HHS Public Access

Author manuscript

Clin Immunol. Author manuscript; available in PMC 2018 October 01.

Published in final edited form as:

Clin Immunol. 2017 October ; 183: 99–108. doi:10.1016/j.clim.2017.08.004.

Adjuvant Formulations for Virus-Like Particle (VLP) Based Vaccines

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Abstract

The development of virus-like particle (VLP) technology has had an enormous impact on modern vaccinology. In order to optimize the efficacy and safety of VLP-based vaccines, adjuvants are included in most vaccine formulations. To date, most licensed VLP-based vaccines utilize the classic aluminum adjuvant compositions. Certain challenging pathogens and weak immune responder subjects may require further optimization of the adjuvant formulation to maximize the magnitude and duration of the protective immunity. Indeed, novel classes of adjuvants such as liposomes, agonists of pathogen recognition receptors, polymeric particles, emulsions, cytokines and bacterial toxins, can be used to optimize the immunostimulatory activity of a VLP-based vaccine.

This review describes the current advances in adjuvant technology for VLP-based vaccines directed at viral diseases, and discusses the basic principles for designing adjuvant formulations for enhancing the vaccine immunogenicity.

Graphical abstract: The roles of adjuvants in VLP-based vaccines

VLPs can be produced from various cellular systems using recombinant DNA technology. Adjuvants play multiple roles in enhancing the efficacy and safety of VLP-based vaccines.

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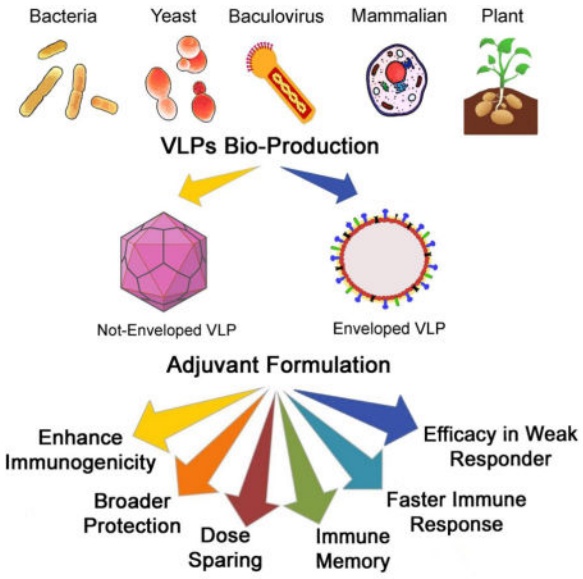
Competing Interest

JMG is the CEO of TechnoVax Inc., VC was an employee of TechnoVax Inc. during the preparation of this manuscript; he is currently an employee of the American Type Culture Collection (ATCC), BEI-Resources. The authors adhere to the journal's policies on sharing data and materials.



1. Introduction

Vaccination represents the most successful and cost-effective intervention in the history of medicine. Global immunization against smallpox led to the complete eradication of the disease and to the prevention of 5 million deaths every year [1]. Most conventional vaccines consisted of live-attenuated, inactivated pathogens or detoxified toxins (toxoids) [2,3]. Such vaccines have been quite successful in protecting the human population against a variety of illnesses, and have dramatically reduced the number of serious infections caused by diseases such as polio, measles, diphtheria, whooping cough, tetanus and yellow fever. Certain live-attenuated viral vaccines, however, are associated with a reversion to virulence resulting in vaccine-associated disease and the risk of transmission [4]. In addition, live-attenuated and inactivated viral vaccine may induce severe side effects in recipients including those without pre-existing conditions [5]. In order to prevent adverse effects, second generation vaccines have been improved using specific pathogen components to formulate subunit vaccines that display highly immunogenic antigens [6]. Subunit vaccines can be generated either by purifying pathogen specific proteins or carbohydrates, or by producing the selected antigen in any of the available cell expression systems using recombinant DNA technologies [7]. Although subunit vaccines may contain strong antigens, they cannot entirely recreate the complexity of the pathogen structure. Indeed, immunization with subunit vaccines containing purified protein/s may not elicit effective humoral and cell mediated immune responses [8]. More structured recombinant vaccine such as viruslike particles (VLPs) show improved efficiency in stimulating the immune system because they resemble the morphology of the native virion displaying a densely repetitive array of epitopes in a limited space [9,10]. Furthermore, VLPs are very safe candidates for vaccine development due to their lack of replicating viral genetic material making them unable to cause disease. During the last decade, advancement in VLP production, purification, and adjuvant optimization has led to the licensing of several VLP-based vaccines for the prevention of infectious diseases such as human papilloma virus (HPV) [10–12], hepatitis B virus (HBV) [13–16], hepatitis E

