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Molecular vaccines for malaria

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Abbreviations: Ad5, adenovirus serotype 5; CSP, circumsporozoite protein; TRAP, thrombospondin-related adhesion protein; SSP2, sporozoite surface protein-2; LSA1, liver stage antigen-1; LSA3, liver stage antigen-3; AMA1, apical membrane antigen-1; MSP 1, merozoite surface protein-1; MSA 2, merozoite surface antigen 2; EMP1, erythrocyte membrane protein-1; EXP1, exported protein-1; Pfs25, surface protein-25; *P*, plasmodium; *Pf, Plasmodium falciparum*; *Py, Plasmodium yoelii*; *Pk, Plasmodium knowlesi*; *Pv, Plasmodium vivax*; *Pb, Plasmodium berghei*; APCs, antigen presenting cells; DCs, dendritic cells; GIA, growth inhibition assays; GPI, glycosylphosphatidylinositol; IFNγ, interferon gamma; TLRs, toll-like receptors; VLPs, virus-like particles; SAPN, self-assembling polypeptide nanoparticle; FMP1, *P. falciparum* $MSP1_{42}$ 3D7 malaria subunit vaccine; FMP2.1, *P. falciparum* AMA1 3D7 malaria subunit vaccine; GAS, genetically attenuated sporozoites; RAS, radiation-attenuated sporozoites; LSA-NRC, *P. falciparum* liver stage antigen 1 3D7 malaria subunit vaccine; ME, multi-epitope string; RTS,S, CSP-based malaria subunit vaccine

The basic premise of vaccination is the triggering of host immune responses leading to the induction of adaptive immunity having sufficient magnitude and duration to provide long term protection. This has been achieved by many licensed vaccines, the majority based on attenuated or inactivated organisms, although often the protective antigens and underlying molecular mechanisms have not been identified. However, this traditional approach has not led to the development of a licensed vaccine for malaria or for several other devastating infectious diseases. Recently, substantial efforts have been focused on applying rational molecular design principles toward the development of novel vaccines for these refractory pathogens. In this review, we discuss the molecular aspects of antigen design, adjuvant advancement and the development of vaccine delivery systems as they are being applied to malaria vaccines.

Introduction

Molecular vaccines, as defined in this review, are vaccines that are rationally designed at the molecular level to induce long term immunity, optimally comprising both antibody and T cell-mediated responses specifically targeting one or more protective antigens. Molecular vaccines are to be contrasted with whole organism vaccines, either attenuated or inactivated, for which underlying molecular components have not been characterized. While attenuated whole organism vaccines constitute the majority of vaccines currently licensed for human use, they can be viewed as occupying one end of a spectrum of approaches to vaccine development, namely the empirical selection and testing of an immunogen without specific knowledge regarding the important constituent antigens or the protective immune responses the antigens induce.[†]

At the other end of the spectrum lie rationally designed molecular (subunit) vaccines. Ideally, these are based on (1) understanding pathogenesis at the molecular level, (2) conceptualization of how pathogenic processes could be interrupted (e.g., blocking binding interactions mediating host cell entry, cytoadherence, nutrient uptake, etc.), (3) identification of specific target molecules based on this concept (e.g., the parasite ligand binding to the host receptor), (4) identification of an immune response able to target the key parasite molecules and interrupt the pathogenic process, and (5) development of an appropriate vaccine delivery system, immunogen(s) and formulation capable of inducing the required, antigen-specific, protective immune responses. **Figure 1** compares these two divergent approaches.

While we have few examples of licensed molecular vaccines, the approach is appealing, especially for potentially lethal pathogens such as HIV where the development of attenuated whole organism vaccines entails daunting safety concerns, and for pathogens where the development of attenuated vaccines is limited by the difficulty with in vitro production such as malaria. Parasites fall primarily into the latter category—they are difficult to culture and in addition, crude preparations of parasite antigens, such as killed parasites or parasite lysates have proven minimally protective, probably because the natural infection itself often fails to induce sterilizing immunity. This lack of naturally-induced sterile protection reflects the many adaptations by parasites enabling them to circumvent the host immune response, thereby permitting establishment of the chronic infections required to achieve transmission.[1]

Malaria represents a poster child for the application of molecular design, due to its supreme public health importance, the

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[†] Although neutralizing antibodies are often a correlate of the protection afforded by whole organism vaccines, this does not mean that they constitute the only protective response; cell-mediated immunity may contribute to protection in many cases.

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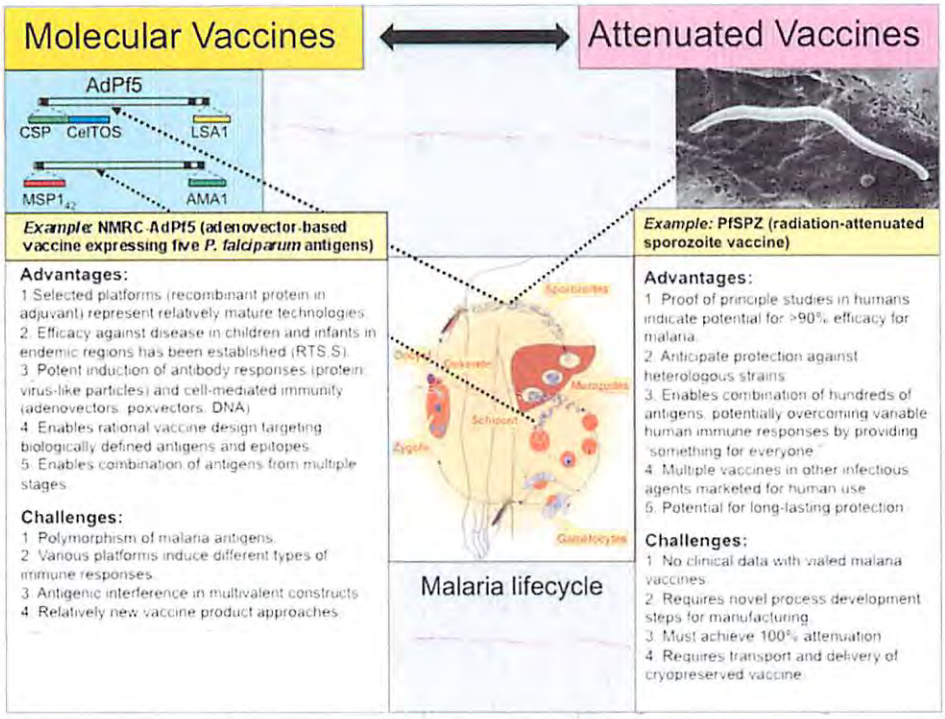


Figure 1. Spectrum of vaccine design. Reproduced in modified form and with permission from Thomson Reuters and Epstein JE, Giersing G, Mullen G, Moorthy V, Richie TL. Malaria Vaccines: Are We Getting Closer? Curr Opin Mol Ther 2007; 9:12-14. Copyright 2007, Thomson Reuters (Scientific) Ltd.

presence of multiple parasite-host molecular interfaces (allowing the conceptualization of many opportunities for molecular-based intervention), good animal models and the ability to safely challenge humans with infectious sporozoites to look for protection, which strongly facilitates the iterative testing and refinement of molecular-based vaccine concepts. Even with a molecular approach, however, malaria's complex life cycle and immune invasion adaptations pose extraordinary challenges for the vaccine developer.

Many of the vaccines discussed in this issue of *Human Vaccines* are molecular in nature, including pregnancy-specific vaccines based on interruption of binding to placental chondroitin sulfate A (reviewed by Menendez et al., 21), transmission-blocking vaccines aimed at inducing antibody responses to sexual stage surface antigens (discussed in reviews by Robert Sinden, p. 3 and Birkett p. 139), asexual stage vaccines aimed at inducing antibody responses to variable antigens expressed on the surface of infected erythrocytes (reviewed by Hviid p. 84) and recombinant protein-based vaccines composed of immunodominant proteins, sometimes with well-described functions (reviewed by Anders et al. p. 39). RTS,S (reviewed by Cohen et al., p. 90) is a particularly elegant example of molecular design applied to a recombinant protein, combining rational antigen choice (the vaccine uses a major surface protein of the sporozoite, called the circumsporozoite protein, CSP) with construction of a fusion gene incorporating the target antigen linked to hepatitis B surface antigen, which facilitates self-assembly of the yeast-produced protein into highly immunogenic particles.[‡] Importantly, RTS,S has shown protection in several Phase 2 field trials and thereby demonstrates the feasibility of developing a molecular vaccine that protects against malaria. Other vaccines reviewed in this issue, such as whole sporozoites genetically attenuated by targeted gene deletion (reviewed by Vaughan et al., p. 107), although based on empiric strategies, incorporate molecular techniques and thus could be considered molecular in design.

To avoid duplication with these accounts of molecular vaccines and molecular approaches, this article focuses on molecular technologies not discussed in detail elsewhere, with an emphasis on gene-based vaccine platforms, such as DNA and viral vectors. It is organized into six sections, the first five emphasizing major vaccine components where molecular design can be applied: (1) antigen design, (2) adjuvant design, (3) vaccine delivery systems, (4) virus-like particles and (5) safety. The review concludes with a discussion of specific challenges in molecular vaccine development for a parasite that has evolved over millions of years to persist in human populations (6).

Antigen Design

Molecular vaccines require specific antigen design considerations, relative to inactivated or attenuated whole organism based

[‡] The mechanism of protection afforded by RTS,S is not well understood; in addition, the vaccine depends upon an adjuvant system that is only partially characterized at the molecular level.

vaccines. For example, both recombinant protein- and gene-based vaccines are candidates for codon optimization or harmonization[††] to enable improved expression in the relevant production cell line or in humans, and may benefit from additional customized alterations for increasing antibody or T cell responses. Several of the most important aspects to antigen design are discussed in this section.

Molecular modification of antigen expression, localization, secondary processing and intrinsic antigenic properties to improve immunogenicity. The low GC content of the *P. falciparum* genome (~20% relative to ~40% for humans) is associated with different patterns of codon utilization compared to the genes of humans. These different codon usage patterns can negatively impact transgene expression and immunogenicity associated with DNA and viral vectors expressing *P. falciparum* genes in vivo. One of the simplest, most effective ways to increase protein expression is to synthesize a new gene encoding the same amino acid sequence, but with codons that are preferentially used by the species receiving the vaccine (e.g., human, mouse, monkey). Codon optimization has been shown to enhance protein expression of HIV1 and malaria genes encoded by DNA vectors by 4–1,000 fold and to improve the immunogenicity of these vectors in mice.[3,4] As a result of these findings, codon optimization has become a standard practice for enhancing the expression and immunogenicity of DNA and viral vaccine vectors. However, recent data indicate that codon optimization may not always provide benefit, particularly for cell-mediated immunity. In a study of DNA vaccines encoding two *P. yoelii* antigens and one *P. falciparum* antigen, antibody responses were enhanced for two of the three constructs while cell-mediated responses, as measured by interferon-gamma (IFNγ) ELISpot, lymphoproliferation and cytotoxic T lymphyocyte assays were stronger with native sequences. Protection against *P. yoelii* sporozoite challenge was also not improved by codon optimization.[5] Thus, the decision whether or not to codon optimize is best made in the context of immunogenicity studies, with knowledge of the immune response required for protection.

A second key feature for gene-based vaccines is targeting genes for intracellular vs. cell surface expression. Secretion or cell surface expression as opposed to intracellular expression of antigens can induce a more robust antibody response in the context of vaccine delivery, as extracellular or surface localization leads to antigen presentation via the exogenous antigen presentation pathway. Many *P. falciparum* proteins, including apical membrane antigen-1 (AMA1), merozoite surface protein-1 (MSP1) and CSP contain functional signal sequences and it may be critical to include these in antigens that are to be delivered by gene-based technologies when antibody responses are the priority, whereas these sequences are often deleted in subunit, protein-based vaccines. Our results indicate that adenovector-mediated expression of AMA1 and $MSP1_{42}$ (42 kilodalton fragment of MSP1) at the cell surface is associated with improved magnitude and functionality of antibody responses relative to intracellular expression.[6] This finding is in agreement with other published data for DNA and poxvirus vectored vaccines. In contrast, induction of AMA1 and $MSP1_{42}$–specific T cell responses following adenovector delivery was not affected by subcellular localization of antigen.

