has been successfully used in several mouse and rabbit immunisation protocols. This adjuvant appears to activate both cytolytic and antibody responses [32].

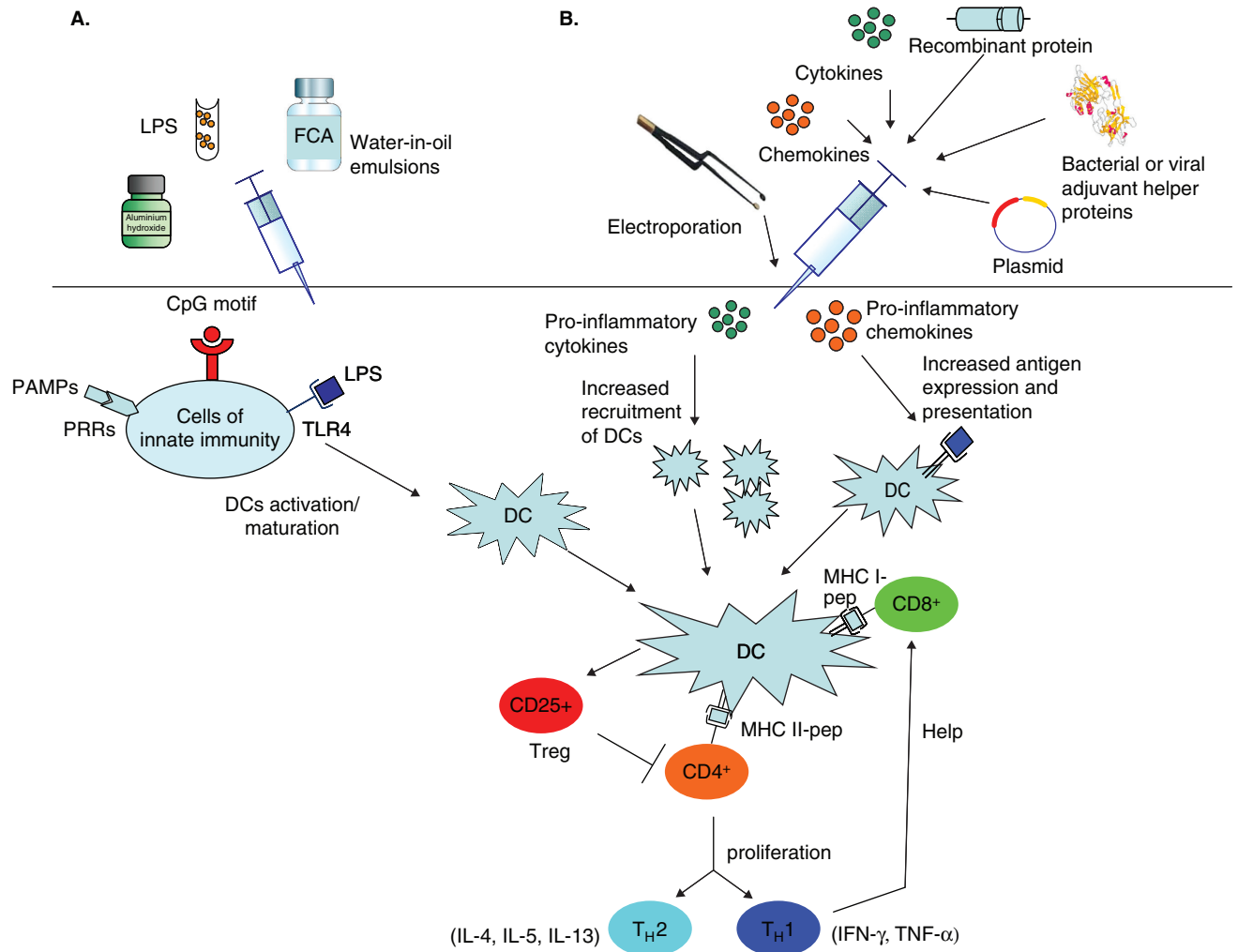


Figure 2. Mechanism of action of adjuvants. Signals elicited by adjuvants stimulating **A)** the innate arm; and **B)** the adaptive arm of the immune system converge on dendritic cells. Efficient DC recruitment, activation and Ag presentation to T cells can radically improve the immune response. CD4⁺ T lymphocytes undergo a clonal expansion into two distinct T-helper subpopulations following contacts with the MHC-peptide complex. T_H1 and T_H2 cells stimulate the humoral and cell-mediated immune response through a different cytokines expression pattern. CD8⁺ T lymphocytes are licensed by dendritic cells to become effector CTLs.

CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; FCA: Freund's complete adjuvant; LPS: Lipopolysaccharide;

PAMP: Pathogen-associated molecular pattern; PRR: Pattern recognition receptor; TLR: Toll-like receptor (TLR is a receptor family belonging to PRRs);

Treg: regulatory T cell; T_H: T helper cell.

Among oil-in-water emulsions, it is worth mentioning MF-59, as it is a highly stable adjuvant and can be used with many different antigens. It consists of small and stable droplets of squalene oil stabilised by addition of emulsifiers. This adjuvant elicits a T_H2-type functional immune response with preferential IgG1 antibody production [33]. MF-59 has been developed at Chiron and has been tested in many human vaccination trials against human papilloma virus, human cytomegalovirus, HBV and HIV-1. MF-59, AS04 with MPL and alum salt are part of already licensed vaccines.

4.2 Bacterial toxins and T helper epitopes

At present, many pathogen-derived genes encoding immune-enhancing proteins are being assessed for their

ability to stimulate the immune system when they are fused with target antigens. Modified bacterial toxins, such as anthrax and pertussis toxins, are being used in vaccination as effective carriers to deliver foreign epitopes that stimulate protective cytotoxic T lymphocyte responses in mammalian cells [34,35]. However, the ability of modified toxins to activate the host immune system does not reside only in the delivery effect exerted on the fused antigen [36].

Among vaccine adjuvants, tetanus toxin Fragment C (FrC) appeared to be a good candidate after proving capable of eliciting the production of antibodies when used as a fusion partner for foreign antigens. In the attempt to generate a vaccine against human schistosomiasis, it was found that the immunogenicity of the *Schistosoma mansoni* glutathione

S-transferase antigen increased when it was administered as genetic fusion to tetanus toxin FrC in a live Aro-attenuated vaccine strain of *Salmonella* [37]. AroA *Salmonella typhimurium* vaccine expressing the protective *Schistosoma mansoni* 28-kDa glutathione *S*-transferase (P28) as a full-length fusion to tetanus toxin FrC, protected mice from *Salmonella* infection, tetanus and schistosomiasis following a single dose of the vaccine [36]. Similar results were obtained by administering intravenously a vaccine construct consisting of multiple tandem copies of the *Schistosoma mansoni* peptide, comprising of amino acids 115 – 131 of P28 glutathione *S*-transferase as C-terminal fusion to tetanus toxin FrC in a live Aro-attenuated vaccine strain of *Salmonella* [38]. In antitumour vaccination, a portion of the tetanus toxin FrC fused with a single antigenic epitope has been demonstrated to induce a cytotoxic T cell response against tumours in vaccinated mice [39]. Similarly, in vaccination against B cell malignancies, DNA vaccines containing the idiotypic determinants of the Ig variable region provided protective immunity against lymphoma when expressed as single-chain variable fragment (sc-Fv) fused to tetanus toxin FrC [40]. Reproducible data from several published papers show that the adjuvant potency of the tetanus toxin FrC relies on a conformational sequence-dependent effect and in the presence of promiscuous T helper epitope complexes within the protein [41]. In tetanus toxin FrC, the presence of at least one T helper epitope, such as the universal p30 T helper epitope (FNNFTVSFWLRVPKVSASHLE aa 947 – 967 of the FrC), which is promiscuous for binding to several human HLA-DR molecules [42], allows activation of CD4⁺ T cells providing substantial help to B and CD8⁺ lymphocytes in the immune response against the antigen [43,44]. The p30 is a strong universally immunogenic T helper epitope; it has been demonstrated that it consists of at least three distinct overlapping helper epitopes, where each epitope is recognised in association with multiple HLA class II alleles [42]. In addition to p30, other T helper determinants with the same ability to bind to the most common HLA molecules have been identified in the region of the FrC spanning from amino acid 865 to 1315. These include the p21TT helper epitope (IREDDNNITLKLDRCNN aa 1064 – 1079) [45], the p23TT epitope (VSIDKFRIFCKALNPK aa 1084 – 1099) [45,46], the pGINGKA epitope (PGINGKAIHLVNNESE aa 916 – 932) [47], the p32TT epitope (LKFIKRYTPNNEID aa 1173 – 1188), and the pGQI epitope (GQIGNDPNRDIL aa 1273 – 1284 [45,46]. As T helper cells support both cell and humoral immunity, it seems that the use of promiscuous T helper peptides capable of activating CD4⁺ lymphocytes, may strongly enhance both types of immune response and this might explain the strong potency of the tetanus toxin FrC as a vaccine adjuvant. Another peculiarity of the tetanus toxin FrC as adjuvant for antitumour vaccination, is related to the use of a specific portion of the sequence that maintains the universal immunogenicity. However, in order to improve