virus (HEV) [17], and influenza [18,19] (Table 1). Furthermore, several clinical trials are currently ongoing for VLP vaccines against influenza [20], norovirus [21], and chikungunya virus (CHIK) [22] (https://clinicaltrials.gov/). Although VLP-based vaccines alone have been shown to be more efficacious than subunit vaccines [23], formulation with adjuvants is relevant to optimize their activity and enhance their potency [24]. Indeed, adjuvants are a class of immunomodulatory molecules [25] able to augment vaccine effectiveness and safety by: 1) enhancing immunogenicity and increasing the duration of protection; 2) broadening the induction of the immune response; 3) reducing vaccine dosage and vaccination cost (antigen sparing); 4) accelerating the immune response; 5) stimulating a stronger immunological memory; 6) improving efficacy in weak responder patients such as neonates, the elderly and immunocompromised individuals (Graphical Abstract). In addition, some adjuvants formulation may also increase VLP-based vaccine stability and play an important role in VLPs delivery [24,26]. Thus, although VLP alone vaccines are generally highly immunogenic, formulation with adjuvant tend to enhance the magnitude, quality and longevity of the elicited immunity. The classical aluminum salts based adjuvants have decades long history of safety and adaptability for numerous vaccine formulations. Indeed, the current licensed VLP-based vaccines are mainly adjuvanted with aluminum (Table 1). Nevertheless, challenging pathogens and weak immunization responders may require an alternative vaccine formulation in order to achieve a superior clinical benefit. A better understanding of the interaction between the pathogen and the immune system has led to the development of novel classes of adjuvants able to induce a more targeted and specific immunological activity. Novel adjuvant formulations can be designed for inducing a tailored immunization response and for improving immunization efficacy and safety for VLP-based vaccines.

2. VLP-based vaccine structure and immunogenicity

VLPs, also referred to as subviral particles, are complex structures generated by the self-assembly of viral structural proteins. The main characteristics of VLPs are their resemblance in morphology, biochemical composition and size (20–200 nm) to the originating native virion but they lack viral genetic material and are therefore unable to replicate or cause infection [23]. Indeed, some VLPs are indistinguishable from the original virion when examined by electron microscopy [26]. The term viral nanoparticle includes both VLPs and multimeric structures of viral proteins that are characterized by a size smaller than < 10 nm and do not exhibit viral morphology [27]. Analogous to the virus families from which they are derived, VLPs can either assemble with a lipid membrane envelope with spherical or polymorphous morphologies, or they can be formed by non-enveloped capsids of defined shape and geometrical symmetry [28,29] (Graphical Abstract). The viral morphologies of VLPs are advantageous for their immunostimulatory activity: i) VLPs are more efficiently recognized by antigen presenting cells (APCs); ii) VLPs are trafficked from the site of injection to the lymph nodes; iii) the VLP structures present a repetitive arrangement of antigens that promote B-cell activation resulting in a stronger humoral immune response, as well as enhanced T-cell stimulation and cellular mediated immunity [30]. Importantly, VLPs can display multiple epitopes in a repetitive and dense array, thus effectively enabling the crosslinking of B cells receptor (BRC) and enhancing B cells activation and response [31].

VLPs can be produced in various heterologous gene expression systems using recombinant DNA technology including (Graphical Abstract): bacteria, yeast, baculovirus systems, mammalian cells, plant cell culture, or plant organisms [32]. Furthermore, a particular class of VLPs called virosomes can be assembled *in vitro* using engineered biomolecules such as liposomes and purified viral proteins [18]. The VLP-based vaccines are not only particularly efficacious and safe, but also suitable for straightforward scalable manufacturing in an economically competitive manner [17]. In addition, VLP production can be achieved using current good manufacture practices (cGMP) without the need for the biological safety containment that is typically required for a live virus vaccine production process [33]. In the case of influenza, for example, new virus strains emerge due to the accumulation of mutations, (antigenic drift) [34] or by genetic reassortment, (antigenic shift) [35] resulting in epidemic outbreaks of recurring frequency and unpredictable intervals. Influenza vaccine manufacturing using the classical embryonated chicken egg system to produce either live-attenuated or split virus vaccines requires a minimum of 6 months for seed selection, optimization, and large-scale vaccine production. In contrast, VLP-based vaccines can be developed and produced in 3–12 weeks, significantly reducing vaccine production timelines. Such flexibility is critical when the emergence of novel epidemic or pandemic flu strains occurs and rapid immunization campaigns must be undertaken [20]. In addition, VLP technology is particularly advantageous for the formulation of multivalent vaccines as for example, the HPV vaccine Gardasil 9 (Merck) which provides protection against 9 different HPV strains as well as in the case of a quadrivalent VLP vaccine for seasonal influenza currently being tested in clinical trials (Medicago, Novavax) [36]. The flexibility of VLP technology allows for the incorporation and display of heterologous antigens or specific epitopes within the structure or on the surface of the particle using either fusion protein or chemical conjugation approach [37,38]. Indeed, different heterologous VLP-based vaccine platforms have been developed and are currently being tested in clinical trials. For instance, the HBV core protein has been employed as a VLP carrier for both the influenza virus antigens [39] and for the first vaccine against the malaria parasite (*Plasmodium falciparium*) named RTS,S (Mosquirix, GSK) [40,41]. Bacteriophage QB protein has been used as a VLP vaccine carrier to immunize against nicotine addiction [42], hypertension [43], melanoma, [44], and *Dermatophagoides pteronyssinus* allergy [45]. Furthermore, the heterologous or chimeric VLP-based vaccine approach has been successfully tested in preclinical studies directed against metabolic diseases such as hypercholesterolemia [46], as well as bacterial infections such as *Borellia burgdorferi* [47], and *Bacillus anthracis* [48]. Recently, another VLP chimeric system has been developed using the chikungunya virus (CHIKV) envelope protein as a scaffolding structure for display of a foreign antigen from *Plasmodium falciparium* [49].

