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The Impact of Immune Responses on the Asexual Erythrocytic Stages of *Plasmodium* and the Implication for Vaccine Development

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1. Introduction

Natural immunity against malaria is acquired after repeated infections for an extended period of time resulting in a state of immunological non-responsiveness against the malaria parasite. This state ultimately prevents the onset of severe disease such as cerebral malaria thereby reducing the risk of death from malaria. Individuals with acquired natural immunity to malaria still harbor parasites (albeit in low densities) in the blood, and therefore natural immunity against malaria is not sterile. For this reason, natural immunity to malaria cannot be compared to immunity achieved against other diseases where the immune response neutralizes and eliminates the pathogen. The second hallmark of natural immunity to malaria is that protection wanes once a "protected" individual leaves the malaria-endemic area indicating that protection depends on continued antigen exposure. Immunity to malaria is stage- and species-specific and distinct immune mechanisms confer protection against the different developmental stages of the parasite. In the case of blood stage infection, passive transfer experiments with purified human immunoglobulins derived from immune individuals living in malaria-endemic areas have demonstrated that antibodies can mediate protection (Cohen et al., 1961; Butcher et al., 1970; Bouharoun-Tayoun et al., 1990). Mechanistic studies revealed that the effect of blood stage-specific antibodies on the asexual erythrocytic parasite depends on their antigen-specificity; antibodies can bind to merozoites, opsonize and target them towards phagocytic cells of the host (Groux and Gysin, 1990), or prevent invasion of new erythrocytes (Perkins, 1991). Once infected, antibodies against asexual blood stage antigens such as Pf332 or MSP-1 inhibit the intra-erythrocytic development of *Plasmodium falciparum* (Ahlborg et al., 1996; Siddique et al., 1998; Bergmann-Leitner et al., 2009). Antibodies directed to antigens expressed by sexual erythrocytic stages (gametocytes) have been shown to prevent transmission of malaria by blocking either the infection of the mosquito or the development in the mosquito (Lavazec and Bourqouin, 2008). As will be outlined in this chapter, it becomes increasingly clear that a blood stage vaccine may never be able to induce sterile protection, but can prevent mortality. Why bother developing blood stage vaccines? Extensive studies characterizing

the leading pre-erythrocytic vaccine RTS,S have shown approximately 40% sterile protection of vaccinated US individuals (Kester et al., 2007). Protection in the field is restricted to reduced mortality and morbidity and requires vaccination of young children that have continued exposure to the parasite. Under these circumstances, partial protection (when defined as time to first clinical episode) was approx 30% (Sacarlal et al., 2009). The limited efficacy of pre-erythrocytic vaccines appears to be the result of the fact that even a few sporozoites which escape the vaccine-induced immune response are still capable of establishing full blown blood stage infection. Combining a pre-erythrocytic vaccine such as RTS,S with a blood stage vaccine would assure that individuals, who did not develop sterile immunity (sterile immunity has never been achieved in field trials) would have a reduced risk of dying as a result of the added blood stage component.

This chapter will highlight the major readout methods that are currently used to gauge the efficacy of blood stage vaccines. In the absence of 'protection' models, the study of blood stage vaccines requires the development of *in vitro* assays that predict vaccine efficacy. In the absence of a definitive correlate of protection, the predictive value of these assays still awaits validation. Various target antigens for vaccine development will be highlighted with a special focus on the Merozoite Surface Protein (MSP)-1. Lastly, we will discuss modes of action of immune effector mechanisms against blood stage parasites and provide a preview of next-generation malaria vaccines.

2. Methodological tools to study anti-parasite activities mediated by *Plasmodium*-specific antibodies

The ability to culture blood stage parasites of *P. falciparum* has greatly assisted in our understanding of events associated with maturation of the intra-erythrocytic parasite (Trager and Jensen, 1976; Haynes and Moch, 2002; Haynes et al., 2002). This is underscored by the lack of knowledge for other *Plasmodium* species that have not been successfully established in culture. Once the liver schizonts have reached maturity, the hepatocyte membrane dissolves and the merosome, *i.e.*, the parasitophorous vacuole which resembles a bag containing infectious merozoites, is released from the liver destined for the lung capillaries where infection of erythrocytes occurs (Baer et al., 2007). The erythrocytic life cycle of *P. falciparum* blood stage parasites ranges from 38-48 hrs. During the first 24 hrs the parasite has a ring-like morphology (ring stage parasite) which subsequently develops into a trophozoite that is clearly distinguishable by the increasing amount of DNA and cytoplasm. Further maturation of the parasite into the schizont stage is marked by DNA segmentation resulting in individual nuclei. Additionally, schizonts have increasing amounts of digested hemoglobin (*aka*, malaria pigment or hemozoin). The schizonts rupture after first dissolving the parasitophorous vacuole and then the erythrocyte membrane, thus releasing 16-32 infectious merozoites per new infected erythrocyte (Cowman and Crabb, 2006). This event typically occurs in post capillary venules where the flow rate of the blood is low thereby favoring the rapid invasion of erythrocytes. Free merozoites have a relatively short lifespan and thus it is thought that the invasion event occurs quickly (within 5 minutes). This poses a significant challenge for effector antibodies which have to engage the parasite during this brief period of vulnerability and to block the interactions between host cells and merozoites. Successful active or passive immunotherapy requires providing an ample supply of antigen-specific, high-affinity antibodies capable of blocking a 'tsunami' of parasites every 38-48

hours. Whether this will be achievable to prevent morbidity or whether blood stage vaccine-induced antibodies will only be able to prevent severe disease and mortality is currently the focus of erythrocytic vaccines. Ideally, *in vitro* methods and/or preclinical models would assist in the down-selection of vaccine candidates. However, currently there is no preclinical model that adequately simulates human malaria. In contrast, there are several *in vitro* methods that have been used for analyzing functional antibodies against blood stage parasites *in vitro*. The following methods have been employed extensively in the evaluation of blood stage vaccines and we outline the advantages and drawbacks of the various methods as well as their predictive value.

