1.1 General introduction

Adapted from: Recent progress in understanding parasite biology, pathology and disease in malaria: translation of knowledge into novel malaria vaccines.

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Res. Adv. in Virology 2, 2007.

INTRODUCTION

Globally, more than three billion people live under the threat of malaria, over one million of whom, mostly young children, succumb to the disease annually. Roughly 60% of clinical cases and over 80% of malaria-related deaths occur in sub-Saharan Africa. Despite the effectiveness of DDT for mosquito control and the availability of anti-malarial drugs, the combination of which effectively contributed to controlling malaria in large parts of Europe, the USA and Northern Australia in the 1960's, in sub-Saharan Africa, as the numbers above suggest, it is far from under control. In fact the situation across the region has progressively worsened in recent years, largely, it is thought, due to the emergence and rapid spread of drug-resistant parasites as well as insecticide-resistant mosquitoes. The gradual loss of effective anti-malarial treatments, combined with lack of timely and adequate investment in alternatives, mean that an effective malaria vaccine has never before been in such great demand. This chapter summarizes recent insights gained into the parasite biology; pathology, host defense mechanisms and disease associated with malaria, and further describe how the advances in these fields have impacted on current thinking on vaccine design. The chapter first describes the parasite life cycle linked to both parasite genomics and proteomics, and their relationships to host defense. The chapter continues with a summary of results obtained thus far in diverse pre-clinical models and early clinical trials using live recombinant malaria vaccines, considered highly promising as vaccine carriers in the fight against the parasite causing malaria.

Parasite species, life cycle, and malaria disease

The parasites causing malaria (*Plasmodium spp.*) belong to the group of the so-called Apicomplexa that includes a number of pathogenic species such as *Toxoplasma*, *Cryptosporidum*, *Eimeria, Babesia* and *Theileria*, all of which are obligate intracellular pathogens descending from a common ancestor. The term Apicomplexa originates from the observation that these parasites, that grow and replicate in a membrane-bound compartment in the host cell called the non-phagosomal parasitophorous vacuole [1], contain a very characteristic feature visible under the electron microscope, namely the presence of a group of organelles localized at the apical end of the parasite. As shown in figure 1, this apicomplex includes a number of organelles including micronemes, rhoptries, the apical polar rings that are composed of microtubules, and in some Apicomplexa a conoid.

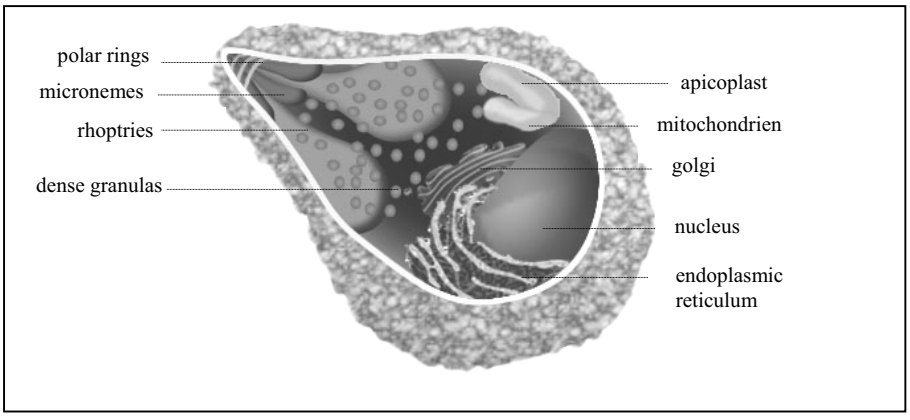

Fig. 1: Schematic representation of the internal structure of *Plasmodium spp*.

The rhoptries, micronemes and dense granules are defined as secretory organelles containing proteins required for mobility and adhesion as well as proteins that contribute to host cell invasion and host cell modification [2]. The genus *Plasmodium* includes around 200 known species able to infect amphibians, reptiles, birds and mammals [3]. The four species causing malaria in humans are *P. falciparum*, *P. vivax*, *P. ovale*, and *P. malariae*. These species, although related, are clearly different in for instance their preference for host red blood cells (RBC), thereby resulting in distinct disease patterns and clinical manifestations. For example, *P. vivax* infects reticulocytes whereas *P. falciparum* invades all RBC. This difference has a significant impact on the severity of clinical symptoms caused by these parasites, since *P.vivax* infection seldom results in death whereas *P. falciparum* accounts for almost all malaria associated death. Next to the human-specific apicomplexa, there are many other species that infect other hosts. For instance, more than 29 species of plasmodia have been identified in monkeys, several of which appear to be closely related to the human species based on their disease characteristics. This close relationship has even resulted in rare cases of symptomatic infection in humans as a result of either experimental, accidental or natural infections with a number of simian species such as *P. simium*, *P. brasilianum*, *P. cynomolgi*, *P. inui* and *P. knowlesi* [4,5]. Understanding the biology, pathology and disease patterns of simian *Plasmodium* species has proven helpful in advancing knowledge of the human plasmodia. *P. cynomolgi*, for example, closely resembles *P. vivax*. Similarly, although they do not faithfully reproduce all the characteristics of human malaria, the use of experimental models with murine *Plasmodium* species has also significantly contributed to a better understanding of parasite biology and the pathology of the disease. Four species of plasmodia are used for experimental infection of mice: *P. yoelii, P. berghei, P chabaudi and P. vinckei*. In inbred laboratory strains of mice, infections can be acute and either lethal or self-curing depending on the combination of plasmodial species and mouse strain used and the murine models are therefore utilized to investigate different aspects of infection. For instance, *P. chabaudi* is useful as a model for investigation of mechanisms of drug resistance since this parasite displays antigenic variation during long lasting, non-lethal infections in rodents. In contrast, *P. berghei* infections are more virulent with lethal outcome and are often used to study experimental cerebral malaria (see later). *P. berghei* has also proven to be an excellent model for research on the developmental biology of plasmodia, whereas *P. yoelii* is extensively used to study the biology of liver and blood stage antigens and their roles in induction of immunity allied to vaccine development. Knowledge of reptilian, avian, and amphibian plasmodia, on the other hand, is comparatively sparse. Elucidating host-parasite interactions in these species could well further contribute to our fundamental understanding of *Plasmodium* parasite biology in general. Since *P. falciparum* accounts for the vast majority of malaria-associated human disease and death, this chapter will focus on knowledge of this particular parasite. A schematic overview of the life cycle of *P. falciparum* is depicted in figure 2. Injection of sporozoites (ca. 10-100 per mosquito) into the skin via the bite of an infected female *Anopheles* mosquito initiates human infection.
Within minutes, sporozoites are carried through the blood stream to the liver where they invade liver cells (hepatocytes). An intracellular differentiation and maturation process ensues with the development of hepatic (exo-erythrocytic) schizonts that rupture after 6-7 days, releasing 20.000-30.000 merozoites per original sporozoite into the bloodstream, each of which is capable of invading circulating RBC [6]. Merozoite development continues within the circulating RBC, during which time the different maturation stages rings, trophozoites, and schizonts can be observed simultaneously. When schizonts rupture, newly formed merozoites are released that invade fresh uninfected RBC, with a consequent and sometimes severe loss of circulating and newly-formed RBC resulting in anemia (see later).

Responding to as yet unknown cues, some merozoites differentiate into male or female gametocytes, the sexual stages that are taken up by a mosquito during a blood meal.

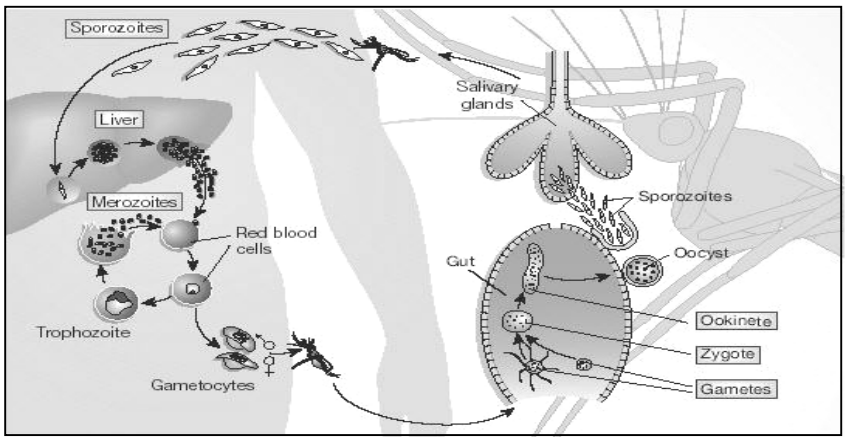

Fig. 2: *Plasmodium* parasite life cycle (Nature; Menard et. al., 2005).

