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Recent progress in adjuvant discovery for peptide-based subunit vaccines

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Abbreviations: APC, antigen presenting cells; ASC, apoptosis-associated speck-like protein containing a CARD (caspase recruitment domain); BPV, bovine papillomavirus; CFA, Complete Freund's adjuvant; COMP, cartilage oligomeric matrix protein; CpG, cytosine–phosphate–guanine; CTL, cytotoxic T lymphocyte; DC, dendritic cells; DDA, dimethyl dioctadecyl ammonium bromide; DNA, deoxyribonucleic acid; DOPE, 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine; DT, diphtheria toxoid; FDA, Food and Drug Administration ; GAS, group A streptococcus; HA, hemagglutanin; HBsAg, gepatitis B surface antigen; HBV, gepatitis B virus; HIV, guman immunodeficiency virus; HPV, guman papillomavirus; HSA, human serum albumin; IFA, Incomplete Freund's adjuvant; IFN, interferon; IL, interleukin; IRIV, immunopotentiating reconstituted influenza virosomes; ISCOM, immunostimulatory complex; LCP, lipid core peptides; LPS, lipopolysaccharide; LRR, leucine-rich repeat; MAP, multiple antigenic peptide; MCC, mono-n-carboxylated chitosan; MDP, muramyl dipeptide; MHC, major histocompatibility complex; MMG, monomycoloyl glycerol; MPL, monophosphoryl lipid A; NA, neuraminidase; NK cell, natural killer cell; NLR, NOD-like receptor; NLRP3, NOD-like receptor family, pyrin domain containing 3; OVA, Ovalbumin; PAMP, pathogen associated molecular patterns; PLA, polylactic acid; PLGA, Poly (lactide-co-glycolide) acid; PRR, pathogen recognition receptors; PtBA, Poly (t-butyl)acrylate; RLR, retinoic acid-inducible gene I (RIG-I)-like receptor; SARS, severe acute respiratory syndrome; SARS-CoV, severe acute respiratory syndrome (SARS) coronavirus; TAT, trans-activating transcriptional activator; TCR, T cell receptor; Th, T helper; TLR, toll-like receptors; TMC, trimethylated chitosan; TT, tetanus toxoid; VLP, virus-like particle

Peptide-based subunit vaccines are of great interest in modern immunotherapy as they are safe, easy to produce and well defined. However, peptide antigens produce a relatively weak immune response, and thus require the use of immunostimulants (adjuvants) for optimal efficacy. Developing a safe and effective adjuvant remains a challenge for peptide-based vaccine design. Recent advances in immunology have allowed researchers to have a better understanding of the immunological implication of related diseases, which facilitates more rational design of adjuvant systems. Understanding the molecular structure of the adjuvants allows the establishment of their structure-activity relationships which is useful for the development of next-generation adjuvants. This review summarizes the current state of adjuvants development in the field of synthetic peptide-based vaccines. The structural, chemical and biological properties of adjuvants associated with their immunomodulatory effects are discussed.

Introduction

Vaccination has emerged as the most effective and economically viable medical discovery to improve public health. The use of vaccines has saved millions of lives globally. Certain diseases such as smallpox, polio and measles have almost been eradicated through vaccination.[1] Despite the remarkable success of vaccination against some types of disease, there are still remaining several infectious diseases for which vaccines are not yet available, including malaria, group A streptococcus (GAS), and human immunodeficiency virus (HIV). Traditionally, most vaccine formulations consisted of live attenuated microorganisms or inactivated microorganisms. However, problems such as unwanted host reactions, reversion to virulence (especially in immunocompromised individuals) and difficulty in culturing the pathogenic microorganisms are often associated with the traditional vaccine approach.[2] The impracticality of traditional vaccines has resulted in the emergence of subunit vaccine that contains purified antigens instead of the whole organisms. Subunit vaccines are generally safe to use as they do not possess the features of original pathogens. In the field of subunit vaccines, protein-based antigens have been investigated extensively. Nevertheless, the development of protein-based subunit vaccines has been hampered by several issues such as complexity in production, instability, and impurity.[3] In addition, the use of proteins as antigens may inadvertently stimulate an autoimmune response as observed in vaccine development for GAS infection.[4]

Other than using the whole protein, it is feasible to identify individual epitopes (such as short peptides) within these proteins that have the capacity to stimulate protective immunity (**Fig. 1**). Thus, peptide-based subunit vaccines may diminish the stimulation of an autoimmune response as they contain a specific, well-defined and synthetically produced fragment of a pathogen.

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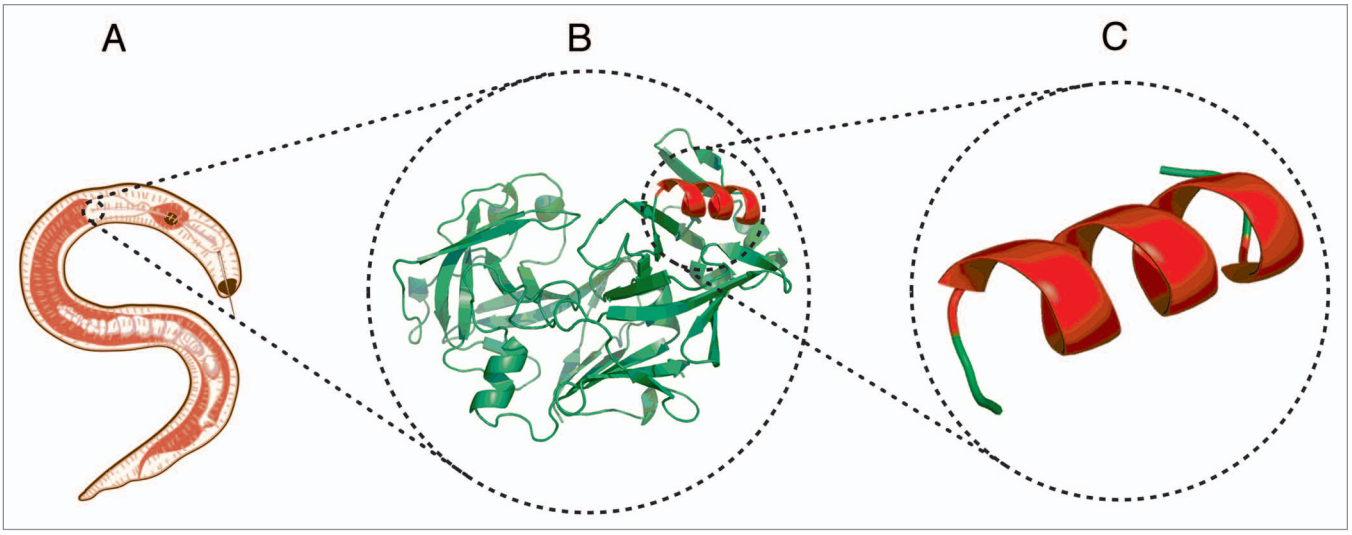

Figure 1. The evolution of vaccines: (**A**) traditional vaccine utilizing a whole pathogen, (**B**) protein-based subunit vaccine, and (**C**) peptide-based subunit vaccine.

Additionally, peptides as antigens are of great interest for development as they are able to induce very specific cell-mediated (T cells) and humoral (antibody) immunity.[5] Unfortunately, peptide-based subunit vaccines are poorly immunogenic by themselves. Thus, the inclusion of immunostimulant (adjuvant) in the vaccine formulation is necessary to induce a more potent immune response. The role of adjuvant in vaccine formulation was originally described as 'substances used in combination with a specific antigen that produced a more robust immune response than the antigen alone'.[6] Many efforts have been made toward the development of an effective adjuvant since 1970s, yet very few of them have been licensed for clinical application (alum, MF59, AS03 and AS04). In order to achieve approval for clinical use, adjuvants need to satisfy strict requirements such as: (1) stimulating a strong humoral/T-cell response; (2) govern long-term immunity; (3) being non-toxic; (4) not inducing autoimmune or allergic responses. The use of the correct adjuvant is critical for the development of synthetic peptide vaccines, because peptides undergo enzymatic degradation even more rapidly than folded protein and are unlikely to be deposited at the administration site. In contrast to protein-based vaccines, peptide antigens usually lack of T helper epitopes which need to be incorporated into the delivery system. Protein antigens can be immunogenic on their own or require a weak immunostimmulant to be immunogenic whereas peptide antigens require stronger adjuvants for effective immunization. For example, alum was found to be an effective adjuvant for protein-based vaccine candidates but was too mild to enhance immune stimulation against peptides alone.[7]

Recent advances in immunology allow the structural characterization of different adjuvants and identification of receptors that are concomitant to their biological activities. This information is useful for the establishment of the structure-activity relationship of adjuvants. The identification of pharmacophores by which different adjuvants stimulate the immune system is important for the design of safer and more potent adjuvants. The focus of this review is to elucidate the structural, chemical and biological properties of selected adjuvants in the context of peptide-based vaccine development. Additionally, the key immunological concepts that govern rational vaccine design will be discussed.

Immunostimulant and the Key Regulators of Immune Responses

The inclusion of adjuvant along with antigens in vaccine formulation is a well-established practice in experimental immunology and represents one of the best methods for developing the next generation of peptide subunit vaccines. Adjuvants can be grouped by their ability to generate immunological 'events' that are necessary to induce the desired immune response (**Fig. 2**). Since some adjuvants act by stimulating more than one immunological pathway, alternative classification by physical and chemical properties may be more useful. Adjuvants may exhibit their immunostimulatory effects via the following mechanisms: (1) providing antigen depot; (2) activation of innate immunity through pathogen recognition receptors (PRR) engagement; (3) co-stimulation of immune cells; (4) immunomodulation, e.g., maturation of Antigen Presenting Cells (APC).

