ADVANCED BIOSYSTEMS
www.adv-biosys.com

Adjuvant and Antigen Systems for Malaria Transmission-Blocking Vaccines

*Wei-Chiao Huang, Zachary R. Sia, and Jonathan F. Lovell**

Malaria is transmitted by protozoan parasites of the *Plasmodium* genus, via mosquito vectors. Highly effective vaccines could be a valuable tool to control the disease, but have remained elusive, in part due to the complex lifecycle of the parasite. Transmission-blocking vaccines (TBVs) take the unconventional approach of targeting the mosquito stages of the parasite life cycle. TBVs are yet to be tested in large-scale human trials, but represent a prominent area of interest for malaria vaccine research and development. Because TBVs rely on passive antibody transfer from a blood meal to the mosquito midgut, techniques to boost host antibody generation are a focus of investigation. In this review, immunostimulants and delivery systems for conjugating, self-assembling, or coadministrating TBV antigens and adjuvants are summarized.

1. Introduction

Malaria is an infectious disease caused by *Plasmodium* parasites. It is transmitted by the *Anopheles* mosquito and impacts numerous populations with significant fatalities worldwide.[1] An estimated 217 million cases of malaria resulted in 445 000 deaths in 2016, with many of the victims being infants.[2] There are more than 100 known *Plasmodium* species, however only five are capable of infecting humans: *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae*, and *P. knowlesi*. Vaccine efforts have focused on *P. falciparum* and *P. vivax*, as they are the cause of most malaria morbidity and mortality.[3] Development of an effective vaccine would be an invaluable tool to help combat the disease, and thus has been the subject of intense research and development.[4] To date the most advanced malaria vaccine is based on the circumsporozoite protein (CSP) and is in late stage clinical trials.[5] This vaccine was developed to target the pre-erythrocytic, sporozoite stage of the disease to prevent infection of the host. However, due to the complexity of the *Plasmodium* parasite and its life cycle, this vaccine alone is likely insufficient to effectively control the spread of malaria. In order to effectively prevent the spread of the disease, a vaccine must significantly reduce the incidence of malaria at the population level, an area in which transmission-blocking vaccines (TBVs) hold promise.[6–9]

W.-C. Huang, Z. R. Sia, Prof. J. F. Lovell
Department of Biomedical Engineering
University at Buffalo
State University of New York
Buffalo, NY 14260, USA
E-mail: jflovell@buffalo.edu

The ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/adbi.201800011.

DOI: 10.1002/adbi.201800011

TBVs aim to reduce malaria incidence by inducing production of antibodies to antigens that are expressed on the parasite within the midgut of the mosquito, thereby blocking parasite development and further spread of the disease. The process of TBV inhibition of parasite transmission from mosquito to host is shown in **Figure 1**. Once immunized with a TBV, the host produces antibodies against specific stages of parasite development. TBVs are not intended to be prophylactic, so an *Anopheles* mosquito carrier can still infect a vaccinated host. However, if another mosquito feeds from the vaccinated human host, it would uptake transmission-blocking antibodies produced by the host along with the parasite. The specific antibody would then interact with the target antigens that are expressed either on gametes, zygotes, or ookinetes to halt the development of mature malaria parasites and block the transmission process from the mosquito to the next human host.

One of the challenges in developing an effective TBV relates to the testing metrics for gauging efficacy in preclinical and early-stage clinical studies. Since TBVs are not intended to induce protection in vaccinated subjects, traditional controlled animal and human infection and challenge experiments are not available. A human transmission-blocking challenge model for malaria has not yet been developed. It is possible to directly assess whether mosquitoes can transmit the disease to healthy animals after feeding on immunized and malaria-infected ones. Although this gives a highly functional measure of vaccine efficacy, there could be differences in terms of both *Plasmodium* and animal host biology between different model systems and humans. More pragmatically, the protein sequences of most TBV antigen targets have limited sequence similarity between plasmodium species, so that a recombinant protein antigen from one species generally cannot be used to immunize against another.

The main goal in assessing a TBV is to quantify how the serum of the immunized host inhibits the development of oocysts in the mosquito midgut post-feeding. The standard membrane feeding assay (SMFA) has been developed and optimized for the purpose of quantifying the transmission reducing activity (TRA) induced by vaccines.[10] The SMFA involves first mixing purified antibodies or serum from immunized subjects in human blood with cultured gametocytes, feeding the sample to *Anopheles* mosquitoes through a membrane, then around a week later dissecting the mosquito midguts to determine numbers of oocysts that have developed.[11] The direct membrane

feeding assay (DMFA) uses the same principle but uses gametocytes obtained directly from infected humans. The SMFA is considered the "gold standard" assay for assessing TBV function and is used widely in preclinical and clinical vaccine development. One limitation of the SMFA is that it is a long, labor-intensive, and technically challenging procedure, so it is not carried out in a large number of laboratories. Throughout all stages in the research and development pipeline, the efficacy of any antiserum or purified IgGs from immunized rodents, rabbits, nonhuman primates, or humans can be assessed using the SMFA.

The inhibition of oocyst development by TBV-induced immunity is thought to be entirely mediated by antibody binding to the parasite, so purified IgG or serum are typically used in the SMFA. Hematological factors such as total lymphocyte counts also can influence transmission blocking,[12] although modulating such factors would likely have too many side effects to be implemented as a transmission-blocking strategy.

2. Transmission-Blocking Antigens

Only a handful of antigens have been widely explored as TBV candidates. As shown in **Table 1**, so far, most of the clinical focus has been on the *P. falciparum* antigens Pfs25 and Pfs230. These and most other TBV antigens are present at the earlier gamete stage (Pfs230) and/or the later ookinete stage (Pfs25). Some studies have been performed with the *P. vivax* antigen Pvs25. Additionally, some antigens originating from the *Anopheles* mosquito midgut known to mediate parasite invasion can be targeted for transmission blocking.

2.1. Gamete Surface Antigens

Gamete surface antigens are expressed during parasite fertilization. They are also present on gametocytes, which circulate in the human host and may naturally boost transmission-blocking antibody production.[16,17] Several TBV antigens are expressed on the *P. falciparum* gamete, such as Pfs230,[18] Pfs48/45, and Pfs47[19] (and their analogues from *P. vivax*[36–38]) as well as HAP2.[20]

Pfs230 and Pfs48/45 have been shown to have a male gamete-specific function presumed to be involved in ligand interactions during fertilization.[21,22] The presence of naturally occurring antibodies against these two proteins in malaria-exposed humans is associated with transmission inhibition.[23] Pfs230 is a 310 kDa surface protein expressed on the *P. falciparum* gametocyte. Pfs230 is a challenging antigen to produce due to its large size and the large number of disulfide bonds involved in its structure.[24] Seven paired domains of Pfs230 have been predicted, and transmission-blocking epitopes are located in these domains. Pfs48/45 is a protein from *P. falciparum* that is expressed during the sexual differentiation of the parasite and plays an important role in fertilization.[25] Monoclonal antibodies which target Pfs48/45 epitopes prevent parasite fertilization.[26,27] Pfs48/45 is a cysteine-rich protein, containing 16 cysteines which form multiple disulfide bonds. Because of this, production of properly folded Pfs48/45 protein is a challenge. The recognition of transmission-blocking Pfs48/45 mAbs is dependent on the properly folded tertiary structure.[27] Expression of various truncated C-terminal portions of the protein has been successfully produced as fusion proteins, with the other protein fragments presumably serving to assist solubilization.[28,29]

Pfs47 is a contiguous paralog of Pfs48/45 and it is located 1.5 kilobases apart from Pfs48/45.[19] It is expressed on the surface of female gametocytes and gametes.[30] Disruption of Pfs47 genes in parasites and monoclonal antibodies against Pfs47 leads to decreased oocyst formation.[31]

HAP2 is also located on the surface of the gamete membrane, and has been investigated as a transmission-blocking antigen.[32]

Wei-Chiao Huang is a doctoral student in the Department of Biomedical Engineering at the State University of New York at Buffalo. She received her M.S. degree in Biomedical Engineering and Environmental Science from the National Tsing Hua University in Taiwan, and worked as a research assistant for three years in liposome design and cell surface engineering at Academia Sinica in Taiwan. Her current research focus is on the design of liposomal adjuvants for malaria transmission-blocking vaccines.

Zachary R. Sia is a doctoral student in the Department of Biomedical Engineering at the State University of New York at Buffalo. He completed a B.S. in Biomedical Engineering at the University of Rochester. His research focuses on the use of functional liposomes as a vaccine adjuvant for infectious disease.

Jonathan Lovell is an associate professor of Biomedical Engineering at the State University of New York at Buffalo. He obtained his Ph.D. degree from University of Toronto at the Institute of Biomaterials and Biomedical Engineering. Dr. Lovell's current research interests include developing and applying new nanoscale delivery systems for recombinant vaccine antigens.

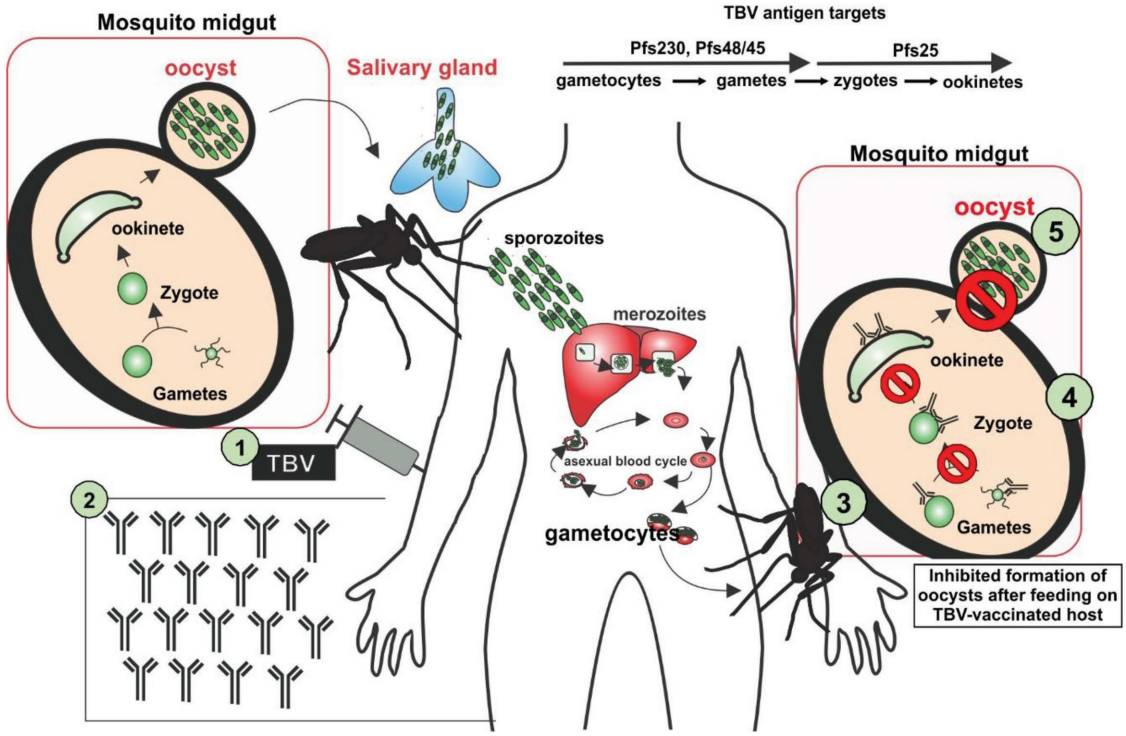

Figure 1. Transmission-blocking vaccine concept. (1) Once a human host is immunized with a TBV, (2) circulating antibodies are generated which recognize transmission-blocking targets on the parasite. (3) When an *Anopheles* mosquito intakes a blood meal from an infected host, both antibodies and gametocytes are ingested. The antibodies are intended to (4) block the gamete fertilization process or block development of ookinetes from zygotes, or (5) prevent the migration of ookinetes out of the midgut to the salivary gland.

HAP2 was originally found in *Arabidopsis thaliana*,[33] and was later also identified as Generative Cell Specific 1 (GSC1) isolated from *Lilium longiflorum* pollen.[20] HAP2 homologs are present in higher plants and several pathogenic protists, including *Plasmodium* species. This antigen has been shown to be a key factor on fertilization and has recently been identified as a eukaryotic class II fusion protein.[34] The role of HAP2 in parasite development has been investigated in *P. berghei,* where it is expressed on the surface of the male gametocyte and microgamete.[35] Targeting of HAP2 in *P. berghei* can affect the fertilization of the sexual stages of parasite by disrupting the ability of male gametes to fuse with female gametes.[32]

The Putative Secreted Ookinete Protein 12 (PSOP12) is a member of the 6-cys family of proteins that play important roles in the recognition and fertilization of gametes.[36] The protein was found to be expressed in both *P. berghei* gametocytes and ookinetes.[37] Several new TBV candidates have also been identified from research with *P. berghei* beyond PSOP12,[38] including PSOP25[39] and Pb51, which are expressed not only on the gametocytes and ookinetes but also on the sporozoites.[40] PbPH, which contains a pleckstrin homology domain, has also been identified as a potential target.[41]

2.2. Ookinete Surface Antigens

These antigens are active on the ookinete surface membrane, likely facilitating interactions between the ookinete and the midgut environment of the mosquito vector.

