

Recombinant modified vaccinia virus Ankara-based malaria vaccines

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Sarah Sebastian and Sarah C. Gilbert*

The Jenner Institute, University of Oxford, Oxford, UK

*Author for correspondence:

Tel.: +44 1865 617605

sarah.gilbert@ndm.ox.ac.uk

A safe and effective malaria vaccine is a crucial part of the roadmap to malaria elimination/eradication by the year 2050. Viral-vectored vaccines based on adenoviruses and modified vaccinia virus Ankara (MVA) expressing malaria immunogens are currently being used in heterologous prime-boost regimes in clinical trials for induction of strong antigen-specific T-cell responses and high-titer antibodies. Recombinant MVA is a safe and well-tolerated attenuated vector that has consistently shown significant boosting potential. Advances have been made in large-scale MVA manufacture as high-yield producer cell lines and high-throughput purification processes have recently been developed. This review describes the use of MVA as malaria vaccine vector in both preclinical and clinical studies in the past 5 years.

KEYWORDS: modified vaccinia virus Ankara (MVA) • malaria • plasmodium • vaccine • clinical trials • heterologous prime-boost

The development of an effective vaccine against malaria remains a high priority on the global health agenda. Despite the use of extensive intervention measures, malaria continues to be a heavy burden in terms of morbidity and mortality, with an estimated 200 million cases and 584,000 deaths in 2013 alone.[1] Out of the five human malaria parasite species, infection with *Plasmodium falciparum* is the most deadly and accounts for approximately 90% of all malaria cases worldwide, while the remaining 10% of cases are attributable to infection with *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. The life cycle of the malaria parasite is complex (reviewed in [2]), involving multiple life stages in both the human host as well as the *Anopheles* mosquito host which acts as a vector; and this complexity has contributed to the difficulty in the development of an effective vaccine.

The *Plasmodium* life cycle is initiated with the bite of an infected mosquito, transferring motile sporozoites from the mosquito salivary gland into the dermis of the human host. The sporozoites then reach the liver through the circulatory system, where they infect hepatocytes, replicate and develop into merozoites.

Once released into the blood, merozoites invade red blood cells and initiate the asexual replication cycle, with each parasite-harboured erythrocyte developing from ring stage to trophozoite stage and finally to schizont stage before releasing a second generation of merozoites. It is this part of the parasite life cycle which is responsible for the clinical malaria symptoms, such as fever, chills and headache. While asexual replication continues, a proportion of invading merozoites develops into male or female gametocytes, initiating the sexual stages of the life cycle. Gametocytes that are taken up from the blood during a second mosquito bite are activated in the mosquito midgut to form gametes, which fertilize to develop into zygotes and then motile ookinetes. These ookinetes traverse the mosquito midgut wall where they develop into sporozoite-containing oocysts. Released sporozoites then invade the mosquito salivary gland, from where they can be injected into a new vertebrate host and thus continue the parasite life cycle. It is of note that during those life cycle stages which expose the parasite to the immune system of the host, *Plasmodium* has become very adept at immune evasion, both by systematically

altering immunogenic epitopes (antigenic variation),[3] or by antigenic polymorphism.[4] Surface antigens on the sporozoite and merozoite, for example, are highly polymorphic among different parasite isolates, whereas sexual-stage surface antigens which are present only in the mosquito are less genetically diverse.

Sporozoites and merozoites are the only extracellular parasite forms in the vertebrate host, accessible to the humoral arm of the immune system for a very short period of time only (seconds to minutes), before they invade a host cell. Despite this and despite parasite immune evasion strategies, neutralizing antibodies to surface antigens of these forms have been shown to confer some protection against disease in humans [5] or animal models.[6–8] GlaxoSmithKline's Mosquirix™, the only malaria vaccine which has progressed to Phase III clinical trials to date (and which has recently received a positive review from the European Medicines Agency), targets the sporozoite stage of the malaria life cycle by inducing antibodies against circumsporozoite protein (CSP), the major protein on the sporozoite surface. Despite reaching an important milestone, the vaccine is only modestly protective against malaria in the target age group (young infants and children), with protection waning within 1 year, emphasizing the need for further vaccine development. Importantly, an ideal malaria vaccine would target several parasite stages at once in order to increase the likelihood of sterilizing protection. Consequently, current malaria vaccine research encompasses pre-erythrocytic, blood-stage, as well as transmission-blocking vaccine candidates. A substantial part of this research focuses on the induction of a humoral immune response, using DNA or protein subunit vaccines. However, the importance of cell-mediated immunity against the parasite has also been amply demonstrated, especially when targeted to the intracellular liver stages of the parasite. A positive correlation between a vaccine-induced T-cell response and protection against infection was first shown in a mouse model, when passively transferred CD8⁺ T-cell clones recognizing a malaria epitope conferred a high degree of protection against malaria challenge.[9] In addition, studies of naturally acquired immunity against malaria in humans found a role for central memory T cells recognizing pre-erythrocytic antigen epitopes in protection against clinical malaria.[10]

A compelling argument can therefore be made that an effective malaria vaccine would greatly benefit from a cytotoxic T-cell inducing component. Vaccines that induce potent CD8⁺ T-cell responses must be processed via the major histocompatibility complex (MHC) class I pathway and while protein subunit vaccines can be introduced into this pathway via exogenous cellular uptake, it is difficult to achieve high levels of cytotoxic T lymphocytes (CTLs) with these vaccines. Recombinant viral vectors, however, deliver their antigenic cargo in the form of nucleic acid, which is then expressed and processed inside the target cell. This direct access to the endogenous MHC class I pathway results in efficient antigen display in the context of MHC class I molecules, which, together with secondary signals, subsequently leads to the activation of

cognate CD8⁺ T-cells. In addition, cells infected with the viral vaccine vector can be engulfed by professional antigen-presenting cells such as dendritic cells, which then also present the foreign antigen on MHC molecules in a process termed cross-presentation. Viral-vectored vaccines are therefore the antigen delivery platform of choice for the induction of high levels of cytotoxic T-cells.

The two most commonly used viral vectors in malaria vaccine research to date are adenoviral vectors and the poxviral vector modified vaccinia Ankara (MVA). The focus of this review will be on recent developments in the field of recombinant MVA-based malaria vaccines.

History of MVA and recombinant MVA production

MVA is a highly attenuated derivative of the vaccinia virus, isolated after more than 570 passages of chorioallantois vaccinia virus Ankara (CVA) in primary chicken embryo fibroblasts (CEF).[11] Compared to its parent, MVA has lost 13% of its genome, including many immunomodulatory genes, leaving it with 178 kbp of linear double-stranded genomic DNA and preventing any possibility for spontaneous reversion to pathogenicity. MVA readily infects a variety of different cell types but is unable to complete its replication cycle to produce infectious progeny in most mammalian cell lines and crucially does not replicate in humans.[12] When used as a vaccine, it efficiently activates a protective immune response, which is thought to be partly due to a potent induction of the innate immune system by the virus itself. Unmodified MVA is currently licensed as a third generation smallpox vaccine (Imvamune®/Imvanex®, Bavarian Nordic), but has also shown great potential as a safe and effective recombinant vaccine vector. Factors contributing to its excellent safety profile, even in the immunocompromised, [13] are its inability to replicate in humans, its genetic stability and its confinement to the cytoplasm of the infected cell, preventing any potential for integration of viral DNA into the host cell genome.

Recombinant MVA is produced by infection of CEF cells with a parent MVA virus immediately followed by transfection of a shuttle plasmid containing the antigen expression cassette flanked by homology arms. These homology regions target the antigen cassette to a specific insertion site in the parental MVA genome and homologous recombination between the two takes place during viral DNA replication of the parental virus. Successfully recombined virus is most commonly selected using fluorescent marker genes, which can later be removed again in order to attain a marker-less virus. Various sites in the MVA genome are available for insertion of foreign genes and the capacity for such cargo appears to be at least 25 kb. All poxviruses encode their own transcription machinery and therefore use their own unique promoter elements; this means that any antigen must be under the control of a poxvirus promoter in order to be expressed by the virus. Numerous promoters have been identified for this purpose, many of which can be classified as either early- or late-stage promoters. It is of interest to note that, for an efficient T-cell induction, strong early expression of

the antigen is essential (within the first 2 h of infection),[14,15] while antibody response seems to be correlated with the cumulative antigen expression levels during the viral life cycle.