3. Role of the Adjuvant in Vaccination

Live attenuated viral vaccines generally do not require an adjuvant because they mimic, albeit in an attenuated form, a natural infection. Vaccines composed of purified viral components like VLPs, however, may not sufficiently trigger the innate immunity response that is required to best stimulate the adaptive immune system. This class of vaccine composition may therefore require the inclusion of adjuvants in order to potentiate their

immunostimulatory activity. Adjuvants are immunomodulatory substances that are admixed with the vaccine to enhance their immunogenicity, potency and efficacy (Graphical Abstract), and have been employed in vaccine formulation for more than 90 years [36,50]. The principal intended use of adjuvant is to further improve the quality, magnitude and duration of an antigen specific immune response. The main mechanism of adjuvant immunomodulatory activity is based on the recruitment of antigen presenting cells (APCs) to the site of injection or administration [51,52]. These APCs play a primary role in immunostimulation by capturing and processing vaccine antigens and migrating to the draining lymph nodes where they stimulate the proliferation and differentiation of T and B cells [53]. Activated CD4+ helper T cells secrete cytokines which are pivotal in signaling and directing stimulation of an specific type of immune response as well as B cell development and antibody production [54]. The specific type of T helper response is important for the efficacy of the protective immunity: the Th1-type response addresses intracellular pathogens such as viruses and intracellular bacteria (e.g. *Mycobacterium tuberculosis* and *Listeria*), and the Th2-type response is involved in the stimulation of the production of antibodies directed against extracellular parasites (e.g. helminth worms). The T follicular helper (Tfh) cells activation stimulates the transformation of B cells into plasma cells for antibody production, while Th17 cell activation plays an important role in the removal of extracellular bacteria and fungi from the skin and mucous membranes. In addition, Cytotoxic T Lymphocytes (CTLs), or CD8+ cells, are essential in blocking viral and intracellular bacteria replication by killing infected cells. During the selection of the most beneficial adjuvant, it is pivotal to examine its specificity and mechanism of action as well as its specific effects on the variety of T cell populations. Most importantly, the adjuvant needs to be well tolerated without provoking significant side effects or undesirable reactogenicity to the vaccinee. Hence, selection of an adjuvant is extremely important not only for potentiating the magnitude and duration of the vaccine specific immunity, but also for maintaining safety and reducing the required vaccine dose (antigen sparing). Vaccine development for challenging pathogens, and immunocompromised responders such as elderly or pediatric patients, can greatly benefit from the advancement of adjuvant technology.

4. VLP-based vaccine adjuvant optimization

As discussed above, VLPs have a bio-particulate structure that is beneficial for APC recognition and uptake as well as BCR crosslinking [23,30,31]. For this reasons VLPs-based vaccine may not necessitate of adjuvants carriers like aluminum salts, which are important for the delivery of subunit vaccines antigens to APC. Another important aspect of VLPs is their resemblance to native virion in morphology and surface antigenic configuration; hence the optimal adjuvant formulation should avoid perturbation of such complex structure. For these reasons, adjuvant formulations for VLP-based vaccines are progressing towards alternative systems from aluminum salts. The liposomes-based adjuvants led to approval of virosomal vaccines against influenza and hepatitis A virus (HAV) [18,19] (Table 1 and 2). Pathogen Recognition Receptors (PRRs) agonist have considerably influenced the VLP-based vaccine formulation area more than any other type of vaccine compositions for viral infections [6]. For instance, the AS04 is a an adjuvant with the ability to stimulate toll-like

receptor 4 (TLR4) and has had a relevant impact in the licensing of two VLPs-based vaccine such as Cervarix and Fendrix [11,16] (Table 1 and 2). Noticeably, VLPs have shown superior stability as compared to recombinant protein vaccines in the particularly harsh mucosal environment. This characteristic is quite advantageous for developing vaccines for mucosal administration such as the oral and nasal route [24]. For instance, the chitosan adjuvant has demonstrated to be quite efficacious for intranasal immunization of the VLP-based vaccine against norovirus [55] (Table 2). In the next section, we describe adjuvants that have been successfully adopted and optimized for VLP-based vaccine formulations (Table 2).