Within the mosquito midgut gametocytes emerge from RBC in response to specific signals that include the drop in temperature, a rise in pH and exposure to mosquito factors such as xanthurenic acid [7-9]. Exflagellation of male gametocytes, whereby they develop thread-like structures, ensues with subsequent fertilization of female gametocytes, leading to a diploid zygote. Within a day, the zygote develops into a so-called motile ookinete, which is able to migrate through the mosquito midgut wall to form an extra cellular oocyst on the external surface. Mitotic division over a period of a few days results in the formation of thousands of sporozoites that migrate to the salivary glands of the mosquito, where they remain ready to infect a human during the mosquito's next blood meal. The parasite life cycle thus reveals that RBC are the cells predominantly affected, thus helping to explain the variety of clinical symptoms associated with malaria, since the hepatocytic phase is clinically silent. The first clinical symptoms of malaria are non-specific and include headache and muscle aches, lost of appetite, nausea, sweating and fever. These early symptoms pose difficulties in early clinical diagnosis as they mimic common diseases such as influenza. In untreated malaria, cycles of sudden coldness followed by rigor, fever and sweating lasting four to six hours typically recur every 1-3 day. These episodes correlate with RBC lysis due to the synchronous multiplication and bursting of schizonts. RBC lysis is associated with two distinct host outcomes. First, the loss of RBC is so profound that it results in anemia [10-12]. Anemia results from direct destruction both of parasite-infected (iRBC) and uninfected RBC (haemolysis), but suppression of the bone marrow's capacity to produce new RBC also contributes to it [13,14]. Severe loss of RBC is associated with oxygen deprivation of organs, leading also to severe disturbance in the body's acid-base balance (metabolic acidosis) and abnormally low levels of glucose [15]. This disease state can lead to organ failure and subsequent death within days. Second, the rupture of iRBC to release newly-formed merozoites is accompanied by the release of a host of RBC- and parasite-derived molecules. Some of these molecules can activate host cells (e.g. macrophages) that then secrete pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) as well as oxygen free radicals. In severe cases it is thought that the consequent disturbance to the normally balanced cytokine milieu leads to an accelerated disease process rather than to control the infection [16-18]. Several studies have shown that significantly

increased levels of pro-inflammatory cytokines such as, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-β) and interleukin-6 (IL-6) is associated with complicated malaria [19-22]. A proportion of individuals with severe disease develop so-called cerebral malaria, characterized by seizures and coma, which is more frequently associated with death than severe anaemia, even when prompt treatment is administered. In cerebral malaria cases, iRBC sequestered in the micro-vasculature of the brain may represent the principal aetiological agent [23,24]. Sequestration of iRBC is thought to be mediated by direct binding of parasite infected RBC to up regulated receptors expressed by blood vascular endothelial cells such as CD36, ICAM-1/VCAM-1, PECAM or E-selectin, which in turn results in induction of nitric oxide (NO) via cytokine release by endothelial cells and leukocytes. It has been hypothesized that high levels of NO in brain tissue is associated with coma in cerebral malaria although this is not a clearly established link [25-27]. To date, most cytoadherence phenomena appear to be mediated by *P. falciparum* erythrocyte membrane protein-1 (PfEMP-1). This molecule is expressed on the surface of iRBC and is pivotal for binding to CD36, thrombospondin (TSP) and intracellular adhesion (I-CAM) molecules receptors on endothelial cells [28,29]. Alternatively, occlusion of the microvasculature via rosetting, giant rosetting (both involving aggregation of iRBC with RBC) and/or auto-agglutination of iRBC, may directly contribute to the acute pathology of severe human cerebral malaria [30].
The next paragraph will detail the current knowledge of the *P. falciparum* genome and proteome in order to better understand which parasite proteins are expressed in the different life-cycle stages of the parasite and the role of such proteins in relation to the observed clinical manifestations and underlying pathology.

Parasite genomics and proteomics
The entire genome sequence of *P. falciparum* was recently elucidated [31] and can be accessed via the *Plasmodium falciparum* Genome Database http://www.plasmodb.org. Briefly, the nuclear genome consists of 14 linear chromosomes ranging in size from 640.000 to 3.3 million base pairs. In addition a mitochondrial genome of 6000 base pairs and a plastid-like circular genome of 35000 base pairs, which resides in the apicoplast have been identified. The *P. falciparum* genome contains 5.268 predicted protein-encoding genes of which to date only 40% code for proteins with known function. Of the genes identified many are involved in cell-to-cell adhesion or invasion of host cells (1.3%), are assumed to have a role in the evasion of the host immune response (3.9%), have important functions in metabolism (8%) or are considered to be associated with functioning of the apicoplast, given the observation that at least 10% of the nuclear encoded proteins are targeted to this organelle. Although the exact role of the apicoplast is unclear it is essential for parasite survival, with functions in the anabolic synthesis of fatty-acids, as well as isoprenoid and heme metabolism [32-34]. Within the sub-telomeric regions of the chromosomes there are clusters of highly variable gene families, representing 5-10% of all genes. These gene families include *var, rif* and *stevor* genes that code for proteins called PfEMP-1, repetitive interspersed family (rifin) and sub-telomeric variable open reading frame (stevor), respectively. Although the function of rifins and stevors are unknown, they are antigenically highly variable, like PfEMP-1 proteins, and are thus thought to be involved in immune evasion [35-37].
The elucidation of the *P. falciparum* genome gene sequence, with the identification of open reading frames subsequently, allows for high-throughput proteomic approaches using different life-cycle stages of *P. falciparum* e.g. sporozoites, merozoites, trophozoites and gametocytes, as starting material [38]. It has become clear, based on such analyses that the sporozoite proteome is markedly different from all other stages, since 49% of sporozoite

proteins are unique in contrast to the 20-30% unique to the other stages. Furthermore, only 25% of the proteins in the sporozoite proteome are expressed in any other stage while between 39% and 56% of the proteins are shared between merozoites, trophozoites and gametocytes. Finally, it is of interest to note that only 152 proteins (6%) were common to all four stages, including housekeeping proteins such as ribosomal proteins, transcription factors, histones and cytoskeletal proteins. Given the importance of an understanding of the role of parasite proteins and their interaction with the mosquito and human host we will now examine the knowledge gathered thus far on protein expression linked to the different life cycle stages.

The sporozoite proteome

The main classes of annotated sporozoite proteins identified are cell surface and organellar proteins. Proteins that ensure mobility of the highly motile sporozoites, including actin and myosin, are abundantly expressed. Since sporozoites possess apicomplex machinery involved in host cell invasion, many proteins associated with rhoptries, micronemes and dense granules are expressed in this stage. Thus far approximately 10 proteins have been identified that seem to be relatively specific for their expression in the sporozoite. Most of these proteins are expressed on the surface of sporozoites but their expression abundance heavily depends on the microenvironment in which the sporozoite resides. For instance, circumsporozoite protein (CSP) is first detected during the oocyst stage in the mosquito, it persists in the sporozoite in the blood, but its expression slowly decreases following hepatocyte invasion and subsequent maturation i.e. in mature hepatic schizonts and merozoites [39]. Another example is Liver Stage Antigen-1 (LSA-1) that is expressed at very low levels in sporozoites but is abundantly expressed following hepatocyte invasion. In contrast glutamate-rich protein (GLURP), for example, is synthesized in all stages of the parasite in the human host. Other proteins expressed in sporozoites include sporozoite surface protein (SSP-2 or TRAP), Sporozoite threonine and asparagines rich protein (STARP), sporozoite and liver stage antigen (SALSA), Liver Stage Antigen-3 (LSA-3), which, in contrast to LSA-1, is expressed during sporozoite and the liver stage, a protein with an altered thrombospondin Type I repeat domain (PfSPATR), a scavenger receptor conserved among all plasmodium species (PxSR), and finally the sporozoite microneme protein essential for cell transversal (SPECT). Several antigens that are predominantly expressed by asexual blood-stage parasites have also been detected during the sporozoite stage, such as erythrocyte binding-like protein (MAEBL) and PfEMP-1. Functionally, it is known that both CSP and TRAP are essential for sporozoite gliding and *in vivo* infectivity of hepatocytes [40]. STARP, expressed during the intrahepatic and early ring stages [41,42] has no known function, but there is evidence that it may play a role in sporozoite invasion, as it contains specific liver cell binding regions [43]. SALSA is continuously expressed by sporozoites and liver stages and was discovered because of its antigenic nature, but its function is also not known. The same is true for both LSA-1 and LSA-3 that are localized within the parasitophorous vacuole surrounding developing exo-erythrocytic parasites [44]. It has been postulated that they have a role in liver schizogony and merozoite release, since they reside in the flocculent material forming a stroma in which merozoites are released into the bloodstream [45]. PfSPATR, a secreted protein with an altered thrombospondin repeat, binds to HepG2 liver cells, suggesting a possible role in hepatocyte invasion [46]. The PxSR protein is expressed in sporozoites of both human and rodent plasmodia. Disruption of the PxSR gene in the *P. berghei* results in parasites that form normal numbers of oocysts, but that fail to produce any sporozoites; it therefore seems to play an essential role in parasite development [47]. SPECT was characterized in the micronemes, one of the secretary organelles of sporozoites, and data thus far suggest a role in hepatocyte invasion [48].

Interestingly, the SPECT2 protein displays features similar to some human complement proteins that are involved in perforation of membranes (MACPF). The human MACPF proteins act by forming pores in the cell membrane, so SPECT2 may be involved in cell-to-cell passage within the liver. This could help to explain the observation that alterations in the SPECT2 gene result in sporozoites that completely lack the ability to migrate through cells and their infectivity for hepatocytes is therefore greatly reduced [48,49]. A second protein, phospholipase (PbPL), is highly conserved amongst plasmodia and is also involved in membrane rupture to facilitate migration of sporozoites through hepatocytes [50]. The so-called *var* genes encode the major iRBC surface-expressed antigen (PfEMP1) of the asexual blood stages of *P. falciparum*. Differential expression of up to 60 diverse *var* genes in each parasite genome yields an almost indefinite number of combinations for PfEMP-1 protein expression. Data thus far strongly indicate that it plays a role in evasion of host immunity during asexual blood stage multiplication, since PfEMP-1 proteins are primarily involved in erythrocyte binding to endothelial receptors. Interestingly, it has been demonstrated that within the sporozoite at least 25 different PfEMP1 iso-forms are expressed, all representing PfEMP-1 proteins that are distinct from any of the PfEMP-1 isoforms expressed by erythrocytic stage. The possible role of PfEMP-1 in sporozoites is currently unknown.

The merozoite proteome

The invasion of RBC by merozoites is a multi-step process, involving several ligand-receptor interactions. The first step is a low-affinity binding interaction between the surfaces of the parasite and the RBC, followed by reorientation of the parasite on the RBC surface. This reorientation brings the apical end of the merozoite into contact with the RBC to form a tight junction at the apex. At this point proteins are secreted from the rhoptries and micronemes leading to the formation of a parasitophorous vacuole surrounding the parasite (figure 3).

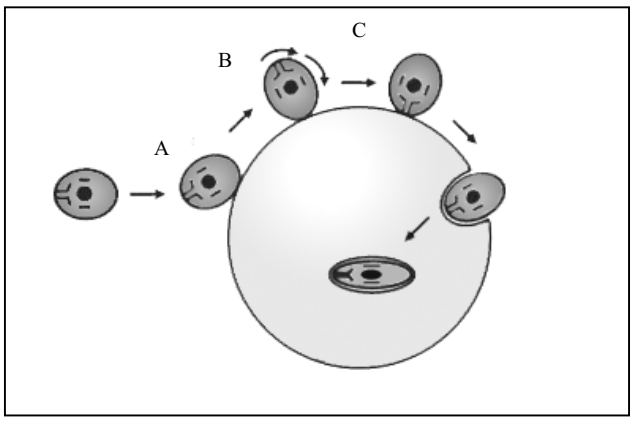

Fig. 3: Schematic overview of merozoite binding to red blood cells and reorientation process.