Pfs25 is a 25 kDa surface protein expressed on the surface of the *P. falciparum* ookinete which enables the migration across the mosquito midgut to form an oocyst.[42] Pfs25 is one of the most well-characterized and explored TBV antigen candidates. Pfs25 is expressed on the surface of both the zygote and ookinete.[25,43] Because Pfs25 is expressed only in the mosquito midgut, associated antibodies are not naturally boosted by the immune system of an infected human host. Pvs25 is a homolog to Pfs25 expressed by *P. vivax* that can induce transmission antibodies with immunization.[44] It is expressed on macrogametes and ookinetes and contains 22 cysteine residues. Clinical-grade, recombinant Pvs25 has been expressed at the large scale in *Saccharomyces cerevisiae* in the form of Pvs25H.[45] Pvs25H is 20.5 kDa and comprises residues 23–195 from Pvs25. Pvs25H formulated with Alhydrogel induced antibodies with weak transmission-blocking activity in clinical trials.[15] Pvs28 is another *P. vivax* surface antigen which is only expressed on the ookinete, and contains 20 cysteine residues.[42] It has also been produced recombinantly by *S. cerevisiae* and can induce transmission-blocking antibodies.[44]

Pfs25 from *P. falciparum* and analogous Pfs25-like proteins from other *Plasmodium* species have protein structures which include four epidermal growth factor (EGF)-like domains and are anchored to the surface of the parasites by glycosylphosphatidylinositol.[46] One transmission-blocking anti-Pfs25 monoclonal antibody, 4B7, recognizes the β-hairpin epitope in $Pfs25_{121–130}$(ILDTSNPVKT).[47,48] A durable antibody response is important, but has proven challenging to achieve.[49] The

Table 1. Clinical activity of transmission-blocking malaria vaccines.

Antigen	Modification	Adjuvant	Dose and schedule	Outcomes	Trial ID
Pfs25 (*P. pastoris)*	EPA toxin conjugate	Alhydrogel	8, 16, or 47 μg day 0, 56, 120, 300	Weak transmission-blocking activity[13]	NCT01434381
Pfs25 (*P. pastoris)*	EPA toxin conjugate	Alhydrogel	47 μg day 0, 56, 112, 480	-	NCT01867463
Pfs25M (*P. pastoris*) Pfs230D1M	EPA toxin conjugate	Alhydrogel	16 μg Pfs25M 15 μg Pfs230D1M day 0, 28, 168, 530	-	NCT02334462
Pfs25 (*P. pastoris*) Pvs25 (*S. cerevisiae*)	–	ISA51	5, 20, 80 μg day 0, 28, 56	Transmission-blocking activity induced Halted due to reactogenicity[14]	NCT00295581
Pvs25 (*S. cerevisiae*)	–	Alhydrogel	5, 20, 80 μg day 0, 28, 180	Weak transmission-blocking activity[15]	-
Pfs25 *(N. benthamiana)*	Virus-like particle	Alhydrogel	2, 10, 30, 100 μg	Weak transmission-blocking activity[a)]	NCT02013687
MVA Pfs25-IMX313	Multimerization (IMX313) Vaccinia viral vector (MVA)	–	5×10^9–10^{10} viral particles	–	NCT02532049
Pfs25	Self-conjugate[a)]	Alhydrogel	10 and 25 μg day 0, 24, 48	Withdrawn	NCT00977899
Pfs25 with Pfs230D1M *(P. pastoris)*	EPA toxin conjugations	AS01	Pfs25: 16 and 47 μg Pfs230: 13 and 40 μg day 0, 28, 168	–	NCT02942277

[a)]Information inferred from the clinicaltrials.gov website.

expression and purification of Pfs25 has been reported in many different expression systems, including yeast,[50–53] plant,[54] *Escherichia coli*,[55] and algae.[56] The immunogenicity of Pfs25 has been studied in nonhuman primates to assess the efficiency of antibody production and safety risks. Immunization of rhesus macaques (*Macaca mulatta*) with a DNA vaccine plasmid encoding Pfs25 or a Pfg27-Pfs25 led to transmission-blocking immunity compared to empty plasmid alone.[57] Another study using a plasmid-encoding Psf25 was delivered with in vivo electroporation to baboons also induced functional antibodies with transmission-blocking activity.[58]

2.3. Midgut Proteins of the Mosquito

Carboxypeptidase and alanyl aminopeptidase N1 (APN1) are midgut glycoprotein targets expressed in the mosquito itself.[59] APN1 is expressed on the surface of *Anopheles* mosquito midguts and plays a role in ookinete invasion.[60] Anti-APN1 IgG has transmission-blocking activity for both *P. berghei* and *P. falciparum*.[61] APN1 comprises a 135 amino acid sequence, however, use of a fragment sequence of 59 amino acids at the N terminus of mature APN1 has been shown to be immunogenic in murine[62] and rabbit[61] studies.

3. TBV Adjuvants and Antigen Approaches

Adjuvants can generally be classified into two groups: immunostimulatory molecules and delivery systems. Immunostimulatory molecules include saponins and molecules which target Toll-like receptors (TLR) ligands, C-type lectin receptors (CLR) ligands, or nucleotide-binding oligomerization domain (NOD)-like receptors. Delivery systems include emulsions, aluminum salts, lipid vesicles, and virus-like particles (VLP). Immunostimulatory and delivery system adjuvants can also be integrated or combined to further increase effectiveness. Many synthetic carriers have been examined for malaria vaccines,[63] such as lipid based adjuvants,[64–66] polymeric particles (such as PLA/PLGA[67] and PLGA[68]), ISCOMs,[69] and virus-like particles.[70,71] Antigen engineering approaches, in which antigens are engineered to express functional domains to form oligomeric structures are used for enhancing immune responses.

3.1. Saponins

Saponins are triterpene or steroid glycosides isolated from plants. Quil-A is a mixture of triterpene glycosides, and is one of the most commonly used saponins in vaccine adjuvants.[72] Due to potential toxicity, it is not used in human vaccines. Saponin-based adjuvants can enhance cell-mediated immunity and antibody production. They can induce cytotoxic CD8+ T lymphocyte responses. Saponins bind cholesterol and have been shown to cause hemolysis of red blood cells.[73] Saponins induce other immune responses such as inflammation[74] and monocyte proliferation.[75]

The saponin QS21 has been explored with TBV antigens. QS21 is a purified component of Quil-A that has lower toxicity and higher adjuvant activity compared to Quil-A.[76,77] Pfs25 was incubated with aluminum hydroxide (alum) or alum/QS21 and administered to rabbits with three injections on day 0, 28, and

36. The rabbits vaccinated with alum/QS21 showed marginally better transmission-blocking activity compared to the group without QS21.[78]

ISCOMs are 40 nm cage-like particles whose principal components are Quil-A and the antigen of interest, together with lipids and cholesterol.[79] ISCOMs can induce both CD8+ and CD4+ cell responses by encouraging MHC class I responses.[80] Previous studies have shown that ISCOMs could destabilize endosomal membranes, leading to greater presentation by MHC molecules.[81] There have been limited studies involving TBV antigens with ISCOMs; however, there have been several studies using non-TBV malaria vaccines developed using ISCOMs, including studies in non-human primate models. For example, the antigen Pf155.RESA which is expressed on merozoites[69] was mixed with ISCOMs to form an immunogenic complex. This approach led to higher antibody production in rabbits and monkeys immunized with this approach compared to Freund's adjuvant.[82]

3.2. Emulsions

Emulsion adjuvants are often used in vaccine research and development. There are generally three types of emulsions: oil-in-water, water-in-oil, and water-in-oil-in-water. The products of oil-in-water emulsions are surfactant-stabilized, oil nanoparticles dispersed in an aqueous water phase. For emulsion systems, the interaction with antigens is caused by hydrophobic and electrostatic force, which could result in interprotein interactions and conformational changes of the antigens.

Emulsion–antigen electrostatic interaction can be influenced with ionic surfactants[83,84] or ionic proteins.[85] The interactions are influenced by the charge of the components, as well as the buffer formulation, pH, and ionic strength.[86,87] Another important factor is the size of the emulsion particle. When the size of the individual emulsion particles decreases, the net surface area is increased, which leads to a greater area available for protein adsorption.[84] Protein-protein interactions are an important factor for proteins in emulsions. Covalent aggregation involving new disulfide bond formation can occur.[88] Precipication of the formulation might occur due to strong surface protein interaction within the emulsion;[89,90] this could be avoided by increasing electrostatic shielding, or by including cosolvents to increase water viscosity.[91] High protein concentration could reduce stability of the emulsion formulation, which might be due to the production of a viscoelastic protein film by interprotein interaction on the single surface of the emulsion.[90] **Figure 2** shows common interactions between antigens and emulsions, including hydrophobic interactions, electrostatic interactions, or protein–protein interactions.[92]

Oil-in-water emulsions can generate strong antibody responses against associated antigens, and stimulate both Th1 and Th2 response.[93] For example, MF59 has been shown to induce recruitment of dendritic cells and monocytes and enhance monocyte differentiation into dendritic cells.[94] Dendritic cells can trigger direct interaction with B cells for initiation of antigen-specific responses.[95]

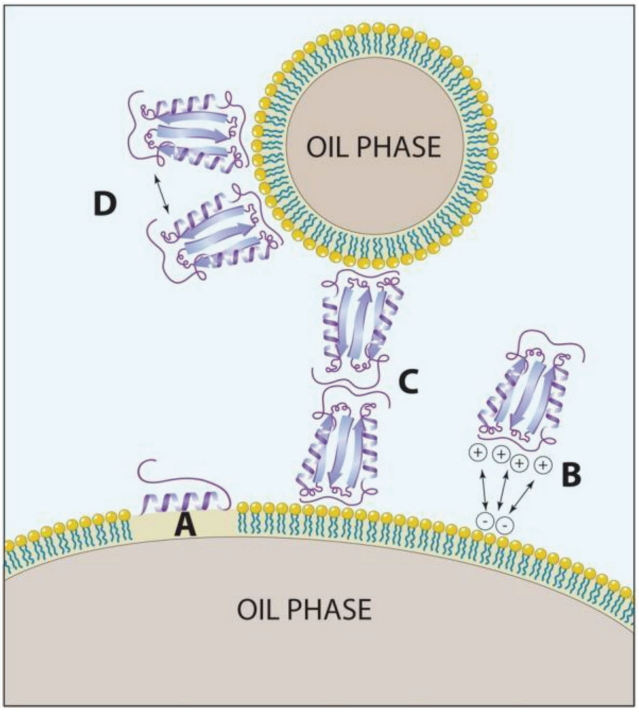

Figure 2. Common protein interactions within emulsion systems. A) Hydrophobic interactions with the oil phase resulting in α-helical transitions. B) Electrostatic interactions binding charged surfaces of antigen and emulsion. C) Protein–protein interactions resulting in flocculation between particles. D) Protein–protein interactions on the surface of the emulsion, resulting in increased surface viscosity. Reproduced with permission.[92] Copyright 2013, MediMedia.

3.2.1. Montanide: ISA720 and ISA51

Montanides (e.g., ISA720 and ISA51) are metabolizable oils, which have similar physical characteristics to incomplete Freund's adjuvant (IFA) but are more biodegradable. Montanides generate a water-in-oil emulsion containing squalene and mannide-monooleate as an emulsifier.[96] The immunogenicity of Pfs48/45 with ISA-51 was studied in Olive baboons; baboons received doses of 50 µg Pfs48/45 with ISA51, administered three times. Antibody titer remained high and stable for five months, with 80% transmission-blocking activity. The antibody titer decreased by 50% after seven months, and transmission-blocking activity dropped below 60%.[97] The montanide adjuvant ISA51 has been used in a malaria vaccine clinical trial with Pfs25; however, unexpected local reactogenicity was observed in volunteers.[14] Pvs25 combined with ISA720 was found to induce transmission-blocking activity in *M. mulatta* monkeys.[98]

3.2.2. MF59

MF59 is an oil-in-water nanoscale emulsion composed of squalene and stabilized by Tween 80 and Span 85.[99] MF59 can stimulate strong T helper cell responses; however, it has limited ability to enhance CD4+ Th1 and Th2 responses. It has also been shown to directly interact with monocytes, macrophages, and

granulocytes to produce cytokines and chemokines for immunostimulation.[100] Limited preclinical studies have been conducted with TBV antigens. In one study, it was found that mice immunized with 50 μg of Pfs25 with MF59 could produce a strong antibody response with transmission-blocking activity.[101] However, other vaccine studies with MF59 have been less promising with non-TBV malaria antigens. A study of mice immunized with MSP-1 protein adjuvanted with MF59 revealed a weak immune response,[102] while another study of mice vaccinated for PvDBP with MF59 adjuvant also showed an antibody production response weaker than that achieved using a montanide adjuvant.[103]

3.3. Aluminum Salts

Insoluble aluminum salts, abbreviated as alum or sometimes Al, can incorporate antigens as well as immunogenic adjuvants. Antigen adsorption onto aluminum salts is easy to achieve, since the aluminum salts are denser than aqueous solutions. The strength of the interaction between antigens and aluminum salts is antigen dependent and can significantly influence the immune response. The mechanism for the adjuvant to associate with the antigen is primarily based on electrostatic interaction,[104] and may also involve hydrogen bonds, van der Waals forces, hydrophobic interaction, and ligand exchange.[105,106] There are two widely used aluminum adjuvants: aluminum hydroxide and aluminum phosphate. At neutral pH, aluminum hydroxide has a positive charge while aluminum phosphate has a negative charge, so the aluminum adjuvant can be selected to compliment the charge of the antigen at neutral pH. One of the well-known brands of aluminum hydroxide adjuvant is Alhydrogel, manufactured by Brenntag.[107] This is a sterilized wet gel suspension of aluminum hydroxide which possesses a positive charge at pH 5–7, adsorbing antigens with negative charges in this pH range. Since alum adsorbs proteins, it prevents antigen precipitation, degradation or adsorption to the storage vessel. Other physical conditions that could affect the antigen–adjuvant interaction include temperature, size of gel particles, and the ionic strength of the mixture.[108,109] The temperature influences the rate of adsorption between antigen and adjuvant, while the size of the gel particles affects the interaction surface area available to interact with antigen.[110] As with emulsion adjuvants, and the pH and ionic strength affect the adsorption by altering the charge on the gel and antigen.