All of the mechanisms involve direct or indirect stimulation of APC, particularly dendritic cells (DC). DC play a critical role in bridging the innate and the adaptive immune system by non-specific internalization and processing of the antigens which are then presented to the sequence-specific T cells. The encountered antigens may constantly be taken up by DC via pinocytosis and/or phagocytosis while their PRR act as a "detector" for infectious agents. Upon stimulation of PRR, naïve DC release various soluble mediators, such as inflammatory cytokines and type 1 interferon (IFN) as part of the innate immune response. The activated DC initiates an adaptive immune response by processing the antigens and presenting them to naïve $CD4^+$ T cells. At the same time, DC upregulate MHC class II and co-stimulatory

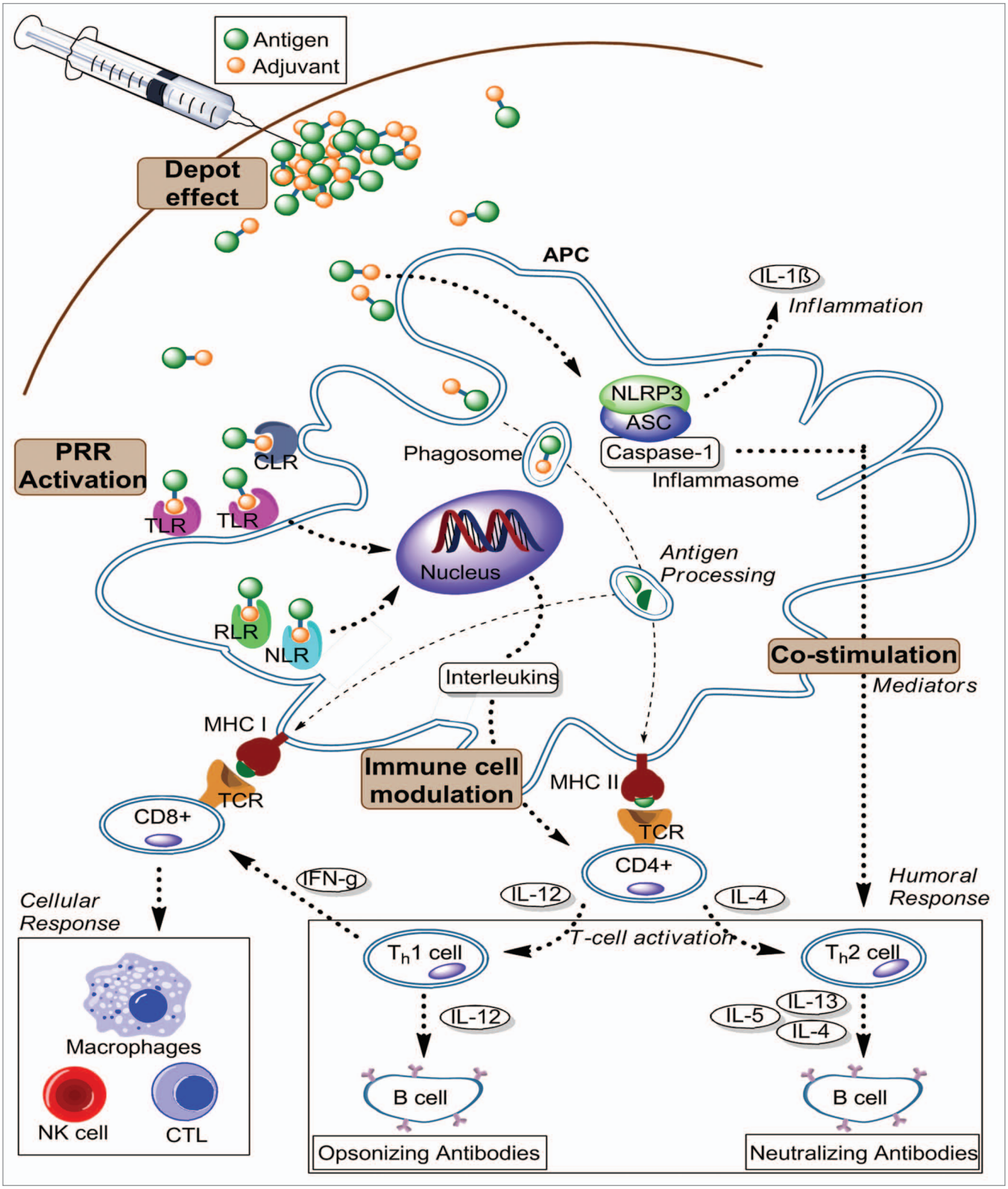

Figure 2. Schematic overview of the immunological cascade induced by adjuvants. These immunological events are essential for enhancing and directing the adaptive immune response against vaccine antigens. The responses are primarily mediated by two main types of lymphocytes, T and B cells. (APC: Antigen Presenting Cell; CTL: Cytotoxic T Lymphocyte; NK cell: Natural killer cell; PRR: Pattern recognition receptor; TLR: Toll-like receptor; RLR: retinoic acid-inducible gene I (RIG-I)-like receptor; NLR: NOD-like receptor; MHC: Major histocompatibility complex; NLRP3: NOD-like receptor family; ASC: The inflammasome adaptor; TCR: T cell receptor; CLR: C-type lectin receptors).

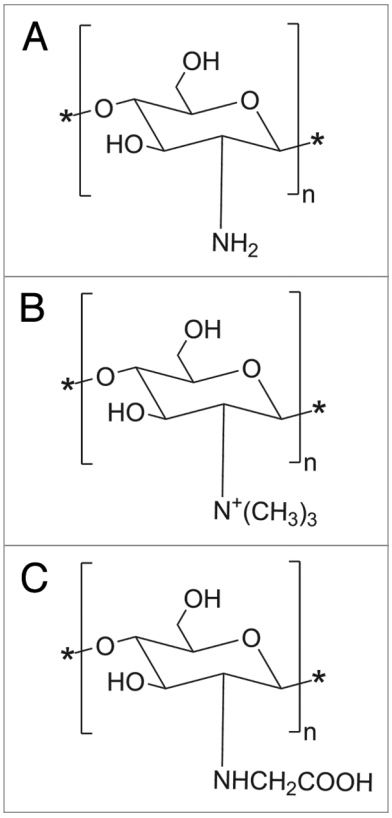

Figure 3. Chemical structure of (a) Chitosan, (b) N-trimethylated chitosan (TMC) and (c) mono-N-carboxymethyl chitosan (MCC).

molecules, inducing interactions between DC and $CD4^+$ T cells. This immunological cascade promotes optimal $CD4^+$ stimulation. However, this immunological cascade is insufficient for $CD8^+$ T cell priming, which is essential for vaccine efficacy against cancer or intracellular pathogens. Cross-presentation is required for dendritic cells to present MHC class I molecule-antigenic peptide complexes to $CD8^+$ T cells to trigger a CTL response. The detailed immunological activities required to generate this response have been extensively reviewed by Joffre and colleagues.[8]

Among the PRR, Toll-Like receptors (TLR) are the most studied receptors and are widely recognized as the main interest of modern adjuvant targeting. TLR are type 1 transmembrane proteins with an extracellular domain of interspersed leucine-rich repeat (LRR) motifs that are involved in recognition of pathogen associated molecular patterns (PAMP), such as lipopolysaccharide (LPS).[9] Engagement of PAMP with TLR will lead to the activation of mitogen-activated protein kinase pathway and NFκβ which results in regulation of pro-inflammatory cytokines (interleukin-1β and TNF-α, and chemokines). TLR activation also plays a major role in initiating adaptive immune response, for instance by directing the immune system toward Th1- and Th2-biased responses.[10] Th1 responses are mediated by the secretion of IFNγ and are thought to be responsible for killing intracellular pathogens. In contrast, Th2 responses are characterized by the secretion of IL-4, IL-5, IL-6, and IL-10, which resulted in the induction of antibodies production. It was shown that vaccine adjuvants that contained TLR ligands were able to induce T cell responses of higher avidity. This prompted extensive research to utilize the TLR ligand as a potential adjuvant. It is believed that adjuvants based on the TLR ligand should be co-delivered with the desire antigen to target the same phagosome cargo at the APC for optimal antigen presentation and subsequent stimulation of antigen-specific T cell responses.[11] Thus the ability of TLR to link innate and adaptive immune responses represents a promising approach for the design of new vaccines.

Various adjuvants execute their effect as a delivery platform for the vaccine antigens. Particulate-based adjuvants such as alum, liposomes and emulsion have been observed to enhance antigen uptake by prolongation of the exposure of antigen to the DC.[12] Two subpopulations of DC have been identified: tissue-resident (immature) DC and migratory (mature) DC. At the administration site, antigens are presented to resident DC located in non-lymphoid tissues close to the mucosal surfaces. Upon antigen encounter, tissue resident DC undergo a maturation process which initiates their migration to the lymph nodes, and thus are subsequently termed migratory DC.[13] Naïve T and B cells do not circulated at the non-lymphoid areas, including most injection sites in the body. They are usually found at secondary lymphoid organs, including the lymph nodes. Therefore, antigens need to reach the lymph nodes via migratory DC in order to be presented to the specific T cells. These types of adjuvants provide a depot formation that trap antigens at injection site, providing a gradual release of antigens to be acquired by migratory DC. Thus, these adjuvants influence the fate of vaccine antigen in time, place and concentration by sustaining the activation of antigen-specific $CD4^+$ T cells and promote uptake of the antigen at injection site. In addition, particulate adjuvants have the ability to bind antigens and form multi-molecular aggregates. The aggregation complex usually exhibits similar dimensions to pathogens, which encourages their uptake into APC via phagocytosis.[14] Hence these adjuvants may contribute to the maturation of APC.

Other adjuvants act as immunopotentiators by having a more direct impact to provoke an immune response. To act this way, adjuvants required interaction with co-stimulatory molecules on APC that contributed to the priming of T helper cells ($CD4^+$).[15] The stimulation of APC provided information to T cells that the presented antigens are proper subjects to prompt an immune response. This facilitates the recognition of antigen for further B cell proliferation and antibody production. The absence of this induction may lead to the immunological tolerance or anergy. Under physiological conditions, proteins fold into a single stable conformation while short peptides derived from a protein are usually unable to preserve their native conformation. However, retaining this native conformation is essential for the activation of B cell receptors to elicit a humoral immune response.[16] Several strategies are available to induce the desired secondary structure of peptide epitopes, including incorporation of additional flanking peptide and hydrophobic moieties such as lipopeptide or polymers.[17-19] Unlike humoral immunity, there is no requirement for the peptide epitope to adopt a specific conformation to stimulate a CTL response. However, APC must process the antigenic

peptides to produce peptides of a defined length.[20] The induction of either CTL or antibody immunity requires additional stimulation of T helper cells. This is typically achieved through the conjugation of the peptide epitope to a carrier protein, such as tetanus toxoid that includes a T-helper epitopes. Alternatively, a universal t-helper such as PADRE (AKFVAAWTLKAAA) can be incorporated into the delivery system.[21]

Adjuvants play a critical role in determining the quality and magnitude of immune response against the antigens. Different pathogens require different types of immune response to target the disease. Thus, the selection of the appropriate type of adjuvant for peptide-based vaccines is important to induce the desired antigen-specific immune response for effective vaccination.