5. Classes of Adjuvants tested for VLP-based vaccines

5.1. Aluminum salt-based adjuvants

Aluminum salt-based adjuvants also referred to as alum, represent one of the first adjuvants developed for vaccine formulation and have been in use since the 1920s [56]. Millions of doses of aluminum adjuvanted vaccines have been administered to humans and other species [36,57]. Aluminum based adjuvant is prepared using insoluble aluminum salts (e.g. aluminum phosphate, aluminium hydroxyl-phosphate, and aluminum hydroxide). Aluminum salts form crystal particles able to absorb to the vaccine components and function as an antigen carrier for the APCs. Because of its prolonged history of safety, aluminum adjuvant has been employed in the formulation of different VLP-based vaccines including HBV, HPV, HEV and influenza (Table 1). The mechanism of action of aluminum is still not completely understood [56]. Multiple reports, however, demonstrate that aluminum functions as a delivery system by binding to the antigen and forming particulate aggregates that stimulate antigen uptake by dendritic cells (DC) at the site of injection. In addition, aluminum induces inflammosome activation and IL-1β cytokine release, mediated by the NOD-like receptor NALP3 [57,58]. It is noteworthy that aluminum salts mainly promote a Th2-biased immune response but also exert a limited effect in stimulating Th1-type immunity and T cell cytotoxic responses. Hence, viral diseases that usually require a Th1-mediated immunity and CTL activation for protection may not fully benefit from employing aluminum as an adjuvant. Importantly, aluminum salts render absorbed vaccines more susceptible to freeze and thaw stress resulting in vaccine structural damages and agglomeration [59,60]. In addition, alum absorption can induce destabilization of the conformational structure of the antigen or epitopes [61].

5.2. Liposomes/Virosomes

Liposomes have shown a very promising activity as an adjuvant for bacterial and viral vaccines. Liposomes are lipid vesicles that demonstrate the ability to encapsulate proteins *via* a membrane-anchoring domain and act as antigen-delivery vehicles. They have also been employed as a scaffolding system for the creation of cell free assembled VLPs called Virosomes [62], which provide an excellent system for antigen delivery to APCs and promote a balanced Th1- and Th2-mediated immunity. Inflexal was the first virosomal based influenza vaccine to be commercialized (Crucell), and has demonstrated acceptable level of efficacy and an excellent safety profile in all demographics including the pediatric population. Such VLP-based vaccine is assembled *in v itro* using recombinant influenza

virus surface glycoproteins hemagglutinin (HA) and neuraminidase (NA), both of which are inserted into the phospho-lipid bilayer of the liposome particles [18]. In addition, the hepatitis A virus (HAV) virosomal vaccine Epaxal (Crucell) has shown a remarkable long lasting protection of at least 30 years against HAV infection in over 95% of immunized healthy subjects [63]. Epaxal is formulated by absorbing the inactivated HAV into an influenza derived virosomes adjuvant system [64].

5.3. Pattern Recognition Receptors (PRRs) Agonist Adjuvants

PRRs are important host sensors that recognize pathogen-associated molecular patterns (PAMPs) displayed by invading pathogens. They are responsible for the stimulation of the innate immune system which is required for the initiation of the adaptive immune response [65]. PRRs include: 1) toll-like receptors (TLR); 2) C-type lectin receptors; 3) RIG-I-like receptors (RIG-I, MDA5); 4) NOD-like receptors (e.g. NALP3); and 5) Cytosolic DNA sensors (e.g. STING). The PRR ligand PAMPs have diverse biochemical compositions and structures with specificity for pathogen constituents such as: 1) nucleic acids, including bacterial and viral DNA, and viral RNA; 2) bacterial cell wall and surface components, including the lipopolysaccharides (LPS), flagellin, peptidoglycans, lipoprotein and lipopeptides; and 3) glycan carbohydrates (e.g. glucan, fucose, and high mannose glycans) present in various pathogens structures such as fungi, mycobacteria and viruses. The PRR agonist adjuvants are often compounds derived from PAMPs or molecular entities mimicking their structure.

5.3.1. Lipid A analogs: MPL and GLA—Lipid A analogs are a potent agonist of the TLR4 and are able to induce a specific Th1-mediated immune response and antibody production [66]. The 3-deacyl-monophosphoryl lipid A (MPL) is a bacterial lipid endotoxin extracted from the Salmonella Minnesota lipopolysaccharides (LPS). Importantly, MPL was the first TLR agonist adjuvant to be licensed for a human vaccine. Currently, Takeda Pharmaceuticals is testing in a phase II clinical trial, a VLP vaccine against norovirus formulated with MPL [67]. Furthermore, MPL has demonstrated an excellent adjuvant activity when admixed with other components in formulations such as the AS04 or AS01 preparations (GSK) (see below).

The glucopyranosyl lipid adjuvant (GLA) is formulated in aqueous nanosuspension (GLA-AF) [68] or in a squalene oil-in-water emulsion (GLA-SE) [69]. The GLA-AF and GLA-SE adjuvants were included in H5-VLP vaccine formulations against influenza, and the efficacy and safety for such vaccine preparations were studied in two different clinical trials sponsored by IDRI (Phase I) and Medicago (Phase II). The results of these clinical trials have not been published at this time.

