Chapter 17

Enabling Vaccine Delivery Platforms and Adjuvants for Malaria

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Additional information is available at the end of the chapter

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Abstract

Enabling vaccine delivery platforms and adjuvants with promising attributes for malaria vaccine development are reviewed within the framework of accessibility, efficacy, clinical status, cost, and cold-chain considerations. An emphasis is placed on commercially available platforms and adjuvants including virus-like particle, nanoparticle, microneedle, and mRNA vaccine delivery platforms as well as lipid vesicle, microparticle, and emulsion-based adjuvants. Strategies for addressing complications of vaccine delivery in endemic regions due to concatenate vaccination and infection, and parasite immune avoidance mechanisms are presented. Additionally, recent findings regarding how malaria infection triggers inflammatory pathways and T cell exhaustion along with negative impacts to the development of effective memory responses are described in a context relevant to vaccine development.

Keywords: *Plasmodium falciparum*, malaria, adjuvant, vaccine, delivery platform, VLP, virus-like particle, microneedle, mRNA, nanoparticle, microparticle, liposome, emulsion, nanoemulsion, TLR agonist, T cell exhaustion, PD-1, immune response, CSP

1. Introduction

Efforts to develop an effective malaria vaccine have been ongoing for decades. In order to promote better coordination and acceleration of malaria vaccine development, the World Health Organization (WHO) initiated a process that resulted in the Malaria Vaccine Technology Roadmap, which was first published in 2006 [1] and updated in 2013 [2]. The vision in the roadmap includes development of vaccines for *Plasmodium falciparum* and *P. vivax* to prevent

disease, death, and malaria transmission as part of enabling malaria eradication efforts. As per the updated Malaria Vaccine Technology Roadmap, the goal of a successful vaccine candidate is ≥75% protective efficacy over 2 years with no more than one annual booster that can be administered to all age groups. Transmission-blocking vaccines were also included in this development effort. Importantly, the Roadmap prioritized access to low cost GMP vaccine manufacture for commercial production, which encompasses a direct pathway to licensure, ability to manufacture at a large scale, as well as vaccine availability and ease of access, inclusive of delivery platforms and adjuvants. With these considerations in mind, we review enabling vaccine delivery platforms and adjuvants with favorable attributes, to both facilitate the fusion of promising malaria targets with novel technology platforms and meet Roadmap vaccine development, efficacy, and accessibility goals.

A brief overview of recent findings regarding development of immune responses during and after malaria infection is relevant to vaccine development (particularly regarding parasite immune evasion mechanisms that engage inflammatory pathways, promote T cell exhaustion, and stimulate regulatory T cell expansion), as vaccination and malaria infection occur concatenately in endemic regions. Traditionally, the intended outcome of any combination of malaria vaccine target and adjuvant/delivery platform would be to elicit as strong of an immunostimulatory response as possible to the Th1 and/or Th2 immune compartments. However, studies of chronic disease in humans and mice, in the cancer and infectious disease fields, have shown that magnitude of the immune stimulus may not be as important as the balance of immune presentation to immune overreaction (e.g., regulatory T cell stimulation and T cell exhaustion/ablation) [3–5]. Recent studies in rodent malaria models have significantly increased our understanding regarding how chronic malaria infection can hamper development of effective Th1 and Th2 immune responses as well as development of B and T cell memory. For example, in a mouse model of severe malaria infection, it has been shown that proinflammatory cytokines (IFN-γ and TNF) and pathways mediating the disease are detrimental to development of humoral response by inhibiting/exhausting T helper cells [6]. That these cytokines are the same as those monitored in regards to enhanced cellular response to many adjuvanted vaccines is of particular concern. Furthermore, in mouse malaria models, chronic malaria infection triggers CD8+ T cell exhaustion (loss of T cell effector function) through a programmed cell death-1 (PD-1) pathway [7, 8]. In humans, the state of T cell exhaustion has been documented following numerous infections, during chronic infection, as well as in cancer patients, with expression of PD-1 as a hallmark exhaustion [9]. Taken together, these phenomena suggest an explanation for some of the difficulties encountered during malaria vaccine development as well as provide insight into why vaccine-induced protective immunity quickly wanes. A knowledge base on preventing T cell exhaustion has been developed in the therapeutic cancer vaccine field, which can be used as a launching point for how to address this issue in malaria vaccine development. One of the key areas of interest is the use of adjuvants to downregulate pathways leading to T cell exhaustion (e.g., PD-1 and LAG-3 inhibitors) [10–12]. Therefore, we suggest that the information presented herein be considered in the context of careful characterization of the mode of action for the combined antigen and adjuvant/delivery technology to overcome the evolving hallmarks of malaria immune evasion.

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1.1. Enabling vaccine delivery platforms

A range of novel vaccine delivery platforms are described herein, including those that can accommodate different antigen/immunogen formats such as recombinant proteins, peptides, epitopes, and/or nuclei acids. Many of these platforms can deliver multiple antigens and in different formats, simultaneously. In describing these platforms, we included state-of-the-art technologies with the potential for major impacts to vaccine delivery where the regulatory and/or licensure pathways may not yet be defined. As there are a significant number of published preclinical and clinical studies on different viral vectored malaria antigens (e.g., adenovirus and modified vaccinia Ankara (MVA) vectors), these platforms have not been detailed herein, but have been recently reviewed elsewhere [13–15]. In addition, several particulate-based platforms that have been used in combination with malaria candidate vaccines, but not described here, have been recently reviewed [16].