Aluminum Salt Adjuvant

Aluminum salts are the most extensively used adjuvants in vaccine formulation and are the only licensed adjuvant for routine human vaccination by FDA. These salts, including aluminum hydroxide [$Al(OH)_3$], aluminum phosphate ($AlPO_4$) and alum precipitated materials are often referred as "alum" in the literature. Presently, there are many marketable vaccines containing alum such as diphteria, tetanus and hepatitis A and B vaccines. The adjuvanting effect of Alum was first discovered by Glenny et al. An alum-precipitated vaccine of diphtheria toxoid provided greater immune protection against challenge than a solution of diphtheria toxoid alone.[22] The adsorption between alum compounds and antigen in vaccine formulation can be predominantly achieved via electrostatic attraction and ligand exchange. It has been hypothesized that antigens need to be adsorbed to the aluminum compounds for alum to act as an effective adjuvant. However, recent findings indicated that antigen adsorption to alum-containing adjuvant may not be necessary to enhance the immune response.[23] Since alum-based adjuvants are composed of very small primary particles they are easily aggregated into a functioning unit in the vaccine formulation and contribute to the uniform distribution of antigen throughout the aluminum-containing vaccine.[24] The resulting aggregate features irregular and porous shapes ranging from 1 to 20 μm in diameter. Some in vitro studies reported that DC is more efficient at internalizing alum-adsorbed antigen through phagocytosis than macropinocitosis in a size-dependent manner.[25] The aggregation behavior of alum allows the antigen to be presented in a multivalent system which enhanced their uptake by DC and improved antigen presentation to the adaptive immune system.[26]

Alum is generally known to provoke a strong Th2 response. It has been shown that administration of ovalbumin (OVA) peptide-alum led to the Th2 effector response that generates production of IL-4, IL-5, and IL-10.[27] However, the underlying mechanism of action at molecular level is still unclear and remains a subject of ongoing investigation. One of the proposed mechanisms for the stimulation of an immune response by alum is its ability to present antigen efficiently to APC. Alum-based adjuvants may provide a depot formation at the injection site, allowing the slow release of the antigen over time. This event may lead to a longer exposure of the antigen to the APC and lymphocytes. It is proposed that antigens are

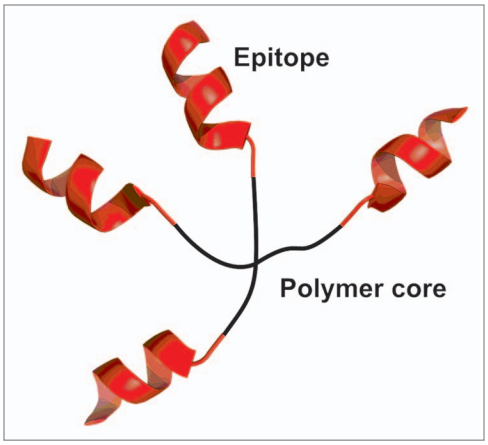

Figure 4. A schematic representation of the four-arm star polymer.

retained at the injection site due to the adsorption force with aluminum salts or by being trapped in the porous spaces within the aggregation particles.[26] Other proposed mechanisms for the adjuvanting activity of alum included the activation of complement cascade,[28] generation of granulomas at the injection site, recruitment of eosinophils and neutrophils, and induction of IL-4 secreting cells in the spleen that are responsible for optimal B cell priming. The immunostimulatory effect of alum has been associated with the activation of NLRP3 inflammasome pathway. It was demonstrated that DT/TT or OVA antigen with alum produced significantly lower antibody titers in NLRP3 deficient mice than in wild-type mice.[29] Additional in vitro studies support this data; Th2 cell priming and antibody response to OVA or human serum albumin (HSA) co-delivered with alum were also reduced in NLRP3 deficient mice.[30] Furthermore, a link between the innate inflammasome pathway and the adaptive humoral response has been found. However, other in vitro data found no difference in antibody response between NLRP3-deficient mice and wild type animals when they were injected with HSA with alum.[31] This study suggested that the NLRP3 inflammasome pathway is dispensable for alum adjuvanticity. These conflicting results may have arisen due to the use of different protocols to conduct the experiment; particularly immunization route and the method of measuring antibody titers. Later findings showed that alum provoked the secretion of uric acid, which may contribute to the activation of NLRP3.[27] Uric acid concentration increased locally after the administration of antigen mixed with alum in the peritoneum. In response to the uric acid secretion, inflammatory monocytes congregated to the injection site, recruited the antigen and process them for further T cell priming. Recently, alum was found to induce cell death that resulted in subsequent release of host cell DNA, thus inducing innate immunity.[32]

Alum has a proven record of safety. However, the use of alum as an adjuvant has several limitations. Alum failed to provide satisfactory immune response when used with certain vaccines such as influenza and typhoid fever.[33,34] Notably, alum adjuvanticity is biased toward Th2 immunity and consequently ineffective to be used for several life-threatening infections such as cancer and

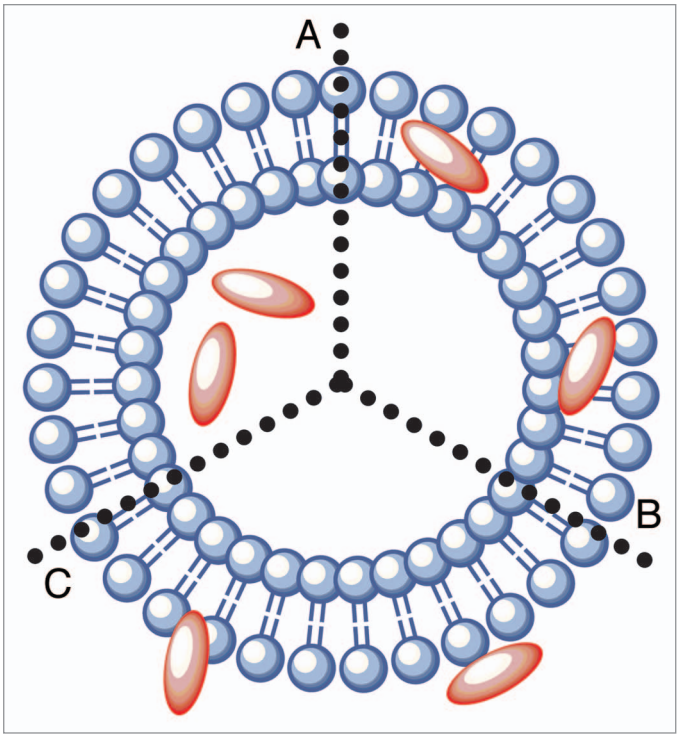

Figure 5. The mode by which antigens are incorporated into liposomes is dependent on their chemical nature. (**A**) Hydrophilic antigens can be entrapped within the aqueous core of liposomes; (**B**) hydrophobic antigens can be conjugated at the liposome surface; (**C**) ampiphilic antigens can be integrated within the phospholipid bilayer.

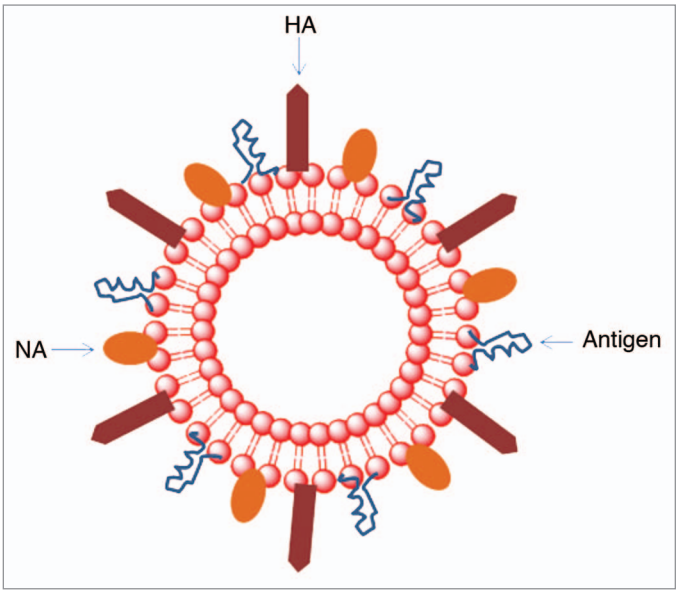

Figure 6. Virosomes are made up of a phospholipid bilayer which is similar to a liposome. This structure provides a platform to hold influenza virus surface protein hemagglutinin (HA) and neuraminidase (NA). Antigens are incorporated into the virosome system.

tuberculosis that required cytotoxic T cell response. It has been reported that alum-based adjuvants are incompatible with small peptide antigens. This is because alum may cause the peptide antigens to be proteolytically degraded more efficiently.[35] As a result, the peptide antigen may lose its ability to stimulate a protective immune response. Partial denaturation of the peptide antigen due to adsorption with alum may also contribute to this incompatibility. Some studies demonstrated that alum did not induce significant antibody titers when conjugated with peptides antigens derived from Malaria parasite and herpes simplex virus proteins.[36,37] Therefore a novel adjuvant needs to be developed to enable the production of vaccines that cannot currently be adjuvanted by alum-based vaccination strategies.