the target antigen presentation, tetanus toxin FrC is restricted to a sequence that does not contain CTL epitopes recognised by the most representative MHC class I molecules [39]. Specific T-cell epitopes of FrC that are universally immunogenic, have also been widely exploited in peptide vaccination as they serve to enhance immune response, particularly against B-cell epitopes. They have been successfully used as vaccine carriers to stimulate humoral response against polysaccharide antigens when used in a string-of-beads fashion [48,49]. The string-of-beads approach is based on the concept that response to the subset of antigens and epitopes, and not the whole organism, can be sufficient for host protection. Furthermore, the availability of bio-informatics sequence analysis tools and databases useful for MHC-binding ligand prediction, can strongly help in designing new vaccines. In multi-epitope vaccination, several CTL epitopes belonging to foreign antigens can be linked to a promiscuous MHC-II binding T helper epitope, to generate a string-of-beads vaccine, with or without intervening spacers [50,51]. In several reports, vaccination with peptides or DNA constructs consisting of a single promiscuous T helper epitope fused to antigen motifs, has also proved to be effective in stimulating the immune system [52-54]; a strong immune response can be triggered when weakly immunogenic CTL epitopes are either co-injected or chemically linked to the T helper epitope.

A synthetic universal pan HLA-DR-binding T helper epitope (PADRE), which binds with high affinity to most of the common HLA-DR alleles of the human population, has been designed and demonstrated to be more potent than the promiscuous tetanus toxin p30 T helper epitope in increasing immunogenicity of CTL and B epitopes [55-57]. The sequence of PADRE has been deduced from the core sequence of the ovalbumin master T helper peptide (aa 323 – 339) and adapted for binding to the more representative human MHC class II molecules [58,59].

4.3 Cytokines, chemokines and dendritic cell-stimulating molecules

Immune response can be enhanced and modulated by molecular adjuvants such as cytokines, chemokines and co-stimulatory molecules. Depending on the type of immune response to be elicited, a specific cytokine or chemokine can be incorporated into a vaccine either as a gene or as a protein. In DNA immunisation this can be achieved through co-delivery of the antigen and the cytokine gene within the same plasmid [60]. Alternatively, the same effect can be achieved by the co-injection of two distinct plasmids coding the antigen and the cytokine molecule, whereas in peptide vaccination cytokines can be given as recombinant proteins in addition to the vaccine.

4.3.1 IL-2

One of the earliest cytokines to be incorporated into a vaccine was IL-2, a well-known T-cell growth factor included

in several immunotherapy protocols. Addition of this cytokine to a plasmid vaccination vector resulted in enhanced antibody responses in low responder mice against malaria [61] and increased production of antibodies directed against the complementary determining hypervariable region 3 of the Ig heavy chain in human B-cell lymphoma [62]. IL-2 showed clinical efficacy in the treatment of metastatic melanoma when used as an adjuvant with a peptide vaccine [63]. It also enhanced the efficacy of a DNA vaccine against a simian immunodeficiency virus when fused with the immunoglobulin Fc fragment, so increasing its half-life [64]. However, the use of IL-2 is now being limited by the emerging evidence that this cytokine can play a major role in maintaining self-tolerance and in supporting survival of CD25⁺ CD4⁺ regulatory T cells (T-regs) [65,66].