Large-scale manufacture of MVA has improved significantly in the past 5 years, particularly with respect to cell culture and purification processes. Primary CEF as producer cells are now being replaced with current good manufacturing practice (cGMP)-compliant suspension cell lines specifically developed for high-yield MVA production (AGE1.CR, QOR2/2E11, EB66).[16–19] For example, the Phase I-tested Ebola vaccine MVA-EBOZ is produced in the AGE1.CR suspension cell line developed by ProBioGen, which was derived from primary duck cells and immortalized with adenoviral E1 proteins.[20] Using a cGMP qualified cell bank for manufacture prevents the possibility of the vaccine being contaminated with adventitious agents which may occasionally be present in primary cells. In addition, ultracentrifugation steps during virus purification are replaced with chromatography-based purification processes, which result in virus preparations containing only trace amounts of host cell protein or DNA.[21,22] It is therefore now possible to manufacture rMVA in a highly efficient, chemically defined production process which is robust and scalable, with the potential of multi-million doses being produced within a few months. MVA is currently supplied in lyophilized form and stored at 4 °C, necessitating a cold chain, but recent advances in thermostabilization techniques have shown that MVA can be vitrified in sugar complexes which allow complete recovery of viral titer and immunogenicity after storage at up to 45 °C for 6 months.[23]

Vaccine targets: choice of parasite stage and antigens

With a genome comprising around 5400 genes, it is not surprising that at each life cycle stage, the malaria parasite proteome is characterized by a unique set of antigens (see Table 1). As a consequence, one of the major challenges in malaria vaccine development has been the choice of vaccine target in terms of life cycle stage and specific antigen. The three main parasite stages targeted as points of intervention in current malaria vaccine research include the pre-erythrocytic (sporozoite and liver-stage), asexual and mosquito stages. Clear advantages of the former are the relatively low numbers of parasites that need to be eliminated at this stage (10–100 sporozoites or infected hepatocytes) and the fact that the blood-stage expansion of the parasite population resulting in symptoms of malaria disease would be prevented. (Sporozoites can be recognized by vaccine-induced host antibodies, while a cytotoxic T-cell response is needed to target the intracellular hepatic stages of the parasite.) A blood-stage malaria vaccine, on the other hand, must necessarily induce an antibody-based immune response, since erythrocytes lack MHC molecules and cannot present foreign antigens to the immune system. Antigenic variation and large parasite numbers are only two of several challenges faced in the development of a blood-stage vaccine; other disadvantages include the fact that such a vaccine would not eliminate the latent hypnozoite liver stage characteristic of *P. vivax* and

P. ovale parasites. Though insufficient by itself, a blood-stage component would nevertheless be of considerable value in a combination vaccine, where it could either act to eliminate any parasites that have escaped from the pre-erythrocytic vaccine component, or limit parasite density in such a way that the formation of sexual stages and therefore transmission to the mosquito would also be significantly reduced. Interest has also recently been renewed in a specific transmission-blocking component as part of a multi-stage malaria vaccine. Antibodies induced against the sexual stages of the parasite can block parasite development in the mosquito vector and thus prevent transmission to the next host. Alternatively, antibodies can also target mosquito proteins which are essential for parasite development. These antibodies would be taken up in the blood meal of the mosquito along with the gametocytes and prevent completion of the mosquito stages of the life cycle. By design, this type of vaccine on its own does not afford protection to the vaccinee directly, but would benefit the local community by reducing the overall transmission potential.

Recombinant MVA is an ideal vaccine vector to address all three life cycle stages of the parasite named above, since it not only elicits a strong antigen-specific CTL response but can also induce significant antibody titers against membrane-bound or secreted antigens. This review will focus on applications of MVA as a malaria vaccine vector within the last 5 years, including both the preclinical setting and clinical trials.

MVA in preclinical research

Pre-erythrocytic vaccines

The first use of recombinant MVA as a vaccine vector against malaria dates to the late 1990s as part of a DNA–MVA prime-boost regimen in mice.[24] In this seminal study, mice of two different strains were found to be completely protected in *P. berghei* sporozoite challenge experiments after receiving a DNA prime and rMVA boost which encoded two *P. berghei* sporozoite and liver-stage antigens [thrombospondin-related adhesion protein (TRAP) and CSP, see Table 1]. The same authors subsequently assessed a variety of vaccination regimes, including homologous and heterologous prime-boosts using virus-like particles, DNA and MVA, targeting the same circumsporozoite (CS) antigen, and found that a recombinant MVA boost consistently increased the CTL response from any priming agent tested.[25] The idea of recombinant MVA as an excellent boosting agent was further reinforced in studies of adenoviral vector-MVA prime-boost regimes, which produced even higher T-cell responses.[26] Subsequently, another group found that a recombinant MVA boost after a priming immunization with a recombinant cold-adapted influenza virus vector also resulted in high numbers of antigen-specific T-cells and protected against infection in a malaria mouse model.[27] This study followed initial work using recombinant replicating vaccinia virus as a booster immunization.[28]

Two studies further investigated the phenotype and functionality of protective T-cells produced in adeno-MVA prime-boost regimens in mice: one observed that interferon-gamma (IFN- γ)

Table 1. Leading *Plasmodium falciparum* vaccine targets in current adeno-MVA regimes, their expression during the parasite life cycle and key facts about their function.

Antigen	Expression in parasite life cycle	Key facts/function	References
Circumsporozoite protein (CSP)	Sporozoite, early-liver stage	<ul style="list-style-type: none"> Major surface protein on sporozoite with rod-like structure (42 kDa) Required for sporozoite development in the mosquito and for adhesion to target cells of vertebrate host Central repeat region contains immunodominant B-cell epitope and C-terminal region contains T-cell epitopes 	[61–63]
Thrombospondin-related adhesive protein (TRAP)	Salivary-gland sporozoites, liver stage	<ul style="list-style-type: none"> 63 kDa transmembrane protein localized in micronemes and on sporozoite surface Essential for sporozoite motility and for invasion of salivary gland and hepatocytes 	[64–67]
Liver-stage antigen 1 (LSA1)	Liver stage	<ul style="list-style-type: none"> 230 kDa protein localized in the parasitophorous vacuole surrounding the developing parasite Unknown function Strong B- and T-cell epitopes in its repeat and non-repeat regions 	[68–72]
Liver-stage associated protein 2 (LSAP2)	Liver stage	<ul style="list-style-type: none"> 35 kDa protein localized at the periphery of the intracellular hepatic parasite Unknown function 	[73]
Merozoite surface protein 1 (MSP1)	Late-liver stage, merozoite	<ul style="list-style-type: none"> Essential for erythrocyte invasion 190 kDa precursor is processed into four fragments on the merozoite surface, these are shed during invasion [except for the C-terminal 19-kDa domain (MSP1₁₉)] 	[74–76]
Apical membrane antigen 1 (AMA1)	Sporozoite, Liver stage, Merozoite	<ul style="list-style-type: none"> Function in erythrocyte attachment 83 kDa precursor protein is converted to PfAMA166, followed by circum-merozoite relocalisation. During invasion, most of ectodomain (PfAMA148) is shed Possible role in hepatocyte invasion, but not essential 	[77–81]
Reticulocyte-binding protein-like homolog (RH5)	Merozoite	<ul style="list-style-type: none"> 60 kDa adhesion protein essential for erythrocyte invasion Interacts with basigin on erythrocyte surface 	[82–84]
Pfs48/45	Gametocyte, gamete	Surface protein with role in fertilization	[85]
Pfs230	Gametocyte, gamete	Surface protein with role in fertilization, Pfs230 on surface of male gametes mediates binding to erythrocytes	[86,87]
Pfs25	Gamete, zygote, ookinete	Surface protein with role in parasite development in mosquito	[88,89]
Pfs28	Gamete, zygote, ookinete	Surface protein with role in parasite development in mosquito	[88,89]

secreting CD8⁺ T-cells and not polyfunctional T-cells, correlated with protection,[29] while the other singled out CD8⁺ T effector memory cells in the blood (and not effector or central memory cells) as predictors of a protective phenotype.[30] The malaria antigen used in both studies was METRAP, a fusion of a multiple epitope (ME) string with the malaria liver-stage antigen TRAP (see Table 1). METRAP is the leading liver-stage malaria immunogen in clinical trials at the moment, after having been shown to be immunogenic and protective in adeno-MVA vaccination schedules in both mice and rhesus macaques.[31]

In the past 5 years, preclinical studies of pre-erythrocytic MVA-containing malaria vaccines have mainly focused on improving the existing MVA vectors encoding the CS protein (rMVA-CS) and the METRAP combined antigen (rMVA-METRAP), and on assessing new liver-stage antigen targets.