2.1 Functional assays capturing the biological activity of blood stage, parasite-specific antibodies

Various methods have been developed that allow the measurement of the biological functionality displayed by either the antibodies themselves (growth inhibition assays (GIA) and invasion inhibition assays (IIA)) or antibodies in collaboration with immune cells expressing Fc-receptors (receptors that naturally bind certain immunoglobulin isotypes in order to eliminate antigens bound by antibodies). Antibodies bind their specific pathogen and form "immune complexes". Depending on the class and isotype of the antibody in the immune complex, this interaction leads to different outcomes: (1) the immune complex activates the complement system, *i.e.* complement fixation (which ultimately results in the lysis of the attached pathogen) or (2) the immune complex binds to Fc-receptors, *i.e.* opsonization (mediating uptake of the bound pathogen by the immune cells) or (3) antibody binds to the antigen and inactivates (neutralizes) the pathogen and thus prevents infection. The functional analysis of anti-blood stage antibodies, especially when obtained from individuals in endemic areas, is complicated by the presence of toxic factors or lipids in sera or plasma (such as anti-malarial drugs and anti-coagulants, or oxidized lipids due to long or inadequate sample storage). Eliminating nonspecific toxicities requires purification of the antibodies from serum or plasma using either bulk enrichment techniques (such as precipitation with ammonium sulfate and caprylic acid) or immunoglobulin purification methods (*e.g.*, the use of Protein A, G , L-columns). It should be noted that most anti-malarial drugs can be removed by simply dialyzing the samples (Sy et al., 1990; Persson et al., 2006) which allows the testing of samples where only small volumes are available as is often the case for pediatric specimen. When comparing various methods in regards to yield, purity, integrity and retention of functional activity of recovered antibodies, we found that some methods lead to the selective enrichment of certain isotypes or in some cases fairly unstable immunoglobulin preparations (Bergmann-Leitner et al., 2008b). Also, not all purification methods perform equally well when purifying immunoglobulins from different species. The choice of purification method greatly influences the results obtained in the functional assay and can lead to significant artifacts.

2.1.1 Microscopic analysis

Microscopic evaluation of blood smears stained with Giemsa is the classic method for detecting parasitized erythrocytes (pRBC) and remains the gold standard in clinical trials. It is also used for studying the effect of antibodies on the morphology of the intra-erythrocytic parasite. To detect parasitemia in the blood, thick film-smears are prepared and 100

microscopic fields are screened for the presence of parasites at a 1000x magnification. For quantitation, 200 leukocytes and all pRBC in the respective fields of a thin film-smear are counted. If nine or less pRBC per 200 leukocytes are detected the quantitation is extended to 500 leukocytes. The equipment is readily available in all clinical labs and is fairly cheap. However, since the analysis is done by humans, it is essential that it is performed in a double-blinded fashion to avoid introduction of bias and subjectivity. Moreover, the microscopists have to be highly trained and experienced in slide reading in order to reliably identify malaria parasites in thick and thin film blood smears. The expertise of microscopists has long been recognized as the key issue for evaluating vaccine and malarial drug trials as quantifying parasitemia of malaria infected individuals commonly defines a study's primary endpoint. Furthermore, manual analysis, *i.e.* individual slide reading, is also rather slow and therefore not suitable for high-throughput screening. The microscopic analysis permits the quantification of parasitemia, but cannot objectively characterize changes in morphology which would indicate developmental growth inhibition or retardation.

2.1.2 Flow cytometric analysis

Flow cytometry is an ideal methodology for the automated and objective analysis of large numbers of cells. It provides information about the number of positively stained cells as well as the intensity of staining (Shapiro, 2004). Staining of mature human erythrocytes with DNA dyes allows for the detection of *Plasmodium* infection as these host cells are devoid of DNA unless parasites are present. This analysis reveals both the percentage parasitemia in the culture (% DNA containing cells) as well as the intracellular DNA content which is indicative of the parasite's maturation stages within the infected cells. Various DNA dyes have been reported to be useful for the detection and quantification of pRBC (reviewed in (Grimberg, 2011)). We have compared several DNA dyes side-by-side (*i.e.,* Syto-16, SybrGreen and Hydroethidine) in order to determine their usefulness in measuring invasion and growth inhibition of anti-blood stage antibodies. Using standard DNA binding dyes such as Syto-16 (Brand et al., 2003) and SybrGreen allows only the quantification of pRBC in culture and does not assess the viability of the intra-erythrocytic parasite (Green et al., 1981; Pang et al., 1999; Tebo et al., 2001; Haynes and Moch, 2002). If invasion occurs but the parasite fails to thrive, then the infected cell is detectable. Thus this approach measures only invasion inhibition since antibodies that block invasion result in either low or no parasitemia. In order to assess actual developmental growth inhibition, a different strategy has to be employed:

(1) Measurement of the DNA content of the pRBC as a correlate of the maturation stage of the parasite (Figure 1). A prerequisite for this approach is the tight synchronization of the parasites – either through repeated Percoll/Sorbitol purification or growth in temperature-cycling incubators - prior to setting up the experiment as the analysis requires setting strict cut-off values (see Fig. 1) for the different maturation stages of the parasite (ring/trophozoite/schizont). Such cut-off values have to be established for every parasite clone and isolate and have to be revised whenever culture conditions (new batch of serum/ culture media) change. Failure to control for these changes and the different growth kinetics of isolates and clones results in inaccurate estimates of parasite growth and yields uninterpretable results.

(2) Use of viability DNA stains such as hydroethidine (HE) (van der Heyde et al., 1995). HE-staining depends on the intracellular conversion of HE into ethidium by parasitic NADPH oxidase and has been described in various protozoan systems including malaria to be a reliable metabolic indicator of parasite viability (Wyatt et al., 1991; van der Heyde et al., 1995). The host erythrocytes' enzymatic activity is not sufficient to convert the dye and, therefore, host cells do not introduce artifacts into this analysis.

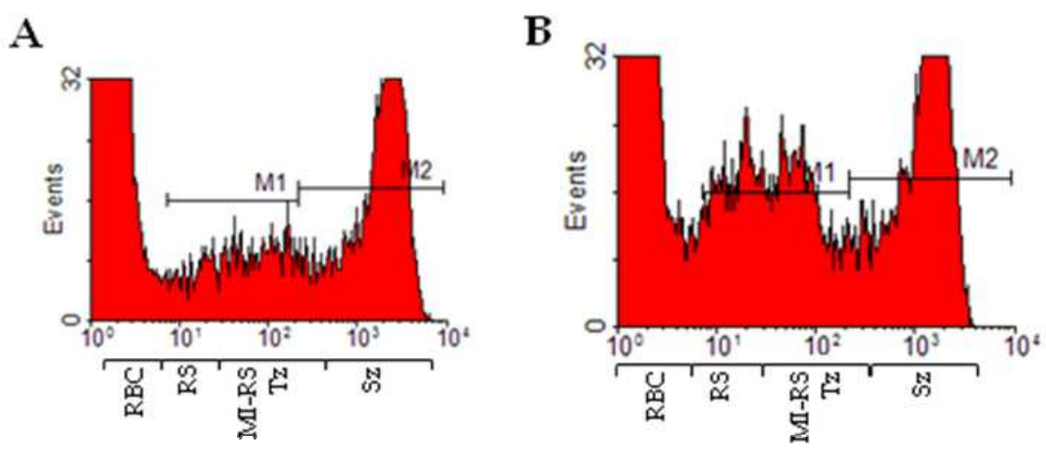

Fig. 1. DNA content of blood cultures infected with *P. falciparum* in the presence of control (malaria-naïve, not growth inhibitory) serum (Panel A) or immune serum (Panel B) determined by flow cytometric analysis. X-axis = Mean fluorescence intensity of Syto-16 DNA staining, Y-axis = arbitrary unit for cell number. Brackets under X-axis indicate the DNA content of uninfected erythrocytes (RBC), ring stage parasites (RS), multiply infected ring stage parasites (MI-RS) and trophozoites (Tz) and schizont stage parasites (Sz). Marker M1 captures young pRBC and M2 mature pRBC and the changes of the proportion have been used to calculate growth inhibition. Panel A serves as reference for healthy, un-impaired parasite growth. Panel B indicates growth retardation as there is a higher prevalence of ring and trophozoite stage parasites compared to the control serum. The difference between the area under the curve for M1 of the test culture and the control culture is used to report the percentage of growth inhibition.

2.1.3 Measurement of invasion inhibition by using transgenic parasites

In an attempt to identify the contribution of MSP-1 mediated invasion inhibition, O'Donnell *et al*. (O'Donnell et al., 2001) developed a *P. falciparum* D10 clone (MAD 20 allele) transgenic in the MSP-1p19 portion where the wildtype sequence was replaced by the p19 of *P. chabaudi*. Testing the inhibitory activity of antibodies against the parental D10 compared to the transgenic parasites identifies the contribution of *P.falciparum* MSP-1 mediated parasite inhibition. Other transgenic parasites have been developed that express the green fluorescence protein to facilitate the detection of the parasites within erythrocytes (O'Donnell et al., 2000). This approach eliminates the need for intracellular DNA staining of pRBCs. Using these parasites requires maintaining an additional parasite strain in the laboratory, which adds cost and labor, but it greatly facilitates the analysis because no additional manipulation of the cultures, *i.e*., DNA staining, is required to perform the

analysis. The analysis would yield similar results as staining with a general DNA dye such as SybrGreen, which allows accurate detection of invasion inhibition while the quantitation of growth inhibition is challenging as outlined above.