Emulsion

There are two types of emulsion used in vaccine formulation; oil-in-water and water-in-oil systems. Most of the water-in-oil emulsion adjuvants work based on delivery system that stimulates the elongation of antigen presentation. The high content of non-degradable oils in water-in-oil formulations is thought to establish an antigen depot at the injection site. The binding force occurred between the antigen and droplet surface enable them to have a longer retention time at the injection site. Moreover, water-in-oil adjuvants are able to protect peptides against enzymatic degradation as emulsion sequesters peptides from peptidase in the body fluids.[38] Complete Freund's adjuvant (CFA) is water-in-oil emulsion that additionally contains heat-killed mycobacteria. CFA has been considered to be the "gold standard" adjuvant in vaccine research as it is more effective than alum and other commercially available adjuvants. An extensive body of research has used CFA as an adjuvant in peptide-based subunit vaccine development. For example, initial study by Frazer and coworkers in the development of therapeutic HPV peptide-based vaccines has utilized CFA to induce immune response against a tumor-derived peptide.[39] Nevertheless, the usage of CFA in vaccine formulation is associated with undesirable side effects such as weight loss, leucocytosis, and granulomatous peritonitis in mice.[40] The side-effects were still observed even when the mice were injected with a low dose of CFA. The undesirable toxicity of CFA led to the development of incomplete Freund's adjuvant (IFA), which excludes the heat-killed mycobacteria. IFA has an improved safety profile but was found to be a relatively poor adjuvant. Modified versions of IFA have been developed: e.g., Montanide ISA 51 (containing mannide monooleate) and Montanide ISA 720 (containing squalene). It has been demonstrated that the use of Montanide ISA 51 in combination with a cocktail of HIV-derived peptide antigen (contain mixture of B and T cell peptide epitopes) enhanced the immunogenicity of the antigenic peptide.[41] However, the vaccine candidate failed to meet safety requirement in clinical trials. Healthy adults that developed higher antibody response were also more likely to produce severe systemic or local adverse reactions. When Montanide ISA 720 was delivered with a malaria vaccine candidate (modified hepatitis B virus core particle (HBc) bearing peptide epitopes derived from circumsporozoite protein (CS) of *Plasmodium*

falciparum), it was found to induce a higher humoral response than alum-derived adjuvant, Alhydrogel.[42] A phase 1 clinical trial of Montanide ISA 720 formulated with peptide antigen showed its ability to stimulate a potent immune response, but a significant number of local reactions such as granuloma, tenderness and erythema were also observed.[43] Recently, a new water-in-oil adjuvant formulation, dubbed "NH_2" has been developed and is comprised of mineral oil and sorbitan monooleate. NH_2 was extensively used as an adjuvant for peptide-based cancer vaccination and outperformed Montanide ISA 51 in the induction of a cellular immune response in mice.[44] The efficacy of the peptide-based vaccine adjuvanted by NH_2 was confirmed in phase-1 clinical trials with advanced cancer patients.

Oil-in-water emulsions are generally considered to be safer than water-in-oil emulsion. MF59 is a representative of oil-in-water type of emulsions that are composed of squalene. MF59 is licensed in Europe as the adjuvant component of influenza vaccines.[45] In contrast to water-in-oil emulsions, MF59 did not provide a long-lived depot at the injection site. Immunofluorescence analysis showed that only 10% of radiolabeled squalene was present at the injection site 6 h after injection.[46] It is proposed that the key element for the adjuvanticity of MF59 is the induction of chemokine secretion at the injection site, resulting in antigen uptake by monocytes.[47] This stimulated the differentiation of monocytes into DC, facilitating their migration to the local lymph nodes where the adaptive immune response was induced against the vaccine antigen. MF59 was shown to induce protective immunity for Alzheimer disease when delivered with full-length amyloid-β peptide [Abeta(42)].[48] The resultant anti-Abeta42 antibody neutralized the cytotoxicity of Abeta42, and higher antibody titers were produced with multiple immunizations. Another oil-in-water emulsion-based adjuvant is AS03, which composed of α-tocopherol, squalene and polysorbate 80. This adjuvant was shown to trigger an equivalent immune response at a 4-fold lower dose than non-adjuvanted antigen.[49] It was reported that AS03 exhibit their adjuvanticity by enhancing antigen migration to lymph node and activating the innate immune system.[50] The immunostimulatory activity of AS03 is associated with the presence of α-tocopherol in their composition. α-Tocopherol is a form of Vitamin E that is known to have immunomodulating effects, including counteracting age-related impairment of naïve T cells and neutrophils.[51] Both the MF59 and AS03 have been extensively used and licensed in Europe as an adjuvant for protein-based subunit vaccine against pandemic influenza. Unfortunately, AS03 has not been studied as an adjuvant for synthetic peptide-based vaccines; even though, peptide epitope derived from influenza protein such as NP_{373}/NP_{458} (fragment from nucleoprotein),[52] synthetic M2e peptide[53] and influenza M1(58-66) peptide[54] were recently discovered.

Polymeric Particles

Polymeric nanoparticle- and microparticle-based adjuvant systems have been widely explored in vaccine formulation.[3] The most common polymers are polylactic acid (PLA), chitosan, poly (lactide-co-glycolide) acid (PLGA), polypeptides, polysterene, polyethylene glycol and their copolymers. The copolymers can serve as antigen reservoirs, allowing a longer release of the antigen over time. The antigen is usually entrapped or adsorbed on the surface of the polymeric particles to create a vaccine formulation. Among them, PLGA is one of the most widely investigated adjuvant for controlled and effective single-dose delivery of vaccine antigens, including synthetic peptides. It has been observed that PLGA polyesters degrade to produce lactic and glycolic acids in vivo, allowing the gradual and continuous release of encapsulated immunogens that promote constant stimulation of APC, thus eliminate the need of multiple immunizations.[55] In addition, PLGA are acquired by DC in culture via phagocytosis which allows further biological processing of the carried antigen.[56] A study conducted by Sharp and coworkers showed that PLGA enhanced IL-1β secretion which consequently triggered caspase-1 activation.[14] This event led to the activation of NALP3 inflammasome that contributes to the induction of

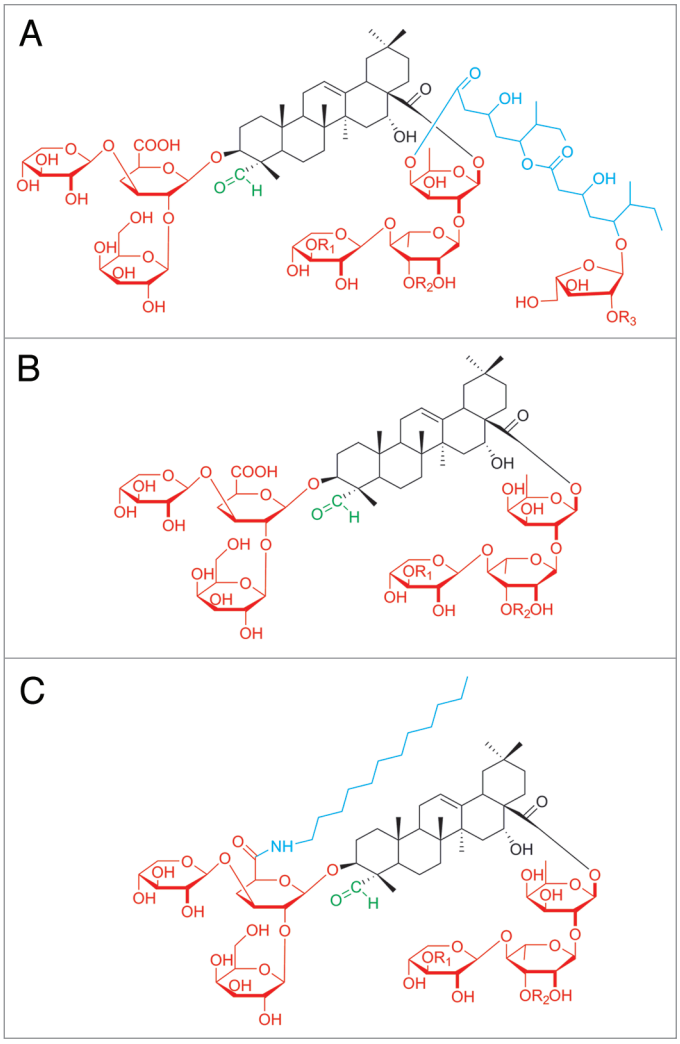

Figure 7. Chemical structure of (**A**) quillaja saponin, (**B**) deacylated saponins, and (**C**) GPI-0100. The lipophilic chain (blue) was mainly responsible for the delivery of antigen, the carbohydrate residues (red) enhanced targeting to the immune cells and the aldehyde group (green) was important for co-stimulation activity.

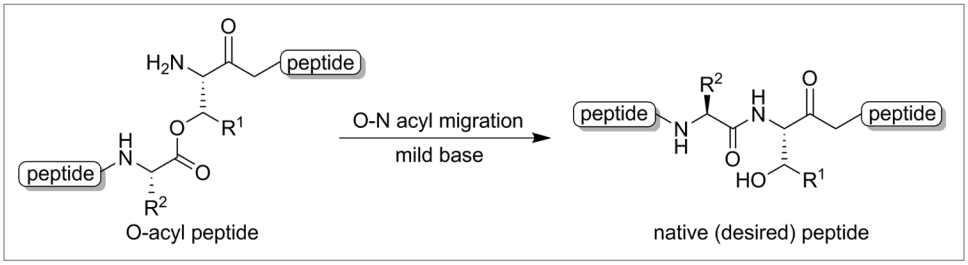

Figure 8. O-N intramolecular acyl migration reactions

adaptive immune responses. Although the presence of a TLR agonist was required to induce the release of IL-1β in vitro, injection of PLGA particles in the absence of a TLR agonist was still able to promote IL-1β secretion. Schwendeman and coworkers have employed a novel formulation of PLGA with magnesium carbonate to improve the delivery of hCG peptide antigens for contraceptive vaccine.[55] Encapsulation of hCG peptide antigen to this new formulation was found to induce a strong immune response after a single-dose immunization in rabbit. PLGA also showed promising result as an adjuvant for peptide-based cancer vaccination. A tumor antigenic peptide encapsulated with PLGA nanoparticles was found to stimulate a strong CTL response in vivo, even at the lower dose of antigen than the IFA-adjuvanted formulation.[57] The use of PLGA microspheres as an adjuvant has reached clinical evaluation for a synthetic HIV peptide vaccine that is employed as an oral immunization strategy.[58] However, in the phase I clinical study PLGA was unable to induce a substantial immune response when administered orally.