Using rMVA-CS as a boosting agent each time, researchers in the Esteban group found that a protective immune response in a mouse-malaria model could be induced by using two quite different priming agents: porcine parvovirus-like particles displaying a CD8⁺ T-cell epitope of the CS protein,[32] or a protein vaccine consisting of a fusion between the oligomerization domain of the vaccinia virus A27 protein and CSP.[33] These findings further confirm the notion of rMVA as a universal boosting agent. Spencer *et al.* also investigated the immunogenicity effect of fusing an enhancing element to the antigen, but in the context of viral vector-delivered antigens.[34] In this study, the liver-stage antigen METRAP is fused to CD74, the MHC class II invariant chain (Ii) and delivered to mice in a chimpanzee adenovirus 63 (ChAd63)–MVA prime-boost. By an as-yet-unknown mechanism, antigen-CD74 fusions had

previously been shown to enhance antigen presentation via MHC class I and concurring with this, the study found a significantly increased CD8⁺ T-cell response against Ii-METRAP in both mice and rhesus macaques, compared to METRAP alone. These encouraging results support the further evaluation of Ii-METRAP as a protective liver-stage immunogen in human clinical trials.

In addition to pre-erythrocytic vaccine studies using *P. falciparum* antigens, progress has also been evident in a recent pre-clinical assessment of a *P. vivax* vaccine.[35] Using ChAd63 and rMVA vectors expressing *P. vivax* TRAP (PvTRAP), the authors were able to induce high antibody titers and T-cell responses in mice. Vaccine efficacy was assessed in mice using a PvTRAP-transgenic *P. berghei* challenge model and protection against infection was found to be correlated with both CD8⁺ T-cells and anti-TRAP antibodies.

Lastly, after a decade focussed on CS and METRAP as the most promising pre-erythrocytic antigens, renewed interest is also being shown in the identification of alternative antigens as an important early stage of malaria vaccine development. A recent study assessed eight *P. falciparum* liver-stage proteins as new vaccine antigens in an adenovirus–MVA vaccination schedule in mice.[36] Of these, two (liver-stage antigen 1, LSA1 and liver-stage associated protein 2, LSAP2) produced T-cell responses superior to those elicited with TRAP or CS, as well as a protective phenotype in challenges with *P. berghei* parasites transgenic for the respective *P. falciparum* antigen. Further preclinical studies to confirm these findings are being undertaken to support a progression of these candidates into clinical trials, possibly as part of a multi-antigen pre-erythrocytic vaccine.

Blood-stage vaccines

Even though the viral-vectored prime-boost schedule including the MVA booster was initially developed to induce high levels of CD8⁺ T-cells, it soon became evident that high antibody titers could also be achieved with this vaccination regime. Understandably, for this to take place, there is a requirement for antigens to either contain a transmembrane domain or an export signal so that the protein will be accessible to extracellular recognition by B-cells once expressed in the target cell. In addition, a prolonged prime-boost interval of 8 or more weeks was found to be essential for a high-titer antibody response. Considering the versatility of the adenovirus–MVA regime, efforts are now underway to achieve immunity against the blood-stage of the malaria parasite using the same prime-boost principle as employed in pre-erythrocytic studies. The leading blood-stage vaccine targets of the last decade, merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) are both abundant merozoite surface proteins, but vaccine efficacy of protein-in-adjuvant formulations in clinical trials has been disappointing, possibly due to antigenic polymorphism and lack of a strong cellular immune response. A strong CD8⁺ T-cell response against MSP1 had previously been shown to reduce liver stage parasite burden, since MSP1 is already expressed during the formation of merozoites at the end of the liver

stage.[37] In addition, AMA1-directed CD4 T-cells have been implicated in blood-stage immunity in a rodent malaria model.[38]

Using a viral-vectored vaccine platform, therefore, one mouse study tested the immunogenicity of an optimized chimeric antigen based on conserved MSP1 sequences and demonstrated an induction of high-titer antibodies with parasite growth-inhibitory activity, as well as a potent cellular immune response.[39] Another mouse study explored the mechanisms of blood-stage protection using a HuAd5–MVA prime-boost regime directed against *P. chabaudi* AMA1.[7] The authors found that depletion of CD4⁺ T-cells after vaccination resulted in a loss of protection against blood-stage challenge. In addition, they were able to confer partial protection to naïve mice by adoptive transfer of either CD4⁺ T-cells or serum of vaccinated mice.

Two further studies tested a combination of recombinant protein and viral vector platforms to assess whether the adenovirus–MVA regime could be improved upon by the addition of a protein-adjuvant component.[8,40] In the first study, recombinant MSP1₁₉ protein (the 19kDa carboxy-terminal region of MSP1) was used as an additional boost after a conventional MSP1-encoding adeno-MVA regime in a three-component schedule, or co-administered with both the adenovirus prime and the MVA boost in a two-component schedule. The three-shot combination vaccine was found to elicit high-titer antibody responses equal to previously tested adenovirus-protein regimes and CD8⁺ T-cell responses comparable to adeno-MVA regimes, an encouraging result in the pervasive quest for vaccines that equally stimulate both arms of the immune system to a high level. Interestingly, the two-shot co-administration regime was shown to be similarly immunogenic and vector potency was unaffected by mixing with recombinant protein. In a subsequent study, these observations were confirmed in rhesus macaques [40]: here, HuAd5 and MVA viral vectors expressing two alleles of AMA1 were boosted with a biallelic recombinant AMA1 protein in adjuvant in a three-shot regime. While the viral vector prime-boost alone was sufficient to elicit strong and long-lasting multifunctional CD8⁺ and CD4⁺ T-cells, the ensuing protein-in-adjuvant boost additionally induced memory B-cells and high-titer IgG responses. Taken together, these studies show that a well-thought-out combination of vaccine platforms has the potential for clinical relevance and should be explored further in human trials.

In another clinically relevant study, investigators tested a HuAd5–MVA regime targeting two separate stages of the *Plasmodium* life cycle, in order to assess possible antigenic competition and immune interference in a combination vaccine.[41] When viral vectors encoding single antigens (pre-erythrocytic CSP or blood-stage MSP1) were co-administered in a prime-boost schedule, no interference was observed for induction of antigen-specific antibodies. However, significant T-cell interference was apparent after MVA boosting, which led to a reduction in protection after sporozoite challenge. Interestingly, this interference could be partially overcome by immunizing mice at separate sites (instead of giving a mixture of the two

MVA vectors). Based on these results, it would be interesting to test whether interference is also seen when two or more antigens are expressed from the same MVA vector.

More recently, reticulocyte binding protein-like homologue 5 (RH5) has entered the stage as a promising new blood-stage vaccine target. RH5 is an essential merozoite invasion protein which is present in all *P. falciparum* isolates tested so far, with very limited sequence polymorphism. Neutralizing antibodies have been shown to inhibit erythrocyte invasion of merozoites with high efficiency at low Ab concentrations and are highly cross-reactive amongst *P. falciparum* isolates. To test the immunogenicity and protective efficacy of RH5-based vaccines *in vivo*, investigators immunized *Aotus* monkeys with several different vaccine regimes, including ChAd63–MVA prime-boost vectors encoding PfRH5.[6] Animals were challenged with *P. falciparum*-infected red blood cells 2 weeks after the last vaccination and parasitemia was assessed each day for 38 days. Parasites were detected in the majority of monkeys post-challenge, but several were able to control and eliminate their parasitemia. The best protective efficacy resulted from a homologous 3-shot prime-boost regime with PfRH5 protein in Freund's adjuvant, which is not approved for use in humans due to its toxicity. The clinically relevant ChAd63–MVA regime also induced significant protective efficacy, although parasitemia levels were higher than for the protein-in-adjuvant vaccine regime. As expected, PfRH5-specific antibodies were found to strongly correlate with challenge outcome, underlining the ability of the viral-vectored prime-boost platform to elicit a relevant and functional antibody response in nonhuman primates.

Lastly, progress is also evident in the development of a blood-stage vaccine against the human malaria parasite *P. vivax*. A unique characteristic of this parasite species is its dependence on the interaction between the Duffy-binding protein region II (DBP_II) and the human host protein Duffy antigen receptor for chemokines (DARC) during merozoite invasion of red blood cells. *Plasmodium vivax* DBP is therefore an obvious vaccine target and recent preclinical assessment of a viral-vectored prime-boost regime in mice and rabbits resulted in high-titer antibodies which were able to bind native antigen from clinical *P. vivax* isolates as well as inhibit DARC-receptor binding in an *in vitro* assay.[42] Based on these encouraging results, a clinical trial of these vectors is now underway.

Transmission-blocking vaccines

Viral-vectored vaccines have only recently begun to be considered for their malaria transmission-blocking potential. Historically, an intensive focus of the transmission blocking vaccine (TBV) field has been on the use of recombinant proteins as inducers of antibodies. The current leading vaccine targets for *P. falciparum* are two gametocyte/gamete surface proteins (Pfs48/45 and Pfs230) and two zygote/ookinete surface proteins (Pfs25 and Pfs28) (see Table 1). All four candidates have been validated in preclinical studies, where antibodies directed toward these antigens were shown to inhibit oocyst formation and subsequent transmission of the parasite from the mosquito to

the host. Development of these targets as clinical vaccine immunogens, however, has been held back by difficulties in large-scale recombinant protein expression of these antigens. The TBV field has therefore begun to explore alternate vaccine platforms, such as antigen-conjugation, virus-like particles and viral vectors.