1.2. Virus-like particles

The ability of viral capsid proteins to self-assemble and incorporate foreign antigens has been exploited in the development of virus-like particles (VLPs) as a vaccine delivery platform [17, 18]. While many VLP platforms for human pathogens have been developed and utilized to deliver native epitopes of the subject virus, other VLP platforms can be used to deliver foreign epitopes; only the latter is discussed herein. Platforms capable of integrating foreign epitopes include human pathogens (e.g., hepatitis B virus (HBV) and human papillomavirus) and many nonhuman pathogens (e.g., bacteriophages, plant viruses, and animal viruses). Foreign antigens can be incorporated into VLPs by genetic modification of the capsid protein at one or more sites or by chemical conjugation of antigen to the capsid protein. Immunogens can also be encapsidated within the VLP lumen. Even with these modifications, capsid proteins can retain the structure and morphology of the originating virus. VLPs are known to induce strong humoral and cellular immune responses as foreign antigens are displayed in a repetitive manner on VLPs, which boost immune responses by facilitating cross-linking of immunoglobulin receptors and B cell activation; VLPs are easily taken up by antigen-presenting cells that ultimately lead to cytokine production, stimulation of CD4+ T helper cells, and induction of potent cytotoxic immune responses through cross-presentation to cytotoxic CD8+ T cells [18–24]. For most VLPs, single-stranded RNA (ssRNA) is packaged within the particles during assembly. The presence of ssRNA is thought to enhance immunity, as this is a natural ligand for Toll-like receptor (TLR) 7 and TLR8 [25]. Of note is that the described plant-based VLP platforms do not meet a strict definition of VLP as they are infectious to and propagate in the organism in which they are grown (but not in humans). For simplicity, and as these platforms are often identified as VLPs in the literature, they are presented together in a single section.

The main drawback of VLP-based vaccines is size constraints of the foreign antigen incorporated or fused to capsid protein, which is dependent on the specific VLP platform. Note that larger antigens can be chemically conjugated to some capsid proteins; however, this is also a drawback as the antigen and capsid must be expressed and purified separately for conjugation, which can elevate cost. Currently, three VLP-based malaria vaccine candidates have been

evaluated in the clinic, and one of these was recently approved for use in humans. These are RTS,S (tradename Mosquirix when in combination with AS01), ICC-1132, and Pfs25 VLP-FhCMB, which are each described below. A recent review of the different GlaskoSmithKline (GSK) adjuvants (AS01, AS02, etc.) can be found elsewhere [26].

1.2.1. Hepatitis virus VLPs

Viruses of the family *Hepadnaviridae*, including the human pathogen HBV, can be utilized as vaccine delivery platforms. These viral particles have both an outer lipid envelope containing a surface antigen (e.g., HBsAg) as well as a nucleocapsid composed of a core protein (e.g., HBcAg). Both the surface protein and core protein can accept foreign peptides and thus be utilized as VLP-based vaccine delivery platforms. RTS,S is composed of the repeat and C-terminal regions of *P. falciparum* circumsporozoite protein (PfCSP) fused to hepatitis B virus surface antigen (HBsAg) and has been licensed in combination with AS01 by the European Medicines Agency (EMA). The vaccine candidate ICC-1132 is comprised of the hepatitis B virus core protein (HBcAg) with PfCSP T cell and B cell epitope insertions. ICC-1132 has been tested in multiple clinical studies [27–30]; however, upon controlled human malaria challenge (CHMI) of vaccinees administered with ICC-1132 formulated in Montanide, no sterile protection was seen [30].

Core protein-based VLPs possess a number of favorable characteristics for vaccine development as compared to surface protein-based VLPs. These include the ability of the recombinant core protein to self-assemble into VLPs, flexibility of the expression system, and increased immunogenicity of the core protein as compared to the surface protein [31–34]. Considerations of immune tolerance issues when using VLPs based on the human pathogen HBV are also important given the number of chronic HBV carriers worldwide. Additionally, as antibodies to the HBcAg serve as the basis for HBV diagnostics, widespread use of HBcAg-based VLPs may compromise use of anti-HBc antibodies to diagnose infection. These concerns have been addressed through use of nonhuman pathogenic hepadnaviruses in VLP platform development, including viruses that infect rodents and ducks [35–37]. In addition to RTS,S and ICC-1132, which are VLPs based on the human hepatitis B virus, a woodchuck hepatitis B virus VLP platform (WHcAg) containing PfCSP T cell and B cell epitopes (developed by VLP Biotech) has shown promise in challenge studies with a rodent malaria model where 80–100% protection was seen with different formulations [38]. In this same study, a WHcAg VLP carrying *P. vivax* circumsporozoite protein (PvCSP) repeat epitopes was used for challenge with a rodent malaria model and 100% protection was seen. A comparative study of antigenicity and immunogenicity of different rodent hepatitis virus core proteins (woodchuck, ground squirrel, and artic squirrel) and HBcAg demonstrated that rodent core proteins are (1) equal in immunogenicity to, or more immunogenic than HBcAg for both B cell and T cell responses, (2) not significantly cross-reactive with the HBcAg for B cell responses and only partially cross-reactive with HBcAg for T cell (CD4) responses, and (3) competent to function as vaccine carrier platforms for heterologous, B cell epitopes [36, 39]. In consideration of cost, WHcAg VLPs can be easily expressed at high levels in *E. coli*. In addition, this platform can

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accommodate insertion of foreign sequences at multiple sites within the coat protein and large inserts at both the N- and C-termini. Note that this platform has not yet been tested in the clinic.