Another factor that could significantly influence immunogenicity is the presence or absence of glycosylation sites. *P. falciparum* parasites do not contain significant amounts of N-linked and O-linked carbohydrates.[7] However, when produced in, or delivered to eukaryotic cells with viral- or plasmid-based delivery systems, many *P. falciparum* proteins, such as AMA1 and MSP1, are expected to be glycosylated. Non-native glycosylation of these antigens could obscure or alter key epitopes derived from the tertiary structure of these blood stage antigens, and induce antibody responses that are inferior to responses that would be observed with non-glycosylated antigens. Consistent with this, it has been reported that a non-glycosylated *P. falciparum* $MSP1_{42}$ subunit protein produced in goat milk was a more effective vaccine candidate than the corresponding native sequence glycosylated version.[8] However, other studies with $MSP1_{42}$, AMA1 and erythrocyte binding antigen-175 (EBA175) subunit protein vaccines and DNA-AMA1 and DNA-$MSP1_{42}$ vectors have indicated that glycosylated proteins can be effective vaccines.[8-11] We have recently shown that adenovectors that express glycosylated versions of $MSP1_{42}$ and AMA1 induced potent functional antibody responses by an in vitro growth inhibition assay (GIA), and that modification of the N-linked glycosylation sites did not improve and in some cases significantly reduced antibody responses.[6] Mutation of glycosylation sites may have destroyed immunogenic epitopes directly or may have had negative effects on protein folding and structure.

The CSP protein contains a glycosylphosphatidylinositol (GPI) anchor, which functions to attach the protein to the plasma membrane of the sporozoite. The immunogenicity of DNA or adenovirus vectors expressing *P. berghei, P. yoelii* and *P. falciparum* CSP can be improved by deletion of the GPI anchor sequence.[12,13] We have demonstrated that an 11 amino acid C-terminal deletion, that removes the GPI anchor sequence, increases *Pf*CSP-specific antibody responses in mice and rabbits following adenovector delivery (JTB, unpublished data).

A poorly understood feature of malaria proteins is the degree to which they have been subject to changes in amino acid sequence in response to immunological selection pressures. Epitopes found within proteins that are functionally important to parasite biology (such as ligands mediating host cell invasion) may have evolved, via changes in amino acid sequence, to minimize the induction of protective immunity. One way this could have happened would have been evolution of reduced affinity for the binding grooves of common major histocompatibility complex (MHC) class I and class II alleles. Another would have been the development of configurations that stimulate regulatory T cells, thereby quelling rather than inducing immunity. Still another predicted response

[††] Optimization here refers to the construction of synthetic genes by selecting for each amino acid the most commonly used codon (i.e., the most commonly used codon by the expressing cell substrate), whereas harmonization refers to tailored selection of codons corresponding in frequency of use to the original codon sequence. Harmonization may be necessary for expression in E. coli in order to slow down translation at key points so as to permit critical folding events within the ribosomal tunnel.[2]

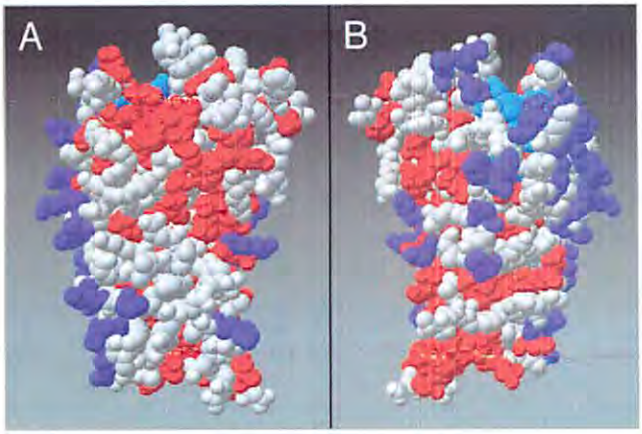


Figure 2. Crystal Structure and Sequence Variability of AMA1, front (A) and rear (B) views of PfAMA1. Residues that are conserved among all Plasmodium species for which sequence is currently available are shown in red. These, along with residues that vary between Plasmodium species (gray), form a core structure that underlies residues that are polymorphic in *P. falciparum* (purple). The polymorphic residues are located exclusively at the molecular surface and show pronounced clustering to the rear face. The putative hydrophobic groove is shown in blue at the top. Original figure by Bart Faber and Henk van Westbroek, using model 1Z40 from the Protein Data Bank (www.pdb.org) provided by Bai et al.[238] The Swiss pdb viewer (http://www.expasy.org/spdbv/) was used to generate the figure.[239]

would be the evolution of highly immunodominant epitopes in other domains of the protein, exemplified by the repeat sequences characterizing many malarial proteins. These immunogenic domains could serve to divert the immune response away from the critical functional epitopes, leaving them "immunologically silent."

Although these various predicted features have not been systematically described for most malaria antigens, there is a growing list of examples concordant with these principles. One is the existence of cryptic epitopes—normally immunologically silent epitopes that when removed from the protein microenvironment can induce protective responses.[14-17] Another is altered peptide ligands (T cell mimotopes)—parasite-derived domains that appear to induce inhibitory rather than protective immune responses.[18-20] These antagonist peptides, which may differ in one or two amino acids from the agonistic peptide, are hypothesized to decrease the threshold of T cell activation, induce T cell anergy, or alter the CD4 T cell phenotype from pro-inflammatory (Th1) to anti-inflammatory (Th2).[1] A particularly interesting example of the interplay of immune selection and epitope sequence and structure is provided by AMA1, described in **Figure 2**. In this case, highly polymorphic residues surround an invariant (and possibly immunologically silent) putative binding site.

Strategies for reversing these parasite adaptations have been explored, but much further work is needed. For example, targeted amino acid substitutions may restore the ability of key epitopes to bind with higher affinity to MHC molecules, or prevent the induction of regulatory T cells. While changing the amino acid structure of a protein might induce immune responses less specific for the native protein, it may be possible to make limited substitutions that enhance rather than reduce the affinity of antibodies or T cells for the native protein (T. Husain, personal communication). Efforts to do this in malaria and other disease models have yielded mixed results,[21, 22] but given the often poor immunogenicity of malaria epitopes, the concept merits further investigation. Additional methods to focus immune responses on protective epitopes are discussed in the next section, in the context of overcoming the challenges provided by antigenic polymorphism.

Molecular approaches to circumventing antigenic polymorphism. The malaria parasite is notable for the antigenic polymorphism exhibited by many of the proteins that have been developed as vaccine candidates. Perhaps the best example is the *P. falciparum* erythrocyte membrane protein-1 (*Pf*EMP1), an immunodominant adhesion protein mediating cytoadherence of parasitized erythrocytes to the vascular endothelium. PfEMP1 is expressed from approximately 60 different paralogous genes encoding immunologically variant versions of the antigen (*var* gene family). These variants are expressed, in a sequentially regulated manner, on the surface of protuberances that appear on the membrane of infected erythrocytes during the late trophozoite stages. This evasion strategy enables the escape of new variants from neutralization by the antibody responses specific for preceding variants, resulting in recurrent waves of parasitemia. Orthologs of the var and similar variant gene families, such as *rif, stevor* and *Pfmc-2TM* are found in many malaria species and appear to be a common adaptation resulting from immune-mediated selection.[23, 24] Strategies for designing vaccines to overcome the polymorphisms presented by variant gene families are discussed in the review by Lars Hviid.

Individual genetic loci also exhibit striking allelic variation, typified by AMA1, the blood stage antigen mentioned above. AMA1 is a promising target antigen for blood stage malaria vaccine development, as demonstrated in animal challenge experiments and in epidemiological studies of humans living in endemic areas. The main issue for vaccine developers is that hundreds of variant alleles of AMA1 have been described (**Box 1**) and antibodies specific for one allele do not efficiently cross-neutralize other strains.[25] Such polymorphism indicates both the potential importance of variant antigens as vaccine candidates (if they were not protective, they would not have been subject to immune selection) as well as the difficulty faced by vaccine developers in constructing a fully cross-protective vaccine.

The Laboratory of Malaria Immunology and Vaccinology (LMIV, formerly the Malaria Vaccine Development Branch) at the National Institutes of Allergy and Infectious Diseases and the Walter Reed Army Institute of Research (WRAIR) have each developed recombinant protein vaccines based on AMA1 and these have been tested by the Malaria Research Training Center (MRTC) in Mali for efficacy against clinical infections in children. The former vaccine did not protect,[26] likely as a result of the extensive allelic diversity present at the study site, and the results of the latter trial are not yet publically available. It will be important in such trials to undertake allelic shift (sieve) analyses, to see if incident infections in the vaccine group demonstrate a paucity

Box 1. AMA1 – A Study in Polymorphism. AMA1 is an 83kDa type 1 integral membrane protein relatively conserved among apicomplexan protozoa. In Plasmodium species, AMA1 is maximally expressed during the formation of merozoites in late schizogony, targeted to the microneme apical organelles and secreted onto the surface of the merozoite at the time of invasion of the host erythrocyte. AMA1-based vaccines provide protection against malaria challenge in animal models (reviewed by Remarque),[240] induce antibodies which inhibit the growth of *P. falciparum* in culture[241] and correlate with protection against clinical malaria in endemic areas.[242-244] AMA1 is also expressed in the sporozoite and liver stages of the parasite,[245] raising the possibility that immune responses to this antigen could simultaneously protect against multiple stages of the parasite life cycle. Although the exact role of AMA1 in malaria biology has not been elucidated, cleavage steps are integral to its release onto the surface of the merozoite[246] and to subsequent erythrocyte invasion.[247,248] Parasites lacking the AMA1 gene, which occurs as a single copy in the genome, are not viable.[249]

The major portion of the molecule, the ectodomain, possesses a remarkable crystal structure characterized by a conserved central core and variable external loops formed by three different interlocking subdomains (**Fig. 2**).[250] A hydrophobic groove on the face is surrounded by polymorphic residues,[238] which may signify epitopes serving to divert the immune response from cryptic epitopes formed by the potential binding site lying within the groove.[27]

AMA1 illustrates the barrier facing the development of molecular vaccines posed by antigenic polymorphism. A review of AMA1 identified 64 polymorphic residues based on sequences listed in GenBank, with only three of the 64 in the conserved C-terminal cytosolic region, nine in the N-terminal pro-sequence and the large majority, 52, in the ectodomain.[251] This diversity likely represents only the tip of the iceberg, as illustrated by studies conducted over three years in Mali revealing equivalent diversity at a single vaccine testing site, including 62 polymorphic amino acid positions (46 dimorphic, 13 trimorphic, 3 tetra-hexamorphic) most of which were clustered on the surface of the ectodomain with the greatest concentration around the edges of the hydrophobic groove. The 506 single strains identified in the 748 infections assessed at a study site in Mali demonstrated 214 unique parasite AMA1 haplotypes.[28]

These estimates of diversity pose an important question for the molecular vaccine enterprise: how can this remarkable polymorphism be reconciled with a subunit vaccine approach?

of the vaccine or other cross-reactive haplotypes relative to those in the control group. Demonstrating allele-specific protection will provide a foundation for building a more broadly protective vaccine by enumerating the number of alleles required.

Dutta et al. at the WRAIR have explored strategies to develop a more broadly protective vaccine using an in vitro GIA and mutant AMA1 proteins. They identified specific polymorphic residues that function as antigenic escape residues (AERs), meaning that polymorphisms at these sites escape cross-protection in the GIA.[27] The primary AERs map to a cluster of polymorphic resides on domain-1 (C1 cluster) that induce strain-specific antibodies. Narrowing down the AMA1 polymorphic sites to a small number of critical AERs is an important step in the molecular design of an AMA1 immunogen(s), because it will facilitate allelic shift analyses during Phase 2b trials.

As a complementary approach, the Malaria Research and Training Centre (MRTC) in Mali in a collaboration with the University of Maryland, examined the probability that the second of two sequential infections experienced by participants in a longitudinal clinical study is symptomatic, based on the degree to which the AMA1 molecule sequenced from the second infection differed from that of the initial infection. This analysis has identified a relationship between the degree of genetic distance between the AMA1 alleles from the first and second infection and the likelihood of clinical illness, with amino acid substitutions within the AERs appearing to be particularly important.[28] If sufficient data are collected from study sites across the world, this approach should enable down-selection of naturally occurring haplotypes maximizing the degree of cross-protection.

Other vaccine developers are also using GIA to help design a broadly protective AMA1 vaccine. The LMIV at NIH in collaboration with MRTC in Mali has applied a clustering algorithm to AMA1 sequence data from isolates around the world and identified six distinct populations based primarily on geographic area that in limited testing have induced non-cross-protective antibodies, thus constituting a minimum requirement for the number variants needed for a vaccine.[29] The Biomedical Primate Research Center (BPRC) in The Netherlands has synthesized three "diversity covering" versions of AMA1 encompassing 97% of the amino acid variability observed in nature and conducted preliminary studies in rabbits showing the induction of cross-inhibitory antibodies.[30] Similar studies are underway at the La Trobe University and at the Walter and Eliza Hall Institute of Medical Research in Australia.