From these observations it can be concluded that proteins expressed on the surface and in the apical organelles of the merozoite are primarily involved in host cell recognition and invasion. Among these proteins, merozoite surface protein 1 (MSP-1) is the most abundant. Although the precise role of MSP-1 in erythrocyte invasion has yet to be defined, the observation that antibodies directed against MSP-1 inhibit cell entry strongly suggests that it plays an important role in RBC invasion [51]. Other proteins expressed on the surface of the merozoite include MSP-2, MSP-4 and MSP-5 whereas MSP-10 and rhoptry-associated membrane antigen (RAMA) reside in organelles at the apical end of the parasite.

Although MSP-1 and MSP-2 are distinct in terms of the levels of protein expressed, both are highly polymorphic, suggesting that they play a role in host immune evasion [31,52-54]. In addition to the proteins expressed on the surface of the merozoite, several other proteins may mediate binding to RBC receptors. These so-called "peripheral proteins", that include the MSP-3/-6 group, MSP-7 family, serine repeat antigen (SERA), acid basic repeat antigen (ABRA) and Pf41 proteases, are secreted into the parasitophorous vacuole during schizont development, and are released upon schizont rupture. Their function has yet to be established since they were identified mainly as a result of their antigenicity. Better defined is the role of apical membrane antigen 1 (AMA-1) that is expressed in micronemes but needs, in order to facilitate invasion, to relocate to the surface of the merozoite for efficient RBC invasion (required for transition of stage A to B in figure 3). The latter was established by the finding that certain antibodies in human sera block parasite re-location rather than preventing initial attachment [55]. Upon correct orientation (phase C in figure 3), the invasion process is initiated, most likely mediated by direct interaction of the apical end of the parasite with RBC receptors. Several proteins expressed within the micronemes, including EBA-175 [56], EBA-140 (also known as BAEBL) [57] and EBA-181 (also known as JSEBL) [58] are involved in RBC receptor binding [59]. EBA-175 and EBA-140 bind glycophorin A [60] and C [61], respectively. In addition, a number of proteins have been found to be transported to and stored within the rhoptry organelles. These proteins can be divided into two groups: low molecular mass rhoptry protein complex consisting of RAP proteins 1, 2 and 3 (Rhoptry-associated proteins) and a high molecular mass rhoptry protein complex, RhopH 1, 2 and 3 (high molecular weight rhoptry proteins). The RhopH proteins can be found in the merozoites and comprise a complex that is involved in erythrocyte binding and parasite growth. Both RhopH and RESA (ring-infected RBC surface antigen) are transferred to new RBC where they are assumed to be involved in cell remodeling.

The trophozoite proteome

Several proteins are uniquely expressed during trophozoite development within iRBC. These proteins are mostly involved in hemoglobin digestion to heme, important to the parasite as a source of amino acids for protein synthesis during growth and maturation [62]. Next to these proteins several proteins expressed uniquely during this stage play an important role in the formation of the parasitophorous vacuole. Additional remodeling of the iRBC occurs when the surface of the host cell becomes decorated with thousands of knob-like structures, consisting of agglomerations of Histidine-Rich Proteins (HRP) [63] on the cytoplasmic side and PfEMP-1 on the external surface [63-66]. As previously mentioned, PfEMP-1 proteins on the iRBC surface are involved in cytoadherence to endothelia and host immunological evasion.

The gametocyte proteome

As mentioned earlier sexually dimorphic gametocytes are found in the human host with, in general, a 1:4 male:female ratio. Both male and female gametocytes are arrested in the cell cycle, precluding maturation in the human host. Further development into gametes only occurs within the mosquito. The differences in the fates of the sexual and asexual stages unsurprisingly result in different patterns of gene expression. In addition, sex-specific expression has also been observed at the level of RNA, mitochondria and ribosomal content. While transcription in the terminally differentiated male gametocytes is 'suppressed', female gametocytes are preparing for continued development into gamete, zygote and ookinete stages. Proteins involved in the early development of the blood stage parasites into gametocytes (gametocytogenesis) include Pfs16 and Pfs27. Targeted disruption of these genes results in complete inhibition of further sexual development [67,68]. Development

into oocysts coincides with abundant expression of the Pfs25 and Pfs28 proteins that are thought to play an important role in ookinete development given that the latter is inhibited in *P. berghei* parasites lacking expression of these proteins [69]. These two proteins are highly conserved amongst plasmodia and are considered major targets for interventions aiming to block parasite transmission. Pfs230 and Pfs48/45 proteins are expressed in the male gametocyte and although their exact role is not yet known it is speculated that Pfs230 is involved in human erythrocyte binding and oocyst production whereas Pfs48/45 might be involved in ligand interactions with the female gametocyte during fertilization [70,71]. Finally, Pfs47 protein is expressed specifically on the surface of female gametocytes, but a specific role has yet to be defined.

Overall it can be concluded that much progress has been made in recent years with the elucidation of the genome of *P. falciparum*, with associated proteomic investigations of gene expression profiles [38,72], and with assignment of functions to *P. falciparum* proteins in relation to disease onset and progression as well as underlying pathology. The next section of this chapter focuses on the interaction between the host and the parasite from an immunological perspective. In this context studies investigating the antibody and T cell response in both naturally infected individuals as well as in human volunteers vaccinated with irradiated sporozoites are described. Such studies have been extremely important in the identification, characterization and subsequent selection of a number of antigenic proteins of *P. falciparum* that have potential as immunological targets in the context of vaccination.

Host defense against *P. falciparum*

Host responses during natural infection

Morbidity and subsequent mortality due to *P. falciparum* occurs primarily in humans that lack acquired immunity to the parasite. Such immunity is acquired only gradually over a period of years and requires continuous exposure even in areas where malaria is endemic and transmission rates are high. This lengthy process is attributed to different factors that include the age of the host as well as genetic variability of both parasite and host [73,74]. For instance, as a strategy for the parasite to evade host immunity, highly variable proteins are expressed on the surface of the iRBC (e.g. PfEMP-1) during asexual multiplication. The high degree of protein polymorphism precludes development of appropriate and timely immune responses capable of controlling the parasite.

Consequently, two human populations are particularly at risk for malaria, namely young children and travelers. Infants up to about 6 months of age have a degree of protection that is commonly attributed to transplacentally transfer of maternal anti-*P. falciparum* antibodies. Since different studies designed to assess this hypothesis have given conflicting results, there remains controversy over its veracity, although it must be said that none have yet assessed anti-PfEMP-1 antibodies, arguably the most relevant, in this context [75-79]. In older infants and in children up to roughly 5 years of age, malaria is one of the major causes of death in areas where the disease is endemic. Theory suggests that protection against severe outcomes such as anemia is acquired rapidly, possibly after only 1 or 2 infections, but in areas where transmission of *P. falciparum* is less frequent, cerebral malaria as an outcome is relatively more common, with consequently higher mortality [80]. Beyond the age of 5 years, clinical immunity is evident, whereby parasitemia in the absence of clinical symptoms is common, and eventually anti-parasite immunity develops such that infection occurs but parasite densities are constrained, often at sub-microscopical levels [81-84]. Paradoxically, immunity acquired against *P. falciparum* during childhood and adolescence can be lost during pregnancy [85]. This phenomenon of pregnancy-associated malaria is attributed to the ability of iRBC expressing particular variants of PfEMP-1 to sequester in the placenta as a result of the expression of a distinct molecule on syncytiotrophoblasts a low-sulphated

form of chondroitin sulphate A (CSA) that differs from forms of CSA expressed in other organs [86]. Pregnancy-associated malaria greatly increases the risk of low birth weight and thus contributes to increased rates of infant morbidity and mortality [87]. Infection with *P. falciparum* during the first and second pregnancies, however, appears sufficient for women to acquire antibody-based immunity that prevents the complications caused primarily by placental infection in subsequent pregnancies, thus protecting the foetus [88,89]. It should be noted that the slow but nevertheless ultimately effective immunity acquired to *P. falciparum* does not protect an individual for life, since individuals who migrate from malaria-endemic to non-endemic regions can again develop clinical symptoms upon return to a malaria-endemic area. Continuous exposure to infection thus appears a requisite for maintenance of fully effective protection.

A prerequisite for controlling the parasite is recognition of target antigenic proteins and induction of antibody- and T-lymphocyte-mediated responses. Studies with sera from naturally-infected individuals have demonstrated that antibodies play an important role in the development of immunity to *P. falciparum* pre-erythrocyte stages (sporozoite and/or liver schizont stage) [90-94], as well as erythrocytic stages (merozoites) [95-102]. Such antibodies can be shown to prevent invasion of liver or RBC *in vitro*. For certain defined parasite proteins, including MSP-1, GLURP and PfEMP-1, it has been established that the prevalence and the levels of specific antibodies acquired as a result of natural exposure is associated with resistance to infection [102-106]. By far the most convincing evidence for the protective role of antibodies comes from experiments involving passive transfer of purified immunoglobulin (IgG) derived from sera of malaria immune adults into non-immune individuals, demonstrating complete clearance, at least temporarily, of parasitemia [107,108].