Alum can stimulate the immune system by triggering monocyte differentiation into dendritic cells. This activation might be due to a direct interaction with NLPR3 inflammasome, or by indirect interaction by releasing uric acid, an endogenous danger signal.[111] In mice, studies have shown that alum tends to induce Th2 response. The activation of Th2 could be due to the activation of NLRP3 inflammasomes to produce IL1β and IL18,[112] which then signals a Th2 response and antibody production.[113]

Alhydrogel has been used extensively for TBV studies. In a human trial, Pvs25 was absorbed onto Alhydrogel and ten volunteers in three groups received different dose of antigens (5, 20, and 80 μg) with intramuscular injections on days 0, 28, and 180. Results showed that oocyst inhibition was achieved by vaccine-induced antibodies, although not at levels sufficient for an effective vaccine.[15] Alhydrogel has been used in conjunction with a number of other adjuvants discussed in this review.

The APN1$^{60–195}$ protein fragment contains linear B cell epitopes and CD4+ T cell epitopes.[110] Female *M. mulatta* received three injections of 50 μg APN1$^{60–195}$ (prime on day 0, boost on day 28 and 70) adsorbed on alum, and it was observed that the anti-APN1 IgG titer remained high until day 150, with no adverse reaction near the injection site.[114]

3.4. CpG Oligodeoxynucleotide (CpG ODN)

The immunogenicity of coadministered DNA-based adjuvants has been found to rely upon the CpG motif, which is an immunostimulatory sequence composed of unmethylated cytosine-phosphate-guanine. When tested with hepatitis B antigens, CpG DNA was found to have mucosal adjuvant properties similar to cholera toxin. CpG ODN is a synthetic oligonucleotide-presenting CpG motifs. The CpG sequence induces immune responses by directly activating B cells, natural killer cells, and antigen-presenting cells[115] and both CD4+ T cells[116] and CD8+ T cells.[117] When Pfs25 was absorbed onto an alum carrier and combined with CpG ODN, there was a significant increase in the Pfs25-specific antibody response.[118]

To further test CpG for TBV applications, a transmission-blocking peptide (NH2-CPLPWELHDGC-COOH) and four other malaria peptides (CSP, MSP-1) were synthesized using Fmoc chemistry. ODN was synthesized with CpG motifs and a nuclease-resistant phosphorothioate backbone. A hydrazone linkage between CpG-ODN and the peptide was formed by introducing free hydrazino groups at the amino terminus of the TBV peptide and other peptides. The final conjugation process of CpG-ODN to peptide was modified at the 5′ end with a C6 linker terminating with an aldehyde group. Microparticles were used to entrap the five peptides with CpG-ODN and were prepared by using poly(lactide-*co*-glycolide) in a water/oil/water emulsion and solvent evaporation. Mice received 10 μg of each peptide. Mice injected with microparticles exhibited enhanced blocking activity compared to mice injected with alum.[119] Another study focused on developing a multistage vaccine, which could target both pre-erythrocytic and sexual stages of *Plasmodium*. Genetically-linked CSP and Pvs25 were transfected in Sf9 cells, which could generate the platform for the baculovirus dual-expression system (BDES).[120] A dose of 1×10^8 PFU of BDES particles was used to immunize mice, and the results showed 82% transmission-blocking activity.

3.5. Lipid-Based Nanoparticles

3.5.1. Liposomal Adjuvants

Liposomal vaccines utilize lipid nanoparticles generally containing different types of lipids forming a bilayer with an aqueous core, and have become a useful carrier system in vaccine development. The major advantages of liposomes are their biocompatibility and flexibility for loading a wide range of cargos (both antigens and adjuvants). The composition of

liposomes or other types of lipid-based vaccines can be easily modified by selecting specific types of lipids with different charges or lipid side-chain lengths. Antigens are generally adsorbed to the surface of liposomes in a poorly defined manner. Antigens can also be entrapped in the core of liposomes or chemically conjugated to the surface, although these approaches are more complex and burdensome. One of the most well-known liposomal malaria vaccine adjuvants is GlaxoSmithKline's liposome-based AS01.[121,122] AS01 contains two active lipid molecular adjuvants embedded in the liposome bilayer: monophosphoryl lipid A (MPL) and QS21. AS01 is used with recombinant full-length CSP in the RTS,S vaccine, and is also used in the Shingrix shingles vaccine.

Glucopyranosyl lipid A (GLA)-LSQ is a liposomal adjuvant composed of QS21 and glucopyranosyl lipid A, which is a synthetic version of MPL. When carrier protein-conjugated Pfs25 was mixed with GLA-LSQ for vaccination, mice vaccinated with the liposomal adjuvant induced a T follicular helper (Tfh) cell response and produced durable, antigen-specific antibodies.[49] In another experiment, Pfs48/45-6C fragments were genetically fused with GMZ2, which itself is a hybrid protein of asexual blood stage antigen and merozoite surface protein 3 (MSP3), forming a conjugate termed GMZ2.6C. Mice received 5 μg of GMZ2.6C with GLA-LSQ or other types of emulsions, such as GLA-SEQ (stable emulsion with QS21) in three immunizations. The results showed that mice vaccinated with the liposomal adjuvant exhibited high titer against sexual and asexual antigens, and based on a splenocyte assay, the mice immunized with GLA-LSQ and GLA-SEQ showed strong IFNγ secretion but not IL-5 secretion.[123]

The interaction of liposome adjuvants with antigens is generally not well-defined and not as easy to characterize as antigen adsorption to alum. In order to conjugate antigens with liposomes, several strategies have been developed. One strategy involves enhanced electrostatic interaction of the antigen with cationic liposomes. An example of this approach is the liposome formulation CFA01, which contains cationic lipids with an immunostimulatory molecule.[124] Besides electrostatic interactions, antigen binding to lipid vesicle surfaces can be achieved by covalent or noncovalent interaction, through different types of conjugation. This strategy involves incorporation of a functionalized lipid into the liposomes, and possibly a functionalized protein or peptide antigen via cloning or chemical conjugation. Interactions involving functionalized lipids can include maleimide lipids which covalently react with thiol-containing antigens. Another conjugation strategy has been to noncovalently bind recombinant his-tagged antigens with liposomes which contain lipids such as 3(nitrilotriacetic acid)-ditetradecylamine (NTA3-DTDA.[125] This approach has more affinity than nickel-chelated lipids with a single Ni-NTA chelator on the lipid head group.[126] Recently, chelated cobalt metal in a porphyrin-phospholipid has been shown to induce very stable noncovalent binding of his-tagged antigens to preformed liposomes on the basis of intra-bilayer his–tag coordination with cobalt–porphyrin.[127]

3.5.2. Bacterial Outer Membrane Vesicles (OMVs)

Bacterial OMVs are around 100 nm, and are produced by gram-negative organisms. The vesicles are usually produced by mucosal pathogens and contain phospholipid and immune stimulators, such as LPS and DNA, which can trigger maturation of immune system as well as cytokine signals. OMVs are potent immunostimulants which can penetrate mucosal membranes. However, they have a complex biochemical composition that is difficult or impossible to precisely quantify. A study involving the combination of OMVs with Pfs48/45 and AnAPN1 was reported.[128] Immunization of mice using antigen-carrying OMVs (with nonspecific binding) produced a similar level of IgG titer compared to mice immunized with antigen combined with cholera toxin or MF59 adjuvants. LPS could target TLR4 receptors and lead to the activation of TRIF and MyD88 pathways which can induce inflammatory cytokines and TNFα.[129]

3.6. Other Types of Nanoparticles

Gold nanoparticles show potential for use in vaccine delivery, offering flexibility in particle size and shape, as well as ease in applying surface modifications.[130–132] Gold nanoparticles can be recognized by dendritic cells and other antigen-presenting cells in order to improve vaccine delivery.[133,134] Recent studies have shown that the size of nanoparticles is an important factor for inducing immune responses, including the interaction with dendritic cells or other antigen-presenting cells, as well as the polarization of T cells response. Pfs25 was covalently immobilized onto differently shaped gold nanoparticles by using 4-aminothiophenol (4-ATP) and a glutaraldehyde spacer. 4-ATP serves as the foundation for the surface modification by binding directly to the gold nanoparticle, due to thiol groups having a strong affinity for gold.[135] Incubation with 4-ATP generated stable amine-functionalized gold nanoparticles.[136] The next step was to incubate the particles with Pfs25 containing glutaraldehyde, which resulted in chemically conjugated Pfs25-gold nanoparticles.[136,137] Mice were immunized with 10 μg Pfs25 containing different shapes of gold nanoparticles as verified by electron microscopy (**Figure 3**). Spherical gold nanoparticles produced more effective antibody compared to the other shapes of the nanoparticles.[137]

Polymer nanoparticles have also been investigated as vaccine delivery adjuvants. A study tested the effectiveness of poly(D,L-lactide-*co*-glycolide) nanoparticles (PLGA-NP) as an adjuvant compared to a nanoemulsion (NE) approach. The NE contained squalane, Span-80, and Tween-80 in saline and was formed by high-pressure homogenization. After mice received 10 μg of Pfs25, the serum was collected and purified to check transmission-blocking activity. Mice immunized with the NE generated better blocking activity compared to the mice that received PLGA-NP.[138] Based on these results, polymer nanoparticles have not yet shown promise as a TBV delivery adjuvant. This might be due to the size of the PLGA-NP significantly increasing, from 260 nm to over 1 μm after incubating with Pfs25, while the size of NE did not increase significantly after mixing with Pfs25.[138] In other studies, PLGA-NP maintained nanoscale-size after mixing with antigen and did show enhanced antibody titers against vaccine antigens, including the hepatitis B core antigen.[139] Therefore, further research is warranted to explore PLGA with transmission-blocking antigens.

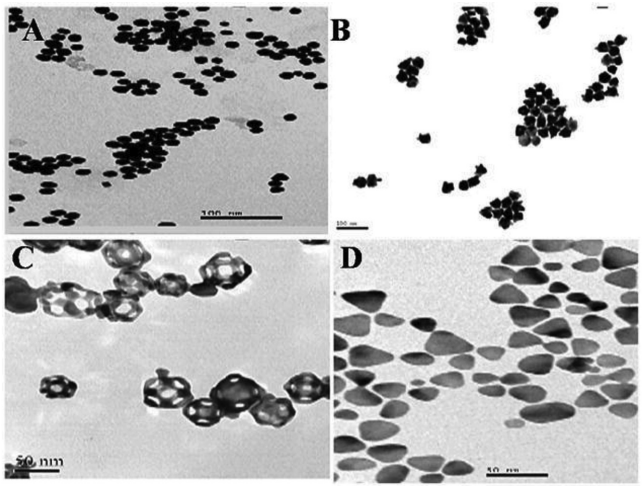

Figure 3. Different shapes of gold nanoparticles used for conjugation with Pfs25. A) Spherical gold nanoparticles, B) star shape gold nanoparticles, C) cage shape gold nanoparticles, and D) triangular nanoparticles. Reproduced with permission.[137] Copyright 2015, Elsevier.

3.7. Microparticle Delivery Systems

Polylactofate (PLE) is a poly(lactide-*co*-glycolide) glycolide derivative is notable for its biocompatibility, biodegradability, and physical properties. Biodegradable microparticles (BMPs) were synthesized by a double emulsion system (solid-in-water-in-oil-in-water), which could produce nanoparticles-in-microparticles, on which were loaded the APN1 mosquito midgut antigen. Mice immunized with APN1-loaded BMPs with alum were compared to APN1-loaded BMPs with IFA. Both APN1-BMP/alum and APN1-BMP/IFA induced transmission-blocking antibody titers capable of effective oocyst inhibition at 60 and 180 d after the immunization.[140] Another microparticle approach is gel core liposomes, which are prepared by a reverse phase evaporation technique. Pfs25 and the polymer polyacrylic acid were added into a lipid mixture in saline with sonication to passively load into the gel core liposomes.[141] The gel core liposomes loaded with Pfs25 produced a higher antibody level and the secretion of IL2 and IFNγ was higher compared to alum, which indicates a stronger cell-mediated immune response. The biodegradable microparticle delivery system can provide a method to slowly release the antigen.