Chitosan (and its derivatives) (**Fig. 3**) is another polymer that attracts great interest in its potential application as a vaccine adjuvant. Chitosan can be obtained via alkaline deacetylation of chitin, a linear β-(1–4)-linked copolymer of D-glucosamine and N-acetyl-D-glucosamine. The chemical versatility of chitosan is dependent on the reactivity of its amine groups. Chitosan as an adjuvant has been extensively investigated as an adjuvant with the aim of developing an anti-GnRH peptide vaccine. Conventional strategies for anti-GnRH vaccination are predominantly based on bonding of GnRH as a hapten to a highly immunogenic carrier protein such as bovine albumin or tetanus toxin. However, the immune responses were primarily directed against the carrier protein instead of the desired antigen. Thus, a peptide-based vaccine was designed to overcome this problem. Formulations of anti-GnRH peptide antigen with different types of chitosan induced high levels of IgG isotype 1 production in rats compared with immunization with CFA.[59] Chitosan with the highest acetylation degree was able to induce an immune response mediated by IgG isotype 2a. Chitosan has also been a popular choice as a delivery platform for mucosal vaccination due to its mucoadhesive properties. Mannosylation of chitosan was reported to further augment its efficacy for mucosal vaccine delivery.[60] However, chitosan has poor solubility that may impair its effectiveness in peptide-based immunotherapy. To increase its water-solubility, various chitosan derivatives such as N-trimethylated chitosan (TMC), mono-n-carboxylated chitosan (MCC) and glycated chitosan (GC) were developed as potential adjuvants.[61]

One of the latest polymer-adjuvant innovations is the development of dendritic polymers and branched polymers with star-like topologies. The dendrimers are composed of repeating units of polymers stemming from a central core. The antigenic molecules can be conjugated at the end of the branching polymers that allows the presentation of multiple epitopes.[62] One example of this approach is the development of a dendritic poly (t-butyl)acrylate (PtBA) polymer that acts as an adjuvant for a GAS vaccine (**Fig. 4**).[18,63,64] The peptide epitope, J14 (GAS M protein derived peptide) conjugated to this polymer readily self-assembles in an aqueous environment to form nanoparticles. The PtBA-antigenic-peptide conjugates induced high antibody titers in mice. The application of PtBA-based dendritic polymers is not only promising for the development of prophylactic vaccines but may also be effective for therapeutic vaccination. The conjugation of a peptide antigen derived from human papillomavirus (HPV) E7 protein to this star polymer system has also been investigated to develop a vaccine candidate against HPV-related cancers.[65] As a result, this vaccine candidate was found to reduce tumor growth and eradicate E7-expressing TC-1 tumors in mice after a single-dose immunization. Interestingly, when acrylate polymer was administered as physical mixture with peptide antigens it failed to induce any immune response.[18]

Parameters such as the size and surface charge of the polymeric particles play a crucial role in their interactions with APCs. Kanchan and Panda demonstrated that PLA-HBsAg nanoparticles (200–600 nm) were taken up by macrophages more efficiently than their microparticle (2–8 μm) counterparts.[66] Additionally, nanoparticles were found to promote higher levels of IFNγ secretion, upregulation of MHC class I molecules, and were biased toward a Th1 immune response. PLGA nanoparticles (300 nm) incorporating a hepatitis B virus peptide antigen produced a strong cell-mediated immune response with a predominant IFNγ profile.[67] Conversely, immunization with microparticles was associated with IL-4 production, upregulation of MHC class II molecules and favored a Th2-biased immune response. In general, charged polymeric particles present antigens more effectively through electrostatic interactions with antigens. A formulation of HIV-1 peptide antigen adsorbed on the surface of anionic PLG microparticles significantly enhanced the immune response.[68] The availability of a variety of polymeric structures with a documented safety profile and flexible chemical modification options makes this system particularly attractive for peptide-based vaccine delivery system.

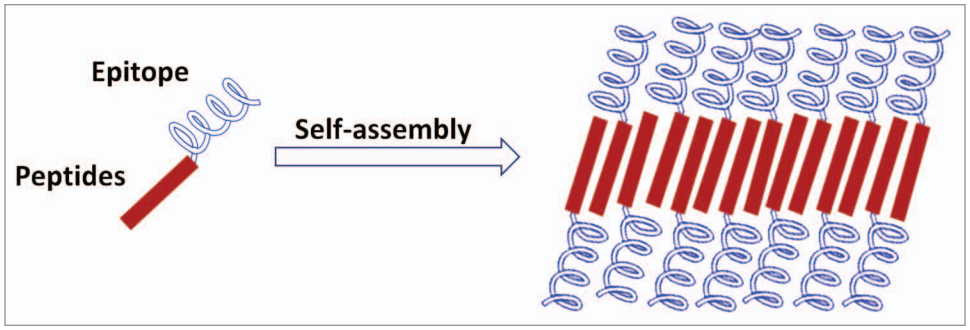

Figure 9. Schematic display of a repetitive antigen in a self-assembling peptide adjuvant system.

Liposomes

Liposomes, lipid bilayers composed of natural or synthetic phospholipids that surround an aqueous core, have been studied extensively for vaccine delivery. In vaccine formulation, the antigenic peptide determinant can be encapsulated within the aqueous core, integrated within the lipid bilayer, or attached to the outer surface of the liposomes (**Fig. 5**). Liposomes can efficiently protect the immunogenic peptide from enzymatic degradation, are easily altered to obtain optimum presentation of the antigen, and can be efficiently taken up by APCs due to their particulate nature.[69] Liposome-antigen complexes induced a significantly higher cellular immune response compared with the use of alum-antigen complexes following subcutaneous vaccination in mice.[70] Liposomal formulation with peptide-based hepatitis C vaccine (HCV) induced a strong CTL response.[71] Peptide based antigens are often favorably adsorbed to the surface of liposome in the formulation. CTL peptide epitopes derived from severe acute respiratory syndrome (SARS) coronavirus (SARS-CoV) adsorbed to the liposomal surface induced effective CTL immune responses in mice.[72] Alternatively, the peptide antigen could be conjugated to lipid moieties and anchored to the liposome.[73] The lipid moieties themselves may also be TLR 2 or TLR 4 agonists and may contribute to the enhancement of the immunogenicity of this liposome-based adjuvant. Although the mechanism of action of liposomes is not completely understood, increased retention time of antigen at the injection site, and enhancing antigen uptake by macrophages are the primary explanations for their adjuvanting role. Cationic liposomes are believed to provide an ideal platform for antigen delivery because they enhance antigen uptake and presentation to the adaptive immune system.[74] This may be due to the present of abundant anionic group on the cell membrane of APCs, which allows efficient chemical attraction with the cationic liposomes.

In addition to charge, liposome composition and size also contribute to the adjuvanticity of liposomes. Inclusion of a fusogenic lipid such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE) within the lipid bilayers induced a superior IgG2a response against OVA peptide, which was indicative of a generating a Th1-biased response.[75] Since liposome uptake occurs via the endocytosis route, size plays a crucial role in determining the immune response. Jamie and colleagues found that larger liposomes (~980 nm) into which the influenza vaccine was incorporated induced a high level of IFN-γ and IgG2a secretion, indicative of a Th1-biased response. In contrast, smaller liposomes (~250 nm) enhanced the Th2-biased response. Thus, larger liposome complexes conferred greater protection against influenza virus challenge in ferrets.[76]

Recent advances in liposome formulation include the incorporation TLR agonist moieties.[69] Inclusion of MPL in cationic dimethyl dioctadecyl ammonium (DDA) liposomes stimulated higher levels of proinflammatory cytokines and chemokines than alum.[77] Furthermore, DDA liposomes formulated with monomycoloyl glycerol (MMG, an apolar lipid derived from the mycobacterial cell wall) were also studied. These complexes were shown to induce a cellular immune response in TLR2/4 knockout mice.[78] This suggest that the these particular TLRs do not play a role in the immunostimulatory effect of DDA liposome:MMG complexes. Another type of liposomal adjuvant is the DiC14-amidine liposomes. DiC14-amidine cationic liposomes were recently shown to promote Th1 responses when mixed with antigen. The presence of DiC14-amidine in the liposome allows them to stimulate the dendritic cells via TLR4 receptors, which resulted in the activation of MyD88 intracellular pathway.[79] Additionally, mannose receptor has been exploited to target APC oriented delivery system. Thomann and coworkers incorporated a mannosylated ligand to the liposome-TLR ligand construct to target the antigen presenting cells.[80] As a result, the mannose-targeted liposomes co-delivered with ErbB2 (protein expressed by variety of tumor cell lines) CTL peptide epitopes displayed high therapeutic efficiency against tumor.[80] Additionally, cell-penetrating peptides such as trans-activating transcriptional activator (TAT) peptide provide promising inclusion in the liposome system to enhance its immunogenicity as observed by Torchilin.[81]

These examples showed that liposome-based systems are promising as effective vaccine adjuvants. The versatility of liposomes in their availability for conjugation with different types of antigen or other immunostimulants offer unique advantages in peptide-based vaccine development.

Virus Like-Particles (VLP) and Virosomes

Virus-like particles (VLP) are composed of several recombinant viral structural proteins that are self-assembled to mimic the conformation of native viruses. VLP are theoretically safe as they lack viral genetic material. VLP have the ideal size (20–100

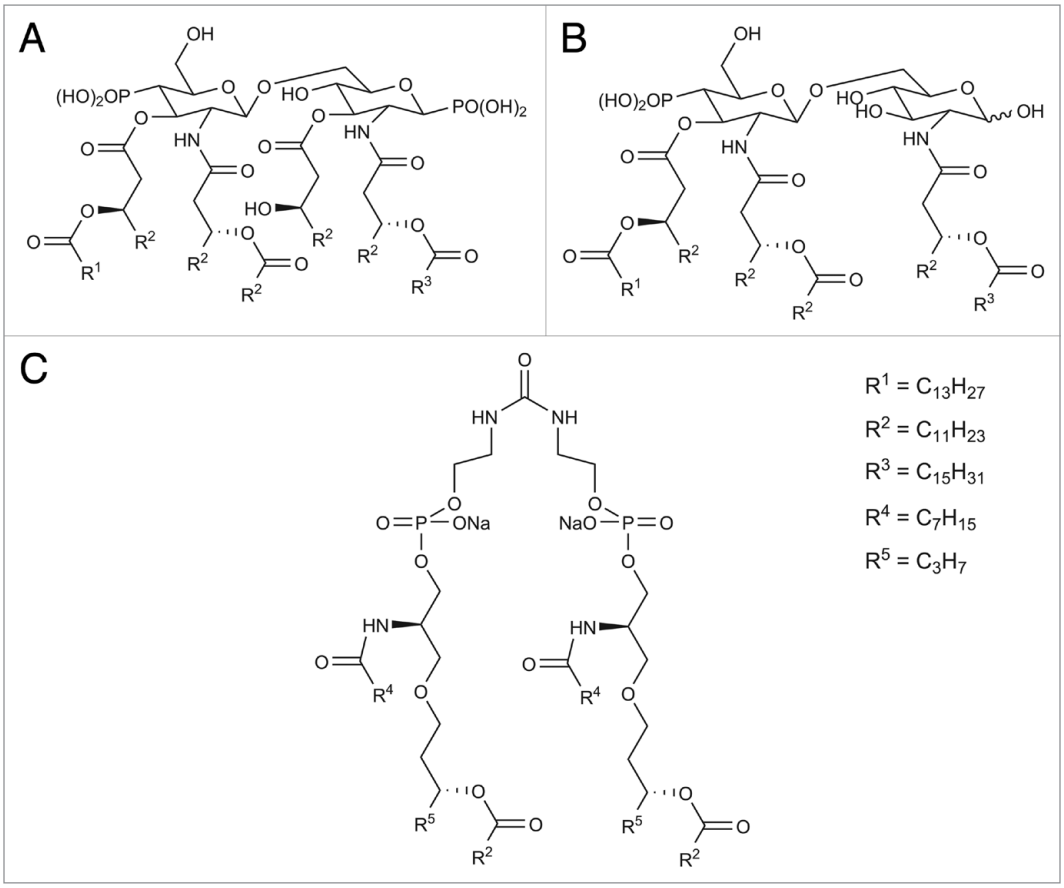

Figure 10. (**A**) Chemical structure of lipid A, (**B**) chemical structure of MPL, and (**C**) and its analog: ER-803022.