The role of T cells in controlling naturally acquired infections with *P. falciparum* is less well defined compared with that of antibodies. Field studies conducted in malaria endemic regions have shown that parasite proteins expressed by pre-erythrocytic stages do induce T cell responses [109-111]. This is not surprising since hepatocytes, in contrast to RBC, for example, do express major histocompatibility (MHC) class I and II molecules, which are a prerequisite for presentation of parasite antigen-derived peptides to $CD8^+$ or $CD4^+$ T cells, respectively [112]. Despite the fact that antigen-specific precursor T cells are only found at low frequencies in the peripheral blood of semi-immune individuals, multiple T cell epitopes of several *P. falciparum* pre-erythrocytic proteins have been described, although, for the most part, their roles in immunity are unknown [111,113-116]. One exception is an LSA-1- specific CTL epitope, restricted by an HLA-bw53 MHC-class-I molecule [117], which was shown to be associated with resistance to severe malaria in Gambian children [118]. A subsequent study in central Africa also revealed a role for IFN-γ-inducing T cell epitopes of LSA-1 in acquired immune protection against malaria in Gabonese children [119]. Indirect lines of evidence for the role of T cells in affording protection against malaria is provided by the observation that T cell epitopes within the *P. falciparum* CSP protein are highly polymorphic. This is strongly suggestive of immune selection pressure, since sequence alterations are likely a mechanism employed by the parasite to escape detection and elimination by the immune system [120]. As for $CD8^+$ T cells, the role of $CD4^+$ T cell responses in naturally acquired human anti-malarial immunity is currently being investigated. In individuals exposed to natural infection, $CD4^+$ T cell responses to multiple pre-erythrocytic stage antigens have been detected [121,122] but again their role in protection has yet to be clearly established. $CD4^+$ T cell IFN-γ responses directed to a conserved epitope in *P. falciparium* CSP have been shown to correlate with protection from natural infection and disease in malaria exposed individuals, thus supporting the hypothesis that $CD4^+$ T cells with specificity for pre-erythrocytic stage antigens have a functional role

to play in anti-malarial immunity in humans [121]. Less clear is a direct role for $CD4^+$ T cells in the context of acquired immunity to asexual blood stages, however, although immunity to the latter that relies apparently exclusively on $CD4^+$ T cells can be induced experimentally in malaria-naïve individuals [123].
Collectively it can be concluded that, during naturally acquired infection, both antibody as well as T cell responses contribute to the host's efforts to control the parasite. Although strong antibody and T cell responses are seen to multiple parasite antigens, defined, precise correlations with protection are lacking, most likely due to the complexity of the host-parasite interactions that primarily reflect the extent of genetic variability on both sides. Despite the lack of understanding, it is generally accepted that both the cellular and humoral arms of the immune system are required for full control of parasite multiplication and prevention of malaria. The next paragraph will explore the knowledge of host immune defense mechanisms gained as a result of immunization with irradiated sporozoites, thus far used only as an experimental vaccination procedure that confers sterile immunity to *P. falciparum*.

Host responses upon vaccination with irradiated sporozoites
In the late 1960s and early 1970s immunization with irradiated plasmodial sporozoites (γ-sporozoites) was shown to induce sterile immunity in rodents [124], non-human primates [125] and humans [126-131]. These studies were pivotal in demonstrating the potential feasibility of the development of a vaccine for *P. falciparum*, provided that the correct combination of parasite proteins in the proper immunological context (i.e. eliciting antibodies or T cells) is presented to the host immune system. The start point in unraveling this scientific challenge, still ongoing, was to understand the mechanism of sterile immunity induced by γ-sporozoites. Growth arrest at the liver schizont stage seemed to be pivotal [132,133]. Other observations showed that, with an appropriate dose of radiation, γ-sporozoites are still capable of invading the liver, to express sporozoite-stage-associated antigens like CSP, SSP2/TRAP, STARP and SALSA, to transform into schizonts and to express the liver-stage antigens LSA-1 and LSA-3, but that they fail to initiate an erythrocytic-stage infection. Sporozoites inactivated by heat or freeze-thawing procedures, on the other hand, and as a consequence unable to invade hepatocytes, failed to induce protective immune responses [134,135]. Thus invasion of hepatocytes is a key event, required to induce the expression of proteins that can subsequently act as targets for an immune response that confers protection. Antibodies induced by γ-sporozoites were shown to recognize CSP and could block sporozoite invasion of liver cells *in vitro* [136]. Thus, these initial studies indicated an important role for antibody-mediated inhibition of parasite invasion of host cells [137]. These findings were strengthened by the demonstration of the protective effects, in rodents as well as non-human primate malaria models, of passive transfer of mainly anti-CSP antibodies [138-140]. A clear role in this system for T cells, in addition to antibody responses, was shown by the full protection conferred to antibody deficient mice by γ-sporozoite immunization [141]. Subsequent studies with γ-sporozoites in humans revealed the involvement of $CD4^+$ and $CD8^+$ T cells with specificity for CSP [142-144], SSP2 [145-147] and LSA-1 [148]. However, a definitive role for T cells was established by either depletion or adoptive transfer of $CD8^+$ T cells in mice prior to γ-sporozoite immunization, demonstrating loss or restoration of protection, respectively [149-153]. Neutralization of IFN-γ by antibody-mediated depletion, neutralizing an effector function of T cells, resulted in full susceptibility of mice to challenge infection, further supporting the involvement of T cells. Further, detailed studies have subsequently been performed to define the role of $CD8^+$ and $CD4^+$ T cells in protection against *Plasmodium*

species. Available information suggests that $CD8^+$ T cells either bind directly to and thereby kill infected hepatocytes that express MHC-restricted parasite antigen-derived epitopes or, possibly, are activated by 'bystander' antigen-presenting cells, releasing cytotoxic molecules (IFN-γ) that lead to death of parasites in infected hepatocytes in the close vicinity [154]. $CD4^+$ T cells most likely contribute to appropriate priming and maintenance of $CD8^+$ T cells, but will also provide help for stimulation of naïve B-cell differentiation into antibody-producing plasma cells. Aside from functional aspects, the dependence of long-lasting protection induced by γ-sporozoite immunization on the generation and maintenance of a population of liver-resident memory $CD8^+$ T cells has also been revealed [155,156]. The role of $CD4^+$ T cells in γ-sporozoite-induced protection has been investigated via either adoptive transfer or depletion. Adoptive transfer experiments demonstrated that CSP-specific $CD4^+$ T cell clones could protect against sporozoite challenge *in vivo*, in the absence of any detectable $CD8^+$ T cell activity [157]. Transfer of $CD4^+$ T cell clones derived from γ-sporozoite-immunized mice to naïve mice also conferred protection against challenge [158] while $CD4^+$ T cell depletion abrogated vaccine-induced protection [159].
A role for gamma-delta (γδ) T cells cannot be excluded, as adoptive transfer of a γδ T cell clone to naïve mice resulted in protection [160]. The abrogated protection in γδ T cell receptor-deficient mice upon live sporozoite challenge lends further support to this idea [161]. Adaptive immune responses i.e. antibodies and T cells with specificity for parasite antigens are considered essential for acquisition of immunity to *P. falciparum*. Innate immune responses, involving, for example, dendritic cells (DC), natural killer cells (NK) and NK T cells, can themselves also generate direct anti-parasitic activity as well as enhancing the adaptive immune response to *Plasmodium* [162-165]. The elucidation of specific and most probably pivotal roles of different DC subsets, for example, is the focus of on-going research.

Selection of parasite protein targets for vaccine development
The range of observations in pre-clinical models, in individuals with natural exposure to malaria, and in individuals vaccinated with γ-sporozoites, as described above, have provided a wealth of information regarding the relative importance of a number of parasite proteins as targets of immunological activity. Based on these knowledge vaccine strategies is being pursued predominantly on two separate but potentially overlapping fronts. First, the finding that irradiated-attenuated sporozoites can confer sterile protection indicates that a multivalent pre-erythrocytic (sporozoite and liver)-stage vaccine might confer protection. In this case, the vaccine should contain proteins such as CSP, TRAP, and STARP to ensure induction of both antibodies and T cells. A vaccine directed against pre-erythrocytic stages is expected to completely inhibit infection or significantly reduce parasite load. A significant reduction in the number of parasites that reach the liver is considered crucial for the clinical outcome of infection, since, in the earliest phase of the infection, the number of merozoites released upon rupture of iRBC is dependent on the number of iRBC, which in turn is related to the number of hepatocytes infected initially by sporozoites. As a consequence such a vaccine is considered beneficial for individuals who have had either limited or no previous exposure to infection, such as infants or travelers. Second, the important role for antibodies suggested by sero-epidemiological studies of naturally infected individuals has triggered investigations into vaccine modalities designed to elicit predominantly antibody responses against the asexual erythrocytic stages of the parasite. For this strategy parasite protein targets include, amongst others, MSP-1 and AMA-1. A vaccine that reproduces aspects of naturally acquired immunity in this context may reduce the density of blood parasites and thereby decrease the severity of clinical disease manifestation. In table 1, a list is provided

of parasite proteins that currently serve as prime targets in vaccines with the expectation that a host will mount a vigorous immune response against this protein or proteins resulting in protection. The identification of a number of *P. falciparum* proteins that are major antigenic targets in natural infections as well as in vaccination studies using γ-sporozoites, have thus provided the basis for the investigation and development of subunit vaccines against malaria.

Parasite stage	**Antigens**
Pre-erythrocytic stage	CSP, TRAP, LSA-1, STARP, SALSA, LSA-3
Erythrocytic stage	MSP-1 to 4, AMA-1, GLURP, SERA-1, RESA, RAP-1, RAP-2, PfEMP-1

Table 1: Prime candidate *P. falciparum* proteins for vaccine development

The next section of this chapter focuses on studies that have been performed using different formulations to present *P. falciparum* antigen targets to the host immune system, studies that have led to the commonly held belief that both antibodies and T cells will need to be elicited by vaccines for effective control of the parasite.

Vaccine strategies for induction of immunity to *P. falciparum*

The evaluation of malaria-naïve sporozoite immunized volunteers has revealed multiple immune mechanisms and target antigens that have encouraged the development of subunit vaccines. Such vaccines consist of protein or peptide fragments of parasite antigens, produced either by recombinant DNA or synthetic peptide technology. To date, most effort has been directed at the development of sub-unit vaccines against the preerythrocytic stages of the malaria parasite in order to intervent early in the parasite life-cycle [166]. Given the perceived importance of CSP, numerous CSP-based peptide and protein vaccines have been assessed in both pre-clinical and clinical studies [167-170]. Results from early clinical trials were rather disappointing as there was no convincing evidence that the immunization-induced enhancement of anti-CSP antibody responses provided any protection. However, further development resulted in a vaccine composed of recombinant CSP fused to the surface antigen of hepatitis B virus, referred to as RTS,S. This recombinant protein particle vaccine was further combined with an oil-in-water-based adjuvant (AS02A). This vaccine is currently the most advanced, having demonstrated protection (30-40%) in human field trials [171,172]. However, the relatively short-lived nature of the protection it induces and the apparent absence of $CD8^+$ T cell responses appear to leave ample room for improvement. Attempts to further improve the immunogenicity of the RTS,S/AS02A regimen are on-going [173].