A radically different strategy is to avoid full length vaccine antigens with their variant sequences, focusing instead on the identification of structurally constrained, conserved epitopes associated with key parasite functions. The conserved, hydrophobic trough of AMA1 may play such a critical role, binding to other molecules involved in merozoite invasion (Fig. 2). The fact that monoclonal antibodies that inhibit invasion in vitro appear to act by hindering this binding interaction[31,32] may indicate that protective immunity could be induced by a vaccine focusing immune responses on a single or small number of epitopes.

Several approaches can be taken to targeting individual conserved epitopes. One is to "cloak" potentially diversionary epitopes (such as those surrounding the hydrophobic trough of AMA1) by coating them with non-immunogenic molecules, allowing the immune system to focus on those conserved epitopes most likely to be protective.[33] Another is to synthesize epitope strings—linked sequences of conserved epitopes designed to bind to an array of MHC molecules, allowing broad population coverage for the vaccine, selecting only the epitopes of interest.[34] These epitope strings can be designed to include complementary sets of variant epitopes from polymorphic antigens, as well as conserved epitopes. Epitope strings, however, do not provide conformational structure, so may not be suitable for inducing antibodies to a putative binding site. A third approach for addressing antigen diversity is represented by "gene shuffling", whereby a series of variant alleles are broken into short segments and then various hybrid alleles are randomly re-assembled. Multiple shuffled products are evaluated via an immunoscreening assay to down-select those inducing the most broadly neutralizing antibodies. Gene shuffling has been applied to malaria vaccines with promising results in pre-clinical studies.[35]

These approaches illustrate the efficiency and versatility of molecular design for malaria vaccine development when applied to overcoming antigenic polymorphism. The techniques potentially (1) focus immune responses on conserved, protective epitopes by eliminating undesired antigens or domains, (2) provide a strategy for inducing, from one vaccine construct, a broadly neutralizing immune response targeting a highly polymorphic antigen, and in the case of epitope strings (3) offer a strategy for combining different antigens from the same or different stages of the malaria parasite life cycle. Antigen combination is further discussed in the next section.

Antigen combination strategies. The feasibility of a highly effective malaria vaccine is strongly supported by the robust (>90%) sterile immunity induced in humans by immunization with the radiation-attenuated sporozoite (RAS) vaccine[36,37] (see review by Stephen Hoffman). This protective immunity appears to target multiple antigens, as evidenced by the identification of genetically restricted responses to pre-erythrocytic (sporozoite and liver) stage antigens other than CSP in the RAS vaccine-immunized volunteers,[34, 38-40] and by the fact that 100% protection can be induced by the RAS vaccine in tolerized mice unable to mount any immune response to CSP.[41]

Additional support for the concept of a multi-antigen vaccine comes from the fact that malaria vaccine candidates based on single antigens have been poorly efficacious, with the exception of the RTS,S vaccine. Based on the high level of protection afforded by RAS and by the closely related approach of gene-knockout attenuation (genetically attenuated sporozoites, or GAS), a highly efficacious malaria vaccine will likely need to target multiple antigens. It is well documented that genetic restriction of host immune responses, sequence polymorphism of critical epitopes targeted by T cells and B cells, allelic variation and antigenic diversity pose enormous obstacles for the development of a malaria vaccine based on only a single antigen.[42] These challenges may be overcome by the inclusion of multiple target antigens into a vaccine.

Doolan et al.[43] showed that inclusion of two antigens in a *P. yoelii* DNA vaccine enhanced protection on multiple genetic backgrounds relative to DNA vaccines based on each of the individual plasmid components. This work suggests that in a heterogenetic human population inclusion of multiple antigens in a malaria vaccine may circumvent the genetic restriction imposed by the host genetic background and improve vaccine efficacy in the population.

Data from the *P. knowlesi* (*Pk*) non-human primate challenge model further supports the contention that candidate malaria vaccines should include multiple antigens.[44-46] Immunization of rhesus monkeys with a DNA prime/poxvirus boost regimen was partially protective only when the DNA and poxviruses expressed four antigens [*Pk*CSP, *Pk* thrombospondin-related adhesive protein/sporozoite surface protein-2 (*Pk*TRAP/SSP2), *Pk*AMA1 and *Pk*MSP1]; substantial delays were observed to >2% parasitemia in most animals and 2/5 animals self-cured their parasitemia. In contrast, regimens where both vectors expressed only one antigen (*Pk*CSP) provided only minor delays to >2% parasitemia and no self-cures.[44] The protection afforded by this four-antigen, multi-stage vaccine was confirmed in a second study where the interval between the DNA prime and the poxvector boost was extended to 44 weeks; in this case 3/5 monkeys were sterilely protected and the remaining two animals show a delay to the first day of parasitemia.[45] Based on these results it would be of interest to develop and test a similar multi-antigen, multi-stage vaccine in humans.

One of the critical factors associated with the combination of antigens is assuring that the selected multi-antigen mixture is internally compatible. Elegant studies immunizing mice with plasmid "cocktails" by Sedegah et al. demonstrated that plasmid mixtures inhibited the immunogenicity of individual plasmids, and that particular plasmids may have been responsible for much of this inhibition. Removing inhibitory plasmids from the cocktail restored the immunogenicity of the remaining plasmids, which then induced antibody and T cell responses equivalent or nearly equivalent to those induced when the antigens were administered by themselves.[47] Transfection studies further indicated that the mixtures may have reduced RNA transcript levels and that this may have been related to competition among the CMV IE promoters used to drive transgene expression.[48]

Another way to combine antigens while potentially avoiding antigenic competition is epitope strings, as discussed in the preceding section. Two such poly-epitope strings representing multiple antigens have been tested in the clinic. The first, consisting of epitopes from several different pre-erythrocytic stage *P. falciparum* proteins, was designed for co-expression with full length TRAP/SSP2. The combined antigen (multi-epitope-TRAP or ME-TRAP), has been delivered via DNA, pox and adenovectors and significantly reduces liver-stage burden in humans following *P. falciparum* sporozoite challenge, showing delays in the onset of parasitemia and sometimes sterile protection when delivered in prime-boost combination, these effects attributed primarily to the TRAP antigen but with the epitope string possibly contributing (see review by Adrian Hill). The Oxford group has also tested a similar polyprotein comprising longer portions of six pre-erythrocytic stage antigens, but without linkage to TRAP/SSP2. Although the latter vaccine was safe and moderately immunogenic in humans,[49] there was no protection against experimental sporozoite challenge (A. Hill, personal communication). Two other polyepitope vaccines are nearing clinical testing. The first is a protein-based construct called FALVAC-1A, being developed by the Center for Disease Control and Prevention.[50] The second, originally developed by the US Navy and Epimmune, Inc, is DNA-based and is now being developed by NIAID for clinical testing, currently scheduled for 2010 (A. Mo, personal communication).

Adjuvant Design

The spectrum of vaccine design discussed in the introduction, with live-attenuated vaccines at one end and rationally designed subunit vaccines at the other simultaneously defines a spectrum in the requirement for adjuvants. Replicating vaccines generally do not require adjuvants to stimulate potent immune responses, while recombinant protein and peptide-based vaccines invariably

do. DNA, viral vectors and virus-like particles possess inherent properties for activating the immune system and traditionally have not required adjuvants. However, it is now clear that their potency may be improved with novel molecular designs, including co-formulation with a variety of immunostimulatory molecules. These and other developments are discussed in this section, beginning with traditional adjuvants and progressing to novel molecular adjuvants for protein-, peptide- and gene-based vaccines, focusing on molecular design.

Adjuvants can be represented by several classes of compounds, such as microbial products, mineral salts, emulsions, microparticles, and liposomes, which can exert their function by various, still poorly characterized mechanisms of action. Some of the modes of action attributed to adjuvants in potentiating immunity, whether innate or adaptive, include antigen uptake by antigen presenting cells, upregulation of antigen presentation and localization to the draining lymph node, increased cellular distress and the antigen depot effect. After decades of intensive investigations, recent advances led to better understanding of the mechanism of action of immunomodulatory compounds as they relate to induction of innate immunity, opening the doorway for improving adjuvant design at the molecular level.

Adjuvants for Proteins and Peptides. Only a handful of adjuvant formulations are currently approved for human use. In the USA, aluminum salts (aluminum hydroxide or aluminum phosphate, generically referred to as alum) are the only example of an FDA approved adjuvant. Alum has been used extensively over several decades in vaccine formulations for diphtheria, tetanus, pertussis, hepatitis A, hepatitis B and other infectious agents. Although able to efficiently enhance humoral immune responses, alum is relatively ineffective for inducing cell-mediated immune responses.

In the European Union (EU), MF59, a squalene-based oil-in-water emulsion, was recently licensed for a flu vaccine formulation (Fluad), representing a second adjuvant approved for human use. GlaxoSmithKline Biologicals (GSK) is also testing novel adjuvants, developing numbered "Adjuvant System" (AS) technologies that are based on combinations of classical adjuvants, such as aluminum salts, oil-in-water (o/w) emulsions, liposomes and immunomodulatory molecules (reviewed in Garcon et al.).[51] The objective is to induce tailored immune responses directed against the pathogen. An example is provided by AS02, which has been tested in several malaria subunit vaccines, including RTS,S, FMP1, FMP2.1 and LSA-NRC.[52] AS02 is an o/w emulsion containing MPL (3-0-desacyl-4'-monophosphoryl lipid A), an immunostimulatory molecule derived from the detoxified cell wall of Gram-negative *Salmonella minnesota* R595 absorbed to alum, and QS21 (saponin), derived from Quil A, a fractionate from the bark of *Quillaja saponaria*, a Chilean tree. AS02 has induced high antibody titers and strong cell-mediated (primarily $CD4^+$ T cell-mediated) immune responses characterized by high levels of interferon-gamma (IFNγ) secretion. Generally, the immunostimulant MPL has been shown to induce the synthesis and release of interleukin-2 (IL-2) and IFNγ, promoting the generation of Th1 responses,[53] while QS21 (saponins) functions mainly through the induction of other cytokines.

In an effort to further strengthen cellular responses, particularly CD8+ T cell responses, GSK has developed a new formulation, composed of liposomes, MPL and QS21, called AS01. In an adjuvant comparison study in rhesus macaques, RTS,S/AS01 elicited higher RTS,S-specific antibody titers and higher numbers of antigen-specific IFNγ producing cells than RTS,S/AS02.[54] Following from these encouraging results, RTS,S/AS01 advanced into Phase 1/2b efficacy trials in pediatric populations and is now transitioning into Phase 3 pre-licensure trials. Another adjuvant formulation in the GSK repertoire, AS04, is a Toll-like receptor (TLR) 4 agonist comprised of MPL in alum. AS04 was recently approved for human use in the EU for hepatitis B virus (Fendrix) and human papilloma virus (Cervarix) vaccines. More recently, AS03, an oil-in-water emulsion, was approved as a component of a pre-pandemic H5N1 vaccine (Prepandrix).

Montanides are biodegradable emulations containing squalene and mannide-monooleate as emulsifier and have similar physical characteristics as incomplete Freunds adjuvant. Some promising results have been reported in previous malaria vaccine trials[55, 56] using Montanide ISA 720 (SEPPIC, Paris, France), possibly due to the slow-release capacity of the inert water-in-oil emulsion and immune stimulating effects of its components. Although very potent immunologically, results from more recent malaria trials using montanides (ISA 51and ISA 720) have led to the conclusion that this class of adjuvant may have limited potential for further development due to local reactogenicity, including the induction of sterile abscesses and erythema multiforme.[57, 58]

Even as these various peptide/protein adjuvants are actively developed, understanding of the molecular mechanisms underlying their immunopotentiating properties remains modest. Development is driven empirically by the safety, immunogenicity and protection identified in pre-clinical and clinical testing, with purposeful molecular design playing a less central role. Several new classes of adjuvants, however, are closely linked to defined molecular pathways. These are best exemplified by TLR agonists, discussed in the next section.

TLR agonists. TLRs are pattern recognition receptors found on different cell types that recognize specific molecular patterns on pathogens like viruses, bacteria, and fungi.[59-61] TLR agonists act as potent adjuvants by activating key antigen presenting cells (APCs), such as dendritic cells (DCs), through their cognate TLRs. These TLR-dependent pathways up-regulate cytokine secretion, increase the expression of MHC class II and co-stimulatory molecules, and promote DC migration to the T cell area of the lymph node.[62] A variety of immunostimulatory TLR agonists targeting specific TLRs are currently being investigated for use as adjuvants in vaccine formulations; for example heat shock proteins (TLR 4), flagellin (TLR 5), porins (TLR 2), LPS (TLR 4), triacylated and diacylated lipopeptides (TLR 1/TLR2 and TLR 2/TLR 6, respectively) and oligodeoxynucleotides (ODNs) such as unmethylated deoxycytosine deoxyguanosine (CpGs) (TLR 9) (reviewed in 63).