2.1.4 Measurement of the metabolic activity of the intra-erythrocytic parasite

An alternative to quantifying either parasitized cells or the DNA content of infected erythrocytes is the measurement of enzymatic activity. The following approaches have been previously described: (1) the quantification of ^{3}H-hypoxanthine incorporation into newly synthesized DNA (Bungener and Nielsen, 1968; Rahman, 1997), (2) the conversion of hydroethidine to ethidium (Wyatt et al., 1991; van der Heyde et al., 1995), and (3) detection/measurement of the parasite-derived lactate dehydrogenase (pLDH) (Makler and Hinrichs, 1993; Prudhomme and Sherman, 1999). Measuring the metabolic activity allows for the detection of parasitized erythrocytes that are viable and metabolically active thus enabling the quantitation of growth inhibition. It should be pointed out that multiple LDH-substrates have been described in the literature for *Plasmodium ssp*. There are, however, qualitative differences between these substrates that will affect the sensitivity and the signal-to-noise ratio. The protocol developed at NIAID/NIH (Kennedy et al., 2002) has demonstrated robustness, reproducibility and specificity (Corran PH, manuscript in preparation). This basic protocol was optimized in our laboratory to accommodate small sample volumes (< 50 µl) and high throughput screening utilizing 384 well plates and assay volumes of ≤ 20 µl (Bergmann-Leitner et al., 2008a).

2.2 The quest for the most sensitive method for identifying anti-parasite activity of anti-malarial antibodies

We have compared four methods in an attempt to identify the most sensitive method capable of measuring an array of functional activities displayed by immune antibodies, namely: (1) GIEMSA-staining of blood smears (with visual detection); (2) flow cytometric analysis using either a standard DNA binding dye (Syto-16) or (3) hydroethidine; and (4) pLDH detection using a substrate specific for the parasitic enzyme LDH (Bergmann-Leitner et al., 2006). The model system to evaluate anti-parasite activities was based on the use of immune sera specific for either AMA-1 (Kennedy et al., 2002) or MSP-1p42 (Angov et al., 2003; Darko et al., 2005) and previously established *P. falciparum* parasite clones (namely 3D7 and FVO). While staining with Giemsa revealed antibody-induced morphological changes in parasite development after exposure to immune serum, directly correlating these changes with parasite viability and thus efficacy of the antibodies is limited due to the subjective nature of the readout method. The DNA dye Syto16 readily permeates membranes of both viable and non-viable cells and thus cannot be used to determine the parasites' viability since any erythrocyte with DNA content will equally be identified as an "infected cell", thus under-estimating the inhibition. Growth inhibition (viability), however, can be determined by either flow cytometric analysis of parasites whose DNA was stained with HE or by measuring the enzymatic activity of pLDH. The conclusion from this comparative study was that the mode of action of antibodies directed against malaria blood stage antigens depends on not only the target antigen but also on the parasite strain. In cases where antibodies primarily mediated invasion inhibition, such as anti-AMA-1 specific antibodies (Triglia et al., 2000; Healer et al., 2004), all four methods yielded similar results.

However, antibodies directed against MSP-1p42 preferentially inhibited invasion or inhibited either parasite growth and development, depending on the parasite test strain. MSP-1 specific antibodies acted on the FVO parasite clone mainly by inhibiting invasion. In contrast, the same antibodies mediated invasion- and growth inhibition in the case of 3D7 parasites (the exact mechanisms involved in this anti-parasite activity will be discussed in section 5). This study demonstrated that readout methods, which can distinguish between invasion inhibition and growth inhibition of the intra-erythrocytic parasite must be employed in order to comprehensively define the antibodies' mechanism of action.

In an effort to increase the sensitivity of the readout methods used to assess the anti-parasite activity through DNA dyes and flow cytometric analysis, multi-cycle experiments have been evaluated (Haynes et al., 2002; Persson et al., 2006), (Bergmann-Leitner, unpublished observations). To this end, cultures were not limited to the length of a single life cycle (40-48 hours duration), but allowed to continue for at least another cycle (72-96 hours duration). Several caveats are associated with this experimental setup: (1) The starting parasitemia of the culture has to be adjusted for each isolate/clone to assure that the parasites will not overgrow after the completion of the first cycle. This would result in the depletion of nutrients and the exhausted culture conditions would result in an overestimation of growth inhibition. One remedy has been to feed the cultures once or repeatedly. This can, however, pose yet another challenge as the exchange of media dilutes the antibodies and without knowing at which stage they will take effect, this manipulation could reduce their biological activity. Replenishing the antibodies when changing the media may lead to an overestimation of the biological effect in cases where the antibodies have already bound to the surface of the pRBC or entered the parasitophorous vacuole. Therefore, adding more antibody artificially increases the total antibody concentration; (2) Outgasing of cultures (*i.e.*, change in the ratio of CO_2 and O_2 in the atmosphere due to exposure to regular air) during the feeding process, can result in reduced invasion or slowed growth; (3) Interrupting invasion events when feeding occurs very closely to the time of schizont rupture. Estimating a safe time frame may be challenging because antibodies may slow the growth of the parasite, but not inhibit it and the growth inhibitory effect would be amplified; and (4) Controlling for proper starting parasitemia and modifying feeding times still leads to reduced multiplication rates in static cultures. Using suspension cultures for larger culture vessels such as 24-well plates and culture flasks yielded similar multiplication rates of the parasites during the second cycle compared to the first cycle (when feeding was also performed). It should be noted, however, that we have been unable to replicate the beneficial effect of suspension cultures when scaling down the assay format to 96 well and 48 well plates (Bergmann-Leitner, unpublished observation).