4. Antigen Engineering

4.1. Virus-Like Particles

VLPs are nanoparticles which resemble viruses with repetitive 3D protein arrangement, but lack pathogenic genetic materials. VLPs can range in size from 20 to 200 nm and can promote enhanced draining of particles to lymph nodes, which is ideal for facilitating uptake into antigen-presenting cells. The highly repetitive fragments could also enhance complement fixation and cross-linking of B cell receptors.[142] To attach the antigen to the VLPs for vaccine development, genetic or chemical conjugation is used. Genetic approaches are suitable for short peptides and proteins, but insertion of a larger sequence fragment can inhibit correct VLP scaffold assembly.[143] For large proteins, chemical conjugation may be a more straightforward option to link the antigen to the scaffold and is based on chemical generation of reactive amino acids on the antigen and scaffold.[144]

Chemical conjugation of TBV antigens was performed using a dual plug-and-display synthetic assembly involving the SpyTag and SpyCatcher peptides. SpyTag is a short peptide that can "plug into" its SpyCatcher protein partner through an isopeptide bond. Neither SpyTag nor SpyCatcher contain a cysteine residue.[145] A set of analogous peptides, SnoopTag and SnoopCatcher have also been used. Expi293 cells were transfected with Pfs25-SpyTag or Pfs28-SnoopTag constructs to produce antigens with reactive amino acids, and were purified by size-exclusion chromatography. VLPs were produced by transfecting IMX313 vectors, with N-terminus binding to SpyCatcher and C-terminus to SnoopCatcher, in *E. coli* to form a VLP containing SpyCatcher–IMX–SnoopCatcher.[146] The conjugation of antigen to VLPs could be attained by forming spontaneous isopeptide bonds; for example, the interaction of the SpyTag antigen with the SpyCatcher domain occurs through a condensation reaction. The SnoopTag–antigen connects with the SpyCatcher domain on the VLP which releases ammonia to form a stable conjugation as shown in **Figure 4**.[146] When mice received vaccine containing 0.7 μg of Pfs25 or Pfs28 antigen with VLPs, the antibody response against both Pfs25 and Pfs28 was greater than the response to the antigen alone.[146] A similar study was performed using another VLP approach termed SpyCatcher-AP205. AP205 is a bacteriophage made by single-stranded RNA (ssRNA), which has a unique sequence in the coat protein that is not similar to other ssRNA phages.[147] The assembled VLPs are very stable and the AP205 coat proteins are able to incorporate large proteins at the N and C terminals,[148] which makes AP205 a good carrier for antigens. SpyCatcher-AP205 was synthesized in *E. coli* and conjugated with a Pfs25-SpyTag. Additionally, in the same study another VLP called Qb was used, with Pfs25–thiol chemically conjugated with the Qb VLP. Mice received 1 μg of the Pfs25 vaccine combined with AP205, Qb, or IMX313. IMX313 is discussed in the following section. The results show a higher anti-Pfs25 IgG titer in all the three VLP groups compared to soluble Pfs25 antigens. However, the Pfs25 conjugated with AP205 showed better blocking efficacy compared to the other two VLP methods.[149] VLPs were generated using the AP205 coat protein and SpyTag/Catcher VLP system by using Pfs48/45 0.6C, which is an antigen containing a transmission-blocking epitope fused in frame with part of the glutamate rich protein (GLURP).[150] GLURP antibodies have been shown to induce antibody-dependent monocyte-mediated inhibition of parasite growth in vitro.[151] Mice immunized with VLP display of Pfs48/45 0.6C had significantly increased specific antibody production compared to mice immunized with the antigen with Montanide ISA720.[150]

In another approach, the Alfalfa mosaic virus coat protein was fused with Pfs25 via a genetic engineering approach to form a 20 nm purified Pfs25-VLP, as shown in **Figure 5**.[70] This VLP was expressed with a plant expression system in *Nicotiana tabacum*. Mice received different doses of Pfs25-VLP (1, 0.1, and 0.01 μg) combined with Alhydrogel. Mice vaccinated with at least 0.1 μg of Pfs25-VLP induced strong

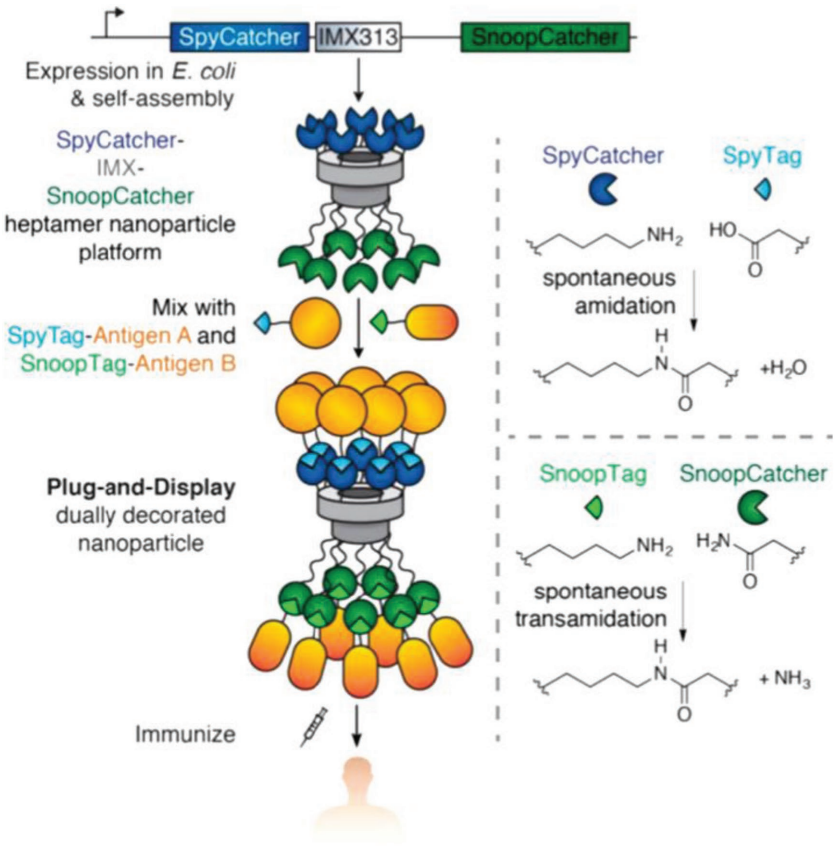

Figure 4. Overview of a dual plug-and-display synthetic assembly. IMX313 is fused at its N-terminus to SpyCatcher and at its C-terminus to SnoopCatcher. Expression in *Escherichia coli* and spontaneous multimerization yields SpyCatcher–IMX–SnoopCatcher nanoparticles. Upon mixing, SpyTag–antigen A forms a spontaneous isopeptide bond with the SpyCatcher domain through a condensation reaction. SnoopTag–antigen B forms a spontaneous isopeptide bond with the SnoopCatcher domain with release of ammonia. Conjugation yields dual-decorated nanoparticles for immunization. Reproduced with permission.[146] Copyright 2017, American Chemical Society.

transmission-blocking activity in mice. This construct has recently entered clinical trials (NCT02013687).

4.2. Multimerization Technology

The multimerization domain IMX31 is a scaffold from the heptameration domain of the complement binding protein C4b. C4b is one of the major complement proteins that regulates the classical complement pathway and activate monocytes.[152] Studies have also shown that C4b can bind to the CD40 receptor and directly activate B cells.[153] Antigens tagged with IMX313 can self-assemble into supramolecular complexes with seven identical subunits. Antigens fused to IMX313 have previously been shown to increase antibody titer as well as enhance antigen localization to lymph nodes for improved immunogenicity.[154,155] For TBV applications, constructs of Pfs25-IMX313 were produced in *P. pastoris* (**Figure 6**), and the Pfs25-IMX313 heptamers were purified for vaccination. Mice were also primed and boosted with a viral vector containing Pfs25-IMX313, which resulted in an improvement in antibody response compared to Pfs25 alone. This approach resulted in a higher germinal center response, a dynamic structure in the spleen and lymph nodes containing a population of antigen-specific B cells that can differentiate B cells into plasma cells or memory B cells.[154] Pfs25-IMX313 has recently entered clinical trials with a viral delivery vector (NCT02532049).

4.3. Antigen Carrier Conjugates

Antigens can be covalently conjugated directly to a wide number of carriers. Outer-membrane protein complex (OMPC) derived from *Neisseria meningitidis* serogroup B can be used as a carrier for the capsular polysaccharide polyribosylribitol phosphate (PRP) of the bacteria capsule.[156] Pfs25 was chemically conjugated with OMPC through a maleimide/thiol reaction, binding the antigen with *N*-ε-[maleimidocaproyloxy] sulfo-succinimide ester to form maleimide-activated Pfs25. The thiol derivative of OMPC was prepared by reacting the protein with N-acetylhomocysteine thiolactone.[157] The complex of Pfs25-OMPC was prepared by mixing maleimide-activated Pfs25 and thiolated-OMPC together. The complex was used for vaccination of mice (0.5 or 2.5 μg doses) and monkeys (4 or 40 μg doses), which resulted in longer-lasting antibody production compared to animals vaccinated with the antigen in ISA51.[158] Another approach attempted to conjugate antigens to the Z domain of *Staphylococcus aureus* protein A, which is an analog of the B domain in the Ig-binding domains (IBDs) and could serve

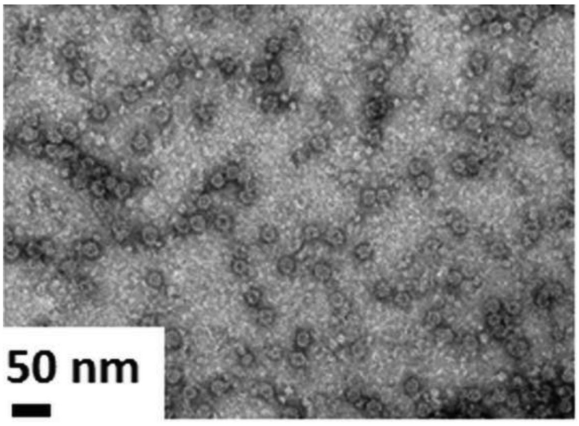

Figure 5. Genetically engineered Pfs25 VLPs. Pfs25 was genetically fused to the Alfalfa mosaic virus coat protein and expressed in plants. The purified VLPs formed 19.3 nm particles that induced transmission-blocking activity in mice when adjuvanted with alum. Reproduced under the terms and conditions of the Creative Commons Attribution License.[70] Copyright 2013, The Authors, Published by PLoS.

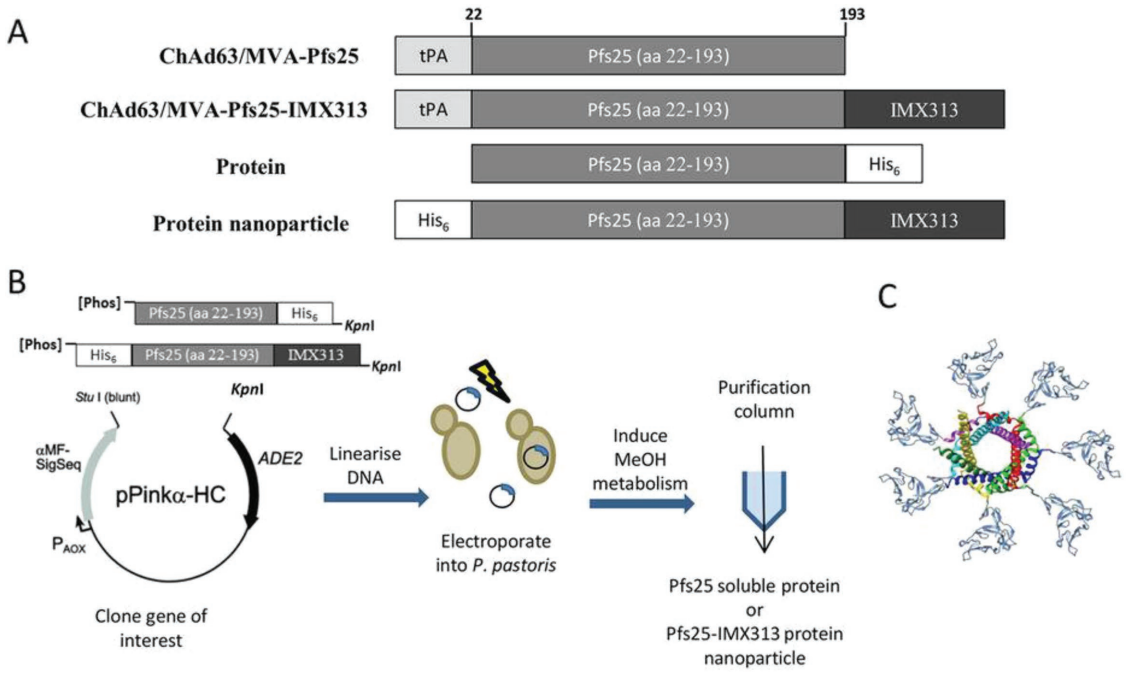

Figure 6. IMX313 multimerization. A) Four constructs used. In viral vectors: (1) Pfs25 (aa 22–194) fused to an N-terminal secretion signal peptide tPA; (2) Pfs25-IMX313 fused to tPA. In *P. pastoris*: (3) Pfs25 (aa 22–194) with a C-terminal hexahistidine (His6) tag; (4) Pfs25 fused to IMX313 with an N-terminal His6 tag. B) Steps in production of Pfs25 and Pfs25-IMX313 in *P. pastoris*. C) Idealized structure of the Pfs25-IMX313 heptamer. Pfs25 (blue) homology model based on the crystal structure of Pvs25 fused N-terminally to IMX313 represented here by the crystal structure of the human C4bp. Reproduced with permission.[154] Copyright 2016, Springer Nature.