nm in diameter) to be efficiently taken up by key immune cells (e.g., DC or macrophages). VLPs also stimulated an efficient CTL response without the need of carrying any genetic information.[82] This is because recombinant VLP may gain access to the cytosol, which allows them to be internalized via endogenous processing pathways within APC, thus activating antigen-specific $CD8^+$ CTL through an event called cross-presentation. This is one of the key factors that make VLP preferable for use as a vector for antigen to stimulate the desired immune response. The particulate nature of VLP allows them to be taken up by APC to provide a long lasting CTL response. Studies of conformation behavior showed that both hepatitis B virus (HBV) core particles and parvovirus VLP exhibit an ordered and repetitive epitope presentation that is useful to overcome B cell tolerance.[83] The antigenic peptides can be incorporated with VLP either by recombination (genetically fused the encoded gene to the formed VLP) or chemical coupling.[84] For example, Pejadar-Gaddy and colleagues constructed bovine papillomavirus (BPV) VLP that were chemically conjugated with a synthetic derivative of human mucin-1 (MUC1) peptide.[85] The subcutaneous administration of this VLP-based vaccine in MUC1 transgenic mice triggered a robust activation of DC and led to the presentation of antigen to both MHC class I and II. This study validated the capabilities of the VLP adjuvant in inducing humoral and cellular immune response. Vaccines-based on VLPs that are currently approved for commercial use are Gardasil and Cervarix. These vaccines are used to provide protection against cervical cancer caused by human papilloma virus (HPV).[86,87]

Virosomes are composed of reconstituted viral envelopes in the absence of a viral genome. Unlike VLPs, virosomes have liposomal components that have viral envelope protein attached in their lipid membrane (**Fig. 6**). Virosomes were constructed using a short-chain phospholipid solubilization and reconstitution method.[88] Antigens were integrated with the virosome by several methods: (1) direct fusion into the membrane; (2) adsorption to the virosomal surface; (3) chemical conjugation to the lipids; and (4) encapsulation within the vesicle. The majority of virosomes that are currently under investigation are based on the reconstitution of influenza virus envelopes, which are called immunopotentiating reconstituted influenza virosomes (IRIV). IRIV contain influenza virus-derived proteins hemagglutanin (HA) and neuraminidase (NA) intercalated in the surface of the phospholipid bilayer. HA is one of the key features of the IRIV system because they can mediate membrane fusion activity.[89] HA binds to monosaccharide sialic acid, which is present on the surface of the APC. This interaction allows IRIV attachment to the APC, which leads to further uptake through endocytosis. The cell begins to digest the content of endosome upon acidification of HA, transforming into endolysosome. NA possesses enzymatic activity that enables the virosome to escape from endolysosome.

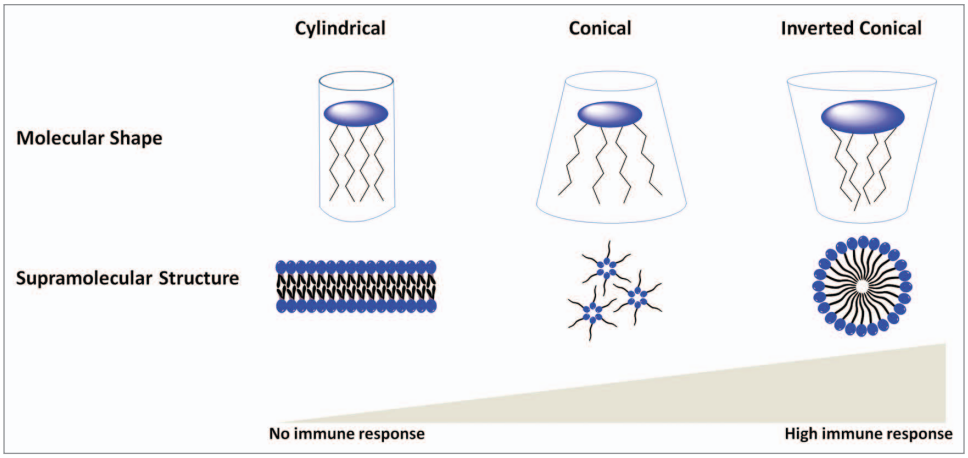

Figure 11. The correlation between supramolecular structures of lipidic chain of Lipid A (from different origins) and their effect on biological activity.

This process allows the release of the antigen into the cytoplasm. Thus, it is believed that virosomes can induce a strong MHC I-restricted CTL response through the delivery of antigen to the APC cytosol, enabling cross-presentation.[90] This phenomenon provides useful insight toward the development of virosome as an adjuvant for cancer immunotherapy.[91] In addition, a fraction of the antigen is still degraded within the endosome, allowing them to be presented to MHC class II molecules and resulting in stimulation of the $CD4^+$ immune response.[90] Moreover, the particulate forms of IRIV enable them to present the antigen to the immune system in a repetitive array to further enhance the immune response.[92] The potential use of virosomes as an adjuvant has been investigated for peptide based vaccines against numerous diseases including malaria,[93] hepatitis C,[94] and melanoma.[95] So far, virosome formulations with synthetic peptides derived from *Plasmodium falciparum* have undergone stage I clinical trials for anti-malaria vaccination.[96] These virosomal malaria vaccines were found to be safe and elicit an appropriate immune response against malaria.

Iscom, Iscomatrix, and Saponins

Immunostimulatory complex (ISCOM) is antigen-containing cage like structure (~40 nm) that is composed of cholesterol, phospholipids and saponin. In ISCOM vaccines, the antigen is incorporated into the structure during the construction process. ISCOMATRIX is a proprietary preformed adjuvant based on ISCOM that is mixed with antigen prior to delivery.[97] ISCOM/ISCOMATRIX adjuvanticity is derived from APC recruitment and activation, prolonged antigen presentation to drain lymph nodes, and inductions of CD8+ cross presentation.[98] A study conducted by Ebert and coworkers showed that the synthetic peptide antigen derived from NYESO-1 formulated with ISCOMATRIX was successfully taken up by DC and cross-presented to MHC I cells, producing a potent T cell immunity.[99]

Quillaja (and its derivatives) are the most common saponins used in ISCOM systems.[100] Quillaja saponins possess a triterpene unit with an aldehyde moiety and two oligosaccharide chains (**Fig. 7A**).[101,102] One of the oligosaccharides is acylated by two repeating units of lipophilic aliphatic acids linked by an ether bond to a fucose. The particulate nature of ISCOM allows them to be efficiently taken up by APCs via endocytosis pathways. The oligosaccharide chain of saponins can mediate their uptake by targeting DEC-205 (a macrophage mannose receptor family of c-type lectin endocytic receptors) on the APCs surface that may result in higher uptake and more competent presentation of antigens to T cells.[103] The aldehyde group plays a critical role in stimulating a Th1-biased immune response by forming an imine (Schiff base) with amino groups on T cell receptors to provide a co-stimulatory signal for T cell activation.[104] The lipophilic acyl side-chain appears to enhance the ability of quillaja saponins to stimulate CTL production, yet al.o contribute to the quillaja saponins' toxicity and instability under physiological conditions.[101,104] Although deacylated saponins (**Fig. 7B**) were proven to be less toxic and capable of stimulating a Th2-biased immune response, they failed to induce Th1 or CTL immune responses.[105] Thus, degradation of quillaja saponins changed the type of stimulated immune response.

To overcome the limitation of quillaja saponins, a derivative called GPI-0100, with a lipophilic moiety (dodecylamide) bound to the glucuronic acid residue, has been constructed (**Fig. 7C**). GPI-0100 was stable, safer than unmodified quillaja saponins, and capable of stimulating a Th1-biased immune response and CTL production.[104] It is believed that the stability of GPI-0100 results from resistance of the dodecylamide moiety to hydrolysis.[104] It was reported that a formulation of peptide-based cancer antigen (MUC1 peptide) with GPI-0100 was superior to quillaja saponins in producing a T cell response, and IgM and IgG antibodies.[106]

Self-Assembling Peptides

Biomaterial constructed from self-assembled peptides received great attention as potential vaccine adjuvants. The constructed biomaterials could either be in the form of tubular, fibrillar or spherical nanostructure by utilizing the known aggregation properties (e.g., helical, β-sheet, etc.) of the selected peptides. Self-assembling peptides provided several advantages including