5.3.2. AS04—AS04 (GSK) is composed of MPL mixed with an aluminum salt (phosphate or hydroxide) [70]. The HPV VLP vaccine against serotypes 16/18 (Cervarix, GSK) formulated with AS04 was shown in preclinical studies and clinical trials to elicit a higher magnitude of neutralizing antibodies against HPV than an analogous formulation containing only aluminum salt [71,72]. Indeed, AS04 has proven the ability to effectively stimulate TLR4, inducing enhanced APC maturation and a Th1–mediated immune response [73]. For

these reasons, Cervarix vaccine has been formulated with the AS04 adjuvant (Table 1). Vaccination against HBV with the standard VLP-based vaccines adjuvanted with alum has showed very high efficacy in healthy subjects. Unfortunately, 30 to 40% of hemodialyzed patients do not seroconvert or develop protective anti-HBsAg immunity following the vaccination schedule with the HBV/VLP vaccine. In addition the duration of immunity is shorter and less robust than the one seen in healthy individuals [16,74]. GSK has further developed a HBV VLP-Based vaccine named Fendrix formulated with the same antigenic composition as the current vaccine (recombinant HBsAg) but adjuvanted with AS04 rather alum. Fendrix has demonstrated the ability to elicit an earlier and higher antibody response in pre and hemodialysis patients as compared to four double doses of standard HBV vaccine, and has also shown a very similar safety profile to the standard HBV vaccine [16,75]. The formulation improvement of HBV vaccine with the AS04 adjuvant represents a case-study demonstrating how adjuvant optimization can increase immunization efficacy not only in healthy subjects but also in weak responders.

5.3.3. AS01/AS02—Adjuvant Systems AS01/AS02 were developed by GSK and contain the immunostimulatory compounds MPL (discussed above in the “Lipid A analogs” section) and the QS21 a natural saponin molecule extracted from the bark of the tree Quillaja saponaria Molina. While AS01 adjuvant is formulated with liposomes, AS02 is an emulsion. AS01/AS02 have very broad immunomodulatory activity. MPL stimulates TLR4 with the subsequent induction of a Th1-mediated response and antibody production (see above), QS21 promotes antibody generation and CD8+ T cell activation, and the liposomes or emulsion stimulate antigen uptake from the APC such as dendritic cells [76,77]. The VLP-based vaccine against malaria RTS,S (Mosquirix, GSK) has shown a better immune response and improved efficacy in several clinical trials when formulated with AS01 in comparison with AS02 [78,79]. AS01 has been tested in clinical trials with the tetravalent HPV VLP-vaccine (GSK) [80] showing a significant effect in broadening vaccine efficacy and enhancing cross-reactivity. In the same study, however, AS01 showed a particularly higher reactogenicity than VLP vaccines formulated with AS04 or AS02.

5.3.4. ISCOMATRIX—ISCOMATRIX (CSL) adjuvant is a particulate complex formed by the combination of saponin the glycoside extracted from Quillaja saponaria Molina (QS21), cholesterol, and phospholipids [81]. The immunomodulatory activity of this adjuvant is due to the stimulation of NALP3 and the inflammosome [82]. ISCOMATRIX delivers vaccine antigens to the APCs, resulting in the stimulation of Th1- and Th2- mediated responses and antibody production. A phase I clinical trial with an H7N9 VLP flu vaccine demonstrated the ability of ISCOMATRIX to enhance seroconversion: 80.6% of the subjects developed hemagglutination-inhibition response when immunized with the VLP-based vaccine containing 5μg Hemagglutinin (HA) and formulated with ISCOMATRIX adjuvant. Only 15.6% of the participants showed seroconversion after immunization without adjuvant even using 9 fold higher dose of the same VLP-based vaccine (45μg of HA) [83]. This study reveals the significant contribution of the ISCOMATRIX adjuvant in enhancing antigen specific immunity in a VLP-based vaccine.

5.3.5. Double stranded RNA analogs (Poly I:C and Poly ICLC)—Double stranded RNA (dsRNA) analogs such as polyinosinic:polycytidylic acid (Poly I:C) activates the intracellular RIG-I-like receptors (RIG-I and MDA5), and the cellular membrane bound TLR3 (toll-like receptor 3) signaling pathways which trigger the production of type I FN (IFN-α and IFN-β). In the context of VLP vaccinology, Poly I:C has been shown to enhance the protective ability of a VLP-based vaccine against a lethal challenge in a mouse model using a mouse-adapted flu virus [84]. In addition, a VLP-based vaccine for Ebola virus was more efficacious in mice and guinea pigs when adjuvanted with Poly ICLC adjuvant (Hiltonol, Oncovir Inc.) which is a stable combination of Poly IC and Poly lysine [85]. The authors have reported that Poly ICLC boosted antigen-specific immunity mediated by poly-functional CD4+ and CD8+ T cell and antibody responses.

5.3.6. Cytidine monophosphate guanosine oligodeoxynucleotide (CpG, CpG ODN)—Cytidine monophosphate guanosine oligodeoxynucleotides (CpG or CpG ODN) are a class of DNA analog composed of unmethylated cytosine and guanine motifs that mimic bacterial DNA and stimulate the innate immune system by binding to the TLR9 and triggering B cell proliferation, dendritic APC maturation, and Th1-mediated immune activation [86]. A foot-and-mouth-disease virus (FMDV) VLP-based vaccine was shown to enhance cell mediated immunity in a guinea pig model [87]. In particular, CpG adjuvant markedly tilted the response toward a Th1 rather than Th2 type of immunity. Furthermore, our laboratory has shown that a CpG adjuvanted VLP vaccine against the 1918 flu virus afforded complete protection from flu virus infection in mice after immunization *via* the intranasal route. [88].