as a ligand for B lymphocytes.[159] The Z domain was genetically conjugated to an α-helical coiled-coil domain[160] of tetrabrachion (TB)[161] or cartilage oligomeric matrix protein (COMP),[162] in order to increase its stability and binding avidity. The Z domain was transfected into and expressed by *E. coli* to produce COMP-IBD fusion proteins. The Pvs25 was then chemically conjugated to COMP-IBD protein using a heterobifunctional cross-linker to form a tricomponent complex (**Figure 7**). Mice received 30 µg of Pvs25 with the tricomponent complex via a subcutaneous route. Mice that were immunized with the tricomponent complex showed enhanced antibody response and better transmission-blocking activity.[163]

Another important use of carrier protein conjugates is related to peptide vaccines. Peptides vaccines have the advantages of low cost, scalability, simplicity in production and characterization, and have well-defined antigen targets. However, they typically lack tertiary conformation and are usually poorly immunogenic. For immunization, peptides are often conjugated to larger proteins that improve peptide vaccine immune responses. Keyhole limpet hemocyanin (KLH) is a large immunogenic carrier protein obtained from the keyhole limpet, a marine organism and is one of the most carriers for peptide conjugation.[164] There has been relatively limited use of petide vaccines in TBVs. Recently, a peptide vaccine based on two short sequences conjugated to KLH (SYHLFKNDNSIKRAKLKC for *P. falciparum* and TYNYFKDDEFIKRAKLKC for *P. berghei*) targeting the conserved Plasmodium fusion loop of HAP2 induced antibodies that inhibited transmission in the SMFA.

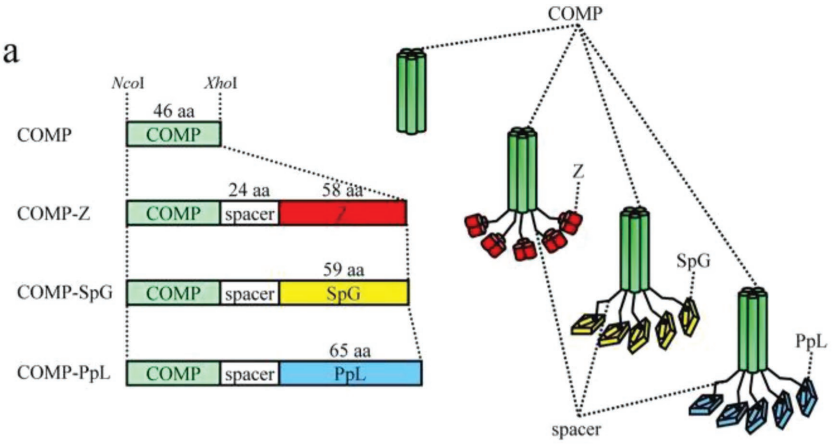

Figure 7. Expression of COMP-IBDs. a) Schematic drawing of the coiled-coil domain of the cartilage oligomeric matrix protein (COMP) fused to immunoglobulin-binding domains (IBDs). The coiled-coil domain (green) is fused to the Z domain (red), a derivative of the B domain of *Staphylococcus aureus* protein A; the B domain of group G *Streptococcus* protein G (yellow); or the B domain of *Peptostreptococcus magnus* protein L (blue). The fusion proteins contain a 24-amino-acid (aa) spacer region between the coiled coil and the IBD. Reproduced with permission.[163] Copyright 2014, American Society for Microbiology.

4.4. Protein Toxin Conjugates

Some toxin proteins can be used as antigen carriers and adjuvants, such as diphtheria toxin, cholera toxin (CtxB), and Endotoxin A (EPA). CRM197 is a commonly used immunogenic, nontoxic form of diphtheria toxin with a single amino acid mutation (glycine 52→glutamic acid).[165] These toxins can serve as mucosal adjuvants in a manner similar to the infection pathway of many pathogens. ADP-ribosylating enterotoxins from *Vibrio cholerae* (cholera toxin)[166] and *E. coli* (heat labile toxin)[167] are well-characterized mucosal adjuvants. Mucosal applications involving Pfs25 have been tested in conjunction with cholera toxin. Specifically, it has been proposed that the β subunit of the CtxB could facilitate immune responses while having a lower toxicity compared to the α subunit of the cholera toxin.[168] In a study, Pfs25 was genetically conjugated to the N-terminal of CtxB, and mice received 20 μg of the conjugated Pfs25-CtxB by oral administration. Specific IgG antibody tier was found to be higher in mice injected with 20 μg of Pfs25-CtxB than Pfs25 alone after the first injection; however, after the third injection, the level of IgG was similar when compared between Pfs25-CtxB and Pfs25 alone.[169]

EPA is a detoxified form of exotoxin A from *Pseudomonas aeruginosa*,[170,171] which has been shown to enhance immune responses. Thiol-modified Pfs25H (Pfs25-SH) and maleimide-modified EPA (EPA-mal) were conjugated to form a 60 nm nanoparticle. Furthermore, mixing the Pfs25-EPA complex with Alhydrogel resulted in adsorption of the complex into a larger particles, which were used for immunization in outbred mice. Mice receiving Pfs25-EPA with Alhydrogel had around 100 times higher IgG production compared to unconjugated Pfs25 with Alhydrogel.[52] Since then, this formulation has entered Phase I clinical trials. Several volunteers observed local and systemic symptoms, and specific antibody titer was not retained after the third booster.[13]

4.5. DNA Vaccines

DNA vaccines introduce genetic materials via DNA plasmids to transform host cells, causing in situ production of the vaccine antigen in host tissues. DNA vaccines were first introduced in the early 1990s, due to their simple design and the ability to induce both cellular and humoral immune responses. Moreover, the platform of DNA vaccines makes it easy to develop a combination vaccine to target multiple stages of malaria parasite by using a single plasmid or a mixture of plasmids which encode antigens present at different stages. DNA, which is highly negatively charged, is frequently complexed with cationic molecules or polymers such as chitosan for vaccination.[172] DNA vaccines encoding Pfs25 and Pvs25 have been developed, and were shown to be immunogenic when administered with in vivo electroporation in mice[173,174] and nonhuman primates.[58] Another report showed the application of a DNA vaccine encoding both Pfs25and Pfs48/45. Immunization with the combination DNA plasmid (Pfs25 with Pfs48/45) elicited more than 95% inhibition compared to mice immunized with a DNA plasmid encoding Pfs48/45 alone. Such results show that combining antigens can enhance transmission-blocking activity.[175]

Another study tested the delivery of plasmids through liposomal carriers. Cationic liposomes were mixed with a Pfs25 plasmid in order to form lipoplexes with different charge ratios. Various charge ratios were achieved by altering the ratio between the cationic lipid head groups and the nucleotide phosphates in the composition of the lipoplexes. Mice were injected with vaccines containing 25 μg of DNA plasmid, and booster injections were administered at three and six weeks after primary injection. Mice immunized with lipoplexes with optimized charge ratios had more effective production of IgG compared to naked plasmid DNA.[176]

5. Conclusion

TBVs proposed as a new tool to control malaria, but have not been tested in large-scale clinical trials yet. Adjuvants and conjugation schemes have been developed that improve antibody generation with TBV antigens. A large amount of TBV research has focused on Pfs25. Since Pfs25 itself is a poor immunogen, various antigen engineering and adjuvant approaches have been developed to enhance its immunogenicity. Other TBVs are also being assessed, including those that are expressed on gametocytes such as Pfs230 and Pfs48/45, which might lead to natural boosting of transmission-blocking immune responses in infected individuals.

Results from initial clinical studies with Pfs25 and Pvs25 imply that new strategies using next-generation adjuvants and delivery systems might be necessary to enhance the production of transmission-blocking antibodies to levels capable of inhibiting the life cycle of the parasite at a mosquito carrier stage. Preliminary published results with toxin conjugates and VLPs do not appear to be highly promising, although several clinical studies are still ongoing (Table 1) and results for these are yet to be reported. It was concluded that a more immunogenic vaccine than Pfs25-EPA/Alhydrogel will be needed to effectively block malaria transmission.[13] Development of new TBV antigens or fragments may be worthwhile, as well as new delivery systems as for improving TBV results. Future TBV approaches could benefit from (i) development of a safe adjuvant with optimized antigen density and dose for vaccination; (ii) a simple method for antigen–adjuvant conjugation; (iii) capacity for large-scale vaccine production and dose-sparing; and (iv) stability at elevated temperatures. If TBVs are validated as an effective tool to control malaria, future endeavors may benefit from multiple transmission-blocking antigen targeting, allowing inhibition of malaria parasites over a range of stages in order to achieve a vaccine effectiveness capable of controlling the transmission of malaria.

Acknowledgements

This study was supported by PATH's Malaria Vaccine Initiative and the National Institutes of Health (R21AI122964 and DP5OD017898). The authors thank Dr. Kazutoyo Miura for valuable feedback.

Conflict of Interest

The authors declare no conflict of interest.

Keywords

adjuvants, antigens, malaria, transmission-blocking, vaccines

Received: January 9, 2018
Revised: July 10, 2018
Published online:

[1] C. J. L. Murray, L. C. Rosenfeld, S. S. Lim, K. G. Andrews, K. J. Foreman, D. Haring, N. Fullman, M. Naghavi, R. Lozano, A. D. Lopez, *Lancet* **2012**, *379*, 413.
[2] WHO, World Health Organisation, **2017**.
[3] D. E. Loy, W. Liu, Y. Li, G. H. Learn, L. J. Plenderleith, S. A. Sundararaman, P. M. Sharp, B. H. Hahn, *Int. J. Parasitol.* **2017**, *47*, 87.
[4] L. Schwartz, G. V. Brown, B. Genton, V. S. Moorthy, *Malar. J.* **2012**, *11*, 11.
[5] S. C. T. P. Rts, *Lancet* **2015**, *386*, 31.
[6] R. Carter, *Vaccine* **2001**, *19*, 2309.
[7] A. E. Barry, A. Arnott, *Front. Immunol.* **2014**, *5*, 359.
[8] C. Arama, M. Troye-Blomberg, *J. Intern. Med.* **2014**, *275*, 456.
[9] J. K. Nunes, C. Woods, T. Carter, T. Raphael, M. J. Morin, D. Diallo, D. Leboulleux, S. Jain, C. Loucq, D. C. Kaslow, A. J. Birkett, *Vaccine* **2014**, *32*, 5531.
[10] M. Van Der Kolk, S. J. De Vlas, A. Saul, M. Van De Vegte-Bolmer, W. M. Eling, W. Sauerwein, *Parasitology* **2005**, *130*, 13.
[11] T. S. Churcher, A. M. Blagborough, M. Delves, C. Ramakrishnan, M. C. Kapulu, A. R. Williams, S. Biswas, D. F. Da, A. Cohuet, R. E. Sinden, *Int. J. Parasitol.* **2012**, *42*, 1037.
[12] C. J. Drakeley, I. Secka, S. Correa, B. M. Greenwood, G. A. T. Targett, *Trop. Med. Int. Health* **2002**, *4*, 131.
[13] K. R. Talaat, R. D. Ellis, J. Hurd, A. Hentrich, E. Gabriel, N. A. Hynes, K. M. Rausch, D. Zhu, O. Muratova, R. Herrera, C. Anderson, D. Jones, J. Aebig, S. Brockley, N. J. MacDonald, X. Wang, M. P. Fay, S. A. Healy, A. P. Durbin, D. L. Narum, Y. Wu, P. E. Duffy, *PLoS One* **2016**, *11*, e0163144.
[14] Y. Wu, R. D. Ellis, D. Shaffer, E. Fontes, E. M. Malkin, S. Mahanty, M. P. Fay, D. Narum, K. Rausch, A. P. Miles, J. Aebig, A. Orcutt, O. Muratova, G. Song, L. Lambert, D. Zhu, K. Miura, C. Long, A. Saul, L. H. Miller, A. P. Durbin, *PLoS One* **2008**, *3*, e2636.
[15] E. M. Malkin, A. P. Durbin, D. J. Diemert, J. Sattabongkot, Y. Wu, K. Miura, C. A. Long, L. Lambert, A. P. Miles, J. Wang, A. Stowers, L. H. Miller, A. Saul, *Vaccine* **2005**, *23*, 3131.
[16] J. Skinner, C. Y. Huang, M. Waisberg, P. L. Felgner, O. K. Doumbo, A. Ongoiba, K. Kayentao, B. Traore, P. D. Crompton, K. C. Williamson, *Infect. Immun.* **2015**, *83*, 4229.
[17] S. Jones, L. Grignard, I. Nebie, J. Chilongola, D. Dodoo, R. Sauerwein, M. Theisen, W. Roeffen, S. K. Singh, R. K. Singh, S. Singh, E. Kyei-Baafour, K. Tetteh, C. Drakeley, T. Bousema, *J. Infect.* **2015**, *71*, 117.
[18] K. C. Williamson, M. D. Criscio, D. C. Kaslow, *Mol. Biochem. Parasitol.* **1993**, *58*, 355.
[19] B. C. van Schaijk, M. R. van Dijk, M. van de Vegte-Bolmer, G. J. van Gemert, M. W. van Dooren, S. Eksi, W. F. Roeffen, C. J. Janse, A. P. Waters, R. W. Sauerwein, *Mol. Biochem. Parasitol.* **2006**, *149*, 216.
[20] T. Mori, H. Kuroiwa, T. Higashiyama, T. Kuroiwa, *Nat. Cell Biol.* **2006**, *8*, 64.
[21] J. Rener, P. M. Graves, R. Carter, J. L. Williams, T. R. Burkot, *J. Exp. Med.* **1983**, *158*, 976.
[22] M. R. van Dijk, C. J. Janse, J. Thompson, A. P. Waters, J. A. Braks, H. J. Dodemont, H. G. Stunnenberg, G. J. van Gemert, R. W. Sauerwein, W. Eling, *Cell* **2001**, *104*, 153.
[23] W. J. R. Stone, J. J. Campo, A. L. Ouédraogo, L. Meerstein-Kessel, I. Morlais, D. Da, A. Cohuet, S. Nsango, C. J. Sutherland, M. van de Vegte-Bolmer, R. Siebelink-Stoter, G.-J. van Gemert, W. Graumans, K. Lanke, A. D. Shandling, J. V. Pablo, A. A. Teng, S. Jones, R. M. de Jong, A. Fabra-García, J. Bradley, W. Roeffen, E. Lasonder, G. Gremo, E. Schwarzer, C. J. Janse, S. K. Singh, M. Theisen, P. Felgner, M. Marti, C. Drakeley, R. Sauerwein, T. Bousema, M. M. Jore, *Nat. Commun.* **2018**, *9*, 558.
[24] C. H. Kocken, J. Jansen, A. M. Kaan, P. J. Beckers, T. Ponnudurai, D. C. Kaslow, R. N. Konings, J. G. Schoenmakers, *Mol. Biochem. Parasitol.* **1993**, *61*, 59.
[25] A. N. Vermeulen, T. Ponnudurai, P. J. Beckers, J. P. Verhave, M. A. Smits, J. H. Meuwissen, *J. Exp. Med.* **1985**, *162*, 1460.
[26] A. N. Vermeulen, W. F. Roeffen, J. B. Henderik, T. Ponnudurai, P. J. Beckers, J. H. Meuwissen, *Dev. Biol. Stand.* **1985**, *62*, 91.
[27] W. Roeffen, T. Lensen, B. Mulder, K. Teelen, R. Sauerwein, J. Van Druten, W. Eling, J. H. Meuwissen, P. J. Beckers, *Am. J. Trop. Med. Hyg.* **1995**, *52*, 60.
[28] S. K. Singh, W. Roeffen, G. Andersen, T. Bousema, M. Christiansen, R. Sauerwein, M. Theisen, *Vaccine* **2015**, *33*, 1981.
[29] W. Roeffen, M. Theisen, M. van de Vegte-Bolmer, G. van Gemert, T. Arens, G. Andersen, M. Christiansen, L. Sevargave, S. K. Singh, S. Kaviraj, R. Sauerwein, *Malar. J.* **2015**, *14*, 443.
[30] J. A. Young, Q. L. Fivelman, P. L. Blair, P. de la Vega, K. G. Le Roch, Y. Zhou, D. J. Carucci, D. A. Baker, E. A. Winzeler, *Mol. Biochem. Parasitol.* **2005**, *143*, 67.
[31] A. Molina-Cruz, L. S. Garver, A. Alabaster, L. Bangiolo, A. Haile, J. Winikor, C. Ortega, B. C. van Schaijk, R. W. Sauerwein, E. Taylor-Salmon, C. Barillas-Mury, *Science* **2013**, *340*, 984.
[32] A. M. Blagborough, R. E. Sinden, *Vaccine* **2009**, *27*, 5187.
[33] M. A. Johnson, K. von Besser, Q. Zhou, E. Smith, G. Aux, D. Patton, J. Z. Levin, D. Preuss, *Genetics* **2004**, *168*, 971.
[34] J. Fédry, Y. Liu, G. Péhau-Arnaudet, J. Pei, W. Li, M. A. Tortorici, F. Traincard, A. Meola, G. Bricogne, N. V. Grishin, W. J. Snell, F. A. Rey, T. Krey, *Cell* **2017**, *168*, 904.
[35] Y. Liu, R. Tewari, J. Ning, A. M. Blagborough, S. Garbom, J. Pei, N. V. Grishin, R. E. Steele, R. E. Sinden, W. J. Snell, O. Billker, *Genes Dev.* **2008**, *22*, 1051.
[36] M. R. van Dijk, B. C. van Schaijk, S. M. Khan, M. W. van Dooren, J. Ramesar, S. Kaczanowski, G. J. van Gemert, H. Kroeze, H. G. Stunnenberg, W. M. Eling, R. W. Sauerwein, A. P. Waters, C. J. Janse, *PLoS Pathog.* **2010**, *6*, e1000853.
[37] A. Ecker, E. S. Bushell, R. Tewari, R. E. Sinden, *Mol. Microbiol.* **2008**, *70*, 209.
[38] K. A. Sala, H. Nishiura, L. M. Upton, S. E. Zakutansky, M. J. Delves, M. Iyori, M. Mizutani, R. E. Sinden, S. Yoshida, A. M. Blagborough, *Vaccine* **2015**, *33*, 437.
[39] W. Zheng, F. Liu, Y. He, Q. Liu, G. B. Humphreys, T. Tsuboi, Q. Fan, E. Luo, Y. Cao, L. Cui, *Parasites Vectors* **2017**, *10*, 8.
[40] J. Wang, W. Zheng, F. Liu, Y. Wang, Y. He, L. Zheng, Q. Fan, E. Luo, Y. Cao, L. Cui, *Malar. J.* **2017**, *16*, 458.
[41] X. Kou, W. Zheng, F. Du, F. Liu, M. Wang, Q. Fan, L. Cui, E. Luo, Y. Cao, *Parasites Vectors* **2016**, *9*, 190.
[42] A. M. Tomas, G. Margos, G. Dimopoulos, L. H. van Lin, T. F. de Koning-Ward, R. Sinha, P. Lupetti, A. L. Beetsma, M. C. Rodriguez, M. Karras, A. Hager, J. Mendoza, G. A. Butcher, F. Kafatos, C. J. Janse, A. P. Waters, R. E. Sinden, *EMBO J.* **2001**, *20*, 3975.
[43] T. Tsuboi, D. C. Kaslow, M. M. Gozar, M. Tachibana, Y. M. Cao, M. Torii, *Mol. Med.* **1998**, *4*, 772.
[44] H. Hisaeda, A. W. Stowers, T. Tsuboi, W. E. Collins, J. S. Sattabongkot, N. Suwanabun, M. Torii, D. C. Kaslow, *Infect. Immun.* **2000**, *68*, 6618.
[45] A. P. Miles, Y. Zhang, A. Saul, A. W. Stowers, *Protein Expression Purif.* **2002**, *25*, 87.