2.3 Functional assays to evaluate antibody dependent cellular cytotoxicity (ADCC)

Early studies have shown that antibodies *per se* may not be sufficient to block the blood stage parasites (Bouharoun-Tayoun et al., 1990; Ouevray et al., 1994) and a cellular component is involved in the antibody-mediated anti-parasitic effect. Compared to classic antibody-dependent cellular cytotoxicity (ADCC), the postulated antibody-dependent cellular inhibition (ADCI) is mediated only by blood monocytes (not macrophages, polymorphonuclear neutrophils (PMN), lymphocytes, platelets) and primarily by cytophilic immunoglobulin classes (particularly IgG3) that target merozoite surface antigens

(Bouharoun-Tayoun et al., 1995; Tebo et al., 2001). The activation of monocytes through FcγRII triggers the release of soluble TNF-α that in turn blocks the development of adjacent intra-erythrocytic parasites (Bouharoun-Tayoun et al., 1995). Furthermore, a role of FcγRI in contributing to clinical malaria has been suggested based on the functional properties of recombinant human antibodies derived from immune Gambian adults (McIntosh et al., 2007). These antibodies were tested in mice transgenic for human Fc-receptors challenged with transgenic rodent malaria parasites (expressing *Pf*MSP-1p19) in an effort to evaluate the role of antibodies in protection. Another study demonstrated that the activation of monocytic cells requires two distinct Fcγ receptors (FcγRII and FcγRIII) simultaneously engaged by a least two cytophilic IgG molecules, which are part of the same immune complexes (Jafarshad et al., 2007). Finally, the concept of the contribution of Fc receptors in natural immunity is supported by epidemiological studies (Shi et al., 2001; Israelsson et al., 2008; Leoratti et al., 2008).

We conclude from these studies that ADCI as an anti-parasite defense mechanism depends on the target antigen, a concept with significant implications for vaccine development (revisited below in section 4). Experiments measuring the ADCI activity of anti-blood stage antibodies are set up in principle as described for growth/invasion inhibition assays (above): parasite cultures are established at a defined percentage of parasitemia in the presence or absence of immune antibodies and – in addition - in the presence of monocytic cells. Readout methods used are the same as for the standard "growth inhibition" assays as described earlier (our lab prefers the pLDH assay for this purpose). Note that the source and quality of the monocytic cells will greatly influence the results obtained in these experiments: (1) freshly isolated PBMC (plastic adherence *vs*. CD14 MACS separated cells *vs*. monocytic tumor cell lines); (2) resting *vs*. cytokine activated cells; or (3) inter-donor variations. When optimizing the ADCI protocol, it is important that the *in vitro* conditions including the cell types of phagocytes used simulate the *in vivo* setting as closely as possible in order to reproduce the conditions in the malaria infected host. Using cancer cells such as the monocytic tumor cell line THP-1 may not adequately mimic the effector population found in the patient. This is underscored by recent findings that infection and acute malaria can drastically change the phenotype of monocytic subpopulations (Chimma et al., 2009). This includes changes in chemokine receptors as well as functional properties. This was evidenced as monocytic cells derived from patients during an acute malaria infection were superior over the monocytes derived from healthy donor individuals. However, other studies have described significant impairments of monocyte functions due to malaria infection, such as their ability to phagocytize (Leitner and Krzych, 1997). This underscores that there is more to monocytic cells than just the expression of Fc-receptors and that these observations warrant the characterization of effector cells used for *in vitro* assays in an effort to minimize *in vitro* artifacts.

To this end, we tested various methods for generating activated monocytic cells *in vitro* and found that the purification of monocytes with magnetic bead labeled anti-CD14 monoclonal antibody resulted in higher and more reproducible ADCI-activities as compared to cells that were isolated by plastic adherence. Moreover, pre-activating the isolated cells overnight with 300 U/ml IFN-γ reduced the inter-assay variability. Pre-activation also decreased the variability observed between different blood donors consistent with the concept that the stimulation "normalizes" the activation state of the cells (data not published).

What role does the parasite isolate or clone play when measuring ADCI activity? Using a sample from an individual from a malaria-endemic area (Lyon et al., 1997) we were able to detect a differential contribution of ADCI in the growth-inhibition of two *P. falciparum* clones: various amounts of serum in the assay in the presence or absence of monocytes were tested against the 3D7 and FVO parasite clones (Figure 2). The main difference in the clones is the fact that they represent the two distinct MSP-1 alleles (the MAD20 and the Wellcome K1 allele, respectively) and they have also been described as invading either through sialic acid independent (3D7) or dependent (FVO) pathways. The inhibitory activity of serum against 3D7 parasites was not increased by the presence of monocytes while the inhibition of FVO parasites could be amplified by adding monocytes (data not published). This synergistic effect of antibodies and monocytes became apparent when limiting the amount of serum in the assay. More studies are needed to determine the underlying mechanism for this differential effect as this may be a matter of antibody fine specificity (discussed in section 5).