5.3.7. Flagellin—Flagellin is a protein derived from the flagella of gram-negative bacteria (e.g. *Salmonella)* and is a potent agonist of TLR5. Dendritic cells and T-lymphocytes express TLR5 and are therefore responsive to flagellin. Indeed, flagellin induces APC maturation and a Th1-mediated immune response followed by B cell activation and antibody production [89]. Recombinant DNA techniques have been used to the generate a flagellin-antigen fusion protein or chimera [89]. VLP-based vaccines incorporating flagellin were developed and tested in preclinical studies for rabies virus and influenza [90,91]. Both studies demonstrated that flagellin augmented the VLP-based vaccine efficacy characterized by a marked Th1-mediated response, and characterized by stronger humoral and cellular immunity. In the context of VLP vaccine against influenza, flagellin as adjuvant appears to elicit a cross-protective hetero-subtypic immune response [91].

5.3.8. Imidazoquinoline: Imiquimod and Resiquimod—The imidazoquinoline compounds imiquimod and resiquimod (R848) are potent TLR7 and TLR7/8 agonists respectively [92], and both were developed by 3M Pharmaceuticals. Imidazoquiline directly activates APCs inducing a Th1-mediated immune response and CD8+ activation [93,94]. Imiquimod is the active ingredient in the topical cream Aldara (3M Pharmaceuticals), which is used for the treatment of genital warts and other skin conditions. In addition, preclinical studies in mice with a HPV 16 VLP vaccine formulated with imiquimod or resiquimod as adjuvants have shown the capability of inducing anti-HPV16 specific antibodies in serum and secretions after mucosal vaccine administration via either the rectal or vaginal routes

[95]. Conversely, an analogous vaccine composition without the adjuvant was unable to elicit an effective immune response. Importantly, this study demonstrates not only that the imidazoquinoline adjuvants enhance immunity toward the associated antigens when delivered via mucosal surfaces, but also that a needle free HPV VLP vaccine administration is possible.

5.4. Chitosan

Chitosan is the deacetylated derivative of the polysaccharide chitin, which is the structural constituent of the exoskeletons of crustaceans and the cell walls of fungi. Chitosan has been reported to have various properties and uses such as adjuvant, matrix for delivery systems, and vaccine stabilizer. Chitosan has the ability to form polymeric particles able to absorb the antigen when admixed in vaccine formulations [96]. In addition, chitosan is an excellent mucosal adjuvant allowing for the delivery of the antigen to local phagocytic cells, leading to strong induction of systemic and mucosal immune responses. The inflammosome is strongly stimulated by chitosan as the result of activation of the NALP3 receptor, triggering a balanced Th1 and Th2 type of T helper cell response. A VLP-based vaccine against norovirus and Norwalk virus was tested in clinical trials with chitosan and MLP adjuvants together [55]: the vaccination significantly protected the participants against Norwalk virus infection and gastroenteritis. Importantly 70% of the vaccinated participants showed virus-specific IgA seroresponse.

5.5. Emulsion adjuvants

Emulsion adjuvants are commonly formulated with oils such as squalene (e.g. MF59), saponin (e.g. ISCOMATRIX), or with mineral oils (e.g. Montanide), and are described as oil-in-water or water-in-oil dispersions. Similar to aluminum salts, emulsion are particulate adjuvants able to stimulate antigens uptake by the APC [97].

5.5.1. MF59—The squalene oil-based emulsion adjuvant MF59 is widely used and was first licensed by Novartis along with a flu vaccine (Fluad) in 1997 [98]. MF59 has demonstrated an excellent safety and efficacy profile when used with influenza vaccines, including in pediatric patients. MF59 appears to have the ability to upregulate production of cytokines and chemokines which attract APCs to the site of injection increasing antigen uptake and subsequent transport to draining lymph nodes [99]. Our group has developed a novel VLP-based vaccine for respiratory syncytial virus (RSV) formulated with AddaVax (InvivoGen) which is analogous to the MF59 adjuvant [100]. Development of an effective RSV vaccine has been hampered by the harmful results of the first clinical trials performed in the 1960s in young children with a formalin inactivated-RSV vaccine (FI-RSV) adjuvanted with alum. Immunized children experienced vaccine enhanced viral disease after RSV natural infection which was characterized by severe bronchiolitis, alveolitis, and pneumonia resulting in an 80% hospitalization rate and 2 fatalities [101,102]. Several reports have shown that FI-RSV vaccine induces a detrimental Th2-mediated immune response, characterized by inflammation and immune cell infiltration inside the lung [103]. Notably, Kim K.H. and co-workers [104] have demonstrated that alum played an important role in exacerbating RSV disease after immunization with the FI-RSV when formulated with this adjuvant. Our preclinical studies in a mouse model demonstrated that RS-VLP formulated with AddaVax

was well tolerated and lacked the lung inflammation and recruited immune cell infiltrate as compared to the FI-RSV vaccine adjuvanted with alum [100]. We conclude that a squalene oil-based adjuvant may prove to be safe and efficacious in a VLP-based RSV vaccine formulation and an excellent candidate for a successful RSV vaccine. In addition, our group has successfully adopted the same adjuvant formulation with AddaVax for an investigational VLP-based vaccine against Zika Virus (ZIKV). Mice immunized with ZIK-VLPs developed very high titers of neutralizing antibodies [105].