The first assessment of a viral-vectored TBV using an adenovirus–MVA platform was reported by Goodman et al.,[43] in a study targeting Pfs25. Antigen-specific antibodies elicited by the vaccine were able to greatly reduce oocyst intensity and prevalence (by 96 and 78%, respectively). A subsequent mouse study compared the transmission-blocking potential of three parasite-derived vaccine candidates (Pfs25, Pfs230 region C and Pfs48/45) and one mosquito-derived antigen (*Anopheles gambiae* alanyl aminopeptidase 1 AgAPN1) in a ChAd63–MVA regimen.[44] Post-boost serum from Pfs25 and Pfs230 C antigens was able to completely inhibit oocyst formation in a direct membrane feeding assay using *P. falciparum*-infected donor blood, at IgG concentrations of 250 µg/ml. No transmission-blocking activity was observed for anti-AgANP1 or anti-Pfs48/45 serum in this assay. Another mosquito-derived antigen was used as a vaccine target by Williams et al.[45] *Anopheles gambiae* serpin-2 (AgSRPN2) is a serine-protease inhibitor involved in the negative regulation of the mosquito immune response and knock-down of SRPN2 has been shown to reduce *Plasmodium* oocyst formation in the mosquito. Disappointingly, antibodies elicited in a prime-boost vaccination using ChAd63 and MVA expressing AgSRPN2 were only able to modestly inhibit oocyst formation in a *P. berghei* model and not at all when tested against *P. falciparum*.

Taken together, these preclinical studies make a convincing argument for a promising future of the adeno-MVA platform in the development of transmission-blocking vaccines.

MVA in clinical trials

One of the overarching challenges in the development of an effective malaria vaccine has been the translation of oftentimes very favorable immunogenicity and protection in mouse models into protective human responses. No definitive correlates of protection have been established for humans, but from previous and ongoing clinical trials it has become apparent that exceptionally high T-cell and antibody levels will be needed for vaccine efficiency.

Initial clinical trials of heterologous prime-boost vaccinations focused on the CS antigen and included priming with DNA or an attenuated fowlpox strain (FP9) and boosting with rMVA. Vaccine regimens were consistently found to be safe, but only elicited modest T-cell responses and afforded no protection upon controlled human malaria infection (sporozoite challenge).[46–48] rMVA encoding CS was also assessed as a possible boosting agent for the subunit vaccine RTS,S, but no augmentation of vaccine efficacy was observed, with protection levels no greater than those seen with RTS,S alone.[49] METRAP, in contrast, was found to be a better immunogen and early heterologous DNA- or FP9-prime, MVA-boost

schedules were able to elicit strong T-cell responses in both malaria-naïve adults and volunteers in endemic countries. Complete protection was observed in some UK volunteers, but efficacy in the field was disappointingly low. The FP9/MVA prime-boost platform was also tested with a polyprotein antigen consisting of a fusion of six *P. falciparum* proteins from different life cycle stages (liver-stage antigen-3, sporozoite threonine and asparagine rich protein, exported protein 1, Pfs16, TRAP and LSA1), but a sporozoite challenge of volunteers unfortunately showed no efficacy of the vaccine.[50] In addition to MVA, one alternative attenuated vaccinia strain has also been used in clinical trials as a vaccine against malaria: the NYVAC strain expressing seven *P. falciparum* genes covering all parasite life cycle stages (CSP, PfSPP2, LSA1, MSP1, SERA, AMA1, Pfs25) was tested in a regimen of three immunizations (at 0, 4 and 26 weeks) in malaria-naïve volunteers, some of whom were subsequently exposed to infected mosquito bites for efficacy testing.[51] This first multistage viral-vectored vaccine against malaria was well tolerated but only elicited disappointingly low antibody titers and CTL responses against pre-erythrocytic antigens were only detected in half of the volunteers. Upon sporozoite challenge of 35 volunteers, complete protection was observed in one case and a delay to parasitemia in the other vaccinated volunteers.

More recently, DNA or FP9 priming agents have been replaced by a priming immunization with recombinant simian adenoviruses. Based on promising preclinical results, the new adeno-MVA platform was first assessed in humans in 2007. Only several years later, ChAd63 and MVA encoding malaria antigens are now regularly advanced into Phase I/II clinical trials, with a total of 21 ongoing or completed trials to date, covering both pre-erythrocytic as well as blood-stage vaccines (see Table 2). The vaccination schedule using this platform typically consists of an adenoviral priming immunization of 5×10^{10} viral particles given intramuscularly (i.m.), followed by 2×10^8 plaque-forming units of rMVA (i.m.) 8 weeks later. If part of the trial, efficacy assessment in the form of controlled human malaria infection generally takes place 2 weeks post-boost. T-cell responses are routinely evaluated via *ex vivo* IFN- γ enzyme-linked immunospot (ELISPOT) and intracellular cytokine staining (ICS), while antibodies are characterized using ELISAs. Clinical trials are generally performed according to a strategy whereby an initial Phase I safety and immunogenicity study in UK volunteers is followed by a small efficacy study also in malaria-naïve volunteers. Only then will larger Phase II trials be conducted in adults in malaria endemic areas. If immunogenic and efficacious in these trials, the vaccine will finally be tested in the target population. For a pre-erythrocytic malaria vaccine, for example, this population consists of young infants and children in malaria endemic areas and vaccination should ideally take place before their first malaria episode.

In a head-to-head comparison of CS and METRAP in the ChAd63-MVA regime in UK volunteers, the latter was found to be more immunogenic and provided better protective efficacy in Phase I sporozoite challenge studies [52] resulting in an

intensified focus on METRAP as the leading pre-erythrocytic vaccine candidate. In the past 5 years, ChAd63-MVA encoding METRAP has therefore been extensively assessed, in naïve adult volunteers in the UK as well as in field trials in Burkina Faso, Senegal, The Gambia, Kenya, in both adults and children. In an initial Phase I route- and dose-finding study in malaria-naïve volunteers, the ChAd63-MVA vaccine was well tolerated and induced IFN- γ -secreting T-cells at an average of between 1000–2000 spot-forming units (SFU) per 10^6 peripheral blood mononuclear cells (PBMC) with intramuscular administration.[53] Interestingly, when some volunteers were re-boosted with the same MVA an average of 33 weeks after the last immunization, a significant increase in ELISPOT responses was observed compared to pre-boosting levels. This result is noteworthy in that the use of MVA as a booster does not seem to induce an anti-vector immune response strong enough to prevent further boosting at a later time point, implying that the adenovirus-MVA platform has a potential for use in multiple unrelated vaccine regimens in the future. ChAd63-MVA encoding METRAP was subsequently assessed in five further published and nine more ongoing or unpublished clinical trials. In an encouraging Phase II efficacy study, three out of 14 vaccinees were sterilely protected when exposed to *P. falciparum*-infected mosquito bites and five more exhibited a delay to blood-stage parasitemia.[54] IFN- γ -producing T-cells were correlated with full or partial protection and reached median levels of >2400 SFU/ 10^6 PBMCs. The same vaccination regimen was then tested in malaria endemic areas (Kenya and The Gambia). It was found to be safe and immunogenic, inducing a median of >1300 SFU/ 10^6 PBMC.[55] In a subsequent Phase IIb assessment of efficacy in Kenyan adults, vaccination reduced the risk of infection by 67% during 8 weeks of monitoring (from first immunization to 2 weeks after the final vaccination).[56] TRAP-specific CD8⁺ T-cells and not antibodies, were again correlated with protective efficacy. Vaccine-induced partial protection in the field as seen in this trial warranted further trials to confirm the results in adults and extend the findings to infants and children. These trials are now underway or have recently been completed.

Since 2010, the ChAd63-MVA vaccine platform is also being tested in clinical trials of the blood-stage vaccine candidates AMA1 and MSP1. In an initial Phase I safety and immunogenicity study in malaria naïve adults, biallelic AMA1 constructs (representing *P. falciparum* strains 3D7 and FVO) expressed from ChAd63 and MVA vectors were well tolerated and induced high-level T-cell responses (median between 1500 and 2000 SFU/ 10^6 PBMC) and antibodies with growth-inhibitory activity.[57] Notably, antibody levels were comparable to those previously observed with AMA1 protein vaccines in alum or Montanide ISA720, but lower than alum + CpG. MSP1 as an immunogen was subsequently tested in an equivalent study and even though very strong T-cell responses were recorded (median >5000 SFU/ 10^6 PBMC), the antibody levels elicited against MSP1 failed to neutralize parasites *in vitro*. [58] Based on encouraging preclinical studies and in the hope of inducing

Table 2. Clinical trials of malaria vaccines containing rMVA, from 2005.