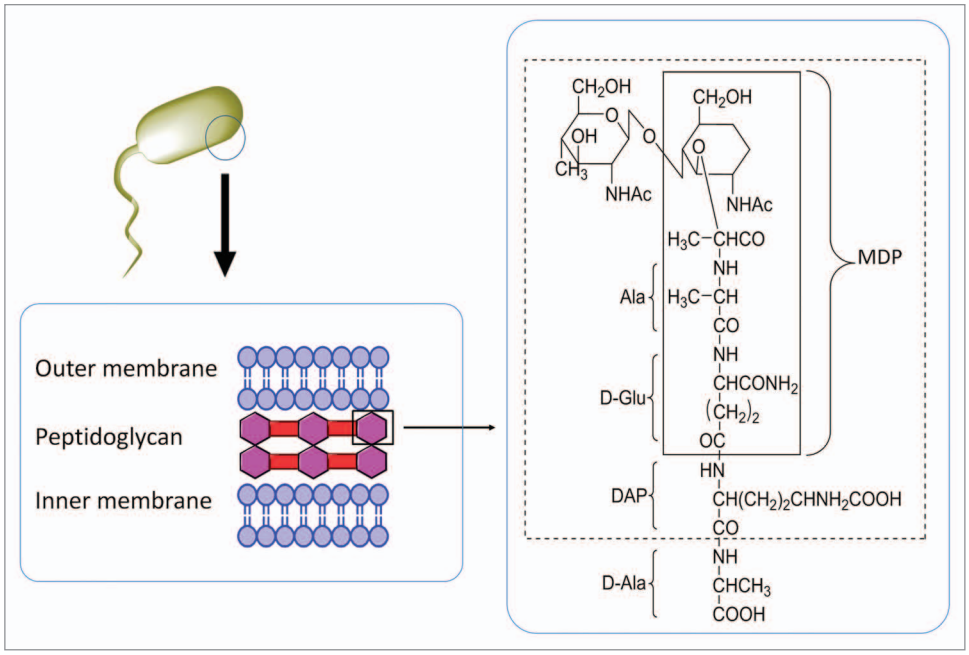

Figure 12. A schematic presentation of muramyl dipeptide (MDP) structure that is derived from a Gram-negative bacterial cell-wall component.

multivalency, biological compatibility, multifunctionality, synthetic definition, molecular specificity and allow control over the nanoscale positioning of antigens.[107]

Burkhard and colleagues have used the pentameric coiled coil oligomerization domain derived from cartilage oligomeric matrix protein (COMP)[108] and a de novo minimal trimeric coiled-coil oligomerization domain[109] to construct self-assembling peptides as a multiple antigen-display platform. Burkhard's group have exploited this system to present a B cell peptide epitope $(DPPPPNPN)_2D$ derived from malaria parasite *Plasmodium berghei* circumsporozoite protein.[110] It was demonstrated that the vaccine formulation successfully induced high antibody titers and conferred durable immune protection. Most of the mice were protected against the parasite challenge for up to 6 mo. This self-assembled peptide-based adjuvant conjugated to $(DPPPPNPN)_2D$ is currently under pre-clinical evaluation in the USA. Additionally, the same research group has also used this system against the severe acute respiratory syndrome (SARS) virus.[111] The antigen tested was HCR1, an α-helical coiled-coil B cell epitope derived from the SARS S protein. The antigen-peptide conjugate self-assembled to form 25 nm nanoparticles and successfully displayed multiple copies of the HRC1 epitope on their surface. Additionally, circular dichroism analysis demonstrated that this system was able to maintain the α-helical conformation of the epitope. The conjugate elicited a specific immune response, with antisera exhibiting neutralization activity against SARS virus in an in vitro infection inhibition assay.[111]

Another self-assembly peptide that is currently under intensive investigation is Q11 (QQKFQFQFEQQ). Q11 is an established fibrilizing peptide that spontaneously self-assembles to form a β-sheet fibrillar network. Rudra and coworkers have recently used Q11 as a platform for antigen delivery.[112] They found that Q11 peptide coupled to a model antigen (OVA peptide) stimulated higher IgG titers than a formulation of the antigen with CFA. Recently, they also conjugated the Q11 peptide to a malaria peptide epitope $(NANP)_3$ derived from *Plasmodium falciparum* circumsporozoite (CS) protein.[113] The $(NANP)_3$-Q11 formulation induced a high antibody titer which lasted up to 40 wk in mice without requiring an additional adjuvant. They found that the antibody response is dependent on $CD4^+$ T cells and the MyD88 pathway. Their study validated that the Q11 peptide is not immunogenic by itself, even in the presence of CFA.

It was also reported that the isopeptide derivative could undergo self-assembly to form fibril-like formation. Toth and coworkers developed an isopeptide using the application of an O-N intramolecular acyl migration strategy (**Fig. 8**).[114] The isopeptide construct was conjugated to the antigenic peptide as a single unit to produce a 'self-adjuvanting vaccine'. They found that the vaccine candidate adopted a β-sheet conformation and aggregated to form fibrils in a pH dependent manner. This discovery provides useful insight into the development of a safe vaccine adjuvant system since it can be produced in a highly controlled manner.

In light of the application of self-assembled peptides as vaccine adjuvants, most studies have demonstrated that their adjuvanticity effects are primarily dominated by the presentation of the epitope on their surface while maintaining the epitope native conformation (**Fig. 9**). Vaccine formulation using self-assembly peptides as adjuvants is a promising approach for the development of next generation peptide-based subunit vaccines as they permit native antigen conformation which is crucial for B cell activation.

Lipopolysaccharides (LPS) and Other Adjuvants Derived from Bacteria

Lipopolysaccharides (LPS) are the outer membrane surface components present in Gram-negative bacteria. They are composed of hydrophilic polysaccharide and a lipophilic phospholipid (lipid A). Lipid A represents the key immunoreactivity components of LPS (**Fig. 10A**). Lipid A is recognized by TLR4 and directs DC toward Th1 immunity.[115] However, the use of lipid A as an adjuvant is often associated with high toxicity. The elimination of the phosphate group and 3-O-deacylation of lipid A yields monophosphoryl lipid A (MPL), which is less toxic yet retains its adjuvant properties (**Fig. 10B**).[116,117] It has been postulated that the β (1–6) diglucosamine backbone and the six hydrophobic chains of MPL are strongly associated with their pharmacophore activity.[116] The amphiphillic properties of MPL allow the formation of supramolecular aggregates in an aqueous environment. The aggregated MPL can be transported to the targeted immune cell membranes by LPS-binding protein. The lipidic chain of MPL can diffuse through the cell membrane and promote TLR-4 activation.[118] However, only conical lipid molecules that form cubic inverted aggregate structures conferred immunostimulatory activity, while cylindrical lipid molecules that bias toward lamellar-like aggregate structures provided low or no immunostimulatory activity (**Fig. 11**).[119] The lipophilic molecule ER-803022, a synthetic analog of MPL, adopts a conical shape (cubic inverted structure) was found to induce immune response by targeting the same LPS receptors (**Fig. 10C**).[120] This data indicates that structural modifications of lipid A (and its derivatives) may influence aggregation behavior, which would influence their binding affinity as TLR4 agonists. Other synthetic analogs of LPS have been also reported.[121] The MPL and its derivative have been studied with the aim of developing peptide-based vaccine. The conjugation of MPL to a peptide epitope enhances the immunogenicity of the vaccine antigen.[122] Different vaccination formulations such as emulsification of CD8 T cell peptide epitope with MPL analogs also induced IFN-γ production and conferred protection against the targeted parasite.[123] Historically, MPL was the first T-cells-stimulating adjuvant that was licensed for use in the routine vaccination. AS04 adjuvant that contain combination of MPL and alum was used as part of the HPV vaccine and recently applied for hepatitis B virus (HBV) vaccine.[115] An adjuvant formulation that includes MPL as the constituents (AS01 and AS02) is currently under clinical evaluation for protein/peptide-based vaccine against malaria and tuberculosis.[124,125]

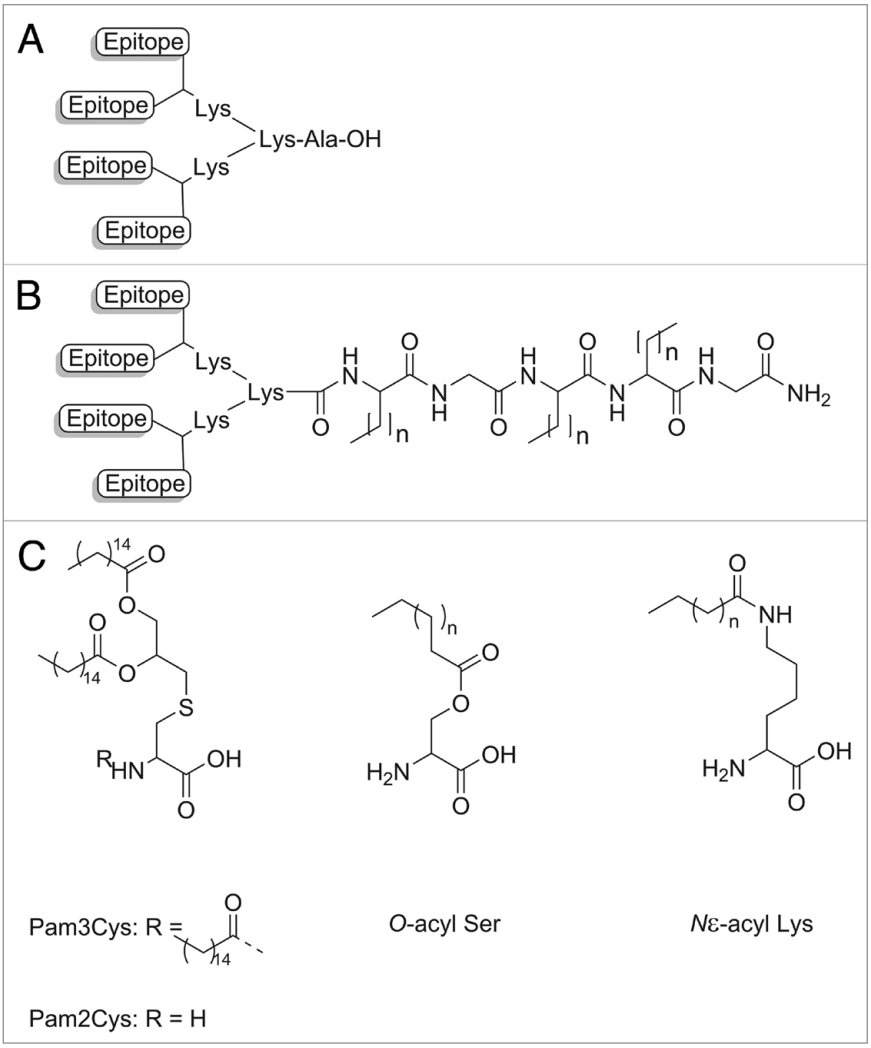

Figure 13. (**A**) A schematic representation of the origin MAP system, (**B**) an example of an LCP core based MAP system (n = total number of carbon molecules than can be varied), and (**C**) other immunostimulating lipid moieties.