1.2.2. Bacteriophage VLPs

VLPs based on ssRNA bacteriophages can be used as vaccine delivery platforms through conjugation of foreign antigens to the coat protein, encapsidation of foreign antigens within the VLP, or genetic insertion of foreign sequences into the coat protein. One application of the last is creation of VLP-based peptide display libraries by the University of New Mexico (based on MS2, PP7, and AP205 bacteriophage VLP platforms) using a series of plasmid vectors that allow insertion of high complexity random sequence peptides into the coat protein enabling construction of libraries with up to 10^{10} to 10^{11} unique random sequence peptide VLP clones with inserts from 6 to 20 base pairs in length [40, 41]. Biopanning can be performed on these libraries using neutralizing or inhibitory antibodies to affinity select VLPs containing mimotopes—peptide sequences that mimics the structure of epitopes. As opposed to traditional peptide display technologies, affinity selected bacteriophage VLPs can be used directly as immunogen without modification. For example, a VLP mimotope to reticulocyte-binding protein homologue 5 (RH5) found through affinity selection of MS2 libraries using a monoclonal antibody able to block parasite invasion of erythrocytes (*in vitro* via growth inhibition assay) was shown to elicit inhibitory antibodies when administered to mice as an immunogen [42]. In addition, MS2 VLP library affinity selection has also been performed with two anti-AMA-1 monoclonal antibodies [42, 43]. Of note is that Agilvax LLC (a startup that was spun out of the Science & Technology Corporation at the University of New Mexico) holds an exclusive license to commercialize this technology for vaccines and immunotherapies based on the MS2 and AP205 VLP platforms. The MS2 VLP platform can also be used to encapsidate RNA and RNA-modified cargo [44]. Similar to the Qbeta VLPs, foreign antigen can be conjugated to AP205 VLPs [45]. Additionally, while most bacteriophage VLPs can only tolerate small foreign insertions, relatively large insertions are tolerated by the AP205 platform [46]. This has been exploited to create a platform whereby antigens can be irreversibly bound to and displayed on AP205 VLPs by simple mixing using SpyCatcher and SpyTag; further, AP205 VLPs utilizing the SpyCatcher/SpyTag system and displaying Pfs25 (a transmission-blocking vaccine target) are immunogenic in mice [47, 48]. Note that use of the SpyCatcher/SpyTag technology might easily be extended to several other platforms included here, in particular to nanoparticles and lipid vesicle-based platforms; however, it will be important to understand if this technology can meet regulatory requirements for clinical use.

1.2.3. Plant-based virus VLPs

A number of plant viruses have been developed into replication-competent platforms whereby VLPs can infect and replicate in plants (generally tobacco or spinach), but not in humans. Two of the more advanced platforms include alfalfa mosaic virus (AlMV) and tobacco mosaic virus (TVM), both of which can be produced at high levels in plants [49]. While the benefits of biologics production in plants include the ability to produce large quantities of material with relatively low cost starting materials, there are logistical challenges in housing and transfecting

plants on a large-scale under conditions that meet GMP requirements as well as development of downstream purification processes to recover VLPs from fibrous plant material [50]. Of note is that significant improvements have been made in plant cell culture for expression of pharmaceutical products (conducted in bioreactors), which has the potential to alleviate some of these challenges and increase the feasibility of using plant VLPs platforms for large-scale vaccine production [50, 51]. Malaria vaccine research efforts have included development of a AlMV VLP containing Pfs25 by Fraunhofer USA Center for Molecular Biotechnology [52], which has been tested in a phase I clinical trial (ClinicalTrials.gov Identifier: NCT02013687); however, results of the trial have not yet been published.

1.3. Polymeric nanoparticle delivery platforms

Polymeric nanoparticles share many of the immunological advantages of VLPs in that their size and structure are similar to that of a pathogen, which leads to interaction with antigen presenting cells [53]. An additional attribute of polymeric nanoparticles is that these can be tailored to a specific purpose through adjusting physical attributes such as size, shape, and charge as well as customizing the type and concentration of polymer [54]. A number of different nanoparticle-based vaccine delivery platforms are available, some of which have been used in the clinic and some of which have thus far only been assessed in animals. The main components of these platforms are biodegradable polymers/composites that function to create a depot for antigen presentation. Immunostimulators can also be incorporated into these delivery platforms. Antigens may be encapsulated or bounded by covalent or noncovalent bonds.

1.3.1. PLGA nanoparticle delivery platforms

Poly-DL-lactide-co-glycolide acid (PLGA) is a biodegradable polymer approved for human use by FDA for several indications. Published applications of PLGA nanoparticles in regards to malaria vaccine development include the targets Pfs25 and PvCSP [55, 56]. PLGA nanoparticle delivery platforms of interest for malaria vaccine development include Selecta Biosciences' synthetic vaccine particle (SVP™) platform and Orbis Biosciences Stratum™ platform. The SVP™ platform in combination with TLR7/8 and TLR9 agonists can enable robust cellular and humoral immune responses, and is flexible regarding how antigens are incorporated [57]. The Stratum™ technology is based on PLGA microspheres that encapsulate aqueous material (including antigens) and degrades in a controlled manner to permit delayed release of encapsulated material [58, 59]. For example, this platform can enable provision of both prime and booster antigen within a single administration with the booster contained in extended release microspheres that degrade 30 days after administration. Clinical studies have been conducted on PLGA-based platforms from both Selecta and Orbis.

1.3.2. Multilayer nanofilm-based nanoparticles

Artificial Cell Technologies, Inc. (ACT) has developed an innovative method for producing nanoparticle vaccines utilizing artificial biofilms comprised of oppositely charged polymers and target antigens constructed on solid $CaCO_3$ cores (nonimmunogenic carrier) using layer-

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by-layer (LbL) fabrication. This platform can accommodate multiple target antigens applied at different layers (depths) within the biofilms as well as immunostimulators, as needed for optimizing the immune response. Additionally, the biolayers can include innate immune stimulants to increase vaccine potency. Biofilm generation and LbL fabrication of nanoparticles have been previously described [60–62]. Of note is that experimental studies have shown that proteins/polypeptides are stabilized in nanofilms by noncovalent interactions, and secondary structure is maintained during the manufacturing process [62]. Additionally, disulfide bonds between cysteine-containing peptides increase nanofilm stability, mimicking the stabilization of the native protein structure [62]. Several ACT nanoparticle constructs, including different B and T cell epitopes of PfCSP, have been developed and tested in mice with encouraging results [63]. While this platform has not yet been assessed in the clinic, ACT is fast approaching intuition of a phase I study with this platform.