Trial title	Trial number	Phase	Antigen in MVA	Start	Reference
A Safety and Efficacy Study of Concomitant Administration of ChAd63/ MVA ME-TRAP + RTS,S	VAC059	I/IIa	ME-TRAP	January 2015	[90]
A Phase I Study to Assess the Safety and Immunogenicity of ChAd63 ME-TRAP – MVA ME-TRAP Heterologous Prime-boost Vaccination Co-administered with EPI Vaccines in Gambian Infants	VAC058	I	ME-TRAP	February 2014	[91]
A Phase Ia Clinical Trial to Assess the Safety and Immunogenicity of New <i>Plasmodium falciparum</i> Malaria Vaccine Candidates ChAd63 RH5 alone and with MVA RH5	VAC057	Ia	RH5	August 2014	[92]
A Safety and Efficacy Study of ChAd63/MVA METRAP + RTS,S	VAC055	I/IIa	ME-TRAP	September 2013	[93]
A Phase I/IIa Sporozoite Challenge Study to Assess the Efficacy of Candidate Combination Malaria Vaccine Approaches Using the ChAd63 and MVA Vectors Encoding the Antigens ME-TRAP, CS and AMA1	VAC052	I/IIa	ME-TRAP, CS, AMA1	January 2013	[94]
Phase Ia Study of ChAd63/MVA PvDBP	VAC051	Ia	PvDBP	April 2013	[95]
A Phase 1/2b Study of an Investigational Malaria Vaccination Strategy in 5–17 Month Old Infants and Children in Burkina Faso	VAC050	I/IIb	ME-TRAP	November 2012	[96]
Adjuvanting Viral Vectored Malaria Vaccines with Matrix M	VAC048	I	ME-TRAP	August 2012	[97]
Efficacy of Candidate Malaria Vaccines in Senegalese Adults	VAC047	IIb	ME-TRAP	August 2012	[98]
Efficacy of Malaria Vaccines in Kenyan Adults	VAC046	IIb	ME-TRAP	March 2012	[56]
A Challenge Study to Assess the Protective Efficacy of Two Malaria Vaccine Candidates (VAC045)	VAC045	I/IIa	ME-TRAP, CS	April 2012	[52]
Safety and Immunogenicity of Malaria Vaccines AdCh63 AMA1, MVA AMA1 and AMA1-C1/Alhydrogel®± CPG 7909	VAC044	Ia	AMA1	June 2011	[59]
Safety and Immunogenicity of Novel Vaccination Schedules with Malaria Vaccines AdCh63 ME-TRAP and MVA ME-TRAP	VAC043	I	ME-TRAP	July 2011	[99]
AdCh63 ME-TRAP and MVA ME-TRAP Malaria Vaccines Evaluation in Healthy Children in a Malaria Endemic Area	VAC042	I	ME-TRAP	October 2011	[100]
AdCh63 ME-TRAP and MVA ME-TRAP Malaria Vaccines Evaluation in Healthy Adults and Children in a Malaria Endemic Area	VAC041	I	ME-TRAP	June 2010	[55,101]
Safety and Immunogenicity of AdCh63 ME-TRAP and MVA ME-TRAP Vaccines in Malaria Endemic Areas	VAC040	I	ME-TRAP	June 2010	[55,101]
Study to Assess Efficacy of New Malaria Vaccine Candidates AdCh63 AMA1, MVA AMA1, AdCh63 MSP1, MVA MSP1, AdCh63 ME-TRAP & MVA ME-TRAP	VAC039	I/IIa	AMA1, MSP1, ME-TRAP	June 2010	[60,102,103]
Safety and Immunogenicity of New Malaria Vaccine Candidate ChAd63 CS Administered alone and with MVA CS	VAC038	Ia	CS	December 2011	[104]
A Study of the Safety and Effectiveness of Two New Malaria Vaccines	VAC037	I/IIa	MSP1	November 2009	[58,60,102]
A Study of AdCh63 AMA1 alone and with MVA AMA1	VAC036	Ia	AMA1	March 2010	[57,102]
A Study to Assess the Effectiveness of a New Malaria Vaccine Candidate by Infecting Vaccinated Volunteers with Malaria Parasites	MAL034	II	ME-TRAP	March 2009	[54,101–103]
A Study of AdCh63 ME–TRAP alone and with MVA ME–TRAP	VAC033	I	ME-TRAP	July 2007	[53]
Efficacy of Combined PEV3A Virosomal Vaccine and FP9-MVA ME-TRAP Prime Boost Regimen	VAC030	I/IIa	ME-TRAP	August 2005	[105]

(continued)

Table 2. (continued).

Trial title	Trial number	Phase	Antigen in MVA	Start	Reference
Safety and Immunogenicity Study of the Malaria Vaccines FP9 PP and MVA PP	VAC027.1	I	Polyprotein	April 2006	[50]
Sporozoite Challenge of Polyprotein Vaccinees	VAC027.2	II	Polyprotein	September 2006	[50]

even higher functional antibodies, a mixed modality vaccine schedule targeting AMA1 was trialled: the ChAd63–MVA platform was combined with a subsequent protein-in-adjuvant boost.[59] A strong antibody response was indeed observed for this triple-immunization schedule, but leaving out the MVA boost (i.e. a ChAd63–prime protein-boost only regimen) elicited the same levels of antibodies and higher levels than ChAd63–MVA alone. Therefore, while the inclusion of an MVA boost did result in the highest T-cell response as expected, MVA did not have an additional enhancing effect on antibody levels.

In order to assess immune interference, another clinical trial assessed the co-administration of a two blood-stage (AMA1 and MSP1) or a blood-stage and a pre-erythrocytic (MSP1 and METRAP) vaccine using the ChAd63–MVA delivery platform.[60] Similar previous preclinical studies in mice had reported immune interference which could however be minimized by separate-site injection of the two vaccines.[41] Disappointingly, in the human clinical trial, investigators still found reduced immune responses despite contralateral arm vaccination, with MSP1-specific responses dominating over AMA1 and METRAP. Upon sporozoite challenge by mosquito bite, no statistically significant efficacy was observed in any vaccinee group, emphasizing the need to further explore mechanisms of immune interference for future multicomponent vaccines. Of additional interest was the finding that despite the induction of considerable cellular immunity against AMA1 and MSP1, there was no significant impact on post-challenge parasite growth during the blood-stage, suggesting that a focus on inducing high-level blood-stage antibodies is a more advisable approach.

Last but not least, after having discussed the adenoviral–MVA delivery platform in the context of pre-erythrocytic and blood-stage clinical trials, it is of interest to note that a clinical trial of ChAd63–MVA encoding the transmission blocking vaccine candidate Pfs25 fused to the multimerization domain IMX313 is slated to start in late 2015.

Expert commentary

The adenovirus prime–MVA boost regime has in recent years become recognized as a leading vaccine delivery platform. Initially developed for induction of strong T-cell responses against intracellular pathogens, it is also capable of eliciting substantial antigen-specific antibody responses and is now being used in clinical vaccine trials against malaria, HIV, influenza and tuberculosis. MVA has persistently been found to be

an excellent heterologous boosting agent and any anti-MVA vector immune response does not prevent homologous re-boosting of the antigen-specific immune response after 6 months. Additional advantages of MVA as a vaccine vector are its excellent safety profile, its large capacity for antigen cargo and its genetic stability. Advances in production methods now mean that MVA can be manufactured in large quantities to GMP standards, in a chemically defined and robust process.

Significant progress has been made in recent years in the development of malaria vaccines based on the ChAd63–MVA prime-boost regime. While efforts are focussed on the leading pre-erythrocytic vaccine candidate METRAP, new candidate antigens (such as LSA1 and LSAP2) are also in the preclinical pipeline. Based on promising immunogenicity data in mice and nonhuman primates, the blood-stage candidate RH5 is also being progressed to the clinic, along with the transmission-blocking candidate Pfs25 and the *P. vivax* DBP antigen. Ultimately, an effective malaria vaccine will contain antigens from multiple life cycle stages of the parasite. While it is imperative that each antigen is first validated independently in prime-boost regimens as is currently the case, its large cargo capacity makes MVA an ideal vector for such a multi-stage, multi-antigen malaria vaccine.

Five-year view

Despite the significant milestone recently reached by the RTS,S-based Mosquirix™, it is unlikely that an extensive roll-out of this vaccine will take place in the next 5 years. It is therefore important that efforts will continue to develop alternative, potentially more efficacious malaria vaccines. Within the next 5 years, results from the latest adenoviral–MVA clinical trials will become available, specifically from those assessing METRAP in Gambian infants and the new blood-stage candidate RH5 and transmission-blocking candidate Pfs25, in UK volunteers. This and further studies with alternative immunogens (such as LSA1 and LSAP2), will provide us with an indication of which antigens to combine in a multi-stage multi-component vectored vaccine. Preclinical testing of such a vaccine will likely begin within this time period. Additionally, efforts are underway to improve the administration schedule of adeno-MVA prime-boost regimens, with regard to timing and combination of injections. Overall, therefore, the next 5 years will bring valuable data and insight into the future shape of an effective malaria vaccine.