The observation that cell-mediated immune responses, principally $CD4^+$ and $CD8^+$ T cells, are implicated in protection against *P. falciparum* liver stage infection has fueled the development of genetic carriers to deliver *P. falciparum* DNA coding for antigenic proteins to a host. The enormous advances in molecular biology and our understanding of the genomes of both viruses and bacteria have led to a wide variety of choices for development of such genetic carriers. Given the fact that viruses engineered as vaccine carriers in general have shown to be capable of inducing strong antigen-specific T cell responses, these carriers have been the main focus of development efforts. Multiple viral vector systems are therefore currently the subject of investigations for their potential as vaccine carriers as components of malaria vaccines. Such vectors include engineered flaviviruses, alphaviruses, baculoviruses, poxviruses and adenoviruses. Among all these, by far the greatest amount of

data has been obtained thus far with poxviruses and adenoviral vectors. The remainder of this chapter will therefore discuss in detail both poxvirus and adenovirus vectors, with only brief descriptions of results obtained with alternative vector systems assessed in the context of malaria vaccine development.

Recombinant poxvirus vectors

Background

The poxviridae comprise a large family of complex linear double stranded DNA viruses with a genome of 130-300 kb in length. The virus particle is approximately 200 nm in diameter, with the core surrounded by a virus envelope (figure 4). The family of poxviridae can be divided into the subfamily chordopoxvirinae (vertebrate poxviruses) and extomopoxvirinae (insect poxviruses). The subfamily chordopoxviridae contains eight genera in which Avipoxvirus and Orthopoxvirus are the most studied. Avipoxviruses affects a great variety of bird species, being named after them e.g. fowlpox, canarypox or pigeonpox viruses, and they are restricted to avian hosts, lacking the ability to replicate in mammalian cells. Attenuated strains of avipoxviruses, produced by repeated passage on chicken embryo fibroblasts, have been extensively used as vaccines in the poultry industry to prevent wild-type virus infection.

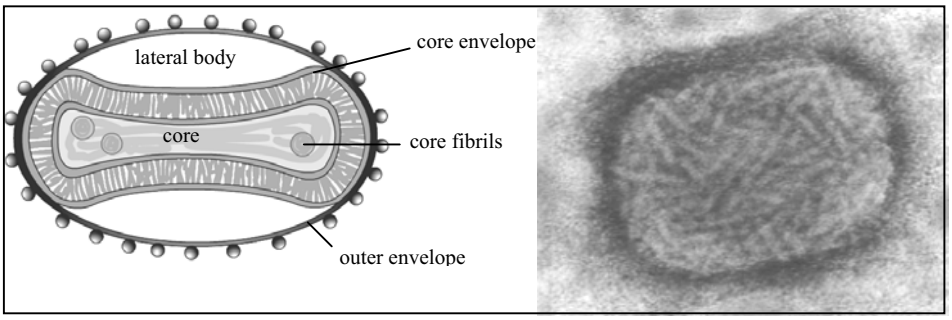

Fig. 4: A schematic overview of the Poxvirus structure (left panel) and in the right panel an electron microscopic image of poxvirus (magnification ± 150.000)

The Orthopoxviridae contains the variola strain, the causes of smallpox, a highly contagious and lethal disease unique to humans. Another important member of this family is vaccinia (cowpox virus), which shows considerable similarity to variola except that it does not cause disease in humans and has a wide vertebrate host range. Vaccinia virus (VV) has therefore been used for decades as a vaccine against smallpox. Although the vaccine has afforded excellent protection, its use has resulted in post-vaccination encephalitis or progressive vaccinia infections in rare cases, predominantly in individuals with an impaired immune system. Demand for a safer vaccine resulted in the development of a modified vaccinia virus [174]. This modified vaccinia virus (MVA) was obtained by passage of the virus more than 500 times on primary chicken embryo fibroblasts, resulting in the progressive deletion of multiple genes within the virus genome and a virus rendered incapable of replication in mammalian cells. This strain was administered as a vaccine to a small human population (120,000 vaccine doses), without apparent side effects [175], prior to the declaration by WHO of the global eradication of smallpox. Despite these improvements a third generation smallpox vaccine strain, NYVAC, was also developed. This vaccine strain was attenuated through molecular biological techniques with the rational deletion of 18 genes in the Copenhagen vaccinia strain. It has been extensively tested in animal models and in humans, demonstrating good safety and immunogenicity profiles [176,177], but has never been used

in vaccination campaigns per se due to the successful eradication of smallpox. Interest in the development of recombinant poxviruses started around the time that smallpox was declared eradicated in 1980, when vaccinia vaccines were no longer needed. The excellent safety profile, immunogenicity and knowledge of the molecular biology of poxvirus replication then led to the development of recombinant vaccinia viral vectors. As viral vectors these attenuated viruses were engineered to express genes coding for antigenic proteins from human pathogens such as influenza, HIV and *P. falciparum*. Characteristics such as the ability to clone large DNA inserts (up to 25Kb) into the genome [178], high-level expression of inserted genes, and cytoplasmic replication without integration, all contributed to the rationale for development of poxviruses as vaccine carriers. The efficacy of recombinant VV, MVA and NYVAC poxviruses has been evaluated to a large extent in tumor and infectious disease models [176,179]. In general, potent and long-lasting immunity could be induced. The immune response includes both antigen-specific humoral [180-183] as well as cellular [184-186] responses. However, the presence of anti-VV antibodies in large segments of populations due to prior smallpox vaccination [176] limited the effectiveness or recombinant vaccinia vectors in humans [177]. Nevertheless, with the eradication of smallpox and hence no further need for vaccination against smallpox, future human generations will not develop anti-poxvirus antibodies and vaccine carriers based upon these viruses are therefore still being actively investigated. However, the high prevalence of neutralizing antibodies against Orthopoxviruses in different populations has led to research into the potential of several Avipox viruses, including canary pox (CPV) and fowl pox (FVP), as vaccine carriers. Such viruses are perceived to be safe since they do not replicate in human cells and they are not susceptible to neutralization by antibodies induced by prior vaccinia immunity [187]. In fact, Avipox vaccine vectors have proven to induce less vector-specific immune responses upon vaccination in comparison to vaccinia strains and are therefore considered suitable for prime-boost vaccination regimens. The most widely used strategy to generate recombinant attenuated poxviruses is based on the insertion of the foreign gene into the locus encoding thymidine kinase (TK). This engineered genome is subsequently introduced into primary chicken fibroblasts, after which the genome is rescued by addition of wild type vaccinia virus (figure 5) [188].

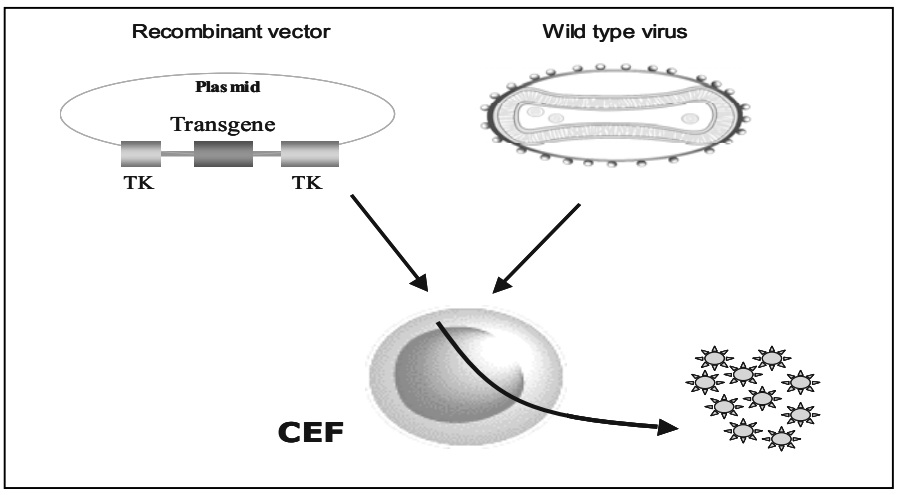

Fig. 5: Schematic representation of poxvirus vector generation.

By targeting of the foreign gene into the TK locus, the gene is disrupted and thus attenuated viral recombinants can be easily selected based on their TK negative phenotype in host cells.

Alternative methods include incorporation of either an additional antibiotic selection marker or a reporter gene, allowing color screening, into the viral genome. Inherent to the vector generation strategy is the use of susceptible cells for propagation of the vector, which to date involves, for example, the use of primary cells such as chicken embryo fibroblast (CEF) or baby hamster cells (BHK21) [189,190]. This represents a serious drawback for the use of poxvirus-based vectors since the use of primary cells requires batch-wise preparation that seriously restricts large-scale virus productions since primary cells cannot be grown continuously in cell culture. Batch-wise production requires campaign-based product manufacturing, which also poses challenges with respect to product quality and reproducibility.

Poxvirus-based malaria vaccines

There have been numerous attempts to assess poxvirus vectors as malaria vaccines both in pre-clinical and in clinical studies.