From the molecular design perspective, TLR agonist delivery can be achieved through direct conjugation to an antigen or through incorporation into targeting vehicles, such as viral particles, liposomes, synthetic microparticles, particulates or

bacterial vector platforms. For example, the 19kDa C-terminal fragment of *P. vivax* MSP1 ($PvMSP1_{19}$) was co-administered with the *Salmonella enterica serovar* typhimurium flagellin (FliC), a TLR 5 agonist, to C57BL/6 mice by either parenteral (s.c.) or mucosal (i.n.) routes. FliC was either mixed or genetically linked to the $PvMSP1_{19}$ protein. This approach elicited strong, long-lasting $MSP1_{19}$-specific antibody responses that were biased toward IgG1 subclass. Inclusion of CpG, a TLR 9 agonist, yielded a shift toward a balanced IgG1/IgG2c ratio and higher cellular responses as measured by IFNγ secretion.[64] In a recent study, a synthetic, FDA approved topical treatment for skin conditions, imiquimod, a TLR 7 agonist, was shown to function as a potent adjuvant for eliciting T cell responses. Topical application of imiquimod at the site of subcutaneous injection of *P. falciparum* CSP peptides elicited parasite-specific humoral immunity that protected C57BL mice against mosquito-bite challenge using transgenic rodent parasites expressing *P. falciparum* CSP repeats.[65]

ODN CpG motifs are recognized by TLR 9 expressed on phagoendosomes of specialized cell subsets, such as B cells and plasmacytoid dendritic cells. CpG ODNs have been primarily used to induce polarized Th1 type immune responses.[66,67] Several studies have shown that antigens and CpG ODN must be co-localized to the same APC to generate the most potent and effective therapeutic antigen-specific immune responses. Delivery vehicles can be used to ensure the co-delivery of antigens and CpG ODN to the same APCs, thus leading to significant increased uptake by APCs. These strategies can result in antigen-specific immune responses that are on the order of 5- to 500-fold greater than for antigen alone. For example, in mice, the co-administration of CpG and recombinant surface protein-25 (Pfs25) led to a 30-fold improvement in antibody titers and avidities compared to rPfs25 plus alum alone.[68] Results from clinical studies using recombinant *P. falciparum* AMA1-C1/Alhydrogel plus CpG 7909 vaccine showed 14-fold-higher AMA1-specific IgG with significantly increased in vitro growth inhibitory activity against homologous parasites compared to AMA1/Alhydrogel alone.[69] Thus, TLR 9 agonists are effective immunomodulatory molecules for use as vaccine adjuvants.

Adjuvant properties of DNA and viral capsids. In contrast to protein-based subunit vaccines, viral, bacterial, and DNA vectors generally do not require formulation in adjuvant for induction of T cell and antibody responses. However, the intrinsic "danger signals" provided by these delivery systems can be improved further by molecular engineering. For example, the addition of unmethylated CpG motifs into the sequence of a DNA vector expressing a tumor associated antigen has been shown to enhance the immunogenicity and efficacy of the vector in a murine melanoma model.[70] Conversely, enzymatic methylation of CpG motifs in a DNA-*P. yoelii* CSP vector decreased the immunogenicity of the vector in mice.[71] CpG activation via a TLR 9-dependent mechanism does not appear to be absolutely essential, however. Multiple signaling pathways may be involved, including two recently discovered pathways utilizing the DNA sensors DNA-dependent activator of interferon regulatory factors (DAI) and absent in melanoma-2 (AIM2).[72] As more is learned about the role that these different pathways play in activating the innate immune system, it may be possible to use this information to develop better DNA vaccine vectors.

Adenovirus-based vectors activate the innate immune system through TLRs and cytoplasmic DNA-sensing, inflammasome-dependent mechanisms.[73-77] This results in the induction of cytokines and chemokines, which potentiate antigen-specific T cell and antibody responses. Moreover, purified adenovector serotype 2 (Ad2) hexon protein has been shown to be a potent adjuvant for induction of antigen specific responses.[78] Other viral vectors, including poxvirus[79,80] and semliki forest virus,[81] can enhance antigen-specific antibody and T cell responses to co-delivered proteins, indicating that these viral vectors also provide adjuvant activity. These intrinsic adjuvant properties of DNA and viral vectors facilitate the induction of robust immune responses to vector-delivered antigens, bypassing the need for adjuvant formulation.

Molecular adjuvants for plasmid and viral vectors. Although plasmid and viral delivery systems do not require adjuvants, co-formulation with an adjuvant can improve the antigen-specific immune responses induced by these vectors. For example, formulation of DNA vaccines with CpG oligonucleotides improved the immunogenicity of DNA plasmids encoding malaria antigens in mice and Aotus monkeys.[82,83] Co-administration of DNA vaccines with plasmids expressing immunostimulatory molecules has likewise improved the immunogenicity of these vaccines in animal models. However, these initial successes have not necessarily translated into humans. For example, when tested in mice, co-delivery of plasmids expressing PyCSP and granulocyte-macrophage colony-stimulating factor (GM-CSF) resulted in more robust antibody and T cell responses, as well as better protection from *P. yoelii* sporozoite challenge relative to *Py*CSP expressing plasmid alone.[84,85] But, when this combination was evaluated in humans, co-delivery of human GM-CSF and *P. falciparum* antigen expressing plasmids did not improve T cell responses relative to the *P. falciparum* antigen expressing plasmids alone, and none of the regimens were protective.[86]

Intervention in costimulatory and inhibitory pathways can modulate immune responses and represent interesting strategies for increasing vaccine responses. The herpes simplex virus glycoprotein D (gD) can enhance T cell and antibody responses to fused antigens in the context of DNA and adenovector delivery.[87] gD functions by blocking inhibitory signals between the herpesvirus entry mediator (HVEM) and B- and T-lymphocyte attenuator (BTLA). This pathway has also been exploited to increase tumor-specific T cell responses. Interactions between HVEM and LIGHT (a lymphotoxin-like inducible protein that competes with glycoprotein D for herpesvirus entry on T cells) provide costimulatory signals for T cell activation. Adenovector-mediated expression of LIGHT within a tumor can induce tumor-specific T cell responses and eradicate spontaneous metastases.[88] In a similar way, increased understanding of the signaling pathways that activate the innate immune system has potential for enhancing the immunogenicity of DNA and viral vector based vaccines. Recently, coexpression of plasmids encoding the TLR-signaling molecules, TRAM and

TAK1, were shown to significantly enhance antigen-specific T cell responses in mice following DNA vaccination.[89]

Adjuvants can act through a variety of mechanisms. For example, since DNA plasmids injected into the skin or muscle are taken up by cells inefficiently, adjuvants that enhance the uptake of the plasmid into the cell are being evaluated. Particles that have a diameter of approximately 1 μm can be internalized by APCs, such as macrophages and DCs. Therefore, one way to potentially enhance DNA vaccines is to formulate the vector onto a particle. DNA vaccines formulated onto cationic microparticles composed of poly lactide-co-glycolide (PLG) or nonionic poloxamer (CRL1005) particles combined with a cationic surfactant can be internalized by APCs and have been shown to be more potent than unformulated DNA vaccines in pre-clinical animal models of HIV and CMV.[90,91] A DNA-CMV vaccine formulated in the CRL1005 adjuvant has also been shown to be safe and moderately immunogenic in a Phase 1 clinical trial.[92] Formulation of DNA vaccines onto PLG and poloxamer particles, however, does not always enhance potency. For example, formulation on PLG microspheres was tested in the rhesus monkey/*P. knowlesi* sporozoite challenge model, using a multistage malaria vaccine administered as three priming doses of DNA, with or without PLG formulation, followed by a poxvector boost. In this study, PLG formulation of the DNA proved less protective than PBS formulation.[84] A similar multivalent DNA vaccine encoding *P. falciparum* antigens was assessed for immunogenicity in rhesus monkeys with and without the poloxamer CRL1005 and the poloxamer did not significantly improve antibody responses or cell-mediated immunity, leading to a "no-go" decision for clinical evaluation.[93]

Other adjuvants for DNA vaccines are also being evaluated. Vaxfectin® is a cationic lipid that binds to negatively charged DNA. A Vaxfectin®-formulated DNA-*Py*CSP vector has been shown to be more immunogenic and efficacious in the *P. yoelii*/ mouse model than the unformulated vector.[94] Similar positive results were obtained in a non-human primate model evaluating the immunogenicity of DNA plasmids expressing malaria antigens that were formulated in Vaxfectin®.[95]

Adenovectors have been adjuvanted by formulation with standard adjuvants and also by modifying capsid components. Formulation of an adenovirus serotype 35 (Ad35)-based vector expressing *Py*CSP with aluminum phosphate adjuvant [AlPO(4)] was shown to increase CSP-specific T cell and antibody responses in mice.[96] Studies in mice and non-human primates indicate that the immunogenicity of Ad35 vectors can be improved by substitution of the Ad35 fiber with the Ad5 fiber.[97,98] The Ad5 fiber shaft has been implicated in DC-targeting and induction of adaptive immunity by Ad5 vectors through binding a heparin-like receptor.[99] These studies suggest that the adjuvant quality of adenovirus capsids may be affected by fiber receptor interactions.

Conjugation of molecular adjuvants to Plasmodium antigens can also enhance immune responses and has the potential to improve malaria vaccines. Fusion of the $MSP1_{19}$ to the complement inhibitor C4-binding protein (C4bp) enhanced antibody responses and protection of mice following challenge with *P. yoelii*-infected erythrocytes.[100] This fusion protein also induced stronger T cell responses, higher titer antibody responses and more robust protection in mice when delivered by an adenovirus vector.[101] The mechanism for the adjuvant activity of C4bp is not known, but may involve antigen oligomerization or targeting of antigen to antigen presenting cells. Conjugation of malaria antigens, Pfs25 and AMA1, to nontoxic *Pseudomonas aeruginosa* ExoProtein S (rEPA) was shown to increase antibody responses by up to 1000-fold.[102] Codelivery of the toll-like receptor (TLR) ligands[103,104] and fusion of antigens to the C3d component of complement[105,106] have shown promise as molecular adjuvants for infectious disease and cancer and may have applications for malaria vaccines.

Antigen Delivery

A highly effective malaria vaccine will likely induce robust protective antibody and T cell responses to multiple malaria antigens expressed at multiple stages in the parasite's life cycle. As discussed in the antigen design section, sequence polymorphism of critical epitopes targeted by T cells and B cells poses enormous obstacles for the development of a malaria vaccine based on only a single antigen.[42] These challenges may be overcome by the inclusion of multiple target antigens into a vaccine, making antigen combination a high priority for vaccine developers. Leading candidate platforms that are capable of inducing responses to multiple antigens are DNA viral and bacterial vectors. Molecular design is an important component for each of these delivery platforms.

Plasmids. In the early 1990s, it was discovered that immunization with a plasmid DNA vector expressing a flu antigen could protect mice against an influenza challenge.[107] Since then, DNA vaccines have been used successfully in many pre-clinical animal studies, including the demonstration that an unadjuvanted DNA plasmid encoding *P. yoelii* CSP could sterilely protect mice against a sporozoite challenge through a $CD8^+$ T cell-dependent mechanism.[108] The early success of DNA vaccines in mice, however, has not translated into humans. Clinical evaluation of first-generation DNA vaccines against HIV and hepatitis B virus indicated that DNA vaccines were safe, but not very immunogenic.[109,110] Similar findings were observed with DNA vaccines against malaria. For example, two Phase 1 studies with a DNA vector expressing a non-codon optimized *Pf*CSP gene[111,112] and a Phase 1/2a study with 5 vectors expressing *Pf*CSP, *Pf*TRAP/SSP2, *P. falciparum* exported protein-1 (*Pf*EXP1), *P. falciparum* liver stage antigen-1 (*Pf*LSA1) or *P. falciparum* liver stage antigen-3 (*Pf*LSA3), with or without a DNA vector expressing human GM-CSF,[86] indicated that DNA vectors could induce malaria-specific CTL or ELISpot responses in about half of the human volunteers, but could not induce detectable malaria-specific antibodies.[86,113,114] Furthermore, the Phase 1/2a trial indicated that three doses of five DNA vaccine vectors expressing different pre-erythrocytic malaria proteins could not protect volunteers against a *P. falciparum* sporozoite challenge. The conclusion from all of these trials was that DNA vaccines were safe, but not very immunogenic or efficacious in humans, even when used in combination.