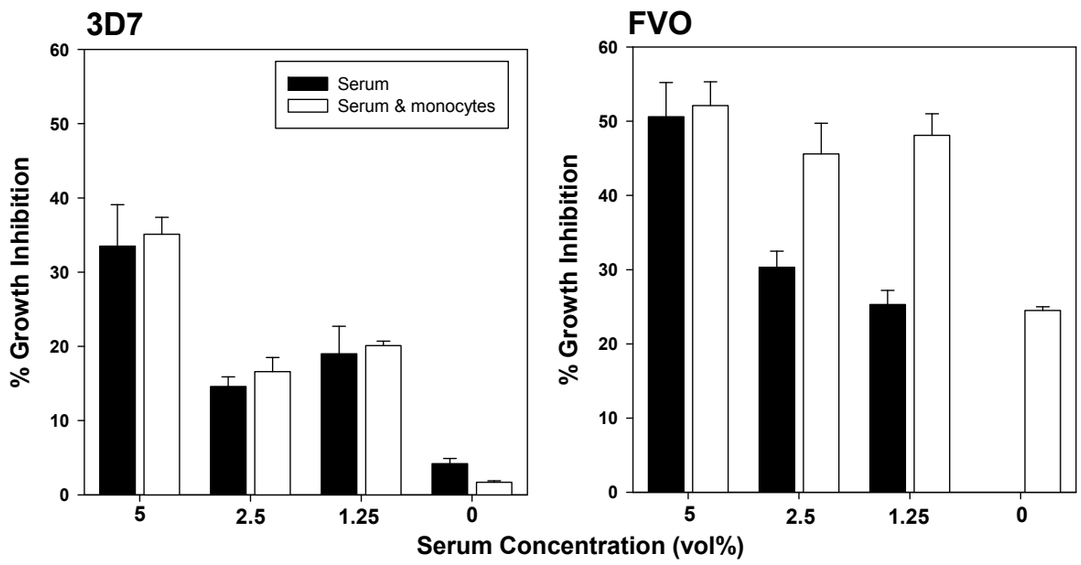

Fig. 2. Effect of inhibitory antibodies and antibody-dependent cellular inhibition on growth of 3D7 (left Panel) and FVO (right Panel) parasite clones. X-axis indicates percentage of human malaria-immune serum in the culture; y-axis shows the % growth inhibition measured by quantifying pLDH. White bars = growth inhibition induced by serum only, black bars = growth inhibition induced by serum and $CD14^+$ IFN-γ activated monocytes. Data expressed as mean % growth inhibition, error bars indicate SD.

3. Anti-parasite activities in sera from individuals living in malaria-endemic areas

Protection against malaria mediated by antibodies recognizing the erythrocytic parasite or the parasitized erythrocyte is mediated by several distinct mechanisms: (1) Binding of the antibody to the surface of merozoites can interfere with the invasion of new erythrocytes and opsonize the merozoite, which results in complement activation and/or phagocytosis; (2) Binding to the merozoite may not suffice to block invasion, but antibodies carried into

the infected RBC by the merozoite can result in growth inhibition; (3) Binding to pRBC can prevent sequestration, thus exposing the pRBC to conditions that are unfavorable for development, which may lead to increased clearance of pRBC by the spleen; and (4) Binding to pRBC can interfere with rosetting, a prerequisite for invasion. Studying the invasion of erythrocytes by merozoites has resulted in the identification of various invasion pathways, which the parasite can utilize. These pathways can be categorized broadly into: (1) sialic acid dependent and (2) sialic acid independent pathways. Field studies of the anti-parasite activity in sera from individuals with acquired natural immunity have shown that one of the earliest mechanisms to block blood stage parasites is to develop antibodies that interfere with the sialic acid dependent invasion (Baum et al., 2003; Nery et al., 2006; Persson et al., 2008). Antibodies obtained from sera of young African children are more likely to block sialic acid dependent pathways while the ability to block sialic acid independent pathways is acquired after years of exposure to the parasite and with age. Antibodies capable of inhibiting rosetting can mediate protection, which reduces the risk of cerebral malaria (Vigan-Womas et al., 2010). Nevertheless, the actual role of antibodies in protecting against disease in the field remains controversial. While they may reduce the parasite load of individuals living in endemic areas, they do not mediate sterile protection or total suppression of parasites in the blood (Genton et al., 2002). Recently, an apical merozoite antigen (AMA)-1 based vaccine that induces high antibody titers, and high GIA responses *in vitro* in human vaccinees (Ellis et al., 2009) was evaluated for its protective effect in a blood challenge. To this end, the vaccinees were immunized and then challenged with blood infected with the 3D7 parasite clone. While the vaccine was able to reduce the multiplication rate *in vivo*, it was unable to mediate complete elimination of the parasites after a blood challenge (Duncan et al., 2011). This may indicate that either a single antigen such as AMA-1 or blood-stage antigens in general may be able to alleviate the morbidity associated with disease (as seen in the case of natural immunity), but may not be able to induce complete protection against disease.