5.5.2. Montanide—Montanide is a water in mineral oil based emulsion adjuvant, and it is commonly formulated as ISA 51 and ISA 720 (SEPPIC) [106]. The uses for Montanide are quite limited in human vaccinology due to its reactogenicity and risk of severe side effects [107,108]. Montanide adjuvants, however, may be suitable for life threatening conditions such as HIV or cancer where the benefits may outweigh the risks. For example, a clinical testing Montanide adjuvant has been performed to treat melanoma using the VLP-based vaccine MelQbG10, a virus-like nano-particle system loaded with the oligonucleotide CpG ODN (Cytos Biotechnology) [109]. Vaccination with Montanide adjuvant induced significant higher T-cell frequencies, resulting in detectable T-cell responses in all participants (11/11), with concomitant generation effector-memory-phenotype T-cells.

5.6. IL-12

IL-12 is secreted mainly from dendritic cells, neutrophils and macrophages, during viral infection, and stimulates a Th1-mediated immune response. IL-12 is able to activate the IL-12 receptor inducing the STAT/Jak pathway by activating the transcription factor STAT4, which is responsible for Th1 development in CD4+ cells [110]. IL-12 has been demonstrated to be an effective vaccine adjuvant against pulmonary pathogens. Notably, IL-12 is particularly effective in enhancing the immunity to associated antigen when administered *via* an intranasal mucosal route. In the initial work that described the efficacy of the IL-12 adjuvant in a VLP based vaccine formulation administered intranasally [111], we studied a VLP vaccine against influenza (H3N2) that afforded a high level of protection in mice when challenged with a lethal dose of virus. Notably, this study revealed that the antibody response was further enhanced when the VLP vaccine was formulated with IL-12 as an adjuvant [111].

5.7. Bacterial toxins : Cholera Toxin (CT) and Heat-labile Inactiv ated Enterotoxins (LT)

*C*holera toxin (CT) and the closely related Escherichia coli heat-labile enterotoxin (LT) are potent mucosal adjuvants [112]. In the past the use of CT and LT in humans was hampered by their extreme toxicity. Subsequently, site-directed mutagenesis has permitted the generation of LT and CT mutants either nontoxic or with a very limited toxicity, but able to exert a strong adjuvanticity at the mucosal level [113]. CT and LT can affect several steps in the induction of a mucosal immune response. They enhance antigen presentation by APCs, stimulate T cell proliferation and cytokine production, and promote isotype differentiation in B cells leading to mucosal IgA production. While CT induces primarily a Th2-type of response, LT can stimulate a more balanced Th1- versus Th2- mediated immunity. CT was tested in preclinical studies as an adjuvant to an influenza VLP-based vaccine and compared to analogous vaccine formulations adjuvanted with alum, CpG or MPL [114]. This study

showed that the CT adjuvanted vaccine afforded greater efficacy than other adjuvants in lowering lung viral loads after challenge. In addition, rotavirus VLP-based vaccines combined with either CT or LT elicited a high level of antibody production when administered *via* the intrarectal route in a murine model [115]. Interestingly, both adjuvant formulations induced a very strong Th17-mediated immune response specific for the viral antigens. LT adjuvant was licensed for a virosomal VLP-based vaccine against influenza in Switzerland (Nasalflu, Berna Biotech). The intranasal administration of the vaccine demonstrated a good profile of safety during the clinical trials, however after the vaccine was licensed and distributed some vaccinee showed a Bell's palsy adverse effect. The vaccine commercialization was withdrawn and the LT adjuvant was suspected to be responsible for the Bell's palsy symptoms [116]. Another study has suggested that using LT adjuvant for intranasal administration is inadvisable [117], nevertheless such adjuvant may demonstrate a good level of tolerability for percutaneous administration [118].