Financial & competing interests disclosure

Both authors are employed by the University of Oxford. SC Gilbert is a named inventor on patent filings related to immunization with vectored vaccines, specifically W02008/122769. The authors have no other

relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

Key issues

- the attenuated poxviral vaccine vector MVA is safe and highly immunogenic in heterologous prime-boost regimens.
- recombinant MVA can be manufactured at large scale with recently improved production processes.
- adenoviral–MVA prime-boost vaccines induce high T-cell responses and significant antibody-titers and are being used in clinical trials for diseases such as malaria, influenza, tuberculosis and HIV.
- malaria immunogens assessed in the clinic cover multiple stages in the parasite life cycle (pre-erythrocytic, blood-stage and mosquito-stage).
- METRAP is the most advanced liver-stage antigen and is currently being tested in an adenoviral–MVA regime in the target population (infants in malaria endemic areas).

References

Papers of special note have been highlighted as:

* of interest

** of considerable interest

- World Malaria Report. World Health Organization. 2014 [cited 2015 Aug 25]. Available from: http://www.who.int/malaria/publications/world_malaria_report_2014/en/.
- Miller LH, Ackerman HC, Su XZ, et al. Malaria biology and disease pathogenesis: insights for new treatments. *Nat Med*. 2013;19(2):156–167.
- Kirkman LA, Deitsch KW. Antigenic variation and the generation of diversity in malaria parasites. *Curr Opin Microbiol*. 2012;15(4):456–462.
- Bolad A, Berzins K. Antigenic diversity of *Plasmodium falciparum* and antibody-mediated parasite neutralization. *Scand J Immunol*. 2000;52(3):233–239.
- Olotu A, Lusingu J, Leach A, et al. Efficacy of RTS,S/AS01E malaria vaccine and exploratory analysis on anti-circumsporozoite antibody titres and protection in children aged 5–17 months in Kenya and Tanzania: a randomised controlled trial. *Lancet Infect Dis*. 2011;11(2):102–109.
- Douglas AD, Baldeviano GC, Lucas CM, et al. A PfrH5-based vaccine is efficacious against Heterologous strain blood-stage *Plasmodium falciparum* infection in *Aotus* monkeys. *Cell Host Microbe*. 2015;17(1):130–139.
- Biswas S, Spencer AJ, Forbes EK, et al. Recombinant viral-vectored vaccines expressing *Plasmodium chabaudi* AS apical membrane antigen 1: mechanisms of vaccine-induced blood-stage protection. *J Immunol*. 2012;188(10):5041–5053.
- Douglas AD, de Cassan SC, Dicks MD, et al. Tailoring subunit vaccine immunogenicity: maximizing antibody and T cell responses by using combinations of adenovirus, poxvirus and protein-adjuvant vaccines against *Plasmodium falciparum* MSP1. *Vaccine*. 2010;28(44):7167–7178.
- Romero P, Maryanski JL, Corradin G, et al. Cloned cytotoxic T cells recognize an epitope in the circumsporozoite protein and protect against malaria. *Nature*. 1989;341(6240):323–326.
- First study to show a correlation between a vaccine-induced T-cell response and protection against malaria in mice.**
- Todryk SM, Bejon P, Mwangi T, et al. Correlation of memory T cell responses against TRAP with protection from clinical malaria, and CD4 CD25 high T cells with susceptibility in Kenyans. *PLoS One*. 2008;3(4):e2027.
- Mayr A, Hochstein-Mintzel V, Stickl H. Abstammung, Eigenschaften und Verwendung des attenuierten Vaccinia-Stammes MVA. *Infection*. 1975;3:6–14.
- Publication describing the derivation of MVA from vaccinia virus.**
- Mayr A, Stickl H, Müller HK, et al. The smallpox vaccination strain MVA: marker, genetic structure, experience gained with the parenteral vaccination and behavior in organisms with a debilitated defence mechanism (author's transl). *Zentralbl Bakteriol B*. 1978;167(5–6):375–390. German.
- Overton ET, Stapleton J, Frank I, et al. Safety and immunogenicity of modified vaccinia Ankara-Bavarian Nordic smallpox vaccine in vaccinia-naïve and experienced human immunodeficiency virus-infected individuals: an open-label, controlled clinical phase II trial. *Open Forum Infect Dis*. 2015;2(2):ofv040.
- Bronte V, Carroll MW, Goletz TJ, et al. Antigen expression by dendritic cells correlates with the therapeutic effectiveness of a model recombinant poxvirus tumor vaccine. *Proc Natl Acad Sci U S A*. 1997;94(7):3183–3188.
- Baur K, Brinkmann K, Schweneker M, et al. Immediate-early expression of a recombinant antigen by modified vaccinia virus Ankara breaks the immunodominance of strong vector-specific B8R antigen in acute and memory CD8 T-cell responses. *J Virol*. 2010;84(17):8743–8752.
- Jordan I, Northoff S, Thiele M, et al. A chemically defined production process for highly attenuated poxviruses. *Biologicals*. 2011;39(1):50–58.
- Jordan I, Vos A, Beilfuss S, et al. An avian cell line designed for production of highly attenuated viruses. *Vaccine*. 2009;27(5):748–756.
- Kraus B, von Fircks S, Feigl S, et al. Avian cell line: technology for large scale vaccine production. *BMC Proc*. 2011;5(Suppl 8):P52.