DNA vaccines have been more successful for veterinary targets. Within the last few years, four plasmid DNA products

have been licensed in the USA, Canada or Australia. A DNA vaccine against West Nile Virus has been developed by Fort Dodge Laboratories for horses. A DNA vaccine against infectious hematopoietic necrosis virus has been developed by Aqua Health, a division of Novartis Animal Health, for salmon.[115] A therapeutic DNA vaccine against oral melanoma has been developed by Merial Animal Health for dogs.[116] A DNA vector expressing a growth hormone to promote growth and reduce mortality has been developed by VGX Pharmaceuticals for pigs.[117] Although the licensing of these veterinary products has bolstered the belief that DNA vaccines may be possible for humans, the clinical experience indicates that the potency of these vaccines will need to be dramatically improved before they can be viable in humans.

DNA vaccines can be delivered by several different systems, including a needle and syringe, a needle-free jet injection device (e.g., Biojector®), a particle mediated needle-free device (e.g., gene gun) or an electroporator. The first DNA-malaria vaccine to be evaluated in humans was injected IM with a needle and syringe.[111] In subsequent trials, DNA-malaria vaccines were also administered IM or IM/intradermally (ID) with a Biojector®, which can deliver intramuscular, subcutaneous or intradermal injections. A direct comparison of these delivery systems indicated that IM administration with a Biojector® elicited higher cellular immune responses than a needle and syringe or IM/ID administration with a Biojector®.[118] DNA vaccines have also been administered by a gene gun, which uses a needle-free device to deliver vaccine coated onto gold beads into the skin. This immunization system, which can deliver the vaccine directly into cells, such as APCs, has been shown to enhance the immunogenicity and efficacy of DNA vaccines.[119] A trivalent DNA-flu HA vaccine formulated onto gold beads and administered with a gene gun has also been shown to partially protect volunteers against an influenza challenge in a recent Phase 1b clinical trial.[120]

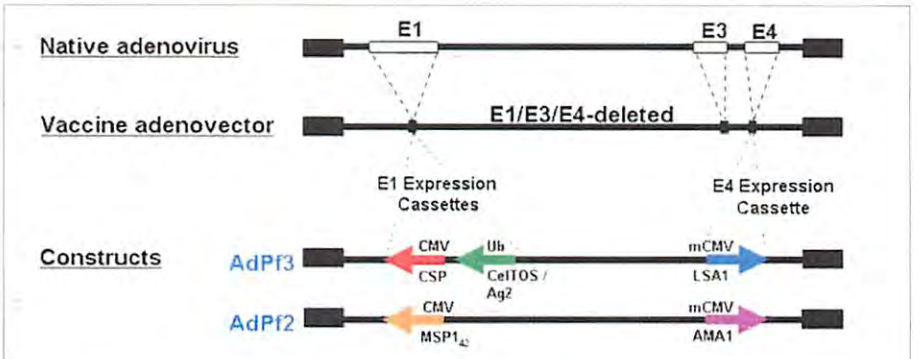
Another promising way to enhance the potency of DNA vaccines is to deliver these vectors with an electroporator. Electroporation involves the administration of brief electrical pulses immediately after intramuscular injection of the vaccine. Electroporation can facilitate cellular uptake and has been shown to enhance the immunogenicity and efficacy of DNA vaccines in pre-clinical animal models.[5] In fact, the DNA-growth hormone vector used to promote growth and reduce mortality in pigs (see above) is delivered by electroporation. Electroporation has also been used to enhance the immunogenicity of a DNA vector expressing a prostate tumor-tetanus toxin fusion protein in a recent Phase 1 trial.[121] Although the pain associated with electroporation was reported to be brief and acceptable, the universal tolerability of electroporation systems for humans has not been determined. Although the initial promise of DNA vaccines has not yet been fulfilled in humans, recent advances to enhance the potency of these vectors by modifying the plasmid backbone, the transgene, the vaccine formulation or the delivery system, and their potential use in prime-boost regimens may eventually make DNA vectors a viable vaccine platform.

Viral vectors. Since malaria vaccines based on viral vectors express high levels of antigen directly in infected cells of the host, antigen is processed and presented to professional antigen presenting cells in a manner that is similar to natural infection. This provides a key advantage over protein subunit-based vaccines and is one of the reasons that viral vectors induce robust T cells responses. Also, appropriate folding of the delivered antigen is facilitated by synthesis within the vaccinated individuals' cells, resulting in the induction of functional antibody responses. In addition, as mentioned above, viral vector delivery systems provide adjuvant activities along with antigen delivery. The leading viral vectors for malaria vaccine development are based on poxvirus and adenovirus, because of their ability to induce potent antigen-specific immune responses in people, as well as their capacity to deliver multiple antigens. Other viral vectored vaccines based on alphavirus[122] and yellow fever virus[123, 124] have induced protective responses in animal models and are also being explored.

Poxvectors. Vectors based on attenuated vaccinia virus NYVAC and modified vaccinia Ankara (MVA), as well as fowlpox have been developed for malaria vaccines. In murine models, vaccination with a NYVAC vector expressing *P. berghei* CSP induced $CD8^+$ T cell-mediated protection in mice.[125] These results prompted the generation of NYVAC-Pf7, a genetically engineered deletion mutant of the Copenhagen strain of vaccinia virus that expresses seven *P. falciparum* antigens derived from multiple stages in the parasite life cycle [CSP, SSP2, LSA1, MSP1, AMA1, serine repeat antigen (SERA), and Pfs25].[126] In the first clinical trial of a poxvirus-based malaria vaccine, NYVAC-Pf7 was well tolerated at doses of 1×10^7 and 1×10^8 plaque forming units and partially protective. One volunteer was completely protected and there was a significant delay in the time to parasite patency in volunteers that received either the low or high dose of vaccine compared with unvaccinated controls.[127]

Prime-boost strategies have significant advantages for malaria vaccine development.[128] Poxvirus vectors, FP9 and MVA, have been used together and with DNA and adenovirus vectors in prime-boost vaccination regimens. These studies demonstrated that the prime-boost approach induced more robust immune responses and superior protective responses in rodent models than either regimen alone. In clinical studies, DNA prime-MVA boost studies employing ME-TRAP antigen[129] induced higher levels of T cells and delays to parasitemia following challenge compared to either modality alone.[130, 131] Prime-boost vaccination with FP9-MVA (also using ME-TRAP) was safe, immunogenic and partially protective in malaria-naïve volunteers receiving experimental challenge, providing sterile protection in a few cases.[132, 133] The immunogenicity of the FP9-MVA (ME-TRAP) prime-boost vaccine was reduced in adults[134] and children[135] in malaria endemic regions in Kenya and provided no evidence of efficacy. The reasons for this discrepancy are not known. (For more details on this approach see the review in this edition by Hill et al., p. 78).

Adenovirus vectors. Adenovirus vectors can be engineered to induce broad and protective T cell and antibody responses in animal models, ranging from mouse to non-human primates.[136-146] Protection from malaria has been observed using adenovectors that express the *P. yoelii*[147, 142, 148] or *P. berghei*[149] CSP antigen in murine models. An Ad5-based HIV vaccine induced antigen-specific $CD4^+$ and $CD8^+$ T cell and antibody responses in a majority



Feature	Optimization	Metric
Codon usage	*Plasmodium* vs mammalian patterns	In vitro expression (mammalian)
Glycosylation status ($MSP1_{42}$ and AMA1)	Native amino acids (glycosylated) vs mutated amino acids (nonglycosylated)	In vitro expression, immunogenicity, Intracellular vs surface localization
Signal sequence ($MSP1_{42}$ and AMA1)	With vs without signal sequence	Intracellular vs surface localization In vitro expression, immunogenicity
GPI anchor (CSP only)	GPI present vs deleted	In vitro expression, immunogenicity
Promoter type	11 promoters compared	In vitro expression, immunogenicity
Casette orientation	5' to 3' vs 3' to 5'	In vitro expression, immunogenicity
Transgene placement	E1 vs E3 vs E4	In vitro expression, immunogenicity
Casette orientation & Transgene placement	Comparison of six trivalent pre-erythrocytic and two bivalent blood stage prototypes	Production yields, genetic stability, In vitro expression, immunogenicity

Figure 3. AdPf5. AdPf5, a pentavalent multistage malaria vaccine candidate comprising a trivalent pre-erythrocytic stage adenovector and a bivalent blood stage adenovector, exemplifies many of the molecular design features discussed in this review. The trivalent vector (AdPf3) expresses three *P. falciparum* (3D7 strain) pre-erythrocytic antigens: *Pf*CSP, *Pf*LSA1 and *Pf*Ag2 (CelTOS). The bivalent vector (AdPf2) expresses two *P. falciparum* (3D7 strain) blood stage antigens: *Pf*$MSP1_{42}$ and *Pf*AMA1. Both vectors are based on an E1/E3/E4-deleted Ad5 vector platform; because both E1 and E4 are required for replication, the vector is multiply replication incompetent. While E3 is not required for replication, its partial deletion increases the carrying capacity of this vector. All five genes, as well as their placement into the adenovector backbone, have been evaluated in multiple configurations in order to optimized several important characteristics. These include: codon optimization (all antigens), inclusion of a heterologous secretion signal ($MSP1_{42}$) and deletion of GPI anchor (CSP). The two blood stage antigens were also evaluated to determine the effect of N-linked glycosylation on induction of functional antibody responses. Mutations in N-linked glysolation sites did not induce better antibody responses in rabbits and were not included in this malaria vaccine candidate. Different promoters are used for each transgene to prevent homologous recombination events. Promoters were selected and positioned in the adenovirus genome for optimal antigen expression in transduced cells and induction of robust antigen-specific T cell responses in mice and growth inhibitory antibody responses in rabbits. These assays showed that both vectors induced robust T cell and antibody responses to each of the five antigens in mice and the bivalent blood-stage vector induced growth inhibitory antibody responses to each antigen in rabbits. Both vectors grow to high titers in 293-ORF6 cells under serum free suspension conditions and are genetically stable. The AdPf5 vaccine comprises two adenovector constructs, AdPf3 (pre-erythrocytic stage vector) and AdPf2 (blood stage vector), which are mixed together prior to intramuscular administration. Key: CMV, human cytomegalovirus promoter; mCMV, murine cytomegalovirus promoter; Ub, ubiquitin promoter. Arrows indicate the direction of transcription.

of volunteers.[150] Similarly, we observed robust $CD4^+$ and $CD8^+$ T cell responses in the first clinical trial of an adenovirus serotype 5 vector (Ad5)-based malaria vaccine candidate expressing CSP and AMA1 antigens. As both $CD4^+$ and $CD8^+$ T cell responses have been implicated in the sterile protection observed following immunization of volunteers with radiation-attenuated sporozoites, these findings suggest that adenovectors might be able to induce the requisite immune responses for protection against malaria.

Multi-gene expressing adenovectors for malaria vaccine development. Adenovirus vectors can be generated that express multiple proteins from different expression cassettes located within the adenovirus genome.[151-154] Vaccines based on these vectors have advantages from both potency and cost-of goods perspectives. Moreover, this multi-gene expression technology is highly flexible, facilitating rapid substitution of different antigens into the multi-gene expressing adenovector after it is developed. A key requirement is that vectors must have sufficient capacity to express multiple antigens. We have generated a multiply deleted Ad5 vector with room for incorporation of up to 9Kb of foreign sequences, by deletion of three Ad5 early regions, including complete deletions of both early regions 1 and 4 (E1 and E4) and a partial deletion of region 3 (E3). E1/E3/E4-deleted vectors can be produced in 293-ORF6 cells, which provide complementation for both E1 and E4 functions[155] and are suitable for cGMP manufacturing of adenovector preparations free from replication competent adenovirus (RCA).[150] These multiply-defective (E1/E3/E4-deleted) vectors express much lower levels of adenovirus genes following infection relative to an E1/E3-deleted vector. Consequently, immunization of volunteers with E1/E3/

E4-deleted vectors did not induce an expansion of adenovirus-specific CD4+ T cells,[156] a characteristic that could have important safety advantages. Recently, we have generated Ad5 vectors that express high levels of three different pre-erythrocytic stage antigens [CSP, LSA1 and cell traversal protein of ookinetes and sporozoites (CelTOS)] from three different expression cassettes located in the E1 and E4 regions of the vector genome (see Fig. 3). In preclinical studies in mice, these vectors induced T cell and antibody responses specific for each of these antigens and the magnitude of these responses was similar to that of control Ad5 vectors that expressed only one antigen.[6] We are currently applying this multi-gene expression technology to alternative serotypes of adenovirus where the prevalence of neutralizing antibodies (NAb) in human populations is very low.