4.3.2 IL-12

IL-12 acts on T and NK cells by inducing the generation of CTL through T_H1 cell activation and IFN- γ production. The beneficial effect of IL-12 in preclinical experimental tumour models suggested the possibility of using IL-12 as an antitumour agent in clinical trials. Despite some toxicity associated with certain doses of IL-12, when administered as a drug in patients affected by melanoma and colon cancer, some clinical responses were observed; this indicates that IL-12 can be used in clinical protocols where a toxic effect of the cytokine could be acceptable in cancer therapy [67,68].

4.3.3 GM-CSF

GM-CSF was identified as the most broadly useful cytokine in inducing a variety of immune responses involving T_H1, T_H2 and CTL cells and for nearly a decade this molecule has been known to augment the immune response. The stimulatory effect is presumed to depend on the role of this molecule in the maturation and function of APCs. GM-CSF has proved to exert a chemoattractant effect on infiltrating APCs when injected into the muscle [69] and to stimulate a potent T_H-dependent immune response when administered with gp120 HIV antigen [70]. In several animal models, GM-CSF increased the tumour immunogenicity. Injection of irradiated tumour cells engineered to secrete GM-CSF resulted in the production of this cytokine by the same malignant cells, and in enhancement of their immunogenicity [71]. GM-CSF is widely used in clinical trials of active immunotherapy against cancer with autologous tumour and dendritic cell vaccines, and it has also proved to be an effective adjuvant also for protein and peptide vaccination [72]. However, despite several clinical applications of GM-CSF, the role of this cytokine is still controversial. Of particular relevance is the finding that GM-CSF is also a tumour-derived factor with immunoregulatory properties on the immune system. In fact, in mice bearing a mammary adenocarcinoma, this cytokine has proved to induce expansion of macrophages with suppressive activity on T and B cells [73]. Further investigations on this

mouse tumour model have demonstrated that GM-CSF seems to be involved in upregulation of the chemokine CCL2 that is responsible for cancer progression [74].

4.3.4 DC-stimulating molecules

Macrophage inflammatory protein (MIP1- α) and fms-like tyrosine kinase 3 ligand (Flt3-L) are, respectively, a chemotactic and growth factor capable of mobilising DCs *in vivo*. Co-delivery of these two factors together with the antigen was responsible for a significant recruitment of DCs at the injection site, resulting in potentiation of the vaccine immunogenicity and protective efficacy [75]. Flt3-L has been shown to retard tumour growth, stimulating a tumour-specific immune response in several mouse tumour models [76,77]. The gene transfer by *in vivo* electroporation resulted in Flt3-L release in blood serum, in an increase in DC number and mobilisation into the tumour site, thus potentiating *in vivo* antigen presentation [78]. Administration of this DC-specific growth factor in a murine liver metastasis model, significantly enhanced the number of DCs and infiltrating CD8⁺ and CD4⁺ lymphocytes [79].

4.3.5 Defensins

Defensins are a family of cationic antimicrobial proteins that are stored in cytoplasmic granules of neutrophils [80]. In 1999, Yang and co-workers observed the recruitment by defensins of human immature DCs and memory peripheral blood T cells [81]. The recruitment of immature DCs and memory T cells by defensins-2 and defensins-3, is mediated through the G_i-protein-coupled receptor (GiPCR) CCR6 [82,83]. α - and β -defensins contribute to innate and adaptive immunity through the production of pro-inflammatory cytokines, as well as of IL-20 and IL-18 [84-86]. They also contribute to the production of both T_H1 and T_H2 cytokines. The chemotactic activity of β -defensins towards immature DCs, also facilitates antigen uptake, processing and presentation, leading to induction of antigen-specific immune responses [87,88].

Tani *et al.* demonstrated that defensins have a potent immunoadjuvant activity, promoting antigen-specific Ig responses and tumour immunity. Administration of defensins in idiotype vaccination against lymphoma, significantly prolonged survival after the tumour challenge [89].

4.3.6 IFN- α

Among the numerous immunomodulator molecules, type I IFN represents a cytokine family with multiple biological activities. IFN- α emerged as a powerful adjuvant, being an activator of B lymphocytes that induces a strong humoral immune response. It has also been shown to stimulate an IgG2a antibody response characteristic of T_H1 immunity when administered intramuscularly with influenza virus vaccine [90,91]. Expression of IFN- α by transfected dendritic cells within brain tumours seems to enhance the antitumour efficacy of peptide-based vaccines [92].