[46] D. C. Kaslow, *Curr. Opin. Immunol.* **1993**, *5*, 557.
[47] A. van Amerongen, R. W. Sauerwein, P. J. Beckers, R. H. Meloen, J. H. Meuwissen, *Parasite Immunol.* **1989**, *11*, 425.
[48] E. A. Stura, A. C. Satterthwait, J. C. Calvo, R. S. Stefanko, J. P. Langeveld, D. C. Kaslow, *Acta Crystallogr., Sect. D: Biol. Crystallogr.* **1994**, *50*, 556.
[49] A. J. Radtke, C. F. Anderson, N. Riteau, K. Rausch, P. Scaria, E. R. Kelnhofer, R. F. Howard, A. Sher, R. N. Germain, P. Duffy, *Sci. Rep.* **2017**, *7*, 40312.
[50] C. W. Tsai, P. F. Duggan, R. L. Shimp Jr., L. H. Miller, D. L. Narum, *J. Biotechnol.* **2006**, *121*, 458.
[51] L. Zou, A. P. Miles, J. Wang, A. W. Stowers, *Vaccine* **2003**, *21*, 1650.
[52] R. L. Shimp, C. Rowe, K. Reiter, B. Chen, V. Nguyen, J. Aebig, K. M. Rausch, K. Kumar, Y. M. Wu, A. J. Jin, D. S. Jones, D. L. Narum, *Vaccine* **2013**, *31*, 2954.
[53] J. Kubler-Kielb, F. Majadly, Y. Wu, D. L. Narum, C. Guo, L. H. Miller, J. Shiloach, J. B. Robbins, R. Schneerson, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 293.
[54] C. E. Farrance, J. A. Chichester, K. Musiychuk, M. Shamloul, A. Rhee, S. D. Manceva, R. M. Jones, T. Mamedov, S. Sharma, V. Mett, S. J. Streatfield, W. Roeffen, M. van de Vegte-Bolmer, R. W. Sauerwein, Y. Wu, O. Muratova, L. Miller, P. Duffy, R. Sinden, V. Yusibov, *Hum. Vaccines* **2011**, 191.
[55] R. Kumar, E. Angov, N. Kumar, *Infect. Immun.* **2014**, *82*, 1453.
[56] J. A. Gregory, F. Li, L. M. Tomosada, C. J. Cox, A. B. Topol, J. M. Vinetz, S. Mayfield, *PLoS One* **2012**, *7*, e37179.
[57] C. Coban, M. T. Philipp, J. E. Purcell, D. B. Keister, M. Okulate, D. S. Martin, N. Kumar, *Infect. Immun.* **2004**, *72*, 253.
[58] R. Kumar, R. Nyakundi, T. Kariuki, H. Ozwara, O. Nyamongo, G. Mlambo, B. Ellefsen, D. Hannaman, N. Kumar, *Vaccine* **2013**, *31*, 3140.
[59] D. K. Mathias, J. G. Jardim, L. A. Parish, J. S. Armistead, H. V. Trinh, C. Kumpitak, J. Sattabongkot, R. R. Dinglasan, *Infect., Genet. Evol.* **2014**, *28*, 635.
[60] J. S. Armistead, S. C. Atkinson, D. K. Mathias, M. M. Sandeu, D. Tao, N. Borhani-Dizaji, B. B. Tarimo, I. Morlais, R. R. Dinglasan, N. A. Borg, *Nat. Struct. Mol. Biol.* **2015**, *22*, 7.
[61] R. R. Dinglasan, D. E. Kalume, S. M. Kanzok, A. K. Ghosh, O. Muratova, A. Pandey, M. Jacobs-Lorena, *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 13461.
[62] D. K. Mathias, J. L. Plieskatt, J. S. Armistead, J. M. Bethony, K. B. Abdul-Majid, A. McMillan, E. Angov, M. J. Aryee, B. Zhan, P. Gillespie, B. Keegan, A. R. Jariwala, W. Rezende, M. E. Bottazzi, D. G. Scorpio, P. J. Hotez, R. R. Dinglasan, *Infect. Immun.* **2012**, *80*, 1606.
[63] L. Powles, S. Xiang, C. Selomulya, M. Plebanski, *Vaccines* **2015**, *3*, 894.
[64] R. L. Richards, M. D. Hayre, W. T. Hockmeyer, C. R. Alving, *Infect. Immun.* **1988**, *56*, 682.
[65] K. White, U. Krzych, D. M. Gordon, T. G. Porter, R. L. Richards, C. R. Alving, C. D. Deal, M. Hollingdale, C. Silverman, D. R. Sylvester, W. R. Ballou, M. Gross, *Vaccine* **1993**, *11*, 1341.
[66] J. N. Verma, M. Rao, S. Amselem, U. Krzych, C. R. Alving, S. J. Green, N. M. Wassef, *Infect. Immun.* **1992**, *60*, 2438.
[67] Y. Men, B. Gander, H. P. Merkle, G. Corradin, *Vaccine* **1996**, *14*, 1442.
[68] D. F. Nixon, C. Hioe, P. D. Chen, Z. Bian, P. Kuebler, M. L. Li, H. Qiu, X. M. Li, M. Singh, J. Richardson, P. McGee, T. Zamb, W. Koff, C. Y. Wang, D. O'Hagan, *Vaccine* **1996**, *14*, 1523.
[69] M. Aikawa, M. Torii, A. Sjolander, K. Berzins, P. Perlmann, L. H. Miller, *Exp. Parasitol.* **1990**, *71*, 326.
[70] R. M. Jones, J. A. Chichester, V. Mett, J. Jaje, S. Tottey, S. Manceva, L. J. Casta, S. K. Gibbs, K. Musiychuk, M. Shamloul, J. Norikane, V. Mett, S. J. Streatfield, M. van de Vegte-Bolmer, W. Roeffen, R. W. Sauerwein, V. Yusibov, *PLoS One* **2013**, *8*, e79538.
[71] K. A. Collins, R. Snaith, M. G. Cottingham, S. C. Gilbert, A. V. S. Hill, *Sci. Rep.* **2017**, *7*, 46621.
[72] M. M. Anisimov, V. V. Shcheglov, S. N. Dzizenko, *Prikl. Biokhim. Mikrobiol.* **1978**, *14*, 573.
[73] C. R. Kensil, *Crit. Rev. Ther. Drug Carrier Syst.* **1996**, *13*, 1.
[74] V. Haridas, C. J. Arntzen, J. U. Gutterman, *Proc. Natl. Acad. Sci. USA* **2001**, *98*, 11557.
[75] F. Delmas, C. Di Giorgio, R. Elias, M. Gasquet, N. Azas, V. Mshvildadze, G. Dekanosidze, E. Kemertelidze, P. Timon-David, *Planta Med.* **2000**, *66*, 343.
[76] C. R. Kensil, U. Patel, M. Lennick, D. Marciani, *J. Immunol.* **1991**, *146*, 431.
[77] C. R. Kensil, J. Y. Wu, C. A. Anderson, D. A. Wheeler, J. Amsden, *Dev. Biol. Stand.* **1998**, *92*, 41.
[78] M. M. Gozar, O. Muratova, D. B. Keister, C. R. Kensil, V. L. Price, D. C. Kaslow, *Exp. Parasitol.* **2001**, *97*, 61.
[79] L. Agrawal, W. Haq, C. V. Hanson, D. N. Rao, *J. Immune Based Ther. Vaccines* **2003**, *1*, 5.
[80] I. D. Davis, W. Chen, H. Jackson, P. Parente, M. Shackleton, W. Hopkins, Q. Chen, N. Dimopoulos, T. Luke, R. Murphy, A. M. Scott, E. Maraskovsky, G. McArthur, D. MacGregor, S. Sturrock, T. Y. Tai, S. Green, A. Cuthbertson, D. Maher, L. Miloradovic, S. V. Mitchell, G. Ritter, A. A. Jungbluth, Y. T. Chen, S. Gnjatic, E. W. Hoffman, L. J. Old, J. S. Cebon, *Proc. Natl. Acad. Sci. USA* **2004**, *101*, 10697.
[81] M. Schnurr, M. Orban, N. C. Robson, A. Shin, H. Braley, D. Airey, J. Cebon, E. Maraskovsky, S. Endres, *J. Immunol.* **2009**, *182*, 1253.
[82] A. Sjolander, M. Hansson, K. Lovgren, B. Wahlin, K. Berzins, P. Perlmann, *Parasite Immunol.* **1993**, *15*, 355.
[83] A. Junghans, C. Champagne, P. Cayot, C. Loupiac, I. Koper, *Langmuir* **2010**, *26*, 12049.
[84] C. B. Chang, C. M. Knobler, W. M. Gelbart, T. G. Mason, *ACS Nano* **2008**, *2*, 281.
[85] J. S. Chen, E. Dickinson, *Colloids Surf., A* **1995**, *100*, 255.
[86] J. Chesko, J. Kazzaz, M. Ugozzoli, T. O'Hagan D, M. Singh, *J. Pharm. Sci.* **2005**, *94*, 2510.
[87] F. Liu, D. Wang, H. Xu, C. Sun, Y. Gao, *Food Chem.* **2016**, *196*, 338.
[88] A. P. Miles, H. A. McClellan, K. M. Rausch, D. M. Zhu, M. D. Whitmore, S. Singh, L. B. Martin, Y. M. Wu, B. K. Giersing, A. W. Stowers, C. A. Long, A. Saul, *Vaccine* **2005**, *23*, 2530.
[89] V. Rampon, C. Genot, A. Riaublanc, M. Anton, M. A. V. Axelos, D. J. McClements, *J. Agric. Food Chem.* **2003**, *51*, 2482.
[90] J. L. Zhai, T. J. Wooster, S. V. Hoffmann, T. H. Lee, M. A. Augustin, M. I. Aguilar, *Langmuir* **2011**, *27*, 9227.
[91] W. Chanasattru, E. A. Decker, D. J. McClements, *Food Res. Int.* **2007**, *40*, 1161.
[92] R. M. Kramer, C. B. Fox, L. Barnes V, Q. M. Dowling, T. S. Vedvick, *Ther. Adv. Vaccines* **2013**, *1*, 13.
[93] G. Ott, G. L. Barchfeld, D. Chernoff, R. Radhakrishnan, P. van Hoogevest, G. Van Nest, *Pharm. Biotechnol.* **1995**, *6*, 277.
[94] A. Seubert, E. Monaci, M. Pizza, D. T. O'Hagan, A. Wack, *J. Immunol.* **2008**, *180*, 5402.
[95] M. Wykes, G. MacPherson, *Immunology* **2000**, *100*, 1.
[96] A. Ricciardi, K. Visitsunthorn, J. P. Dalton, M. Ndao, *BMC Infect. Dis.* **2016**, *16*, 112.
[97] D. R. Chowdhury, E. Angov, T. Kariuki, N. Kumar, *PLoS One* **2009**, *4*, e6352.
[98] W. E. Collins, J. W. Barnwell, J. S. Sullivan, D. Nace, T. Williams, A. Bounngaseng, J. Roberts, E. Strobert, H. McClure, A. Saul, C. A. Long, *Am. J. Trop. Med. Hyg.* **2006**, *74*, 215.
[99] D. T. O'Hagan, G. S. Ott, E. De Gregorio, A. Seubert, *Vaccine* **2012**, *30*, 4341.
[100] S. Calabro, M. Tortoli, B. C. Baudner, A. Pacitto, M. Cortese, D. T. O'Hagan, E. De Gregorio, A. Seubert, A. Wack, *Vaccine* **2011**, *29*, 1812.

[101] P. J. Barr, K. M. Green, H. L. Gibson, I. C. Bathurst, I. A. Quakyi, D. C. Kaslow, *J. Exp. Med.* **1991**, *174*, 1203.
[102] S. Sachdeva, A. Mohmmed, P. V. Dasaradhi, B. S. Crabb, A. Katyal, P. Malhotra, V. S. Chauhan, *Vaccine* **2006**, *24*, 2007.
[103] S. S. Yazdani, A. R. Shakri, P. Mukherjee, S. K. Baniwal, C. E. Chitnis, *Vaccine* **2004**, *22*, 3727.
[104] R. H. al-Shakhshir, F. E. Regnier, J. L. White, S. L. Hem, *Vaccine* **1995**, *13*, 41.
[105] S. Iyer, R. S. Robinett, H. HogenEsch, S. L. Hem, *Vaccine* **2004**, *22*, 1475.
[106] L. J. Peek, T. T. Martin, C. E. Nation, S. A. Pegram, C. R. Middaugh, *J. Pharm. Sci.* **2007**, *96*, 547.
[107] D. Luo, K. A. Carter, J. F. Lovell, *Wiley Interdiscip. Rev.: Nanomed. Nanobiotechnol.* **2015**, *7*, 169.
[108] R. K. Gupta, B. E. Rost, E. Relyveld, G. R. Siber, *Pharm. Biotechnol.* **1995**, *6*, 229.
[109] S. L. Hem, J. L. White, *Pharm. Biotechnol.* **1995**, *6*, 249.
[110] S. C. Atkinson, J. S. Armistead, D. K. Mathias, M. M. Sandeu, D. Tao, N. Borhani-Dizaji, B. B. Tarimo, I. Morlais, R. R. Dinglasan, N. A. Borg, *Nat. Struct. Mol. Biol.* **2015**, *22*, 532.
[111] B. N. Lambrecht, M. Kool, M. A. Willart, H. Hammad, *Curr. Opin. Immunol.* **2009**, *21*, 23.
[112] H. Li, S. Nookala, F. Re, *J. Immunol.* **2007**, *178*, 5271.
[113] S. Romagnani, *Inflammatory Bowel Dis.* **1999**, *5*, 285.
[114] J. S. Armistead, I. Morlais, D. K. Mathias, J. G. Jardim, J. Joy, A. Fridman, A. C. Finnefrock, A. Bagchi, M. Plebanski, D. G. Scorpio, T. S. Churcher, N. A. Borg, J. Sattabongkot, R. R. Dinglasan, *Infect. Immun.* **2014**, *82*, 818.
[115] M. J. McCluskie, H. L. Davis, *J. Immunol.* **1998**, *161*, 4463.
[116] G. Sen, Q. Chen, C. M. Snapper, *Infect. Immun.* **2006**, *74*, 2177.
[117] S. Rothenfusser, V. Hornung, M. Ayyoub, S. Britsch, A. Towarowski, A. Krug, A. Sarris, N. Lubenow, D. Speiser, S. Endres, G. Hartmann, *Blood* **2004**, *103*, 2162.
[118] C. Coban, K. J. Ishii, A. W. Stowers, D. B. Keister, D. M. Klinman, N. Kumar, *Infect. Immun.* **2004**, *72*, 584.
[119] A. A. Bhat, R. K. Seth, J. Babu, S. Biswas, D. N. Rao, *Int. Immunopharmacol.* **2009**, *9*, 1197.
[120] M. Mizutani, M. Iyori, A. M. Blagborough, S. Fukumoto, T. Funatsu, R. E. Sinden, S. Yoshida, *Infect. Immun.* **2014**, *82*, 10.
[121] S. Mian-McCarthy, S. T. Agnandji, B. Lell, J. F. Fernandes, B. P. Abossolo, B. G. N. O. Methogo, A. L. Kabwende, A. A. Adegnika, B. Mordmuller, S. Issifou, P. G. Kremsner, J. Sacarlal, P. Aide, M. Lanaspa, J. J. Aponte, S. Machevo, S. Acacio, H. Bulo, B. Sigauque, E. Macete, P. Alonso, S. Abdulla, N. Salim, R. Minja, M. Mpina, S. Ahmed, A. M. Ali, A. T. Mtoro, A. S. Hamad, P. Mutani, M. Tanner, H. Tinto, U. D'Alessandro, H. Sorgho, I. Valea, B. Bihoun, I. Guiraud, B. Kabore, O. Sombie, R. T. Guiguemde, J. B. Ouedraogo, M. J. Hamel, S. Kariuki, M. Oneko, C. Odero, K. Otieno, N. Awino, M. McMorrow, V. Muturi-Kioi, K. F. Laserson, L. Slutsker, W. Otieno, L. Otieno, N. Otsyula, S. Gondi, A. Otieno, V. Owira, E. Oguk, G. Odongo, J. W. Woods, B. Ogutu, P. Njuguna, R. Chilengi, P. Akoo, C. Kerubo, C. Maingi, T. Lang, A. Olotu, P. Bejon, K. Marsh, G. Mwanbingu, S. Owusu-Agyei, K. P. Asante, K. Osei-Kwakye, O. Boahen, D. Dosoo, I. Asante, G. Adjei, E. Kwara, D. Chandramohan, B. Greenwood, J. Lusingu, S. Gesase, A. Malabeja, O. Abdul, C. Mahende, E. Liheluka, L. Malle, M. Lemnge, T. G. Theander, C. Drakeley, D. Ansong, T. Agbenyega, S. Adjei, H. O. Boateng, T. Rettig, J. Bawa, J. Sylverken, D. Sambian, A. Sarfo, A. Agyekum, F. Martinson, I. Hoffman, T. Mvalo, P. Kamthunzi, R. Nkomo, T. Tembo, G. Tegha, M. Tsidya, J. Kilembe, C. Chawinga, W. R. Ballou, J. Cohen, Y. Guerra, E. Jongert, D. Lapierre, A. Leach, M. Lievens, O. Ofori-Anyinam, A. Olivier, J. Vekemans, T. Carter, D. Kaslow, D. Leboulleux, C. Loucq, A. Radford, B. Savarese, D. Schellenberg, M. Sillman, P. Vansadia, R. S. C. T. Partnership, *N. Engl. J. Med.* **2012**, *367*, 2284.
[122] P. A. Minsoko, B. Lell, J. F. Fernandes, B. P. Abossolo, A. L. Kabwende, A. A. Adegnika, B. Mordmuller, S. Issifou, P. G. Kremsner, M. M. Loembe, J. Sacarlal, P. Aide, L. Madrid, M. Lanaspa, S. Mandjate, J. J. Aponte, H. Bulo, A. Nhama, E. Macete, P. Alonso, S. Abdulla, N. Salim, A. T. Mtoro, P. Mutani, M. Tanner, C. Mavere, G. Mwangoka, O. Lweno, O. A. Juma, S. Shekalaghe, H. Tinto, U. D'Alessandro, H. Sorgho, I. Valea, J. B. Ouedraogo, P. Lompo, S. Diallo, O. Traore, A. Bassole, E. Dao, M. J. Hamel, S. Kariuki, M. Oneko, C. Odero, K. Otieno, N. Awino, V. Muturi-Kioi, J. Omoto, K. F. Laserson, L. Slutsker, W. Otieno, L. Otieno, N. Otsyula, S. Gondi, A. Otieno, B. Ogutu, J. Ochola, I. Onyango, J. Oyieko, P. Njuguna, R. Chilengi, P. Akoo, C. Kerubo, C. Maingi, A. Olotu, P. Bejon, K. Marsh, G. Mwabingu, J. Gitaka, S. Owusu-Agyei, K. P. Asante, O. Boahen, D. Dosoo, G. Adjei, E. Adeniji, A. K. Yawson, K. Kayan, D. Chandramohan, B. Greenwood, J. Lusingu, S. Gesase, A. Malabeja, O. Abdul, C. Mahende, E. Liheluka, M. Lemnge, T. G. Theander, C. Drakeley, J. Mbwana, D. Ansong, T. Agbenyega, S. Adjei, H. O. Boateng, T. Rettig, J. Bawa, J. Sylverken, D. Sambian, A. Sarfo, A. Agyekum, F. Martinson, I. Hoffman, T. Mvalo, P. Kamthunzi, R. Nkomo, T. Tembo, G. T. M. Tsidya, J. Kilembe, C. Chawinga, W. R. Ballou, J. Cohen, Y. Guerra, E. Jongert, D. Lapierre, A. Leach, M. Lievens, O. Ofori-Anyinam, A. Olivier, J. Vekemans, D. Kaslow, D. Leboulleux, B. Savarese, D. Schellenberg, R. S. C. T. Partnership, *PLoS Med.* **2014**, *11*, e1001685.
[123] S. L. Baldwin, W. Roeffen, S. K. Singh, R. W. Tiendrebeogo, M. Christiansen, E. Beebe, D. Carter, C. B. Fox, R. F. Howard, S. G. Reed, R. Sauerwein, M. Theisen, *Vaccine* **2016**, *34*, 2207.
[124] M. Henriksen-Lacey, D. Christensen, V. W. Bramwell, T. Lindenstrom, E. M. Agger, P. Andersen, Y. Perrie, *J. Controlled Release* **2010**, *145*, 102.
[125] E. Shahum, H. M. Therien, *Int. J. Immunopharmacol.* **1995**, *17*, 9.
[126] S. Pejawar-Gaddy, J. M. Kovacs, D. H. Barouch, B. Chen, D. J. Irvine, *Bioconjugate Chem.* **2014**, *25*, 1470.
[127] S. Shao, J. M. Geng, H. A. Yi, S. Gogia, S. Neelamegham, A. Jacobs, J. F. Lovell, *Nat. Chem.* **2015**, *7*, 438.
[128] M. Pritsch, N. Ben-Khaled, M. Chaloupka, S. Kobold, N. Berens-Riha, A. Peter, G. Liegl, S. Schubert, M. Hoelscher, T. Loscher, A. Wieser, *J. Immunol. Res.* **2016**, *2016*, 3576028.
[129] V. Mata-Haro, C. Cekic, M. Martin, P. M. Chilton, C. R. Casella, T. C. Mitchell, *Science* **2007**, *316*, 1628.
[130] S. D. Perrault, W. C. Chan, *J. Am. Chem. Soc.* **2009**, *131*, 17042.
[131] T. K. Sau, C. J. Murphy, *J. Am. Chem. Soc.* **2004**, *126*, 8648.
[132] S. Rana, A. Bajaj, R. Mout, V. M. Rotello, *Adv. Drug Delivery Rev.* **2012**, *64*, 200.
[133] N. G. Bastus, E. Sanchez-Tillo, S. Pujals, C. Farrera, M. J. Kogan, E. Giralt, A. Celada, J. Lloberas, V. Puntes, *Mol. Immunol.* **2009**, *46*, 743.
[134] N. G. Bastus, E. Sanchez-Tillo, S. Pujals, C. Farrera, C. Lopez, E. Giralt, A. Celada, J. Lloberas, V. Puntes, *ACS Nano* **2009**, *3*, 1335.
[135] E. Pensa, E. Cortes, G. Corthey, P. Carro, C. Vericat, M. H. Fonticelli, G. Benitez, A. A. Rubert, R. C. Salvarezza, *Acc. Chem. Res.* **2012**, *45*, 1183.
[136] B. M. DeRussy, M. A. Aylward, Z. Fan, P. C. Ray, R. Tandon, *Sci. Rep.* **2014**, *4*, 5550.
[137] R. Kumar, P. C. Ray, D. Datta, G. P. Bansal, E. Angov, N. Kumar, *Vaccine* **2015**, *33*, 5064.
[138] R. Kumar, G. Ledet, R. Graves, D. Datta, S. Robinson, G. P. Bansal, T. Mandal, N. Kumar, *Pharm. Res.* **2015**, *32*, 3827.
[139] C. S. Chong, M. Cao, W. W. Wong, K. P. Fischer, W. R. Addison, G. S. Kwon, D. L. Tyrrell, J. Samuel, *J. Controlled Release* **2005**, *102*, 85.
[140] R. R. Dinglasan, J. S. Armistead, J. F. Nyland, X. Jiang, H. Q. Mao, *Curr. Mol. Med.* **2013**, *13*, 479.