Adenovector neutralizing antibodies (NAb): prevalence and impact on vaccine safety and efficacy. Adenovirus vectors derived from Ad5 have been a leading platform for the generation of molecular vaccines. However, forty to sixty percent of the United States population possesses NAb to Ad5[99, 157] and the frequency is even higher in Africa, where malaria is endemic.[158-160] In animal models including mice[161] and non-human primates,[162] prior exposure to Ad5 diminished T cell responses to the vaccine antigens delivered using Ad5 based vaccines. As expected, due to the subtype specificity of adenovirus Nab,[163] pre-existing Ad5 NAb were not effective in reducing vaccine responses to other adenovirus vaccines based on alternative human[157, 164] or simian serotypes.[165-168]

Reports addressing the impact of pre-existing Ad5 neutralizing antibody on adenovector vaccine immunogenicity in the clinic (with HIV vaccines) indicated that volunteers possessing high titers of Ad5 neutralizing antibody were capable of mounting significant antigen-specific humoral and cellular responses.[150, 169, 170] However, the magnitude of the T cell responses in individuals with pre-existing Ad5 NAb was approximately three-fold lower than those of the Ad5-seronegative volunteers.

The most conservative path forward for adenovirus-based vectors is to advance vaccines based on alternative serotypes of adenovirus where the prevalence of NAb is low. However, finding a highly immunogenic substitute for Ad5 has not been straightforward. To date other serotypes of adenovirus (Ad35, Ad11, Ad26, Ad48, Ad49, Ad50 and chimpanzee adenovectors) have generally under-performed Ad5 vectors in terms of induced immunogenicity to their payload antigen.[97, 147, 161, 164, 171, 172]

Capsid-modified adenovectors. An alternative strategy for avoiding preexisting Ad5 NAb is to modify the NAb binding sites on the Ad5 capsid. The majority of serotype-specific neutralizing antibodies recognize determinants on the hexon and fiber capsid proteins,[160, 173, 174] and hexon-specific neutralizing antibodies appear to be the most prevalent and potent in vivo.[164] Epitopes targeted by these hexon-specific neutralizing antibodies have been mapped to the hypervariable regions (HVR) of the hexon protein[174, 175] contained within exposed loops at the surface of the capsid. Roberts et al., 2006 demonstrated that it is possible to replace each of the 9 HVR of the Ad5 hexon with those derived from Ad48, a rare adenovirus serotype.[176] Induction of transgene-specific immune responses was unaffected by Ad5-specific NAb in murine or non-human primate vaccine models.

GenVec, Inc. has developed a hexon-modified adenovector vaccine platform for malaria vaccine development that avoids preexisting anti-adenovirus immunity prevalent in human populations by replacing the Ad5 HVR sequences with those derived from Ad43. This Ad5 hexon-modified vector is not neutralized by hexon-specific Ad5 neutralizing antibodies in vitro using sera from mice, rabbits and human volunteers. Furthermore, the hexon-modified adenovector that expresses PyCSP is as immunogenic in mice as an unmodified Ad5 vector and can induce robust T cell responses in mice with high levels of preexisting anti-Ad5 Nab. In addition, the hexon-modified vector effectively boosted Ad5 vector primed T cell and antibody responses.[177]

Adenovirus capsids can also be modified to increase the immunogenicity of the vector. We have demonstrated that the introduction of a DC targeting ligand into the fiber improved DC transduction and antigen-specific T cell responses.[98] Also, B cell epitopes can be inserted into the hexon, fiber and penton base and displayed on the capsid to induce protective immune responses in mice.[146, 178-180]

Bacterial Vectors. Currently, there are three commercially available live bacterial vaccine vectors: *Salmonella enteric* serovar Typhi Ty21a, *Vibrio cholera* CVD 103-HgR, and *Mycobacterium bovis* BCG. Live attenuated bacteria are an attractive vectored platform for expression, delivery and eliciting host immunity to foreign antigens. The bacterial vector itself mimics natural infection in the host, exhibiting tissue tropisms that enable the targeting of vaccine antigens to various cellular compartments, thereby inducing mucosal, humoral and cellular immune responses. This approach has been widely used for expression and delivery of heterologous targets derived from various bacteria, fungi, viruses and parasites in clinical and veterinary applications, particularly for gastrointestinal, genital and respiratory pathogens. The specific advantages of this approach include (1) the relative low cost and potential for large scale manufacture of target antigen by the "bacterial factory," (2) the presence of natural immunostimulatory molecules, such as LPS and flagellin, thus obviating the requirement for inclusion of classical adjuvants (see section on TLR agonists), (3) sensitivity to antibiotics for eradication if indicated and (4) tropism for dendritic cells and macrophages. Concomitantly, safety considerations for live attenuated bacterial vaccines focus on the possibility of undesirable systemic dissemination, toxic bacterial byproducts (LPS), transmission through contact to either healthy or immune compromised recipients, and/or the potential for environmental contamination.

Vaccination using attenuated or killed bacterial vectors that serve as antigen vehicles can provide a dual-pronged immunization strategy -- to elicit protective responses against the target antigen and the vehicle itself. These attenuated bacterial vaccine vectors can stimulate potent immune responses by inducing the production of cytokine cascades, including tumor necrosis factor alpha (TNFα), IFNγ, IL-12 and other proinflammatory mediators such as NO.[181] The type of immunity elicited by these bacterial vectors is strongly dependent on the bacterium itself, the route of delivery, as well as other biological indicators

associated with the target antigen. For example, the intracellular pathogen *Listeria monocytogenes* induces both mucosal and systemic immunity. In murine models, using either *E. coli* β-gal expression or oral administration of *L. monocytogenes* expressing HIV-1 gag, both mucosal and systemic $CD8^+$ cellular immune responses were detected against the target antigens.[182, 183]

Nearly two decades ago, an attenuated *Salmonella typhi* was used to elicit a human serologic or cytotoxic T lymphocyte response to a foreign target antigen, *P. falciparum* CSP.[184] The attenuated *S. typhi* vaccine strain CVD 908, harboring deletion mutations in *aroC* and *aroD*, was shown to be well-tolerated and highly immunogenic, eliciting impressive serum antibodies, mucosal IgA and cell-mediated immune responses. A decade later, when *P. falciparum* $MSP1_{19}$ was genetically fused to fragment C of tetanus toxin and then expressed within attenuated *S. typhi* CVD 908, under nonreductive intracellular bacterial conditions, chimeric C-$MSP1_{19}$ did not fold to form the properly disulfide bridged epidermal growth factor (EGF)-like domains of MSP1. Disulfide dependent monoclonal antibodies failed to recognize these conformational domains in immunoblots under non-denatured conditions on bacterial lysates. These results demonstrate that proper targeting of the antigen to the appropriate cellular compartment is required.[185] In a recent report, *S. enterica* serovar Typhi strains were used for expressing and exporting truncated *P. falciparum* CSP B-cell and T-cell immunodominant epitopes fused to *S. enterica* serovar Typhi cytolysin A (ClyA), which were then used in a heterologous prime-boost approach with a DNA-vectored vaccine. Interestingly, mice that were primed with serovar Typhi expressing CSP epitopes and boosted with DNA plasmid induced high antibody titers to NANP, predominantly IgG2a, and high frequencies of IFNγ producing T cells, while mice receiving only DNA vectored vaccine did not induce any antibodies.[186] In a recent published report, investigators showed that the genetic detoxification of the *S. enterica* serovar Typhimurium to improve its safety profile led to a serendipitous enhancement in antigen-specific immune responses.[187] Additional modifications may lead to safer and more effective *S. typhi* vaccine platform.

Other bacterial vectors have been used for targeting malarial epitopes, including a recombinant *Mycobacterium bovis* bacille Calmette-Guèrin (rBCG) vector expressing epitopes from *P. falciparum* EBA 175 and CSP (F2R(II)EBA and (NANP)3 respectively) as well as two T cell epitopes of the *M. tuberculosis* ESAT-6 antigen.[188] Immunized BALB/c mice showed an enhanced antibody response to all IgG subclasses, as well as an enhanced splenocyte proliferative response against both malarial epitopes. Combining immunization platforms, for instance, a prime-boost vaccination strategy that encompasses the benefits of BCG with that of a potent boosting agent like MVA, could provide a strategy not only to broaden anti-mycobacterial immunity, thus reducing the burden of TB disease, but also to target malaria, HIV or other concurrent pathogenic infections.[189] Specifically for TB, when administered in healthy adults, this vaccination strategy induced good $CD4^+$ T cell responses with the capacity to produce effector cytokines and to proliferate following antigen stimulation.

Previously, *ompA* genes of *Escherichia coli* and *Shigella dysenteriae* have been used to construct enterobacterial surface expression vectors for foreign genes. These fusion constructs have been widely used as a vector system in experimental models and have been used to transfer target antigens, such as β-galactosidase, foot-and-mouth disease virus (FMDV) and malaria antigens, to the bacterial surface as insertions into OmpA. Several strains of attenuated *Shigella*, such as *S. flexneri* 2a, have been developed and used in various human clinical trials with good tolerability.[190] Alternatively, food-grade gram-positive bacteria such as *Lactococcus lacti* have advantages over other attenuated bacterial vaccine delivery vehicles primarily because of their inherently greater safety profiles. *P. falciparum* merozoite surface protein-2 (MSP2) expressed in recombinant *L. lactis*, either intracellularly or covalently anchored to peptidoglycan on the cell wall, elicited serum IgG antibodies that reacted with native MSP2 on the surface of *P. falciparum* merozoites, as detected by immunofluorescence.[191]

A eukaryotic vector has also been used to deliver malaria antigens. In one published study, recombinant attenuated *Toxoplasma gondii* expressing the *P. yoelii* CSP proved highly effective in priming for $CD8^+$ T cell-dependent protective immunity in mice.[192]

Virus-Like Particles

Virus-like particles (VLPs) are non-infective, self-assembling viral envelope proteins that are devoid of genetic material. They have features relevant to both antigen design and to antigen delivery, on the one hand providing a macromolecular scaffolding for the array of antigen, and on the other triggering host immunity by taking advantage of natural immunostimulatory properties of the virus particles that they mimic.

VLPs are highly repetitive and ordered structures that retain their morphology similar to infective particles and have been shown to stimulate both potent humoral and cellular immune responses, although they are most effective at stimulating humoral immunity.[193] Two examples of successful application of this approach are the hepatitis B virus (HBV) vaccine, a formulation prepared from VLPs assembled in yeast expressing the hepatitis B surface antigen (HBsAg), and the human papillomavirus (HPV) vaccine, a quadrivalent formulation also prepared from VLPs assembled in yeast expressing the L1 major capsid protein of HPV strains 6, 11, 16 and 18. Each of the vaccines targets the homologous infectious agent (the agent from which the structural components of the VLP are derived). VLPs made by the expression of recombinant capsid proteins in baculovirus-infected insect cells are also under study as potential subunit vaccines against norovirus, rotavirus, hepatitis C virus, hepatitis E virus, and human immunodeficiency virus (all homologous targets).[194-197]

Molecular design and engineering have provided the means for converting these VLPs into platforms for presenting heterologous antigens. Principally, the development of such platforms requires having sufficient structural information to allow for the rational design and incorporation of foreign antigens into self-organizing capsid proteins without compromising the capacity

to assemble into a VLP. Two basic approaches are used. The first is the creation of a fusion gene expressing the heterologous antigen in frame at an appropriate point within the envelope protein to result in display on the VLP surface. The resulting product may require mixing with unmodified envelope protein to achieve stable self-assembly, and because of structural constraints may only be able to tolerate the introduction of short foreign peptide sequences, such as an antigenic epitope. The second strategy is to allow the unmodified envelope protein to assemble into VLPs as the first step, and then heterologous antigen is coupled to the particle as a second step. The latter technique generally does not interfere with particle stability, allowing decoration with large protein domains and even full-length proteins.[198] Whichever technique is used, the density of the heterologous array on the surface is critical to potency. It is hypothesized that array density determines the efficiency with which the receptors of inquiring lymphocytes are cross-linked. As a result, low density arrays, such as the RTS,S vaccine, are not ideal for inducing immunity in the absence of adjuvant. The size of the VLP, which varies from 22 to 150nm, is less important for achieving potency than the array density.

The list of VLPs that have been studied is extensive, and includes those assembled with the capsid proteins from mammalian (HBV, HPV, parvovirus B19), avian (Flock House virus, FHV), plant (papaya mosaic virus, cucumber mosaic virus), and yeast (TY-1, L-A) viruses, as well as bacteriophage (MS2, Q-beta). The two VLPs studied most extensively as a platform for malaria vaccines are based on the hepatitis B core (c) and surface (s) proteins.