1.4. Microneedle platforms

Recent advances have increased interest in intradermal/transdermal vaccine delivery due to an improved understanding of the high immune response achievable within the skin, based on plasticity and high numbers of antigen-presenting cells (APCs) within this tissue, including Langerhans cells, dendritic cells, and macrophages [64–66]. Traditional intradermal vaccine delivery, using the Mantoux or skin scarification methods, requires special training to perform correctly and can be inaccurate regarding the dose of vaccine that is delivered or difficult to achieve based on volume limitations (skin scarification). Therefore, platforms that provide easy and accurate transdermal delivery of vaccines are of high interest [67, 68]. Microneedle arrays described herein are solid-state platforms that either integrate vaccine components into a biodegradable polymer, which is subsequently formed into an array, or are manufactured from nonbiodegradable materials into an array and then coated with vaccine components. In either case, size and length of the microneedle array is controlled so that temporary pores are created in the stratum corneum (the protective outer layer of the skin), and the vaccine is administered to the desired depth. In addition to the benefit of "needleless" administration, the depth at which microneedles penetrate does not reach underlying blood vessels or pain receptors. Stabilization of antigens and adjuvants on or within the microneedle array is also of benefit, particularly in regards to cold-chain requirements. One potential drawback of these platforms is that relatively high concentrations of antigen and adjuvant must be possible in order to achieve a relevant dosing range for most vaccines. Also of note is that development costs for microneedle platforms that incorporate antigens are higher than those where antigens are coated on the microarrays. In addition, consideration must be given to the administration device used to place the array regarding cost and ease of use. However, cost savings are achieved with these platforms because a needle and syringe is not required for administration.

Viral vectored ME-TRAP, PbCSP, and PyMSP-1 have been assessed with silicone microneedle arrays in mice where increased immune response to the target antigen (compared to the vector) and protective efficacy were found [69–71]. However, better responses were found using a mixed administration regimen where antigen(s) were first given via the microneedle platform and then boosted via the intradermal route. For these studies, antigen was applied to the skin

just prior to application of the microarray rather than being incorporated into or coated on to the microneedle array itself.

1.4.1. Natural polymeric microneedles

Silk fibroin is a biocompatible, biodegradable block copolymer that self-assembles into β-sheets separated by flexible hydrophilic spacers. This natural polymer is approved by FDA for human use in medical devices such as wound dressings and sutures, and GMP grade silk fibroin is available from Vaxess Technologies. Significant progress has been made by Vaxess in developing a consistent, repeatable manufacturing process for silk fibroin microneedles [72]. Additionally, several protein immobilization strategies can be used with this polymer including adsorption, covalent bonding, entrapment, and encapsulation [73]. One attribute of this platform that is highly attractive for malaria vaccine development is that silk fibroin microneedles can be designed such that an initial bolus of vaccine is delivered upon administration followed by low-level sustained release of vaccine over a period of several weeks or longer [74]. Note that this microneedle platform has not yet been tested in the clinic.

1.4.2. Synthetic polymeric microneedles

Microneedle arrays can also be constructed from synthetic biodegradable polymeric materials such as PLGA. Corium International has a vaccine-in-tip platform where antigen and adjuvant are combined with MicroCor excipients and then the solution is cast into molds to create the microstructure array (MSA) [75]. A PLGA backing layer is applied and the patch integrated into an applicator. The MicroCor platform is designed such that the needles fully dissolve over a period of several minutes after which the backing is removed. Arrays can also be constructed from nonbiodegradable polymers, which are then coated with antigen and adjuvant. Such a platform is available from 3M's drug delivery systems division whereby arrays are molded from medical grade liquid crystalline polymer and substances (antigens, adjuvants, etc.) are coated on the microneedles using a dip coating process [76]. This type of array is designed to be left *in situ* for a short period of time and then removed once the coating has dissolved. Note that liquid crystalline polymer is not biodegradable. Microneedle platforms from both Corium and 3M have been tested in the clinic.

1.5. mRNA-based vaccine delivery platforms

Recent advances in mRNA vaccine delivery have elevated these platforms to the point at which feasibility of mRNA-based vaccines has been demonstrated in the clinic. The CureVac RNActive platform uses mRNA for vaccine delivery and relies on sequence modifications at the 5′ and 3′ ends to enhance protein expression and inclusion of a protamine sequence to increase immunogenicity [77]. An RNActive prostate cancer vaccine has been tested in the clinic with encouraging results regarding safety and immunogenicity, where induction of both Th1 and Th2 responses was seen [78]. There are significant advantages in using a nucleic acid-based platform including (1) the cost benefits of neither having to manufacture/purify antigen nor formulate with adjuvant, (2) the possibility for development of multivalent vaccines without concerns regarding formulation, and (3) quick man-

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ufacturing speeds where gene synthesis to completion of GMP production can take less than 2 months. A parallel mRNA-based delivery platform has been developed by Moderna; however, relatively limited information is available regarding Moderna's mRNA Therapeutics™ platform with the published studies relating to injection of mRNA into the heart (in a mouse model) to treat myocardial infarction [79, 80].

1.6. Densigen™ platform

The Densigen™ platform (available from Altimmune) is based on rationally designed long, fully synthetic peptides (30–40mers) containing natural clusters of CD4+ and CD8+ T cell epitopes (termed densigens). A proprietary bioinformatics approach is applied to select the most immunogenic and conserved domains. Densigens are conjugated to a fluorocarbon moiety, which allows the densigens to self-assemble into micelle-based nanoparticles. The self-adjuvanting properties of densigens are thought to be attributed to persistence of the nanoparticles at the administration site (depot effect) and resistance of the nanoparticles to proteolytic degradation [81]. Multiple densigens can be incorporated into a single formulation. A Phase I clinical study with a densigen-based influenza vaccine demonstrated good immunogenicity to all six peptides contained in the vaccine (across divergent influenza strains) [82]. In addition, a Phase 1 study of an HBV therapeutic densigen vaccine (HepTcell, which is composed of nine densigens) is ongoing (clinicaltrials.gov identifier: NCT02496897).