Table 1. Reported vaccine adjuvants.

Adjuvant type	Examples	Effects	Ref.
Adjuvants targeting innate immunity			
PAMPs	LPS	↑ pro-inflammatory cytokines and chemokines (leading to maturation DCs, NK cells, B and T lymphocytes)	[4,5]
	MPL		
	Mannans		
	Bacterial DNA		
	Ds RNA	TLR9; + T _H 1 cytokines (as IFN-γ)	[7]
	CpG motif		
	CpG-DNA	↑ TLR	[3]
	FCA	Stimulation and activation DCs	[14,15]
Cochleate and PL			
Delivery systems			
Electroporation	Local tissue damage	Antigen presentation due to site-specific inflammation	[23,24]
Water-in-oil	FCA	Recruitment of APCs at the injection site	[3,18]
Mineral salt	Aluminium hydroxide	Antigen delivery mechanism	[21]
	Calcium salts		
Particulate vehicles	Microparticles	Stimulation of antigen uptake	[19]
	Liposomes		
	Virosomes		
	Immunostimulatory complexes		
Adjuvants targeting adaptive immunity			
Cytokines and chemokines	IL-2	++ CTLs	[61-63]
	IL-12	↑ CTLs; ↑ IFN-γ	[67,68]
	GM-CSF	↑ T _H immune response; attract APCs	[69-72]
	MIP1-α	↑ DCs	[75-78]
	Flt3L	↑ Chemokines	
		↑ DCs	
		↑ <i>In vivo</i> antigen presentation	[80-88]
	Defensins	↑ DCs	
Water soluble adjuvants	QS-21	↑ T _H 1; ↑ T _H 2	[29,30]
	OM-174	↑ CTLs	[32]
Oil-in-water emulsions	MF-59	↑ T _H 2	[33]
T helper peptide from modified bacterial toxins	Anthrax	↑ CTLs	[34,35]
	Pertussis		
	Tetatus toxin FrC	↑ CTLs; ↑ T _H cells	[39-44]

+ Proliferation; ↑ Upregulation; APC: Antigen-presenting cell; CTL: Cytotoxic T lymphocyte; DC: Dendritic cell; FrC: Portion of fragment C of tetanus toxin;

FCA: Freund's complete adjuvant; Flt3-L: Fms-like tyrosine kinase 3 ligand; LPS: Lipopolysaccharide; MIP1-α: Macrophage inflammatory protein;

MPL: Monophosphoryl lipid A; NK: Natural killer cell; PAMP: Pathogen-associated molecular pattern; PL: Proteoliposome; T_H: T helper cell;

TLR9: Toll-like receptor 9.

5. Conclusion

The increased knowledge of the mechanisms regulating the immune response and the recognition of the importance of signals that integrate the innate and adaptive immune systems, has sparked scientists' enthusiasm in the search for new effective adjuvants for vaccination.

The discovery of several new immune receptors and molecular factors able to activate immune cells has led to the development of a plethora of candidate adjuvants (Table 1). All these factors, substances and delivery systems are now part of the new class of vaccine adjuvants and immune potentiators. Although the

use of adjuvants in humans might be associated with local pain and a mild inflammatory response, the evaluation of the risk/benefit ratio will reflect a compromise between the requirement for a good adjuvanticity and an acceptably low level of adverse reactions in vaccination protocols.

6. Expert opinion

The new generation vaccines against tumours and infectious diseases are based on the subunit and multi-epitope approach, implying the selection of a specific target against which the immune response is directed. Although these vaccines have

gained great specificity, they lack the innate immunity signals that are indispensable for eliciting a strong immune response in the host.

There is a general consensus in designing new vaccines, in order to elicit strong inflammatory signals targeted at APCs linking the two arms of the immune system. For this reason the current trend is to combine subunit vaccines with adjuvants capable of stimulating the innate compartment of the immune system. Understanding the specific combination of innate immune receptors (PRRs) and DC subsets activated by these receptors, and how this mechanism affects the adaptive immunity, will be of great importance to develop new vaccines. The future vaccines will probably consist of antigens combined with multiple, may be as yet undiscovered, PRR ligands and factors able to activate DCs. A promising vaccination strategy for both infectious and cancer diseases, seems to be the genetic immunisation by DNA vectors coupled to innovative adjuvants.

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