Hepatitis B virus core protein (HBc) was demonstrated to be an effective malaria vaccine platform in preclinical models, where high levels of anti-CS repeat antibodies protected animals from malaria challenge.[199] In a recent nonrandomized, non-blinded Phase I safety study using ICC-1132 (Malariavax), a recombinant VLP comprised of hepatitis B core antigen engineered to express the central repeat regions from *P. falciparum* CSP protein, an immunodominant B [$(NANP)_3$] epitope, an HLA-restricted CD4 (NANPNVDPNANP) epitope and a universal T cell epitope (amino acids 326—345) was found to be well tolerated, but poorly immunogenic when formulated with alhydrogel.[200]

Alternatively, the *P. falciparum* CSP-based vaccine, RTS,S, developed by GSK, is composed of a hepatitis B virus surface antigen, which contains the CSP repeat and C terminal region (amino acids 207—395) of the *P. falciparum* NF54 isolate (3D7 clone). RTS,S is the most advanced malaria vaccine, currently in pivotal Phase 3 efficacy trials in sub-Saharan Africa. In combination with GSK proprietary adjuvant systems, this vaccine has demonstrated significant protective efficacy against malarial disease and severe malaria.[201,202] Adjuvanted RTS,S has been shown to induce both a strong antibody response and stimulate Th1 cells—a subset of helper T cells—that participate in cell-mediated immunity.[203] It is the relative success of RTS,S that has provided impetus for researchers to consider, develop and evaluate alternative particulate-based vaccine platforms.

Another particle approach, virosomes, represents a variation on VLPs. Virosomes are made up of liposomes containing viral envelope proteins and have been previously developed for many enveloped-viruses, i.e., semiliki forest, measles, rubella, herpes simplex, rabies, sindbis, Epstein-Barr, HIV and vesicular stomatitis virus. All influenza virosomes are unilamellar vesicles having a diameter of 100-200nm depending on lipid composition. The major advantages of virosomes include their immunogenicity in the absence of adjuvants and their relatively simple production compared to traditional recombinant protein-based processes. Virosomes are thought to enhance immune responses against co-delivered target antigens at multiple levels. Virosomes can interact with APCs and B lymphocytes to present the target antigen to T lymphocytes to induce both antigen-specific B cell and $CD4^+$ T cell responses. Optimally, virosomes can act as carriers of B cell epitopes, but are also promising for the delivery of carbohydrates, DNA, siRNA, and drugs.

Virosomes can be derived solely from virion lipids or from virion plus supplementary lipids (e.g., Immunopotentiating Reconstituted Influenza Virosomes, IRIV). The final composition of lipids forming the IRIV can impact not only the physiochemical properties of the resultant virosome, but also immunological properties. IRIV act as antigen carriers through encapsulation or direct association, providing significant flexibility for formulation. Pevion Biotech has developed optimized formulations of IRIV designed for specific applications (reviewed in Moser et al.).[204] AMA-49-CPE is an IRIV that was evaluated as the carrier and adjuvant system for a *P. falciparum* vaccine that targets both the exo-erythrocytic and erythrocytic stages based on two malaria peptide antigens (AMA1 and UK39, a circumsporozoite protein) previously shown to induce parasite inhibitory activities.[205] Although this vaccine approach was found to be safe and highly immunogenic in a placebo-controlled, randomized clinical Phase 1a safety study conducted at the University Hospital of Basel,[206] no sterile protection was observed against a homologous sporozoite challenge in a subsequent clinical Phase 2a trial.[207] However, some evidence for the development of blood stage immunity was reported, as shown by a reduction in parasite growth rates and the observation of crisis forms in the blood from some volunteers. A Phase 1b study to evaluate the safety and immunogenicity of this vaccine in Tanzanian adults and children has been completed and final results are awaited (a collaboration between the Bagamoyo Research and Training Unit and the Swiss Tropical Institute).

Another variation on molecularly designed particulate vaccines is represented by self-assembling polypeptide nanoparticle (SAPNs) (Fig. 4 and Box 2). As with VLPs and virosomes, the repetitive display of B cell epitopes on the nanoparticle may lead to the induction of a strong humoral immune response. Additional engineering to include the pan-DR T cell epitope, PADRE, or other relevant helper sequences into the peptide design may influence the immunogenicity of the nanoparticle constructs. These peptide nanoparticles have a reported icosahedral symmetry which mimics viral protein capsids, but can be specifically designed to vary in size and the density of the surface array. Since these peptide nanoparticles are recombinant peptide-based vaccines, the safety risks of live-attenuated vaccines are avoided. In studies using an experimental murine model of malaria, SAPNs

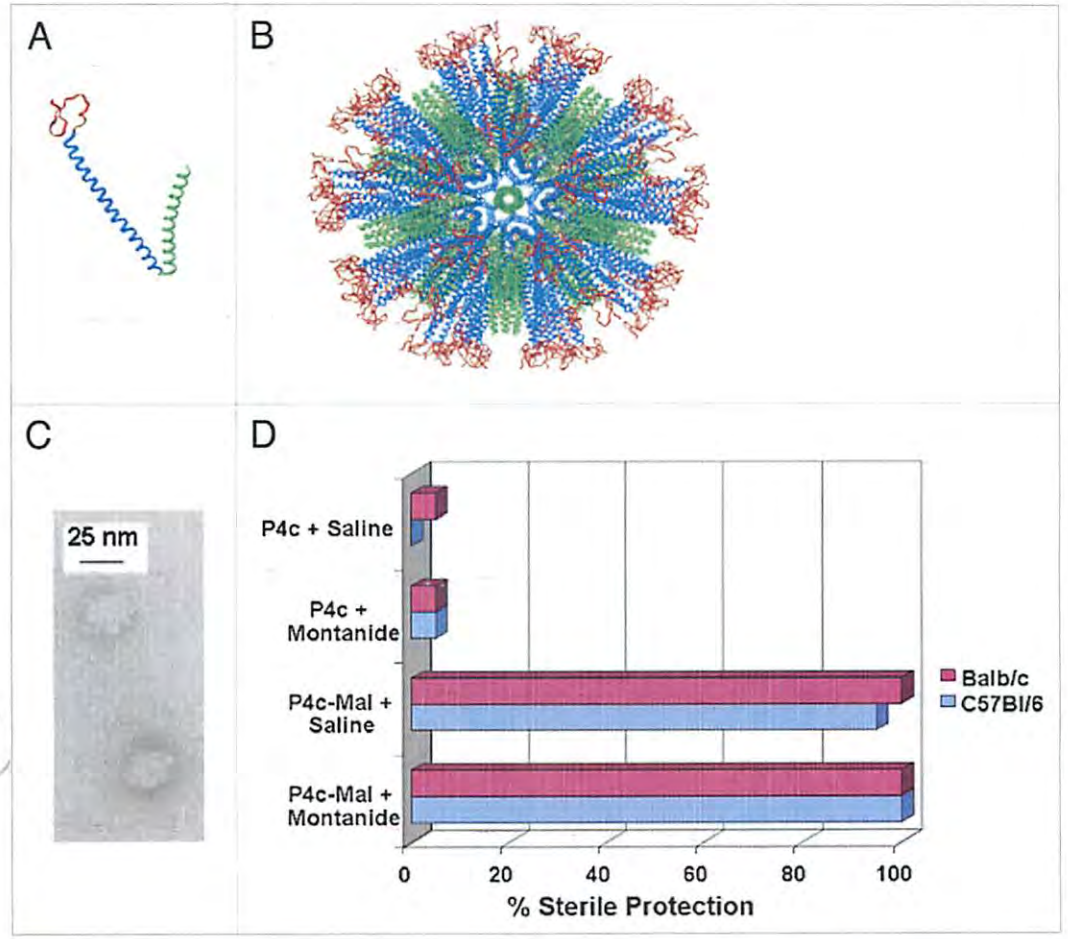


Fig 4. Self-assembling polypeptide nanoparticles (SAPN). (A) Monomeric building block of P4c-Mal composed of a modified pentameric coiled-coil domain from cartilage oligomeric matrix protein (COMP) (green) and a trimeric de novo designed coiled-coil domain (blue) which is extended by the sequence of *P. berghei* CSP repeat region, $(DPPPPNPN)_2D$ (red). (B) Computer model of the complete peptide nanoparticle (P4c-Mal), containing the repeat region of *P. berghei* CSP, with icosahedral symmetry. The calculated diameters of these particles are about 25nm and have a molecular weight of 14.8 kDa. (C) Transmission electron microscopy image of P4c-Mal nanoparticles at 242 000×. The sample was negatively stained with 1% uranyl acetate. Sample concentration was 0.076 mg/mL and the buffer was 20 mM Tris pH 7.5, 150 mM NaCl, 10% glycerol. Nanoparticle size ranges are from 25 to 30 nm. (D) BALB/c and C57BL/6 mice were immunized three times with P4c-Mal or P4c (empty) SAPNs at 10μg dose in the presence or absence of adjuvant, Montanide ISA 720, at two week intervals and challenged with 4000 (for BALB/c) and 1000 (for C57BL/6) P. berghei sporozoites by intravenous injection two weeks post final immunization. SAPN P4c-MAL induced approximately 100% sterile protection in both BALB/c and C57BL/6 mice regardless of adjuvant inclusion.[208] These excellent protection results support the further development and evaluation of SAPNs for malaria.

designed to express peptide epitopes from the repeat region of the *P. berghei* CSP provided nearly 100% protection against sporozoite challenge with *P. berghei* sporozoites without the requirement for additional adjuvant (Fig. 4).[208] Furthermore, a SAPN displaying the *P. falciparum* CSP repeat epitope completely protected mice from challenge using a *P. falciparum* CSP transgenic *P. berghei* parasite without the requirement for additional adjuvant (D. Lanar, personal communication).

Safety of Molecular Vaccines

Establishing safety is the most important development objective for a malaria vaccine assuming that it offers significant protection. Because the intended target populations are healthy individuals, including small children, infants, travelers, pregnant women and females entering puberty, tolerability should be acceptable and the safety profile excellent.

The clinical assessment of molecular vaccines, as with any vaccine, aims to identify adverse events stemming from the injected material. Such hazards fall into two categories—adverse events that are anticipated, given what is known about the vaccine, its formulation and mechanism of action, and adverse events that are unexpected. The occurrence of the former can be specifically sought in clinical trials through testing or other design features built into the trials. The latter cannot be anticipated and dictates that early studies should comprise small numbers of volunteers subject to intense follow-up in order to safely identify unknown risks.

Box 2. Self-assembling peptide nanoparticles: Novel vaccine platforms based on virus-like particles are gaining increased interest in the field of vaccine development. Peptide-based nanoparticles represent an ideal repetitive epitope display system and a platform for the development of highly tailored recombinant vaccines. These peptide nanoparticles have a regular polyhedral symmetry that mimics the icosahedral architecture of viruses. As such, epitope display approaches have been shown to strongly increase the immunogenicity to a given epitope.
The fundamental structure of SAPNs is derived from the molecular design of a monomeric linear polypeptide having about 100 amino acids (**Fig. 4**). Each linear polypeptide is comprised of a pentameric and a trimeric coiled-coil oligomerization domain joined by a two-glycine residue flexible linker. These alpha-helical coiled-coils represent a common protein oligomerization motif stabilized through hydrophobic interactions occurring along their coiled-coil interface. Both N- and C-terminal ends of the polypeptides in the assembled complex are exposed on the surface, allowing for presentation of B cell epitopes in a repetitive antigen display. Peptide epitopes of B cell, T helper, or CTL specificity can be molecularly incorporated into polypeptides by the cloning of their nucleotide sequences into an expression plasmid used to express the polypeptides. Following expression in *E. coli*, the protein can be purified, refolded, and assembled into nanoparticles having a diameter of approximately 25 nm. By taking advantage of the increasing knowledge of this structural class of protein motifs, a growing number of biological and therapeutic applications are being considered.[208]

Molecular vaccines based on genetic approaches potentially entail additional hazards beyond those of the injected material (vaccine substance), resulting from the fact that RNA transcripts are synthesized in vivo following injection and host cell entry, and these in turn lead to the expression of protein products, including both transgene antigens and incidentally expressed vector proteins. Because these products are expressed intracellularly, the safety of a given genetic vaccine cannot be demonstrated by clinical studies of the exogenous protein encoded by the transgene, which will remain primarily in the interstitial tissues following administration. Thus, a candidate malaria antigen that has been studied in humans as a recombinant protein requires new toxicology studies if subsequently developed as a DNA- or virally-vectored vaccine.