2. Enabling vaccine adjuvant platforms

Malaria vaccine enabling adjuvant platforms and immunostimulators are detailed in this section. Many of these can be combined with different immunogen formats and vaccine delivery platforms. We describe novel adjuvants as well as those where studies have been performed with malaria vaccine candidates. Also noted is if an adjuvant has been or can be combined with additional immunostimulators. Similar to the vaccine delivery platforms, we included state-of-the-art technologies where the regulatory and/or licensure pathways may not yet be defined.

2.1. Lipid vesicle-based platforms

Several different classes of lipids can be incorporated into vesicle-based vaccine adjuvant and delivery platforms. These include traditional liposomes (phospholipids as well as anionic, neutral, and cationic lipids from bacteria and eukaryotes), lipids derived from viral envelopes (virosomes), and lipids from Archaea (archaeosomes). The versatility and plasticity of lipid vesicle-based platforms are a major advantage as liposomal compositions can be customized to achieve desired characteristics including lipid type(s), charge, size, antigen association type, and inclusion of adjuvants and immunostimulators [83, 84]. Depending on the chemical properties of the liposomes, antigens may be entrapped in the aqueous core, intercalated into the lipid bilayer, and/or attached to the liposome surface by adsorption or conjugation. Additionally, different antigens/adjuvants can be combined to tailor liposomal vaccines for

specific applications [85, 86]. In general, these systems provide adjuvant activity by enhancing antigen delivery to effector cells and/or by potentiating immune responses. Of note is that some of the platforms in this section can serve as both adjuvants as well as delivery platforms, depending on how antigen is incorporated. A comprehensive review of liposome vaccine delivery platforms is provided in [87].

2.1.1. Aqueous liposomal platforms

Several aqueous liposomal platforms have been built around the capabilities of cholesterol to stabilize lipid bilayers and QS-21 to create pores in lipid bilayers through association with cholesterol. In addition to these properties, QS-21 has also been shown to stimulate Th1-type responses and production of antigen-specific cytotoxic T lymphocytes (CTLs) [88]. The molecular stability of QS-21 is increased when incorporated into liposomes [86], as free molecules undergo deacylation above pH 6 and at temperatures problematic for vaccine administration in warm climates where cold-chain may not be maintained [88]. Aqueous liposomal formulations containing QS-21 provided in a format that can be directly mixed with antigen include AS01 from GSK (which also contains monophosphoryl lipid A – MPLA), GLA-LSQ developed by IDRI (which contains a synthetic form of MPLA known as GLA), ALF-Q developed by WRAIR (which contains MPLA), and Matrix-M from Novavax, previously known as AbISCO-100 (which is a unique 40-nm-sized complex and can be combined with immunostimulators such as TLR agonists [89]). These adjuvants are known to promote elicitation of both Th1 and Th2 immune responses [86, 90, 91]. One of the biggest drawbacks for several of these adjuvants is limited access/availability. However, GMP grade MPLA and QS-21 are available from Avanti Polar Lipids, Inc. and Agenus Inc., respectively. In addition, GMP grade lipids suitable for liposomal preparations are available from Avanti Polar Lipids.

Multiple clinical studies combining malaria vaccines/candidates (RTS,S, LSA-1, AMA-1, MSP-1, and CelTOS) with this class of adjuvants have been performed [16]. The different levels of protection seen with RTS,S/AS01 are well documented in the literature, and therefore not reviewed here. A high level of protective efficacy has not been found with any of the other malaria antigens combined with AS01, which has been recently reviewed in Ref. [16]. Preclinical studies have been conducted with full length PfCSP recombinant protein adjuvanted with GLA-LSQ where 40% sterile protection was seen upon challenge in a mouse malaria model [92]. This adjuvant has also been used in combination with PfCelTOS recombinant protein where a statistically significant reduction in liver load was found in challenged mice [93]. In addition, a clinical study with GLA-LSQ and the placental malaria vaccine candidate PAMVAC (VAR2CSA) has been registered on clinicaltrials.gov (identifier: NCT02647489); however, no published information is available. ALF-Q is a relative newcomer, and there are currently no published studies using ALF-Q in combination with malaria vaccine candidates. Matrix-M has been tested in preclinical studies with a variety of antigens including viral-vectored Pv Duffy binding protein (DBP) vaccine candidates. High levels of *in vitro* erythrocyte binding inhibition (>90%) were achieved with Matrix-M formulations in this study [94]. Additionally, Matrix-M has been assessed in a Phase I clinical study in combination with chimpanzee adenovirus 63

(ChAd63) ME-TRAP and MVA ME-TRAP (ClinicalTrials.gov identifier: NCT01669512); however, study results have not yet been published.

A platform related to Matrix-M and also comprised of lipids, cholesterol, and QS21 is immune stimulating complex (ISCOM) technology. ISCOMs are spherical open cage-like structures formed by cholesterol binding QS-21 and then stabilized with phospholipids [95]. Similar to Matrix-M, ISCOMs have a mean diameter of ~40 nm; however, this technology incorporates antigens into the ISCOM such that they are displayed in a multimeric fashion on the particles. ISCOMs are known to traffic antigen into the cytosol of dendritic cells, stimulate both Th1 and Th2 immune responses, and link innate and adaptive immune responses *in vivo* in a MyD88-dependent manner [96, 97]. The major drawback of this platform is that the type and amount of antigen displayed can be limiting; however, researchers have developed additional methods for incorporating antigen into ISCOMs, including using cationic ISCOMATRIX particles to attract anionic proteins, adding a lipid tail to the antigen, or using a fusion protein strategy to add hydrophobic peptide tags to the antigen [98]. Although modification of a vaccine candidate for inclusion in such a platform can add a layer of complexity and additional expense to the development process, ISCOMs might be of interest to adjuvant malaria immunogens containing a GPI-anchor. No studies with ISCOMs and GPI-anchored proteins were found in the literature. ISCOMs are commercially available from CSL Behring as ISCOMATRIX.