[141] S. Tiwari, A. K. Goyal, N. Mishra, K. Khatri, B. Vaidya, A. Mehta, Y. Wu, S. P. Vyas, *J. Controlled Release* **2009**, *140*, 157.
[142] M. F. Bachmann, G. T. Jennings, *Nat. Rev. Immunol.* **2010**, *10*, 787.
[143] P. Pushko, P. Pumpens, E. Grens, *Intervirology* **2013**, *56*, 141.
[144] E. Strable, M. G. Finn, *Curr. Top. Microbiol. Immunol.* **2009**, *327*, 1.
[145] S. C. Reddington, M. Howarth, *Curr. Opin. Chem. Biol.* **2015**, *29*, 94.
[146] K. D. Brune, C. M. Buldun, Y. Y. Li, I. J. Taylor, F. Brod, S. Biswas, M. Howarth, *Bioconjugate Chem.* **2017**, *28*, 1544.
[147] J. Klovins, G. P. Overbeek, S. H. E. van den Worm, H. W. Ackermann, J. van Duin, *J. Gen. Virol.* **2002**, *83*, 1523.
[148] A. C. Tissot, R. Renhofa, N. Schmitz, I. Cielens, E. Meijerink, V. Ose, G. T. Jennings, P. Saudan, P. Pumpens, M. F. Bachmann, *PLoS One* **2010**, *5*, e9809.
[149] D. B. Leneghan, K. Miura, I. J. Taylor, Y. Li, J. Jin, K. D. Brune, M. F. Bachmann, M. Howarth, C. A. Long, S. Biswas, *Sci. Rep.* **2017**, *7*, 3811.
[150] S. K. Singh, S. Thrane, C. M. Janitzek, M. A. Nielsen, T. G. Theander, M. Theisen, A. Salanti, A. F. Sander, *Vaccine* **2017**, *35*, 3726.
[151] M. Theisen, S. Soe, C. Oeuvray, A. W. Thomas, J. Vuust, S. Danielsen, S. Jepsen, P. Druilhe, *Infect. Immun.* **1998**, *66*, 11.
[152] D. F. Lappin, K. Whaley, *Biochem. J.* **1990**, *271*, 767.
[153] S. R. Brodeur, F. Angelini, L. B. Bacharier, A. M. Blom, E. Mizoguchi, H. Fujiwara, A. Plebani, L. D. Notarangelo, B. Dahlback, E. Tsitsikov, R. S. Geha, *Immunity* **2003**, *18*, 837.
[154] Y. Li, D. B. Leneghan, K. Miura, D. Nikolaeva, I. J. Brian, M. D. Dicks, A. J. Fyfe, S. E. Zakutansky, S. de Cassan, C. A. Long, S. J. Draper, A. V. Hill, F. Hill, S. Biswas, *Sci. Rep.* **2016**, *6*, 18848.
[155] A. J. Spencer, F. Hill, J. D. Honeycutt, M. G. Cottingham, M. Bregu, C. S. Rollier, J. Furze, S. J. Draper, K. C. Sogaard, S. C. Gilbert, D. H. Wyllie, A. V. Hill, *PLoS One* **2012**, *7*, e33555.
[156] S. Marburg, D. Jorn, R. L. Tolman, B. Arison, J. Mccauley, P. J. Kniskern, A. Hagopian, P. P. Vella, *J. Am. Chem. Soc.* **1986**, *108*, 5282.
[157] W. J. Leanza, L. S. Chupak, R. L. Tolman, S. Marburg, *Bioconjugate Chem.* **1992**, *3*, 514.
[158] Y. Wu, C. Przysiecki, E. Flanagan, S. N. Bello-Irizarry, R. Ionescu, O. Muratova, G. Dobrescu, L. Lambert, D. Keister, Y. Rippeon, C. A. Long, L. Shi, M. Caulfield, A. Shaw, A. Saul, J. Shiver, L. H. Miller, *Proc. Natl. Acad. Sci. USA* **2006**, *103*, 18243.
[159] M. Tashiro, R. Tejero, D. E. Zimmerman, B. Celda, B. Nilsson, G. T. Montelione, *J. Mol. Biol.* **1997**, *272*, 573.
[160] A. N. Lupas, M. Gruber, *Adv. Protein Chem.* **2005**, *70*, 37.
[161] J. Peters, M. Nitsch, B. Kuhlmorgen, R. Golbik, A. Lupas, J. Kellermann, H. Engelhardt, J. P. Pfander, S. Muller, K. Goldie, A. Engel, K. O. Stetter, W. Baumeister, *J. Mol. Biol.* **1995**, *245*, 385.
[162] V. P. Efimov, A. Lustig, J. Engel, *FEBS Lett.* **1994**, *341*, 54.
[163] T. Arakawa, T. Tsuboi, J. Sattabongkot, K. Sakao, M. Torii, T. Miyata, *Clin. Vaccine Immunol.* **2014**, *21*, 561.
[164] F. Angrisano, K. A. Sala, D. F. Da, Y. Liu, J. Pei, N. V. Grishin, W. J. Snell, A. M. Blagborough, *Cell Rep.* **2017**, *21*, 2868.
[165] M. Bröker, P. Costantino, L. DeTora, E. D. McIntosh, R. Rappuoli, *Biologicals* **2011**, *39*, 195.
[166] J. Sanchez, J. Holmgren, *Indian J. Med. Res.* **2011**, *133*, 153.
[167] V. P. da Hora, F. R. Conceicao, O. A. Dellagostin, D. L. Doolan, *Vaccine* **2011**, *29*, 1538.
[168] I. Lonnroth, J. Holmgren, *J. Gen. Microbiol.* **1973**, *76*, 417.
[169] J. A. Gregory, A. B. Topol, D. Z. Doerner, S. Mayfield, *Appl. Environ. Microbiol.* **2013**, *79*, 9.
[170] F. Qian, Y. M. Wu, O. Muratova, H. Zhou, G. Dobrescu, P. Duggan, L. Lynn, G. H. Song, Y. L. Zhang, K. Reiter, N. MacDonald, D. L. Narum, C. A. Long, L. H. Miller, A. Saul, G. E. D. Mullen, *Vaccine* **2007**, *25*, 3923.
[171] F. Qian, K. Reiter, Y. L. Zhang, R. L. Shimp, V. Nguyen, J. A. Aebig, K. M. Rausch, D. M. Zhu, L. Lambert, G. E. D. Mullen, L. B. Martin, C. A. Long, L. H. Miller, D. L. Narum, *PLoS One* **2012**, *7*, e36996.
[172] H.-Q. Mao, K. Roy, V. L. Troung-Le, K. A. Janes, K. Y. Lin, Y. Wang, J. T. August, K. W. Leong, *J. Controlled Release* **2001**, *70*, 399.
[173] D. Datta, G. P. Bansal, R. Kumar, B. Ellefsen, D. Hannaman, N. Kumar, *Clin. Vaccine Immunol.* **2015**, *22*, 1013.
[174] C. A. Lobo, R. Dhar, N. Kumar, *Infect. Immun.* **1999**, *67*, 1688.
[175] D. Datta, G. P. Bansal, D. L. Gerloff, B. Ellefsen, D. Hannaman, N. Kumar, *Vaccine* **2017**, *35*, 264.
[176] Z. Heidari, J. S. Arora, D. Datta, V. T. John, N. Kumar, G. P. Bansal, *Pharm. Res.* **2017**, *34*, 1796.