19. Brown SW, Mehtali M. The avian EB66(R) cell line, application to vaccines, and therapeutic protein production. *PDA J Pharm Sci Technol*. 2010;64(5):419–425.
20. A Phase I study to assess Ebola vaccines cAd3-EBO Z and MVA-EBO Z. Available from: <https://clinicaltrials.gov/ct2/show/NCT02451891>.
21. Wolff MW, Siewert C, Hansen SP, et al. Purification of cell culture-derived modified vaccinia Ankara virus by pseudo-affinity membrane adsorbers and hydrophobic interaction chromatography. *Biotechnol Bioeng*. 2010;107(2):312–320.
22. Wolff MW, Siewert C, Lehmann S, et al. Capturing of cell culture-derived modified vaccinia Ankara virus by ion exchange and pseudo-affinity membrane adsorbers. *Biotechnol Bioeng*. 2010;105(4):761–769.
23. Alcock R, Cottingham MG, Rollier CS, et al. Long-term thermostabilization of live poxviral and adenoviral vaccine vectors at supraphysiological temperatures in carbohydrate glass. *Sci Transl Med*. 2010;2(19):19ra12.
24. Schneider J, Gilbert SC, Blanchard TJ, et al. Enhanced immunogenicity for CD8+ T cell induction and complete protective efficacy of malaria DNA vaccination by boosting with modified vaccinia virus Ankara. *Nat Med*. 1998;4(4):397–402.
- ** First use of recombinant MVA as a vaccine vector against malaria in mice.**
25. Gilbert SC, Schneider J, Plebanski M, et al. Ty virus-like particles, DNA vaccines and modified vaccinia virus Ankara: comparisons and combinations. *Biol Chem*. 1999;380(3):299–303.
26. Gilbert SC, Schneider J, Hannan CM, et al. Enhanced CD8 T cell immunogenicity and protective efficacy in a mouse malaria model using a recombinant adenoviral vaccine in heterologous prime-boost immunisation regimes. *Vaccine*. 2002;20(7–8):1039–1045.
27. Gonzalez-Aseguinolaza G, Nakaya Y, Molano A, et al. Induction of protective immunity against malaria by priming-boosting immunization with recombinant cold-adapted influenza and modified vaccinia Ankara viruses expressing a CD8+T-cell epitope derived from the circumsporozoite protein of *Plasmodium yoelii*. *J Virol*. 2003;77(21):11859–11866.
28. Li S, Rodrigues M, Rodriguez D, et al. Priming with recombinant influenza virus followed by administration of recombinant vaccinia virus induces CD8+ T-cell-mediated protective immunity against malaria. *Proc Natl Acad Sci U S A*. 1993;90(11):5214–5218.
29. Reyes-Sandoval A, Berthoud T, Alder N, et al. Prime-boost immunization with adenoviral and modified vaccinia virus Ankara vectors enhances the durability and polyfunctionality of protective malaria CD8+ T-cell responses. *Infect Immun*. 2010;78(1):145–153.
30. Reyes-Sandoval A, Wyllie DH, Bauza K, et al. CD8+ T effector memory cells protect against liver-stage malaria. *J Immunol*. 2011;187(3):1347–1357.
31. Capone S, Reyes-Sandoval A, Naddeo M, et al. Immune responses against a liver-stage malaria antigen induced by simian adenoviral vector AdCh63 and MVA prime-boost immunisation in non-human primates. *Vaccine*. 2010;29(2):256–265.
32. Rodriguez D, Gonzalez-Aseguinolaza G, Rodriguez JR, et al. Vaccine efficacy against malaria by the combination of porcine parvovirus-like particles and vaccinia virus vectors expressing CS of *Plasmodium*. *PLoS One*. 2012;7(4):e34445.
33. Vijayan A, Gomez CE, Espinosa DA, et al. Adjuvant-like effect of vaccinia virus 14K protein: a case study with malaria vaccine based on the circumsporozoite protein. *J Immunol*. 2012;188(12):6407–6417.
34. Spencer AJ, Hill F, Honeycutt JD, et al. Fusion of the *Mycobacterium tuberculosis* antigen 85A to an oligomerization domain enhances its immunogenicity in both mice and non-human primates. *PLoS One*. 2012;7(3):e33555.
35. Bauza K, Malinauskas T, Pfander C, et al. Efficacy of a *Plasmodium vivax* malaria vaccine using ChAd63 and modified vaccinia Ankara expressing thrombospondin-related anonymous protein as assessed with transgenic *Plasmodium berghei* parasites. *Infect Immun*. 2014;82(3):1277–1286.
36. Longley RJ, Salman AM, Cottingham MG, et al. Comparative assessment of vaccine vectors encoding ten malaria antigens identifies two protective liver-stage candidates. *Sci Rep*. 2015;5:11820.
37. Draper SJ, Goodman AL, Biswas S, et al. Recombinant viral vaccines expressing merozoite surface protein-1 induce antibody- and T cell-mediated multistage protection against malaria. *Cell Host Microbe*. 2009;5(1):95–105.
38. Xu H, Hodder AN, Yan H, et al. CD4+ T cells acting independently of antibody contribute to protective immunity to *Plasmodium chabaudi* infection after apical membrane antigen 1 immunization. *J Immunol*. 2000;165(1):389–396.
39. Goodman AL, Epp C, Moss D, et al. New candidate vaccines against blood-stage *Plasmodium falciparum* malaria: prime-boost immunization regimens incorporating human and simian adenoviral vectors and poxviral vectors expressing an optimized antigen based on merozoite surface protein 1. *Infect Immun*. 2010;78(11):4601–4612.
40. Draper SJ, Biswas S, Spencer AJ, et al. Enhancing blood-stage malaria subunit vaccine immunogenicity in rhesus macaques by combining adenovirus, poxvirus, and protein-in-adjuvant vaccines. *J Immunol*. 2010;185(12):7583–7595.
41. Forbes EK, Biswas S, Collins KA, et al. Combining liver- and blood-stage malaria viral-vectored vaccines: investigating mechanisms of CD8+ T cell interference. *J Immunol*. 2011;187(7):3738–3750.
42. de Cassan SC, Shakri AR, Llewellyn D, et al. Preclinical assessment of viral vectored and protein vaccines targeting the Duffy-binding protein Region II of *Plasmodium vivax*. *Front Immunol*. 2015;6:348.
43. Goodman AL, Blagborough AM, Biswas S, et al. A viral vectored prime-boost immunization regime targeting the malaria Pf525 antigen induces transmission-blocking activity. *PLoS One*. 2011;6(12):e29428.
44. Kapulu MC, Da DF, Miura K, et al. Comparative assessment of transmission-blocking vaccine candidates against *Plasmodium falciparum*. *Sci Rep*. 2015;5:11193.
45. Williams AR, Zakutansky SE, Miura K, et al. Immunisation against a serine protease inhibitor reduces intensity of *Plasmodium berghei* infection in mosquitoes. *Int J Parasitol*. 2013;43(11):869–874.
46. Dunachie SJ, Walther M, Epstein JE, et al. A DNA prime-modified vaccinia virus Ankara boost vaccine encoding thrombospondin-related adhesion protein but not circumsporozoite protein partially protects healthy malaria-naïve adults against *Plasmodium falciparum* sporozoite challenge. *Infect Immun*. 2006;74(10):5933–5942.

47. Walther M, Thompson FM, Dunachie S, et al. Safety, immunogenicity, and efficacy of prime-boost immunization with recombinant poxvirus FP9 and modified vaccinia virus Ankara encoding the full-length *Plasmodium falciparum* circumsporozoite protein. *Infect Immun*. 2006;74(5):2706–2716.
48. Imoukhuede EB, Berthoud T, Milligan P, et al. Safety and immunogenicity of the malaria candidate vaccines FP9 CS and MVA CS in adult Gambian men. *Vaccine*. 2006;24(42–43):6526–6533.
49. Dunachie SJ, Walther M, Vuola JM, et al. A clinical trial of prime-boost immunisation with the candidate malaria vaccines RTS,S/AS02A and MVA-CS. *Vaccine*. 2006;24(15):2850–2859.
50. Porter DW, Thompson FM, Berthoud TK, et al. A human Phase I/IIa malaria challenge trial of a polyprotein malaria vaccine. *Vaccine*. 2011;29(43):7514–7522.
51. Ockenhouse CF, Sun PF, Lanar DE, et al. Phase I/IIa safety, immunogenicity, and efficacy trial of NYVAC-Pf7, a pox-vectored, multiantigen, multistage vaccine candidate for *Plasmodium falciparum* malaria. *J Infect Dis*. 1998;177(6):1664–1673.
52. Hodgson SH, Ewer KJ, Bliss CM, et al. Evaluation of the efficacy of ChAd63-MVA vectored vaccines expressing circumsporozoite protein and ME-TRAP against controlled human malaria infection in malaria-naïve individuals. *J Infect Dis*. 2015;211(7):1076–1086.
- * **Comparison of CS and METRAP in the ChAd63-MVA regime (immunogenicity and efficacy).**
53. O'Hara GA, Duncan CJ, Ewer KJ, et al. Clinical assessment of a recombinant simian adenovirus ChAd63: a potent new vaccine vector. *J Infect Dis*. 2012;205(5):772–781.
54. Ewer KJ, O'Hara GA, Duncan CJ, et al. Protective CD8+ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. *Nat Commun*. 2013;4:2836.
55. Ogowang C, Afolabi M, Kimani D, et al. Safety and immunogenicity of heterologous prime-boost immunisation with *Plasmodium falciparum* malaria candidate vaccines, ChAd63 ME-TRAP and MVA ME-TRAP, in healthy Gambian and Kenyan adults. *PLoS One*. 2013;8(3):e57726.
56. Ogowang C, Kimani D, Edwards NJ, et al. Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against *Plasmodium falciparum* infection in Kenyan adults. *Sci Transl Med*. 2015;7(286):286re5.
57. Sheehy SH, Duncan CJ, Elias SC, et al. Phase Ia clinical evaluation of the safety and immunogenicity of the *Plasmodium falciparum* blood-stage antigen AMA1 in ChAd63 and MVA vaccine vectors. *PLoS One*. 2012;7(2):e31208.
58. Sheehy SH, Duncan CJ, Elias SC, et al. Phase Ia clinical evaluation of the *Plasmodium falciparum* blood-stage antigen MSP1 in ChAd63 and MVA vaccine vectors. *Mol Ther*. 2011;19(12):2269–2276.
59. Hodgson SH, Choudhary P, Elias SC, et al. Combining viral vectored and protein-in-adjuvant vaccines against the blood-stage malaria antigen AMA1: report on a phase Ia clinical trial. *Mol Ther*. 2014;22(12):2142–2154.
- * **Clinical trial of mixed modality malaria vaccine: ChAd63-MVA combined with protein-in-adjuvant boost.**
60. Sheehy SH, Duncan CJ, Elias SC, et al. ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. *Mol Ther*. 2012;20(12):2355–2368.
61. Menard R, Sultan AA, Cortes C, et al. Circumsporozoite protein is required for development of malaria sporozoites in mosquitoes. *Nature*. 1997;385(6614):336–340.
62. Coppi A, Natarajan R, Pradel G, et al. The malaria circumsporozoite protein has two functional domains, each with distinct roles as sporozoites journey from mosquito to mammalian host. *J Exp Med*. 2011;208(2):341–356.
63. Plassmeyer ML, Reiter K, Shimp RL Jr., et al. Structure of the *Plasmodium falciparum* circumsporozoite protein, a leading malaria vaccine candidate. *J Biol Chem*. 2009;284(39):26951–26963.
64. Ghosh AK, Devenport M, Jethwaney D, et al. Malaria parasite invasion of the mosquito salivary gland requires interaction between the *Plasmodium* TRAP and the *Anopheles saglin* proteins. *PLoS Pathog*. 2009;5(1):e1000265.
65. Sultan AA, Thathy V, Frevert U, et al. TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites. *Cell*. 1997;90(3):511–522.
66. Akhouri RR, Sharma A, Malhotra P, et al. Role of *Plasmodium falciparum* thrombospondin-related anonymous protein in host-cell interactions. *Malar J*. 2008;7:63.
67. Robson KJ, Frevert U, Reckmann I, et al. Thrombospondin-related adhesive protein (TRAP) of *Plasmodium falciparum*: expression during sporozoite ontogeny and binding to human hepatocytes. *Embo J*. 1995;14(16):3883–3894.
68. Guerin-Marchand C, Druilhe P, Galey B, et al. A liver-stage-specific antigen of *Plasmodium falciparum* characterized by gene cloning. *Nature*. 1987;329(6135):164–167.
69. Fidock DA, Gras-Masse H, Lepers JP, et al. *Plasmodium falciparum* liver stage antigen-1 is well conserved and contains potent B and T cell determinants. *J Immunol*. 1994;153(1):190–204.
70. Kurtis JD, Hollingdale MR, Luty AJ, et al. Pre-erythrocytic immunity to *Plasmodium falciparum*: the case for an LSA-1 vaccine. *Trends Parasitol*. 2001;17(5):219–223.
71. Hillier CJ, Ware LA, Barbosa A, et al. Process development and analysis of liver-stage antigen 1, a preerythrocyte-stage protein-based vaccine for *Plasmodium falciparum*. *Infect Immun*. 2005;73(4):2109–2115.
72. Mikolajczak SA, Sacci JB Jr., De La Vega P, et al. Disruption of the *Plasmodium falciparum* liver-stage antigen-1 locus causes a differentiation defect in late liver-stage parasites. *Cell Microbiol*. 2011;13(8):1250–1260.
73. Siau A, Silvie O, Franetich JF, et al. Temperature shift and host cell contact up-regulate sporozoite expression of *Plasmodium falciparum* genes involved in hepatocyte infection. *PLoS Pathog*. 2008;4(8):e1000121.
74. Holder AA, Blackman MJ, Burghaus PA, et al. A malaria merozoite surface protein (MSP1)-structure, processing and function. *Mem Inst Oswaldo Cruz*. 1992;87(Suppl 3):37–42.
75. Blackman MJ, Heidrich HG, Donachie S, et al. A single fragment of a malaria merozoite surface protein remains on the parasite during red cell invasion and is the target of invasion-inhibiting antibodies. *J Exp Med*. 1990;172(1):379–382.
76. Sacci JB Jr., Ribeiro JM, Huang F, et al. Transcriptional analysis of in vivo *Plasmodium yoelii* liver stage gene expression. *Mol Biochem Parasitol*. 2005;142(2):177–183.