Another aqueous liposomal platform of potential interest is the adjuvant CAF01, developed by Statens Serum Institute. A clinical study conducted with this adjuvant demonstrated long-lived Th1 responses over a 3-year monitoring period (ClinicalTrials.gov identifier: NCT00922363) [99]. This adjuvant is comprised of two synthetic components, cationic liposomes and a glycolipid immunomodulator (synthetic mycobacterial cell wall cord factor). In a recent preclinical mouse study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared head-to-head [100]. Of the five adjuvants tested, CAF01 was the only adjuvant to elicit a Th1 immune response when formulated with each of the three antigens. In addition, the CAF01 formulation demonstrated the highest reduction of *M. tuberculosis* and *Chlamydia trachomatis* in challenge models. However, in most cases, the humoral response elicited was similar to the no adjuvant and/or alum formulations. Note that MF59®, GLA-SE, and IC31® are discussed in subsequent sections.

2.1.2. Lipid-in-oil platforms

The DepoVax™ platform, developed by ImmunoVaccine Inc., contains lipids, cholesterol, oil, emulsifier, and an immunostimulant (e.g., polyIC or Pam_3Cys) [101]. This unique lipid-in-oil platform is designed to present antigen(s) and adjuvant(s) at a long lasting depot that effectively attracts APCs and from which antigen is released over an extended period of time, from weeks to months [102]. Based on this long lasting depot, a single dose of vaccine formulated in DepoVax has been shown to be superior to multiple doses of the same vaccine formulated in traditional adjuvants [103, 104]. In addition, DepoVax has been shown to not only promote Th2 responses, but also enhance Th1 immune responses without triggering

regulatory T cell [101]. DepoVax has been used in the clinic as part of a Phase I/II study for a cancer vaccine (ClinicalTrials.gov identifier: NCT01095848) [105]. Of note is that there are no aqueous components in this formulation; therefore, antigen is lyophilized for use with DepoVax and components are mixed and emulsified prior to administration using materials provided as part of an administration kit.

2.1.3. Virosomes

Virosomes are liposomes prepared by reconstituting virus envelope phospholipids. Those from influenza virus are the most common with the virosome physicochemical properties modulated by the amount and type of lipids used [106]. In contrast to liposomes, virosomes can contain functional viral glycoproteins (i.e., influenza virus hemagglutinin and neuraminidase) within the phospholipid bilayer membrane, which enhance immunogenicity. Additionally, virosomes can induce both B and T cell responses through antigen presentation in the context of both MHC-I and MHC-II [87, 106, 107]. Antigen can be encapsulated within the virosomes, conjugated to phospholipids, or adsorbed to the virosome surface [108]. Malaria antigens have been incorporated into influenza virosomes including epitopes from AMA-1, CSP, MSP-3, and GLURP [109–111]. Clinical studies were performed with virosomes containing an AMA-1 peptide from domain 3 (PEV301) and a CSP repeat region peptide constituting constrained NPNA units (PEV302). In a Phase 1a trial, volunteers immunized with PEV301, PEV302, or a combination of the two (PEV301 + PEV 302) had good seroconversion and long-lived humoral responses when assessed at a 1-year follow-up [112–114]. A Phase 1b trial was conducted in a malaria-endemic area where adults and children were immunized with PEV3B (a formulation including both the subject AMA-1 and CSP epitopes) or a comparator virosome-based vaccine to influenza, Inflexal®V [115]. While not statistically significant, the malaria incidence rate in children administered PEV3B was lower than children given Inflexal®V (67% versus 80%, respectively) over the 1-year follow-up period.

2.1.4. Archaeosomes

Species of the domain *Archaea* contain unique polar lipids that have adjuvanting properties when used as liposomes (archaeosomes) containing encapsulated antigens. The lipid backbones found in *Archaea* have a higher resistance to acid hydrolysis compared to those from *Eukarya* and *Bacteria*, and surface tension and permeability characteristics of archaeosomes differ in comparison to traditional liposomes; these properties are also thought to increase adjuvanting potential of archaeosomes [116]. The type of immune response generated with archaeosomes can be manipulated by changing composition of the head groups attached to archaeol, and lipids from some *Archaea* have been used to chemically synthesize additional lipid types with interesting characteristics [117, 118]. Several studies with archaeosomes and encapsulated ovalbumin (for cancer applications) have shown elicitation of both Th1 and Th2 immune responses as well as the ability to modulate the immune response by varying the types of polar lipids within the archaeosomes [117–119]. As this is a relatively new platform, a pathway to regulatory approval for clinical testing and licensure is needed.

2.2. Polymeric microparticle-based adjuvants

Advax™ (developed by Vaxine) is a microparticle-based adjuvant comprised of microcrystalline delta inulin, a plant-derived polysaccharide. Of note is that delta inulin is insoluble at body temperature. This adjuvant generates Th1 and Th2 immune responses and activates the alternative compliment pathway [120]. However, Advax™ does not activate nuclear factor-kappa B (NFkB) so an inflammatory response is not seen with this adjuvant [121]. Given the mounting data regarding the ability of malaria to hinder development of effective immune responses, adjuvants that do not trigger inflammatory responses are of interest, particularly for use in malaria-endemic regions. Although there are no published studies with Advax™ and malaria antigens, preclinical assessments have been conducted with a variety vaccine candidates formulated in this adjuvant [121]. In addition, this adjuvant has been assessed in multiple clinical studies [122, 123].