Initial studies used live vaccinia virus vectors carrying plasmodial antigens to immunize rabbits against *P. knowlesi* CS [191], or *P. falciparum* RESA protein [192], with clear induction of antibody responses in both cases. Mice immunized with recombinant vaccinia expressing full length PfSSP2 (TRAP) developed antigen-specific $CD8^+$ T cell responses [145]. Despite these encouraging immunological results, subsequent vaccination and challenge infection studies in mice and monkeys were disappointing, failing to demonstrate protection [193-195]. Whether this was due to an inappropriate selection of *P. falciparum* antigen or a problem of the vector itself remains obscure, given that vaccinia viral vectors expressing rabies antigens, as well as antigens from several other human pathogens, induced protective immunity in pre-clinical models [196-201]. Several of these studies did, however, also demonstrate that priming vaccination induced high titer anti-vector immune responses that inhibited the response to booster vaccination. As such, these experiments provided clear evidence that an ideal vector would be one for which efficient priming is not constrained by any pre-existing anti-vector antibodies, as well as one that triggers only low-level anti-vector immunity upon priming such that a booster vaccination with the same vector will be effective. In this respect both MVA and vaccinia vectors are hampered by pre-existing immunity in the human population. However, as explained earlier, the eradication of smallpox will result in a progressively larger human population with no pre-existing antibodies against either MVA or vaccinia. With this in mind, such vectors are still being actively investigated in clinical settings (see later), in some cases alone but predominantly in so-called heterologous prime-boost combinations. The latter refers to a regimen that employs immunologically distinct recombinant vaccine carriers or vaccine formats for priming and boosting in order to avoid the sort of loss of booster vaccine potency seen with homologous e.g. poxvector-poxvector prime-boost vaccinations. The rationale for the heterologous prime-boost combination approach was based on studies with plasmodial gene-containing constructs that convincingly demonstrated them to be significantly more immunogenic. For instance, prime-boost studies with recombinant influenza virus priming followed by recombinant vaccinia virus or recombinant vaccinia Ankara virus (MVA) boosting, elicited high levels of CSP-specific $CD8^+$ T cells and conferred protection against sporozoite challenge infection [202-204]. All single modality vaccinations, including homologous prime-boost combinations, were consistently less immunogenic. Interestingly, protection was only conferred with recombinant vaccine virus boost following influenza vector prime, but not when the order of immunization was reversed, indicating the importance of the choice of vector for priming and boosting components [202,204]. Other pre-clinical studies in mice and non-human primates have used the concept of heterologous prime-boost vaccination, with MVA as one of the components, and, for instance, naked

DNA or recombinant adenoviral vectors expressing pre-erythrocytic stage antigens such as CS, TRAP and LSA-1, or recombinant protein. All these studies were consistent in reporting the induction of high levels of $CD8^+$ T cell responses and protection against sporozoite-induced challenge infection [205-210]. Based on these encouraging pre-clinical studies a number of clinical studies involving MVA have been performed. One of the first studies utilized DNA or MVA vaccine as a stand alone or in a DNA prime MVA-boost regimen [211]. Both vaccine carriers contained identical sequence corresponding to full-length TRAP antigen extended with a string of 20 T and B cell epitopes derived from major pre-erythrocytic proteins such as CSP, LSA-1, LSA-3 and STARP, respectively (the vaccine referred to as ME-TRAP). These studies showed that both DNA and MVA were capable of inducing modest T cell responses to the inserted parasite antigens, but the vaccination regimen of DNA priming followed by MVA boosting resulted in a 5-10 fold higher magnitude of T cell responses when compared to either carrier given alone. In addition, it was shown that T cell responses induced by the DNA-prime, MVA-boost regimen comprised both $CD4^+$ and $CD8^+$ T cell subsets, and that partial protection was observed following *P. falciparum* sporozoite challenge infection. This same vaccine regimen was further tested in field trials, showing that vaccination with MVA, in either the presence or absence of a DNA prime, could boost pre-existing T cell responses in naturally infected individuals [212]. Nevertheless, the DNA prime in non-exposed individuals had shown a significant effect and the ME.TRAP DNA-MVA vaccine progressed into Phase IIa studies (portfolio malaria vaccines 2006: http://www.who.int/).

Like MVA, the replication-incompetent NYVAC vector has been extensively studied for its safety and potency profile as a vaccine carrier. Pre-clinical studies in mice with NYVAC expressing CSP resulted in high levels of T cell-mediated protection (60-100%) upon sporozoite challenge [213]. With the aim of further increasing the NYVAC vaccine potency, DNA coding for seven proteins (CSP, SSP2, LSA-1, MSP-1, SERA, AMA-1 and Pfs25) were incorporated into the NYVAC vector (vaccine referred to as NYVAC-Pf7). Evaluation of NYVAC-Pf7 in pre-clinical studies demonstrated the vaccine's safety and its ability to induce antibody responses against most of the incorporated parasite antigens [214]. Based on these results NYVAC-Pf7 progressed to Phase I/IIa safety and efficacy trials in healthy malaria-naïve volunteers. Although it induced both humoral and cellular immune responses, the levels of immunity obtained resulted in protection of only 1 of 35 immunized individuals subsequently challenged with infected mosquitoes. Importantly, this study nevertheless indicated that the presence of pre-existing anti-vaccinia antibodies, due to prior smallpox vaccination of volunteers, might interfere with the potency of the vaccine [215]. The observed effect of pre-existing immunity on vaccinia, MVA and NYVAC vectors fueled further research into the utility of alternative poxvirus-based vectors derived from other genera such as the Avipox viruses. The latter viruses, as described previously, can only replicate in avian cells and should not be hampered by neutralizing antibodies against MVA, vaccinia or NYVAC since they are an immunologically distinct class of poxviruses. These are considered important features both for the safety and the potency profiles of these viruses as vaccine carriers. Amongst the Avipox viruses, FP9 (an attenuated strain of fowl pox) is so far the most extensively studied vector in the malaria field. Pre-clinical studies in mice, with FP9 expressing *P.berghei* CSP have shown clear induction of $CD8^+$ T cell responses and demonstrated that FP9 vector based priming followed by MVA boosting provides a high level of protection against sporozoite challenge. In fact the FP9-MVA vaccine conferred significantly higher protection to mice (67.5%) compared to the next best alternative i.e. the DNA-MVA vaccine, which in these studies resulted in only 12.5% protection [216]. Nevertheless, both the DNA-MVA as well as the FP9-MVA vaccine regimens progressed into clinical trials. The clinical trials with healthy malaria-naïve adult

volunteers using DNA, MVA and FP9 combinations have shown excellent safety and immunogenicity, inducing T cell responses after DNA priming with MVA boosting, as well as after FP9 priming with MVA boosting [212,217]. Subsequent studies in human volunteers vaccinated with the FP9 ME.TRAP prime and MVA boost showed enhanced T cell mediated protection (40%) using the human malaria challenge model [218]. Studies conducted in adults and children in endemic areas also revealed an excellent safety profile for FP9 and MVA [218,219], but immunogenicity in a Phase IIb follow-up study was comparatively poor and no protection against malaria was observed. These studies provided evidence; in addition, that cross-reactivity between different poxvirus vectors might influence the efficacy of a booster vaccine.
In conclusion, studies using poxviruses have resulted in two candidate vaccine regimens for malaria, namely DNA-MVA and FP9-MVA. The challenges that remain using these types of vaccines are many and include (i) circumventing the pre-existing immunity against poxviruses in order to achieve accurate vaccine dose control, (ii) increasing the efficacy of the vaccine at safe dosages since protection above 40% has not yet been shown even in a controlled challenge environment, (iii) testing the vaccine in the target population of newborns. Over and above these challenges for poxvirus vector-based malaria vaccines, there remain technological hurdles associated with the need to produce poxvirus vectors and naked DNA at a scale and affordable price suitable for production of the hundreds of millions of vaccine doses required for the people most in need of a malaria vaccine.

Recombinant adenoviral vectors

Background

The Adenoviridae family is divided into two genera: Mastadenovirus (mammalian) and Aviadenovirus (avian). Adenoviruses are species-specific and different serotypes have been isolated from a variety of mammalian species. To date, 51 different human adenovirus serotypes have been identified and, based on similarities in genome organization and hemagglutinin activity, grouped into six subgenera (A-F). Besides human adenoviruses, approximately 50 non-human adenoviruses have been isolated from different species including mice, frog, cats, deer, macaques, sheep, cows and chimpanzees. Adenoviruses are medium sized, non-enveloped double strand DNA viruses with spiked icosahedral morphology. The virus capsid (70 to 100 nm) is made up of 252 capsomeres: 240 hexons forming the faces and 12 pentons at the vertices (figure 6).

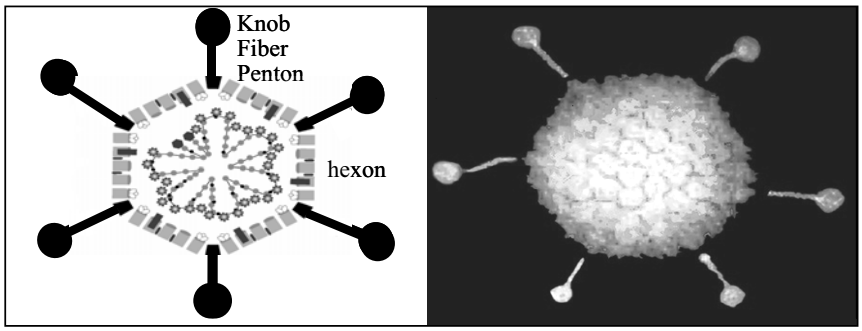

Fig. 6: A schematic overview of the Adenovirus structure (left panel) and in the right panel an electron microscopic image of adenovirus (magnification ±150.000).

Each penton consists of a base and a fiber with a terminal knob, which is involved in primary attachment of the virus to its specific receptor on the cell surface [220,221]. In general, adenoviruses are associated with respiratory illness, such as the common cold and

severe complications only occur in patients with an impaired immune system [222]. Due to their association with sub-clinical disease only, adenoviruses are considered safe viral vector systems. Other features contributing to the rationale for using adenoviruses as vectors are their ability to infect a wide variety of cells, their capacity to infect both dividing and non-dividing cells, the high level of expression of inserted transgenes, and the lack of chromosomal integration.
The classical method to generate replication-incompetent adenoviral vectors is to delete the region encoding E1 proteins, as they are involved in transactivation of all other viral promoters that are engaged in virus replication (extensively reviewed in [223]). In order to rescue the virus, the entire genome must be introduced into E1-expressing cell lines. However, systems have also been developed whereby the entire genome is not present on one plasmid but on two plasmids and intracellular homologous recombination ensures the formation of the complete genome. An example of an adenovirus plasmid system involving DNA recombination in mammalian cells is schematically depicted figure 7. Here, one plasmid carries 5000 bp of the left end of the adenovirus genome and a second plasmid (cosmid vector) carries the remainder of the adenovirus genome (31kb). The first plasmid contains an expression cassette, which usually consists of a strong promoter e.g. the cytomegalovirus (CMV) promoter, a poly-linker allowing insertion of DNA coding for the desired antigen and a poly-adenylation signal derived from, for instance, SV40 or the bovine growth hormone gene (BGH). The expression cassette is located in the former E1-containing region [224]. Heterologous recombination between the plasmid and cosmid within an E1 expressing cell line results in the formation of the entire genome, which subsequently can replicate in the nucleus to form E1-deleted progeny.