It is well established that flagellin (structural protein present on bacterial flagellum) is potent activator of broad range of immune cells. Flagellin showed promise as an adjuvant; intramuscular administration of recombinant flagellin with influenza virus peptide epitopes stimulated both humoral and cellular responses against influenza challenge.[126] Interestingly, the same study found that this formulation also provided some protection against Influenza A virus subtype (H5N1). The use of flagellin as an adjuvant for cancer vaccination via the intranasal route showed promising results. For example, the intranasal immunization with flagellin-adjuvanted peptide-based HPV antigen elicited a robust cellular immune response that managed to suppress tumor growth in a mouse model.[127] The strong immune response stimulated by flagellin was mediated by the activation of TLR5. The flexibility of flagellin structure contributes to the ease of fusion with peptide antigen, making it possible to create a powerful peptide vaccine against a variety of diseases including tumors.

Several other adjuvants based on bacterial components are currently under development. One of these adjuvants is a synthetic oligonucleotide (ODN), CpG-ODN. CpG-ODN contains unmethylated CpG motifs that resemble the structure of bacterial DNA. CpG-ODNs are known to activate TLR9, which located within the APC.[128] TLR9 signaling can lead to the maturation of dendritic cells and enhance B cell differentiation into antibody-secreting plasma cells.[128] The use of CpG-DNA as an adjuvant was found to enhance the immunogenicity of the immunodominant CD8 T cell peptide epitopes.[129] They found that the CD8 T cell peptide epitopes alone were able to be presented by immature DC in lymph nodes, but failed to stimulate a CTL response. However, administration of the same peptide epitope with CpG-DNA induced the maturation of DC, which led to direct stimulation of cytolytic T cells. Thus CpG-DNA was able to promote a CTL response against MHC class 1 restricted T cell epitopes without requiring Th cell activation. It was determined that the CPG-ODNs need to be in close proximity (physical contact) with the antigen to provide maximum immunostimulatory effect.[130] These results were supported by the findings of Daftarian et al., where CpG-ODN covalently linked to the peptide epitope stimulated greater epitope recognition than a non-linked formulation of the same molecules.[131]

Peptidoglycans, a bacterial cell wall component, are highly investigated as potential immunostimulants. Adjuvants based on peptidoglycan constituents, known as muramyl dipeptide (MDP) (**Fig. 12**) were found to modulate the immune response. It was reported that MDP and their derivatives confer their adjuvanticity effect by activating the NF-κB pathway through the NOD2 receptor.[132] The same group also found that a tetrasachharide that contained MDP promoted additional production of IgG2b subclasses compared with MDP alone, which instead induced IgG1 production. However, MDP does not stimulate strong immune response as other TLR agonists under a variety of immunization conditions.[133] Thus, MDP is a less favorable adjuvant for peptide-based antigens which require a relatively strong immune modulator to exhibit potency.

The use of bacterial components as adjuvants is effective because bacteria represent a foreign antigen threat and trigger an immune response. However, if these components are not synthetically produced, it will be challenging to control the level of potential bacterial contaminants. Thus, the purity of the bacterial components must be carefully evaluated during their isolation and production.

Lipopeptides and MAP System

Lipidation of antigenic peptides is a promising strategy to improve their potency for stimulating immune responses. Lipopeptide carrier systems can stimulate TLR2 activation, improve peptide diffusion across epithelial barriers, and enhance peptide stability from enzymatic degradation.[134] Furthermore, lipidation may also contribute to the enhancement of peptide secondary structure. Lipopeptides can undergo aggregation and thus exhibit protein analogous micelles properties which enable the short peptides to regain their native conformation.[5] Lipidation provides "self-adjuvanting vaccine" properties as the antigen and lipid-based adjuvant are incorporated into a single construct. Thus, the same APCs encounter both the antigen and adjuvant. It was reported that the antigen and TLR agonist should be colocalized in a similar phagosome for effective MHC–II antigen presentation.[135] Physical mixture of lipids and peptides failed to stimulate immune response.[136] Varieties type of lipid moieties conjugated to peptide antigen have been studied. The simplest designs of lipopeptide vaccine are comprised of a fatty acid moiety conjugated to an antigenic peptide epitope. Jackson and colleagues have demonstrated that a T cell peptide epitope derived from influenza virus when covalently linked to Pam_3Cys stimulated a T cell mediated immune response.[137] Although proven to enhance the immunogenicity of peptide antigens, lipopeptides comprised of Pam_3Cys have usually poor water-solubility. A similar derivative, Pam_2Cys, lacking one palmitic acid group, was conjugated to a B cell peptide epitope for contraceptive vaccination[138] and T cell peptide epitope for hepatitis C virus vaccine.[139] These conjugates upon imminization in mice enhanced the antigen-specific immune responses and shown significantly better solubility than Pam_3Cys analogs.[136]

Lipopeptides have been also used in conjugation with the multiple antigen pepetide system (MAP), which allows incorporation of multiple copies of the peptide antigen in a single construct. The MAP system uses lysine as a core dendrimer because it has two functional side-chains available for branching purposes, the α- and ε-amino groups (**Fig. 13A**). Kamo and coworkers showed that a linear peptide that was ineffective at raising antibodies could become immunogenic when presented in MAP format.[140] Lipid core peptides (LCP) that use the functionalities of lipoamino acids in the MAP system have been extensively studied to develop an efficient vaccine delivery system (**Fig. 13B**).[141] Lipoamino acids are recognized as TLR2 agonists and their lipophilicity can be tailored by varying the length of the alkyl side chain.[142] It was demonstrated that an LCP constructed with the peptide epitope (J8) derived from GAS M protein as an antigen induced IgG antibodies, heterologous opsonic antibodies, and conferred complete protection against GAS challenge in mice without any additional adjuvant.[143] LCP constructs were reported as vaccine candidates against wide a range of diseases including GAS,[144] cancer,[145] malaria,[146] and hookworm.[19] Other lipid moieties such as palmatic acid derivatives (Pam3Cys and Pam2Cys), O-acyl serine, N-acyl lysine, and glycolipid compounds that were found to stimulate innate immunity were also investigated for this purpose (**Fig. 13C**).[121,147]

Generation of an optimal response to a B cell epitope requires recognition of the antigenic fragments by T helper cells. The activated T helper cells can deliver activating signals to B cells, subsequently stimulating B cell proliferation into antibody-secreting cells. Thus an effective adjuvant to target a humoral response should activate both B cell and T helper cells. The MAP system can meet this requirement because it has the ability to incorporate multiple epitopes in the same construct. MAP that possesses T helper epitopes and the antigenic determinants has been widely investigated. The inclusion of a universal T helper epitope, along with peptide antigen derived from malaria parasite

enhanced the immune response against a peptide antigen that was found to be ineffective when used alone.[148] Incorporation of T-helper epitope into the LCP and Pam2Cys-MAP constructs allowed induction of a potent immune response in an outbred mouse population.[149,150]

The incorporation of functional immune components into a MAP system can be exploited to design a more potent peptide-based vaccine. Vaccine efficacy can be optimised by altering the ratio of B cell epitopes and other functional components to develop a safer, more cost-effective vaccine.

Concluding Remarks

Stimulation of a potent and long-term immune response against protective antigens is crucial for the creation of any vaccine. In particular, peptide-based antigens usually target a specific immune response. However, peptides are not able to trigger the recruitment and activation of cells involved in the non-specific immune response (innate immunity), which potentiate the activation of a specific response (adaptive immunity). This issue must be addressed through the development of next generation adjuvants. An adjuvant should not only act as a delivery system but also should stimulate an innate immune response. However, safety needs to be considered in the development of strong immunoadjuvants, as modern adjuvants usually induce inflammatory cytokines such as TNF and IL-1. Excessive proinflammatory responses may lead to undesirable side-effects which have been observed with squalene oil emulsions that triggered autoimmune disease in animal models.

The activation of APCs, especially DCs is paramount to the development of an effective adjuvant. DC activation can result in enhanced antigen uptake, antigen migration to the draining lymph nodes, acquisition of co-stimulatory molecules and enhanced antigen presentation to T cells. DC activation can be stimulated via several mechanisms. One mechanisms of interest is to engage with the TLR that are presented by the DC. TLR activation can lead to the generation of co-stimulatory molecules (CD40, CD80, CD70) and trigger the release of Th1 cytokines such as IL-1, IL-2, IL-6, and TNF. The incorporation of antigen and TLR agonist in the same construct would contribute to a greater immune response. This is due to the fact that some of the endosomal organelles of DC may express TLRs, which would enable antigen processing and DCs activation to occur simultaneously.

Additionally, the construction of adjuvant by mimicking natural pathogens is a promising approach as pathogens are easily recognized by cells of the innate immune system. Constructed adjuvants that have similar properties to pathogens (e.g., particulate behavior, size, and repetitive display of antigenic epitopes) are easily taken up by DC to undergo further biological processing. It has been demonstrated that the particulate nature of certain adjuvant system such as virosomes, liposomes, polymeric nanoparticles, and ISCOM help to boost their uptake by DC and appears to trigger antigen cross-presentation and priming of $CD8^+$ T cells.

The combination of adjuvants in formulations has brought a new era to modern vaccine development. It is clearly shown that adjuvants may confer their adjuvanticity effect via different mechanisms. Thus, combining two or more adjuvants may result in a more potent immune response through a synergystic effect. This strategy has already been proven to work, with recent licensed vaccine formulations such as Gardasil (VLP and alum) and Cervarix (MPL and alum) which incorporate a combination of adjuvants. With increased interest in targeting TLR, it would be a promising option to combine multiple TLR agonists in a vaccine formulation in order to exploit their synergistic effects on cytokine production, ultimately stimulating a superior immune response. In addition, multiple TLR agonists can also be conjugated with a particulate based adjuvant to enhance their uptake by DC and gain the benefit of multiple antigenic displays for the development of a next generation adjuvant system. However, the possibility of stimulating an excessive inflammatory response needs to be considered when combining several adjuvants. Thus, optimizing the balance between an effective immunostimulant and potentially excessive inflammatory responses would be crucial in moving toward this approach.

Growing knowledge about the stimulation of immunological events associated with an adjuvants mode of action, especially the identification of particular signaling pathways that lead to an adaptive immune response, provides hope that effective peptide vaccines can be developed in the near future.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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