2.3. Emulsion-based adjuvants

All of the adjuvants described in this section are oil-in-water emulsions. Those that include solvents and surfactants form nanoemulsions, which facilitate antigen uptake by dendritic cells [124]. Formulation of malaria vaccines with emulsion-based adjuvants is of interest in part because there are examples of a high level of sterile protection in clinical studies with CHMI challenge when RTS,S was combined with an oil-in-water emulsion-based adjuvant [125, 126]. The GSK adjuvants AS02 and AS03 are both oil-in-water emulsions. AS03 contains squalene, vitamin E, and Tween 80, while AS02 contains these components plus MPLA and QS21. In a clinical study with CHMI where RTS,S was combined with either AS02 or AS03, sterile protection was seen in 6/7 (86%) volunteers given RTS,S/AS02, and 2/7 (29%) volunteers given RTS,S/AS03 [126]. However, 6 months after the last vaccination only 1/5 (20%) of the volunteers given RTS,S/AS02 showed sterile protection upon a second CHMI [125]. For these volunteers, the factors contributing to loss of protective efficacy in such a short period of time is unclear but may relate to challenge and the ability of malaria parasites to compromise development of long-term immunity. In light of these and similar results, improvements in sustaining established immunity must be made regarding development of an effective malaria vaccine.

2.3.1. MF59

MF59 is an oil-in-water nanoemulsion that consists of squalene oil, Tween 80, and sorbitan trioleate (Span 85). MF59 (available from Novartis) is licensed in Europe as a clinical vaccine adjuvant for influenza and has been intramuscularly administered to millions of people ranging in age from children to elderly adults. This adjuvant has been assessed in animal studies with MSP-1 and PvDBP malaria antigens; however, immunogenicity and binding assay (PvDBP) results were poor in comparison to other adjuvants [127–129]. While these results were discouraging, using MF59 in combination with small molecule immunostimulators, particularly those known to induce a Th1 response such as CpG (which has been previously used MF59 with encouraging results [130]), may improve efficacy with malaria antigens. In a recent study, five approved or clinically tested adjuvants

(Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, only MF59 and GLA-SE were able to induce inhibitory titers to influenza; however, inhibitory titers with MF59 were >60% higher than with GLA-SE. Note that MF59 formulations did not induce a Th1 immune response for any of the antigens.

2.3.2. NanoStat™ platform

NanoStat™ (available from NanoBio Corporation) is an oil-in-water nanoemulsion composed of soybean oil, ethanol, Tween 80, cetylpyridinium chloride, and water. Note that these components are quite inexpensive as compared to those of some of the other adjuvants described herein. The adjuvant activity of this nanoemulsion is dependent on the nanodroplet structure and positive charge, which enables the penetration of the mucous layer, binding to cell membranes, and cellular uptake [124, 131]. In mice, NanoStat™ has been shown to produce systemic and mucosal immune responses including MyD88-independent Ab responses and MyD88-dependent Th-1 and Th-17 cell-mediated responses [132]. While most of the published research (including a Phase I clinical [133]) is with NanoStat™ formulated for intranasal delivery, a formulation that contains the same components in proportions tailored for intramuscular administration is also available. These adjuvants can be combined with small molecule immunostimulators. Currently, there are no published studies of NanoStat™ used in combination with malaria antigens.

2.3.3. GLA-SE

GLA-SE (developed by IDRI) is an oil-in-water emulsion containing squalene and glucopyranosyl lipid A (GLA), a synthetic Toll-like receptor 4 (TLR4) agonist that is similar to MPLA. This adjuvant is known to generate both Th1 and Th2 immune responses. Multiple clinical studies have been conducted with GLA-SE formulated vaccines [134–136] including several with malaria antigens; however, results from the latter have not yet been published. Preclinical studies with GLA-SE have been conducted with a number of malaria vaccine candidates. As an example, in a study conducted with a full length PfCSP recombinant protein (produced by Pfenex) adjuvanted with GLA-SE, 50% sterile protection was seen upon challenge in a mouse malaria model (Pb/PfCSP repeats and C-terminal region replacement) [92]. With a different full length PfCSP recombinant protein (produced by WRAIR), 60% protection was seen upon challenge in a different mouse malaria model (Pb/PfCSP full-length replacement) [137]. This adjuvant has also been used in combination with PfCelTOS recombinant protein in preclinical studies, where a statistically significant reduction in liver load was found in challenged mice [93]. A clinical study with PfCelTOS formulated in GLA-SE has been conducted, but data have not yet been published (ClinicalTrials.gov identifier: NCT01540474). Additionally, in a preclinical study with PvRII (region II of PvDBP) where moderate levels of in vitro erythrocyte binding inhibition (>50%) was achieved with GLA-SE, slightly higher levels (>60%) were seen when GLA-SE was combined with the TLR 7/8 agonist R848 [138].

In a recent study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, only GLA-SE demonstrated statistically significant inhibition in all three challenge models. However, it was not the best performer in any of the models.

2.3.4. Nutritive immune-enhancing delivery system (NIDS)

NIDS (developed by Epitogenesis, Inc.) is an oil-in-water nanoemulsion containing vitamin A, a polyphenol-flavonoid, catechin hydrate, Tween 80, and mustard oil that were originally developed to boost mucosal immune responses to a variety of antigens without triggering inflammatory responses [139]. All the adjuvant components are generally regarded as safe (GRAS) by the FDA and are available at GMP-grade. Note that these components are quite inexpensive as compared to those of some of the other adjuvants described herein. The NIDS platform allows modification of the NIDS components toward more Th1 or Th2 responses [140]. In addition, this adjuvant can be administered systemically (e.g., IM injection) or delivered via a mucosal route. Although this adjuvant has not yet be assessed in the clinic and no preclinical studies have been published that use NIDS in combination with malaria antigens, it is worth consideration regarding both its low cost and ability to adjuvant in the absence of triggering an inflammatory response.