Each molecular vaccine platform carries its own particular safety risks. For example, viral proteins and nucleic acids present in viral vectors display a variety of pathogen-associated molecular patterns that trigger specific host pattern recognition receptors to activate the innate immune system. Viral proteins may trigger TLR 2 and 4 receptors, viral RNA TLR 3, 7 and 8 receptors, and viral DNA the TLR 9 receptor. Each platform can be expected to induce different patterns of immune activation and cytokine release based on its effects on the innate immune system. As an example of side effects consistent with these known pathways, we have observed that most recipients of our Ad5-vectored malaria vaccine, NMRC-M3V-Ad-PfCA, demonstrate transient dose-dependent reductions in circulating neutrophils and lymphocytes, likely due to margination and diapedesis into the interstitial space of peripherally circulating white cells in response to the danger signal and resulting cytokine cascade engendered by the viral capsid or its DNA.[209] This occurs during the first few days following administration of the vaccine and parallels published data on the side effects of CpG 7909, a TLR9 agonist, when administered subcutaneously.[210] Likely also associated with the intrinsic inflammatory properties of the vaccine components, adenovirus-vectored vaccines cause fever and other systemic reactions, generally with doses of 1×10^{11} particle units or higher. Additional safety concerns are discussed in Box 3.

In addition to these side effects that are consistent with our understanding of the vaccine vector, vaccines may cause a range of unexpected, idiosyncratic reactions based on environmentally-induced or genetic differences among individual recipients which are difficult to predict. For example, the poxvirus platform became the subject of concern when sporadic cases of myocarditis were linked to the administration of replicating vaccinia virus as part of a smallpox immunization campaign (CDC 2003). However, a subsequent study of volunteers receiving MVA and other non-replicating poxvectors indicated that this concern did not extend to these related poxvirus platforms. Most recently, the Merck STEP trial suggested a link between naturally acquired immunity to Ad5 (acquired following exposure to wild-type virus earlier in life) and an increased risk of HIV acquisition in uncircumcised men receiving the Ad5-vectored HIV vaccine (see Box 3).[211] No plausible mechanism to explain this finding has been established since the STEP trial results were announced in November, 2007. The finding itself remains unconfirmed by other studies, as investigators search to identify covariates affecting the results of the STEP trial that may have contributed to this finding.

Particularly because of unpredicted side effects such as those just described, each candidate malaria vaccine based on molecular approaches needs to be examined carefully for toxicity, although when a platform is shared by two vaccines (e.g., identical DNA backbones, adenovirus or poxvirus vector systems, etc.), it is appropriate to cross-reference nonclinical and clinical studies in support of safety. This is true, for example, of the cell substrate used to produce a vaccine: once safety is demonstrated and the cell line is qualified for production of vaccines for use in healthy humans (such as for the PER.C6 or 293-ORF6 cell lines used to produce adenovirus vectors), retesting the cell line for each vaccine may not be required. In addition to careful evaluation in nonclinical studies, the vaccine itself should be designed with safety considerations prominently in mind. For example, many malaria antigens contain sequences sufficiently similar to human sequences (e.g., thrombospondin-like domains[212,213]) that it is appropriate to conduct BLAST searches[#] against the human genome in order to avoid sequences in the vaccine that, due to homology, might favor integration into the host genome or the breaking of self-tolerance.

Once a new vaccine or previously-tested vaccine based on a new cell substrate has demonstrated safety in appropriate nonclinical studies and is advanced to clinical testing, it is important to evaluate safety in a very small number of individuals, with a delay before commencing further immunizations. An example where this procedure was not followed involved the administration of TGN1412, a humanized monoclonal antibody designed as an agonist to the CD28 receptor on T lymphocytes. Six of the eight

[#] Basic Local Alignment Search Tool

Box 3. Adenovector Safety: Each molecular platform, and potentially each molecular vaccine, is characterized by theoretical safety concerns that may or may not turn out to be significant in vivo. These concerns must be rationally addressed prior to clinical testing, potentially by conducting nonclinical studies, and then monitored closely, to the extent possible, in Phase I studies. Here are several concerns relating to adenovirus vectors:

Integration potential: Although adenoviruses infect a wide range of cell types, their genome remains episomal in the infected host cell and they lack native integration machinery. A report from the European Medicines Agency classified adenovirus as a non-integrating vector.[252] However, studies have shown that replication-incompetent adenoviral vectors can randomly integrate into host chromosomes, at frequencies of $10^{-2} - 10^{-5}$ per infected cell, when studied in vitro in replicating cells where active selection was used to detect these events.[253, 254] This low rate has limited the usefulness of unmodified, non-replicating adenoviruses as vehicles for effecting permanent gene transfer, but represents an advantage for their use as vaccine vectors where long-term expression is not required. The risk of insertional mutagenesis in neighboring genes resulting from integration events would be expected to be lower than the rate of integration, since only a small proportion of integration events would be expected to result in mutagenesis.

Latency and transformation: Wild-type adenoviruses can remain latent in tissues and have been detected in tumors as well as in normal tissues, although there is no definitive evidence that vectors such as Ad5 (Group C adenoviruses) play a causative role in human cancer,[255-259] nor is there evidence of germline transduction.[260, 261] Adenoviruses are also reported to have transforming (immortalizing) potential in vitro, including Group C adenoviruses.[262,263] One approach to addressing this concern is to delete the regions of the virus associated with transformation, notably the E1A and E1B genes.[264] Most adenovectors currently in the clinic have the E1 region of the virus removed and replaced with the expression cassette for the transgene. The AdPf5 (**Fig. 3**) vaccine also has the E4 and portions of the E3 genes removed.

Merck STEP Trial: The findings in the STEP trial (see text and Buchbinder et al.[211]) raise central questions about the safety of adenovirus vectors. Do they indeed increase the risk of HIV infection in those seropositive for anti-Ad neutralizing antibodies? Since the signal was detected only in uncircumcised men, could it relate, for example to the presence of CD4+ T cells and virus incubating under the foreskin following exposure through sexual contact, and thus remain restricted to this age, sex and risk group? Is the risk peculiar to Ad5 or would it apply to all viral vectors? Would the risk disappear with adenovectors that express significantly lower levels of viral proteins than the Merck vector, such as the E1/E3/E4-deleted AdPf5 vector? Until these and additional questions are answered, it will be prudent to restrict the testing of adenovectors to seronegative individuals or, if seropositive, to those individuals who know and have control over their risk for HIV exposure.

healthy adult subjects receiving the injection in an initial immunization session developed multi-organ failure associated with a systemic inflammatory response syndrome and nearly died, despite reportedly unremarkable toxicity observed in rabbits and monkeys receiving up to 500 times the human dose.[214] Although this was a therapeutic monoclonal antibody, not a vaccine, the principle applies equally to molecular vaccines.

With these considerations in mind, molecular vaccines have generally been well-tolerated and safe in humans, both malaria-naïve research subjects tested in the US and Europe, and in malaria-exposed residents of endemic areas. This includes vaccines based on DNA,[111, 112] poxvirus,[127, 215-218] and adenovirus vectors (only results of adenovirally-vectored non-malaria vaccines are currently published[150, 170, 219-221]) and prime-boost combinations of DNA and poxvirus vectors or DNA and recombinant protein[130, 222-224]. Protein-based malaria vaccines employing molecular approaches covered in this review have also generally proven safe and well-tolerated, including virus like particles,[200,225-227] virosomes[206, 207] and bacterial vectors.[184] Theoretical concerns regarding the induction of autoimmunity (e.g., anti-double stranded DNA antibodies), integration into the host genome or germline, or persistence of transgene expression with resulting untoward effects, have not materialized. As the electroporation of DNA plasmids to increase cellular uptake and expression undergoes clinical testing, the question of integration into host genomic DNA must remain open, as electroporation may increase this risk.[228] Viral vectors appear more reactogenic than naked DNA, but still carry an acceptable tolerability profile. The most extensively studied of virally vectored vaccine platforms, MVA, has been administered as a prevention for smallpox (thus without transgenes) to over 120,000 previously unvaccinated individuals, including elderly individuals, alcoholics and others with compromised immunity. Only a small proportion showed any systemic side effects, such as fever and there were no indications of serious reactions associated with the vaccine.[229] In addition to these clinical studies, a substantial body of literature has been accumulated regarding animal toxicology, biodistribution and genomic integration (or lack thereof) of gene-based vaccines to support the good-safety profiles observed in humans.[230-233]

Molecularly-based adjuvants provide a different set of challenges. As with adjuvants used with protein-based vaccines, adjuvants for gene-based vaccines require extensive safety evaluation. After nonclinical studies indicated that the oligonucleotide CPG 7909 significantly enhanced the immunogenicity of the recombinant protein-based malaria vaccine, AMA1-C1,[234, 235] subsequent clinic testing raised concerns regarding the frequency of allergic reactions[69] and this in turn led to identification and testing of less allergenic formulations.[236] A major step in establishing safety of immunomodulatory adjuvant systems occurred with the co-administration of plasmids encoding human GM-CSF to healthy adults as an immunostimulant to improve the potency of the pentavalent MuStDO5 malaria vaccine (a mixture of five plasmids encoding pre-erythrocytic stage antigens).[86] Although a potentially adverse inflammatory response might have been anticipated following the injection of this human cytokine, the vaccine/adjuvant combination was well-tolerated, even if non-protective.[237] One of the ways that the safety profile of this vaccine was assessed pre-clinically was to immunize mice with the vaccine in a standard toxicology study, but instead of co-administration with a plasmid encoding human GM-CSF, the mice were given a plasmid encoding murine GM-CSF to better reproduce a host-appropriate inflammatory response.[231] This illustrates that careful study design based on purported mechanism may be helpful in improving the predictive ability of non-clinical studies. The case of TGN1412 monoclonal antibody cited above provides another example where potential species-specific toxicity profiles misled investigators, with benign results in animal models failing to predict life-threatening toxicity in humans.[214] This principle is an important consideration for the immunomodulatory components of a vaccine, since many of these compounds act via a species-specific mechanism.

Potential rare side effects of molecular vaccines will remain a major concern, as their identification ultimately relies upon the larger sample sizes associated with Phase 3 testing and licensure, milestones not yet achieved by any gene-based vaccine. Post-marketing surveillance, key to this process, will be hampered for malaria vaccines, since many of the target populations reside in resource-poor countries lacking the capacity to adequately monitor and report serious adverse events post licensure.

Looking to the Future

Many vaccine developers are currently focusing on molecular vaccines. One of the most important reasons for this is their potential for improved safety and efficacy, especially for the difficult infectious disease targets, such as malaria, HIV and tuberculosis, where traditional approaches have failed to deliver effective vaccines or carry unacceptable safety risks. It is comforting for the customer and satisfying for the vaccine developer to conceive and build a vaccine based on known biological mechanisms and precisely defined molecular targets. This type of immunogen can be manufactured according to tightly controlled, reproducible processes, yielding a drug substance where nearly every attribute is defined. Ultimately, the comprehensive understanding intrinsic to a well-conceived molecular vaccine should improve both safety and efficacy, thereby meeting the major goals for vaccine developers.

Another important benefit of molecular vaccines is their agility. Knowing the mechanism of action allows the design of functional assays for monitoring vaccine efficacy that can promptly detect vaccine resistance by the pathogen and enable modification of the vaccine in a timely way to compensate. A re-engineered immunogen can be placed into the existing expression cassette, and the improved product manufactured by the same processes as the original product.

Ultimately, however, molecular vaccines may be needed because they are the only way to protect against difficult pathogens, including chronic infections like malaria and HIV, where the pathogen successfully manipulates the immune system to its advantage, as well as emerging threats, such as pandemic influenza. While attempting to design and license molecular vaccines for these pathogens, significant obstacles have been encountered, such as antigenic polymorphism, the existence of redundant molecular pathways, and host genetic restriction. These obstacles in turn have led to major improvements in molecular technologies. We now have multiple promising platforms to optimize, a variety of excellent models for testing, and burgeoning knowledge of innate and acquired immune responses to inform development efforts.

As illustrated in this review, malaria is playing a crucial role in the development of molecular vaccines. Many novel technologies, such as the evaluation of a plasmid encoding human GM-CSF in healthy adults, have first been tested against Plasmodium. Malaria has the advantage of providing an ethical human challenge model, presenting vaccine developers with rapid feedback regarding efficacy, thereby accelerating the testing of new vaccine concepts.

Looking to the future, one of the main challenges for vaccine developers is the complexity of malaria proteins as antigens. The best malaria antigens may prove difficult for the immune system to recognize, either because of diversionary strategies or for other reasons, such as host mimicry (hiding under the umbrella of self-tolerance), or because evolution has favored amino acid sequences that bind poorly to MHC molecules. For example, the parasite exposes immunodominant domains (such as the NANP repeat of *P. falciparum* CSP) that, while inducing partially protective immune responses, also may serve as decoys diverting the immune response away from subdominant responses that are potentially more protective. Moreover, epitopes may have evolved to stimulate regulatory T cell responses, suppressing rather than enhancing immunity. The success of molecular vaccines for malaria will likely require progress in many areas, including the identification of new antigens, the optimization of vaccine platforms able to deliver multiple antigens, the development of new, safer adjuvants and immunostimulants, and the redesign of antigenic targets to improve immunogenicity.

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