6. Conclusions and Future Perspectives

To the best of our knowledge, this is the first review focused specifically on adjuvant technologies and formulations for VLP-based vaccines. We have described distinct and innovative adjuvants that were successfully adopted for enhancing the potency and effectiveness of VLP-based vaccines (Table 1 and 2). The VLP-based vaccine technology has made remarkable progress in the last 10 years and has proven to afford excellent efficacy, tolerability and safety. Flexible manufacturing systems of non-infectious vaccine products reduce operation costs as well as risks to personnel and the environment while enhancing production capacity. VLP-based vaccines have demonstrated enhanced immunogenicity as compared to classical subunit vaccines, and a higher-level of safety than attenuated live virus vaccines. Moreover, adjuvant selection for VLP-based vaccine formulations is an important factor in maximizing their efficacy, potency and safety (Graphical Abstract). Aluminum salts represent the most frequently used adjuvant in vaccine formulations including the VLP-based vaccines (Table 1), however, new classes of adjuvants are needed not only to achieve greater effectiveness and safety but also to tailor their use based on mechanism of action, antigen characteristics and pathogen specific protective requirements. Advancing the development of new adjuvants should bring forth better choices for the vaccine field particularly for those vaccines that need improvement (e.g. influenza and malaria), vaccines under development (e.g. Zika virus and Ebola virus), and especially for vaccines against challenging pathogens (e.g. respiratory syncytial virus and norovirus) (Table 2). Difficult to control viral diseases may require specific and robust stimulation of the Th1-mediated immune response, which cannot be achieved ideally with the classical aluminum adjuvant. Adjuvant technology is a very broad and dynamic field, and we anticipate that novel adjuvants such as the plant polysaccharide inulin [119], the TLR9 agonist IC31 [120], the liposome based adjuvants CAF01 [121], the TLR4 agonist RC-529 [122], or the oil-in-water emulsion AS03 [123], will be further advanced and employed for VLP-based vaccines formulations.

Historically, adjuvant development has been an empirical enterprise, however new knowledge in complementary fields (e.g. immunology, virology, medicinal chemistry, etc.) has provided the foundation for a more rationale approach to the field of adjuvant research

and development. This emphasis should generate more effective and safer compounds and therefore enhance the prospect for better vaccines.

Acknowledgments

We thank Drs. George Martin and Katherine Frederick for helpful discussions and critical review of the manuscript.

Funding

This work was supported by a Small Business Innovation Research (SBIR) grant from the National Institute of Health (NIH-R43AI108099) to J.M.G.

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Highlights

- Virus-like particle (VLP) technology has significantly contributed to modern vaccinology
- Adjuvant formulation is fundamental for VLP-based vaccines efficacy and safety
- Novel classes of adjuvants have been developed for enhancing vaccine immunity
- We discuss progress in adjuvant development and formulation for VLP based vaccines
- Advances in these fields should bring forward more effective and safer vaccines

Table 1

Commercial VLP-based vaccines

Several VLP-based vaccines have been licensed for the prevention of viral diseases and they are broadly used due to their excellent level of protection, safety and affordability.

Virus Species	Assembly System	Antigen/s	Adjuvant	Commercial Name (Company)	Ref.
HPV	Baculovirus	Capsid protein L1	AS04	Cervarix (GSK)	11
HPV	Yeast	Capsid protein L1	Aluminum	Gardasil (Merck)	12
HBV	Yeast	HBsAg	Aluminum	Engerix-B (GSK)	13
HBV	Yeast	HBsAg	Aluminum	Recombivax HB (Merck)	14
HBV	Mammalian	HBsAg	Aluminum	GenHevac B (Pasteur)	15
HBV	Yeast	HBsAg	AS04	Fendrix (GSK)	16
HEV	E. Coli	Capsid protein CP	Aluminum	Hecolin (Xiamen Innovax Biotech)	17
Influenza A	Cell Free	HA and NA glycoproteins	Liposome	Inflexal V (Crucell)	18
HAV	Cell Free	Inactivated HAV	Virosome	Epaxal (Crucell)	19

Table 2

Adjuvants employed for the development of VLP-based vaccines

Different classes of adjuvant are utilized for modulating vaccine immunity and optimizing efficacy and tolerability of novel VLP-based vaccines.

Adjuvant	Receptor	T cell Response	VLP-based Vaccine	Development Stage	Ref.
Aluminum	NALP3	Th2	HPV, HBV, HEV	Approved Vaccines	12–15,17
Liposomes, Virosomes	?	Th1, Th2	Influenza, HAV	Approved Vaccines	18–19
MPL	TLR4	Th1	Norovirus	Phase II Clinical Trial	67
GLA-AF, GLA-SE	TLR4	Th1	Influenza	Phase II Clinical Trial	68–69
AS04	TLR4	Th1, CD8+	HPV, HBV	Approved Vaccines	71, 74
AS01	TLR4	Th1, CD8+	Malaria	Approved Vaccine	78–80
AS02	TLR4	Th1, CD8+	HPV	Phase I Clinical Trial	80
ISCOMATRIX	NALP3	Th1, Th2, CD8+	Influenza	Phase I Clinical Trial	83
Poly IC, Poly ICLC	TLR3, RIG-I	Th1, CD8+	Influenza, Ebola	Preclinical	84–85
CpG, GpG ODN	TLR9	Th1, CD8+	FMDV, Influenza	Preclinical	87–88
Flagellin	TLR5	Th1, Th2	Rabies, Influenza	Preclinical	90–91
Imidazoquinoline	TLR7/8	Th1, CD8+	HPV	Preclinical	95
Chitosan	NALP3	Th1, Th2	Norovirus	Phase I Clinical Trial	55
Squalene oil-based	?	Th1, Th2	HRSV, ZIKV	Preclinical	100, 105
IL-12	IL-12R	Th1, NK cells	Influenza	Preclinical	111
CT, LT	?	Th1, Th2, Th17	Influenza, Rotavirus	Preclinical	114–115