Longitudinal studies in hyper-endemic malaria transmission areas have revealed factors related to the development (or lack thereof) of malaria-specific antibodies. Due to overlapping malaria infections in high transmission areas, where infections can occur frequently or even daily, it is difficult to study immune responses elicited by and maintained after a discrete infection. Studies in high transmission areas have a limited ability to consider age because participants suffer many unrelated infections during their first 5 years of life, including viral, bacterial and other parasitic diseases. One example is nematode infections, which are quite common in malaria-endemic areas and predispose the host to Th2-type immune responses. Exposure to malaria is an important potential confounder in immune-epidemiological studies. Therefore, the inadequate measurement and adjustment for differences in exposure may lead to the underestimation of the strength of associations between immunological variables and malaria incidence (Kinyanjui et al., 2009). The strongest evidence that antibodies are important mediators of naturally acquired immunity is from passive transfer experiments of antibody from immune adults used to treat children with severe *P. falciparum* malaria (Cohen et al., 1961; McGregor and Carrington, 1963; Bouharoun-Tayoun et al., 1990). IgG and IgM antibodies present in sera of malaria-exposed individuals recognize and bind directly to trophozoites or schizonts. Neutralization and agglutination of merozoites and pRBC by these antibodies are reported as possible protective mechanisms during *Plasmodium* infection (Cohen and Butcher, 1971).

IgM antibodies are characterized by lower affinity for antigen compared to their IgG counterparts because little or no somatic hypermutation and clonal selection has occurred. IgM antibodies form pentamers to compensate for the lower affinity, which results in a higher overall avidity due to increased number of binding sites. Evidence for a protective role of IgM against malaria infection (Wahlgren et al., 1986; Boudin et al., 1993) or against severe malaria (Brasseur et al., 1990) has been reported.

Evidence from field studies in Ghana (Dodoo et al., 2000), Senegal (Oeuvray et al., 2000) and East Asia (Soe et al., 2004) suggest that cytophilic antibodies are associated with a lower risk for subsequent clinical malaria episodes. These isotypes are also associated with the antibody-dependent cellular inhibition as discussed above. The importance of immunoglobulin isotypes in addition to the antigen specificity of the humoral response is underscored by studies that report an association between the levels of noncytophilic antibodies and clinical malaria incidence. IgG4 antibodies are preferentially induced after repeated exposure (Aalberse et al., 1983a; Aalberse et al., 1983b) and this isotype is associated with enhanced risk of infection and with a high risk of clinical malaria episodes (Aucan et al., 2000).

The attitude towards the use of measuring growth inhibition as an immune correlate has changed over the past five years. Invasion or growth inhibitory (GIA) activity was considered a reliable predictor of vaccine efficacy and the results from these assays were used to down-select potential vaccine candidates. Initially, GIA activity was determined by using purified immunoglobulins from preclinical or clinical trials at high concentrations and from these studies, strong inhibition was frequently observed. However, the same vaccines that generated very "high" GIA activities in rabbits and other preclinical models failed to induce protection in naïve US individuals (Spring et al., 2009; Duncan et al., 2011). In residents from malaria-endemic areas, GIA activity is, however, a common factor associated with protection against clinical disease (Dent et al., 2008). In this study, malaria-infected participants were drug-cured and followed up until their next malaria infection. Participants whose sera exhibited higher growth inhibition prior to drug cure were protected for significantly longer periods of time than those participants whose antibodies mediated only low GIA activities. Moreover, an age-dependent effect was observed in that GIA activity inversely correlated with the age of study participants. Another confounding factor is the historical exposure of study participants as vaccine efficacy differs dramatically between individuals with no prior malaria history *vs.* residents from malaria-endemic areas. For example, an AMA-1 vaccine tested in US naïve study participants resulted in relatively high GIA activities, but the same vaccine failed to induce either GIA activity or protection as defined by time to the next malaria episode when given to individuals in Mali (Miura et al., 2011).

Review of the scientific literature on blood stage malaria vaccines reveals no straightforward strategy for evaluating a successful blood stage vaccine. A variety of reasons may be responsible for the contradictory outcomes.

- Differences in the transmission rates (intensity and stability) and the short lifespan of malaria specific antibodies can skew the measurements and conclusions. Therefore, directly comparing study results from different geographic regions that are not matched by transmission rates and seasons should be avoided. Transmission rates may have an impact on the clinical outcome of malaria episodes as well as on the induction of natural immunity.

- Differences in the kinetics of induction of natural immunity (mainly caused by high transmission rates) may influence the immune factors mediating protection.
- Differences in the definitions of clinical malaria and study endpoints. In some settings, are the individuals with no signs of malaria truly protected or simply not exposed to the parasite?
- The quality of plate antigens used for the ELISA: Proteins used for *in vitro* analyses may be of inferior quality due to misfolding or truncation. Recombinant proteins may not represent the native antigen structure. Moreover, for the analysis of MSP-1 specific immune responses induced by natural exposure to the parasite, most investigators have focused on the MSP-1p19 fragment rather than the MSP-1p42 protein, which is initially presented on the merozoite surface.
- Mismatching of recombinant antigens and parasite phenotype: In many studies, the parasite variant prevalent in the study area is not matched with the recombinant protein used for the *in vitro* analysis (*e.g.*, plate antigen for ELISA assay).
- Differences in the age of study participants: concentrations of malaria-specific antibodies are age dependent and, therefore, only comparisons of study participants with similar ages are valid.
- Lack of information about study participants' conditions such as the use of bed nets, use of anti-malarial drugs or folk-medicine, co-infection with other parasites, bacteria and viruses.
- Incompatibility in study design: in some cases study participants are pre-treated with anti-malarial drugs to clear parasites from the circulation prior to immunization while in other studies vaccines are administered while study participants have – in some cases significant numbers of- parasites in the blood. Therefore, it is inappropriate to compare vaccine potency and efficacy between such studies.