77. Silvie O, Franetich JF, Charrin S, et al. A role for apical membrane antigen 1 during invasion of hepatocytes by *Plasmodium falciparum* sporozoites. *J Biol Chem.* 2004;279(10):9490–9496.
78. Pizarro JC, Vulliez-Le Normand B, Chesne-Seck ML, et al. Crystal structure of the malaria vaccine candidate apical membrane antigen 1. *Science.* 2005;308(5720):408–411.
79. Remarque EJ, Faber BW, Kocken CH, et al. Apical membrane antigen 1: a malaria vaccine candidate in review. *Trends Parasitol.* 2008;24(2):74–84.
80. Mitchell GH, Thomas AW, Margos G, et al. Apical membrane antigen 1, a major malaria vaccine candidate, mediates the close attachment of invasive merozoites to host red blood cells. *Infect Immun.* 2004;72(1):154–158.
81. Bargieri DY, Andenmatten N, Lagal V, et al. Apical membrane antigen 1 mediates apicomplexan parasite attachment but is dispensable for host cell invasion. *Nat Commun.* 2013;4:2552.
82. Wright KE, Hjerrild KA, Bartlett J, et al. Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature.* 2014;515(7527):427–430.
83. Rodriguez M, Lustigman S, Montero E, et al. PfrRH5: a novel reticulocyte-binding family homolog of *Plasmodium falciparum* that binds to the erythrocyte, and an investigation of its receptor. *PLoS One.* 2008;3(10):e3300.
84. Baum J, Chen L, Healer J, et al. Reticulocyte-binding protein homologue 5 - an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J Parasitol.* 2009;39(3):371–380.
85. van Dijk MR, Janse CJ, Thompson J, et al. A central role for P48/45 in malaria parasite male gamete fertility. *Cell.* 2001;104(1):153–164.
86. Gerloff DL, Creasey A, Maslau S, et al. Structural models for the protein family characterized by gamete surface protein Pfs230 of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A.* 2005;102(38):13598–13603.
87. Eksi S, Czesny B, van Gemert GJ, et al. Malaria transmission-blocking antigen, Pfs230, mediates human red blood cell binding to exflagellating male parasites and oocyst production. *Mol Microbiol.* 2006;61(4):991–998.
88. Tomas AM, Margos G, Dimopoulos G, et al. P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *Embo J.* 2001;20(15):3975–3983.
89. Baton LA, Ranford-Cartwright LC. Do malaria ookinete surface proteins P25 and P28 mediate parasite entry into mosquito midgut epithelial cells? *Malar J.* 2005;4:15.
90. A safety and efficacy study of concomitant administration of ChAd63/MVA ME-TRAP + RTS,S. Available from: <https://clinicaltrials.gov/ct2/show/NCT02252640>.
91. A Phase I study to assess the safety and immunogenicity of ChAd63 ME-TRAP - MVA ME-TRAP heterologous prime-boost vaccination co-administered with EPI vaccines in Gambian infants. Available from: <https://clinicaltrials.gov/ct2/show/NCT02083887>.
92. A Phase Ia clinical trial to assess the safety and immunogenicity of new *Plasmodium falciparum* malaria vaccine candidates ChAd63 RH5 alone and with MVA RH5. Available from: <https://clinicaltrials.gov/ct2/show/NCT02181088>.
93. A safety and efficacy study of ChAd63/MVA METRAP + RTS,S. Available from: <https://clinicaltrials.gov/ct2/show/NCT01883609>.
94. A Phase I/IIa sporozoite challenge study to assess the efficacy of candidate combination malaria vaccine approaches using the ChAd63 and MVA vectors encoding the antigens ME-TRAP, CS and AMA1. Available from: <https://clinicaltrials.gov/ct2/show/NCT01739036>.
95. Phase Ia study of ChAd63/MVA PvDBP. Available from: <https://clinicaltrials.gov/ct2/show/NCT01816113>.
96. A Phase 1/2b study of an investigational malaria vaccination strategy in 5-17 month old infants and children in Burkina Faso. Available from: <https://clinicaltrials.gov/ct2/show/NCT01635647>.
97. Adjuvanting viral vectored malaria vaccines with Matrix M. Available from: <https://clinicaltrials.gov/ct2/show/NCT01669512>.
98. Efficacy of candidate malaria vaccines in Senegalese adults. Available from: <https://clinicaltrials.gov/ct2/show/NCT01658696>.
99. Safety and immunogenicity of novel vaccination schedules with malaria vaccines AdCh63 ME-TRAP and MVA ME-TRAP. Available from: <https://clinicaltrials.gov/ct2/show/NCT01364883>.
100. AdCh63 ME-TRAP and MVA ME-TRAP malaria vaccines evaluation in healthy children in a malaria endemic area. Available from: <https://clinicaltrials.gov/ct2/show/NCT01450293>.
101. Kimani D, Jagne YJ, Cox M, et al. Translating the immunogenicity of prime-boost immunization with ChAd63 and MVA ME-TRAP from malaria naive to malaria-endemic populations. *Mol Ther.* 2014;22(11):1992–2003.
102. Biswas S, Choudhary P, Elias SC, et al. Assessment of humoral immune responses to blood-stage malaria antigens following ChAd63-MVA immunization, controlled human malaria infection and natural exposure. *PLoS One.* 2014;9(9):e107903.
103. Elias SC, Collins KA, Halstead FD, et al. Assessment of immune interference, antagonism, and diversion following human immunization with biallelic blood-stage malaria viral-vectored vaccines and controlled malaria infection. *J Immunol.* 2013;190(3):1135–1147.
104. De Barra E, Hodgson SH, Ewer KJ, et al. A phase Ia study to assess the safety and immunogenicity of new malaria vaccine candidates ChAd63 CS administered alone and with MVA CS. *PLoS One.* 2014;9(12):e115161.
105. Thompson FM, Porter DW, Okitsu SL, et al. Evidence of blood stage efficacy with a virosomal malaria vaccine in a phase IIa clinical trial. *PLoS One.* 2008;3(1):e1493.