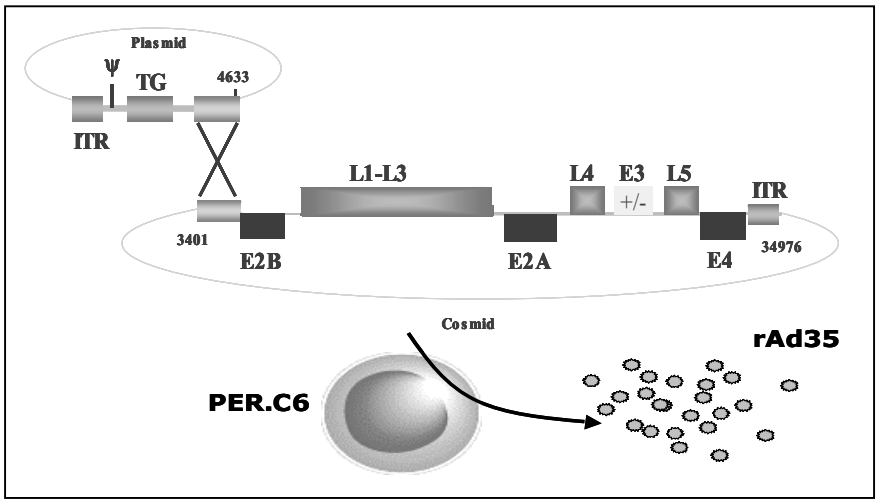

Fig. 7: Schematic representation of adenovirus vector generation.

There are several cell lines available that over-express the adenovirus E1 proteins, including HEK293 [225], 911 cells [226] and PER.C6 cells [227]. Of these cell lines the latter is the only one suitable for large-scale production of E1-deleted adenoviral vectors. This is because PER.C6 cells do not allow the formation of replication-competent adenoviruses (RCA) due to the lack of any overlap in vector sequences with E1 sequences located with in the cell genome (figure 7). In other cell lines, the presence of identical viral sequences in both vector and the E1-coding construct in the cellular genome triggers recombination and thus significantly increases the risk of generation of replication-competent adenoviruses

[228,229]. The presence of replication-competent adenovirus in clinical lots is undesirable since it could result in increased vector shedding by the patient [230] or enhancement of inflammatory responses [231]. Delivery of intact E1 sequences at high doses to vaccinees is also undesirable given the transformation potential of E1 proteins. In contrast to poxviruses, the adenoviral vector system is scalable to large volumes as it makes use of the PER.C6 cells that were purposely engineered to grow indefinitely. As such qualified master cell banks provide an excellent starting point for large scale manufacturing ensuring product quality and control. Also, given the enormous yield of recombinant adenovirus on a cell line like PER.C6, vaccines based on adenoviruses are considered highly cost-effective even for diseases like HIV and malaria, which require hundreds of millions of doses.

Adenovirus-based malaria vaccines
Replication-incompetent adenoviral vectors are being tested as subunit vaccines for a variety of diseases in both pre-clinical and clinical studies [232-235]. All the studies so far conducted have used the human recombinant adenovirus derived from serotype 5 (rAd5), mainly because this serotype was the first to be discovered, resulting in the availability of the genome sequence. Pre-clinical studies have shown that vaccines based on rAd5 vectors elicit high-frequency cellular immune responses against a variety of pathogenic antigens [236,237]. In the context of malaria, the T cell and antibody responses obtained using rAd5 carrying CSP of the murine *P. yoelii* strain were even higher than those induced by vaccination with irradiated sporozoites [238,239]. More importantly, vaccination with rAd5CSP conferred very high levels of protection (>90%) in mice [239]. These promising results have triggered the development of adenoviral vectors carrying multiple *P. falciparum* antigens for reasons outlined earlier. One of the rAd5-based vaccines contains pre-erythrocytic stage antigens CSP, SPSS-2/TRAP and LSA-1, whereas another contains the two erythrocytic antigens AMA-1 and MSP-1 [240]. These vectors wait further pre-clinical testing. Likewise, Phase I/IIa studies using a mixture of two rAd5-based vaccines (one carrying CSP, the other AMA-1) have recently been initiated. Although results obtained with adenovirus vectors show great promise in pre-clinical models, the data from clinical trials recently initiated are pivotal to better understand the strengths and weaknesses of for recombinant Adenovirus based vectors.

Emerging viral vector systems
As described earlier, a number of viral vectors other than pox- and adeno-viruses are being explored as carriers for malaria vaccines. However, many of these vector systems are poorly scalable and need far more process development including, for instance, state-of-the-art purification, in order to be developed as vaccine carriers that can be scaled for the sort of mass vaccination campaigns needed for malaria. Also, literature describing the potency of these vectors in the malaria field is scarce and it therefore remains to be seen whether any will progress to clinical trials of there safety and efficacy profiles. This paragraph details the data obtained thus far in the malaria field using recombinant flavivirus vectors, recombinant alphavirus vectors, and baculovirus vectors.

Flavivirus vectors
The flavivirus family consists of about 70 viruses, subdivided into 9 species, among which are the Yellow fever virus (YFV), Dengue virus (DE) and Japanese encephalitis virus (JE). Flaviviruses are small, single positive stranded RNA viruses with an icosahedral capsid of 40-60 nm in diameter, surrounded by an envelope. As for poxviruses, flaviviruses were first utilized as live attenuated vaccines following repeated cell passage. To date Yellow fever virus (YFV) strain 17D, derived from the attenuated Asibi strain, represents a highly

efficacious registered vaccine for yellow fever with one single subcutaneous administration required to confer life-long immunity. This vaccine has also demonstrated an excellent safety record in infants older than 9 months of age, having been used for vaccination of millions of people over the last 70 years. These observations have significantly contributed to the development of chimeric flaviviruses for vaccination against other members of this family including Dengue, Japanese encephalitis (JE) and west-nile (WN). For this purpose the envelope (E) protein or pre-membrane (prME) protein genes from yellow fever strain 17D were deleted and exchanged with the envelope (E) protein or pre-membrane (prME) protein genes from other flaviviruses. These live attenuated recombinant vaccines have been tested in preclinical models showing good safety profiles in both mice and monkeys [241,242]. Importantly, recombinant 17D carrying the PrME and E protein from Dengue virus elicited high-level neutralizing antibody responses in non-human primates, which conferred protection upon subsequent challenge with live Dengue virus [243]. Based on such data, as well as excellent safety and immunogenicity data using a 17D strain carrying prME/E from Japanese encephalitis virus flaviviruses, clinical trials have begun to further evaluate the safety and efficacy of these 17D vector-based vaccines in humans [244,245]. In addition, YF viruses can be used to express non-flavivirus genes by introducing these genes within the non-structural or E genes of the 17D strain. For instance within the field of malaria, a YF 17D chimeric vector, expressing one immunogenic epitope derived from the repeat region of the *P. falciparum* CSP gene in the 17D E protein coding domain, has been constructed. This vaccine proved immunogenic in mice, inducing high levels of anti-CSP antibodies [246]. Since only B-cell epitopes were introduced it is not expected that this vaccine will elicit strong T-cell responses. Also, protection against challenge infection was not determined in these studies, so the functional capacity of the antibodies in vivo remains unknown. In a follow-up study, a recombinant YF 17D vector was generated in which a CTL epitope of *P. yoelii* was inserted between the non-structural proteins NS2B and NS3 of the YF 17D strain (see figure 8).

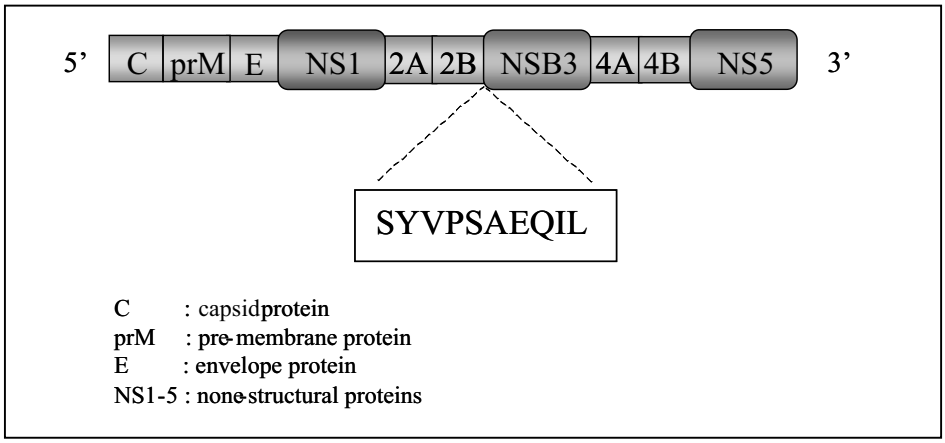

Fig. 8: Schematic representation of recombinant Yellow fever virus poly protein indicating the insert position of a *P.yoelii* CS epitope.

Pre-clinical testing of this vaccine in mice demonstrated the ability of the YF 17D vector to induce strong T-cell responses conferring protection (~70%) upon live sporozoite challenge [247]. These results are highly promising but have not yet been followed up by studies in non-human primates or human clinical trials. The numerous technological hurdles involved with use of the YFV 17D vector may be the reason for this. These technological hurdles

include restricted scale of manufacturing due to transgene instability, and limited insertion capacity of foreign genes, thereby preventing the use of full-length plasmodial antigens [240]. Furthermore, it remains unknown whether pre-existing immunity in human populations vaccinated against yellow fever with the attenuated 17D vaccine will hamper the use of the modified YFV strain 17D vectors. Finally, the use of the attenuated YFV strain 17D in newborns has been associated with adverse effects such as post-vaccination encephalitis or neurotropic accidents [248]. Since newborns represent an important target group for malaria vaccination, careful studies will need to be designed in order to investigate safety aspects of 17D strain-based vaccines in this population.