2.4. Small molecule adjuvants and immunostimulants

Several small molecule-based adjuvants and immunostimulators relevant to malaria vaccine development are described herein. Note that although a number of TLR agonists have been used in preclinical (and in some cases clinical) assessments of malaria vaccine candidates, these have been thoroughly reviewed elsewhere [141, 142]. Such TLR agonists include MPLA/GLA (TLR4 agonists), CpG oligodeoxynucleotides (ssDNA containing cytosines and guanines, which are TLR9 agonists), Poly:IC (dsRNA, which is a TLR3 agonist), Pam3Cys (lipopeptide and TLR1/2 agonist), as well as imiquimod and resiquimod (TLR 7/8 agonists), none of which are detailed herein.

2.4.1. IC31® adjuvant platform

IC31® (developed by Valneva) is a two-component adjuvant comprised of a polycationic peptide (poly-L-arginine) and ODN1a (a TLR9 agonist). Activity of this adjuvant includes recruitment of MHC class II at the injection site as well as migration of antigen to the draining lymph node [26]. The poly-L-arginine contributes to development of humoral and Th2 immune responses [143], while ODN1a is a single-stranded DNA oligonucleotide that stimulates Th1 responses [144]. No studies of malaria vaccine candidates formulated IC31® have been published. In a recent study, five approved or clinically tested adjuvants (Alum, MF59®, GLA-SE, IC31®, and CAF01) were each combined with antigens from *M. tuberculosis*, influenza, and chlamydia and compared in head-to-head mouse studies [100]. Of the five adjuvants tested, IC31® elicited Th1 and Th2 immune responses and demonstrated a statistically significant

reduction (but not the biggest reduction) of *M. tuberculosis* and *C. trachomatis* in challenge models. This adjuvant has been assessed in the clinic [145–149].

2.4.2. 7DW8-5

7DW8-5 (developed by ADARC) is a glycolipid α-galactosylceramide (α-GalCer) analog identified as part of structure-activity relationship (SAR) screening of α-GalCer analogs for increased adjuvant activity as compared to the parent molecule [150]. 7DW8-5 induces Th1 immune responses by binding CD1d (nonclassical MHC proteins expressed on APCs that present lipid antigens), stimulating natural killer cells, and inducing dendritic cell activation/maturation as well as dendritic cell trafficking to the draining lymph node [150, 151]. This adjuvant has been assessed in preclinical studies with several malaria vaccine candidates including CSP, AMA-1, and irradiated sporozoites [151–153]. In formulations with 7DW8-5, enhanced Th1 responses were found as well significantly reduced liver load [153] and a high level of sterile protection (90%) upon challenge [151].

2.4.3. Saponins (QS-21)

Saponins (particularly QS-21), which can be extracted from the bark the *Quillaja saponaria* Molina tree or be semisynthetic, are of interest because they have been shown to stimulate Th1-type responses and production of antigen-specific cytotoxic T lymphocytes (CTLs) [88]. However, a major concern regarding use of QS-21 in malaria vaccines is instability at pH above six and at elevated temperatures [88]. While it is possible to stabilize QS-21 (e.g., in lipids with cholesterol), this small molecule is not recommended for malaria vaccines formulations (intended for use in endemic regions) without assessments of stability at elevated temperatures in the selected formulation.

3. Conclusions

In recent years, there has been a proliferation of novel and promising adjuvant and vaccine delivery systems that together cast a wide net over the effector targets offered by our current understanding of immune system's pathways. Accordingly, this review surveys the field of vaccine delivery platforms and adjuvants in the context of their potential utility for improving the sterile or protective immunity conferred by malaria vaccine candidates. These technologies have the potential to positively affect the induction of immune response elicited by a vaccine candidate through more effective antigen delivery and presentation, ability to present multiple epitopes/copies of epitopes, and mobilizing different components of the immune system appropriate to the antigen and the malarial life-cycle stage being targeted.

Indeed, the judicious selection of vaccine delivery platforms and adjuvants is a necessary part of the malaria vaccine development process. For example, RTS,S was tested with a range of different adjuvants in both preclinical and clinical studies prior to section of AS01 for the commercial formulation. Additionally, while many other malaria vaccine targets have since been tested with AS01, none of these has achieved the same level of protective efficacy seen

with RTS,S/AS01 [16]. These data demonstrate the necessity of evaluating malaria vaccine targets with a range of delivery platforms and adjuvants prior to selection of the platform(s)/adjuvant(s) for clinical testing. In addition, testing multiple formulations will likely be necessary to sufficiently evaluate efficacy. In endemic regions, this process is compounded by the parasite's ability to hamper development of effective, long-lived immune responses as the intersection of vaccination and infection varies greatly depending on the level of malaria transmission. Several strategies for addressing these complications have been presented including, (1) platforms capable of sustained antigen release, (2) adjuvants that function without triggering inflammatory immune responses, and (3) use of blockage inhibitors to reduce T cell exhaustion.

Additional considerations include affordability and cold-chain requirements. As the principal target populations for vaccination against malaria are individuals residing in endemic regions, primarily Africa and other developing countries, an effective vaccine must be relatively inexpensive to manufacture, store, and deliver. Several of the enabling technologies presented are relatively inexpensive. Additionally, some have the potential to stabilize the vaccine formulation for room temperature storage and transport. However, accessibility is problematic for several of these technologies, and there is a significant cost component regarding the development process, particularly for the vaccine delivery platforms. Due to the complex nature of malaria vaccine development, it is clear that a team approach capable of tapping into expertise in the commercial, academic, government, and nonprofit sectors to efficiently assemble the right combination of vaccine development and delivery technologies is critical to success of malaria vaccine development strategies.

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