Alphavirus vectors

The alphaviruses belong to the family of *Togaviridae* and contain well-known species like Sindbis virus (SIN), Semliki Forest virus (SFV), and Venezuelan equine encephalitis virus (VEE). The genome of alphaviruses consists of a linear, single positive stranded RNA of approximately 10.000-20.000 bp. Alphaviruses and flaviviruses have similarities as they are both enveloped with an icosahedral structure. Features contributing to the use of alphaviruses as vectors for gene therapy and vaccine carriers include their ability to infect a wide variety of both vertebrate and invertebrate cells. Furthermore, alphaviruses exclusively replicate within the cytoplasm of the host cell, which leads to a transient but high expression of heterologous proteins. Since DNA integration into the host cell genome cannot occur, and pre-existing immunity is absent because most alphaviruses do not infect humans, alphavirus vectors are considered as safe and extremely suitable for vaccination purposes.

Alphavirus expression vectors can be generated in three different ways (figure 9): (i) as live attenuated virus, (ii) as replication-deficient RNA vectors, and (iii) as DNA-based vectors (extensively reviewed in [249]).

Briefly, replication-competent vectors contain the full-length viral genome and an additional promotor for the transcription of the inserted heterologous gene. Within mammalian host cells continuous production of viral particles takes place together with expression of the inserted protein. In contrast, replication-deficient vectors are only partially equipped with non-structural genes and an additional set of structural genes provided by RNA co-transfection or packaging cell lines are required to form virus particles (RNA replicons). Alternatively, alphavirus RNA can be introduced into cells as DNA-based alphavirus vectors. With the utility of eukaryotic RNA polymerase II expression type promotors, self-amplifying replicons can be formed (DNA replicons). In general, alphavirus vaccine development is mainly focused on the last two particle-based systems since they represent replication-incompetent vectors with reliable safety profiles.

In the last decade replicon-based vaccine vectors have emerged as viral systems for the development of vaccines against diverse intracellular pathogens, such as HIV and para-influenza [250 ,251]. Pre-clinical studies using these vaccine vectors have demonstrated that alphavirus replicons are highly immunogenic and protection was observed in virus challenge models [252-254]. Recently, clinical trials have been initiated to evaluate alphavirus-based vaccines for HIV, cytomegalovirus and influenza, but data is as yet not available. A limited number of studies report the use of alphavirus vectors as malaria vaccines. For instance, the efficacy of a sindbis virus-based vaccine has been assessed in the *P. yoelii* mouse model.

This study showed that immunization with recombinant sindbis virus expressing a CSP T-cell epitope, induced strong $CD8^+$ T cell responses and conferred protection after live sporozoite challenge [255,256]. Another pre-clinical study, conducted in mice, rats and rabbits, evaluated the efficacy of a SFV RNA replicon-based vaccine expressing part of the PfEMP1 protein. Clearly detectable levels of anti-PfEMP-1 antibodies were generated upon prime with SFV RNA particles and recombinant protein boost.

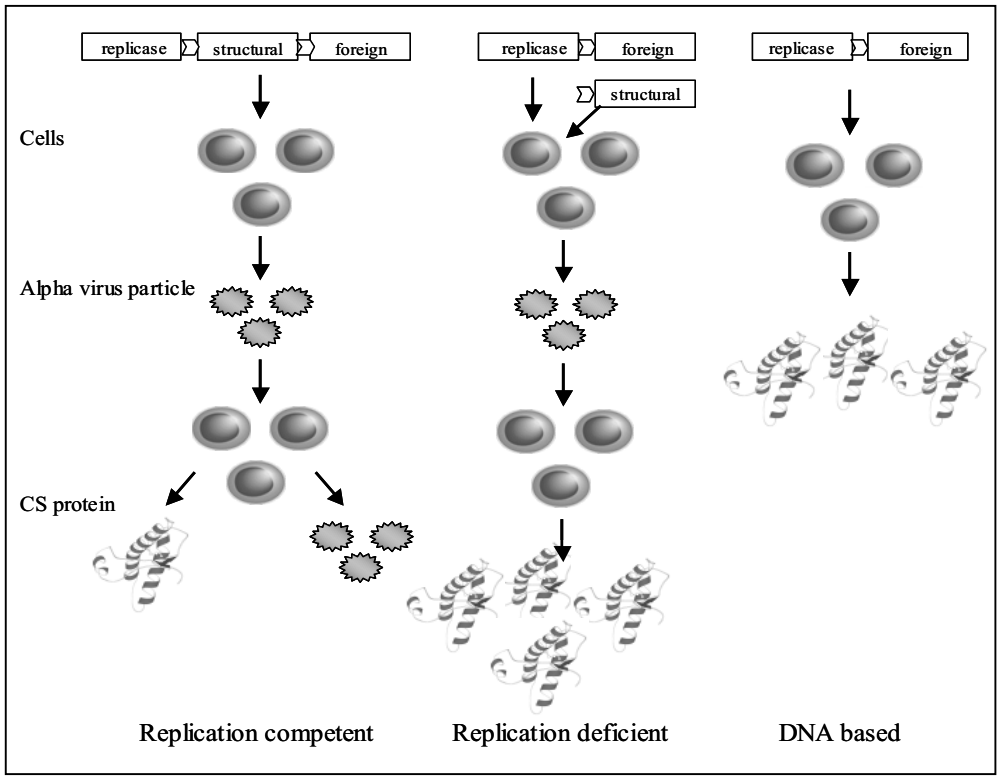

Fig. 9: Schematic representation of alphavirus vectors.

These antibodies were further shown to recognize parasite infected erythrocytes and to prevent the sequestration of *P. falciparum*-infected erythrocytes in mice [256]. These studies did not show the effect of the SFV vaccine alone since these studies primarily aimed to investigate the antigenicity of PfEMP-1. Although such results showed promise, no further studies with alphavirus based malaria vaccines have been reported yet.
In conclusion, the exploration of alphaviral vectors as vaccine carriers is still in the early stages with very limited clinical experience thus far. Therefore additional studies are required to further establish the safety and efficacy of alphavirus-based vaccines in humans. As described earlier for flavivirus-based vaccines, technical hurdles in manufacturing alphavirus-based vaccines will need to be solved first, especially in terms of scale reproducibility and ensuring that replication-competent alphavirus vector induction is avoided.

Baculovirus vectors
Baculoviruses are enveloped insect viruses consisting of a double stranded DNA genome of 130 kb that belong to the genus of Nucleopolyhedro viridae. Viral vectors derived from the *Autographa californica* nuclear polyhedrosis virus (*Ac*NPV) are commonly used as tools for protein production in insect cells. Although its host specificity was originally thought to be restricted to cells derived from arthropods, more recently it was established that several mammalian cells are also susceptible to baculovirus infection [257-259]. For instance it was found that baculovirus vector could efficiently transduce human derived dendritic cells, a desired target cell population for adequate immune induction [260]. Because of the latter

finding, baculoviruses have been explored as vectors for gene transfer in mammalian cells. Additional advantages for the use of recombinant baculovirus vectors include the capacity to insert large heterologous genes and the perceived safety profile, since baculovirus does not replicate in mammalian cells and induces only negligible viral protein expression in such cells. Indeed, studies to determine pre-existing anti-baculovirus immunity in humans confirmed the absent of both baculovirus-specific T cells as well as antibodies in human blood samples [260]. First vaccination studies with a recombinant baculovirus vector expressing pseudorabies virus glycoprotein B have been shown to elicit measurable humoral responses against the introduced viral protein, showing the rationale for using this baculovirus as a vaccine carrier [261]. Within the malaria field recombinant baculovirus vectors engineered to display on their surface a *P. berghei*-derived CSP demonstrated the capacity to induce high levels of CSP-specific antibodies in mice. Moreover, it was shown that upon challenge with sporozoite-infected mosquitos 70-90% of the mice were at least partially protected, as shown by significantly decreased clinical symptoms and parasite loads [262]. A recombinant baculovirus vector has also been generated, engineered either to carry the *P. falciparum*-derived CSP on the surface or to express the CSP gene from the baculovirus genome. Naturally, a baculovector expressing a gene of interest located in its genome has major advantages in manufacturing processes compared with creating a complex of a baculovector and an antigenic protein. In addition, these studies proved that baculovirus vectors could efficiently induce both CSP-specific antibody and T cell responses ($CD4^+$ and $CD8^+$) in mice when the antigen is expressed from the baculovirus genome. Notwithstanding the existing data, the further application of baculovirus vectors as vaccine carriers is much less well-defined than any other viral vector tested in the malaria field thus far. Therefor further characterization is required to better understand the position of baculovirus as a 'mature' vaccine carrier in comparison with the other vaccine carriers described in this section.

Conclusion

The last three decades have been marked by tremendous progress in understanding malarial disease, parasite biology and pathology. This basic knowledge has been translated into detailed information on a range, arguably still rather limited, of *P. falciparum* proteins that are known to play pivotal roles in induction of host immune responses, and that are thus considered excellent candidates for the development of vaccines. Undoubtedly, the availability of the entire genome of *P. falciparum*, combined with powerful proteomic technology that allows rapid identification of protein expression profiles, will lead in the near future to the identification of additional *P. falciparum* proteins that could serve as targets of new vaccines. Likewise, both animal and human experimental infection models that allow robust testing of vaccine candidates are established and are now routinely used, providing more rapid assessments of the value of candidate malaria vaccines. It has also become clear that in order to effectively control *P. falciparum*, a vaccine will need to elicit both neutralizing antibodies and strong T-cell responses such that protection is long lasting. These insights have shifted current thinking of vaccine design from proteins, usually most efficient at eliciting strong antibody responses only, to vaccine modalities that can also elicit T-cells. As described here, these new generation vaccines include a variety of live replication-deficient or attenuated viruses engineered to carry *P. falciparum* antigens, and the pre-clinical data generated thus far with several of these carriers are extremely promising. Based on these data early clinical trials are now testing the safety and immunogenicity of these entirely novel vaccine modalities. Although further development of these vaccine carriers in the malaria field still requires many technological hurdles to be overcome, the investment is clearly warranted. The combination of the scientific advances

described here strongly supports the belief that we are entering an exciting decade, which will undoubtedly reveal the value of many of these novel virus-based vaccine carriers in the battle against malaria.

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