4. Identification of antigens that are targeted by immune responses in immune individuals

The merozoite consists of the merozoite surface coat, the micronemes, the rhoptries, apicoplast and a nucleus. From each of these components several antigens have been isolated and their immunological potential and biological function has been evaluated.

4.1 Merozoite surface coat antigens

Several antigens expressed on the merozoite surface coat have been evaluated as vaccine candidates. Highlighted in this section are the most frequently targeted of those antigens:

- MSP-1: the properties and functions of this antigen will be discussed in detail in Section 5 since this antigen represents a major blood stage vaccine candidate (Ockenhouse et al., 2006; Ogutu et al., 2009).
- MSP-2: immunization with the recombinant protein MSP-2 protects non-human primates and has yielded allele-specific reduced parasite density in field studies (Genton et al., 2002). Much of the development of MSP-2 as a vaccine in the field has been hampered by the fact that MSP-2 is an unstructured protein that tends to form amyloid fibrils in solution (Genton et al., 2003). Therefore, quality control of manufactured vaccines is difficult without a known structure or conformation-dependent antibodies.

- MSP-3: this protein is non-covalently attached to the surface of the merozoite. This is different from MSP-1 and MSP-2, which are GPI-anchored proteins. Disruption of the MSP-3 gene is not lethal while genetic knockouts of MSP-1 and MSP-2 are. Similar to MSP-1, allelic dimorphism has been reported. MSP-3 specific antibodies found in sera from malaria-endemic areas are associated with protective immunity (Soe et al., 2004). MSP-3 based vaccines have been designed as long peptides representing the conserved C-terminus of the antigen (Druilhe et al., 2005; Sirima et al., 2007). Anti-parasite activity of MSP-3 specific antibodies was not evident unless monocytic cells were used in the assay (ADCI).
- Glutamate-rich protein (GLURP): this antigen is not only expressed on the merozoite surface, but also on the liver stage parasite making it an attractive potential target for a multi-stage vaccine. The N-terminal region of the antigen is quite conserved and thus synthetic peptides representing the N-terminal non-repeat region have been tested in the clinic. Antibodies induced by these GLURP-peptides were able to inhibit parasite growth in the presence of monocytes indicating that - similar to MSP-3 - anti-GLURP antibodies act through ADCI (Hermsen et al., 2007). Several clinical studies have been conducted with GLURP alone (Hermsen et al., 2007) or in combination with MSP-3 (Esen et al., 2009; Belard et al., 2011).

4.2 Antigens within merozoite organelles

Antigens found in the apical organelles of the merozoites such as the apical membrane antigen (AMA)-1 or the erythrocyte binding antigen (EBA-175 RII) have long been studied due to their immunogenicity or biological function. While the exact function of AMA-1 is unknown, EBA-175 has been better characterized. EBA-175 is found in the micronemes of the merozoite and is secreted by merozoites in order to bind to erythrocytes that are ready for invasion. This binding facilitates the attachment of merozoites to the coated erythrocytes in a strain-specific manner (Camus and Hadley, 1985). EBA-175 is a member of a family of binding proteins such as EBA-140, EBA-181, MAEBL; all of these antigens share a receptor binding domain (Region (R)-II). The analysis of sera from malaria-endemic areas for the presence of EBA-175 specific antibodies revealed some association with protection in children that have higher antibody titers (reviewed in (Fowkes et al., 2010)). Recently, an EBA-175 based vaccine yielded some clinical efficacy (El Sahly et al., 2010).

AMA-1 is a highly polymorphic antigen generated in the rhoptries of the merozoites and it appears on the parasite's surface just before invasion occurs. AMA-1 is highly immunogenic and anti-AMA-1 antibody responses are found in sera from individuals living in malaria-endemic areas. Another promising feature of this antigen is the fact that AMA-1 is expressed on sporozoites and thus the antigen could act as a pre-erythrocytic as well as an erythrocytic vaccine. Analysis of AMA-1 specific antibodies in growth inhibition assays provided even more promise as the antibody activity is typically very high compared to other blood stage antigens. A clinical study in which volunteers were challenged by mosquito bite revealed that vaccination with AMA-1 was unable to provide sterile protection and only yielded a limited delay in the development of parasitemia in vaccinees compared to challenge control subjects (Spring et al., 2009). Characterization of the AMA-1 gene sequence revealed a fatal characteristic of this antigen which will likely preclude its use as a malaria vaccine in the field: well over 150 allelic variants of the antigen have been reported (Takala et al., 2009) and

humoral responses against AMA-1 indicate that immunity is allele-specific and therefore, an AMA-based vaccine would primarily provide strain-specific protection at best. Efforts are underway to develop AMA-1 vaccines that induce allele-cross-reactive responses to overcome this limitation (Remarque et al., 2008; Dutta et al., 2010). However, the success of any AMA-1 based malaria vaccines is also impeded by the fact that sera from malaria-endemic areas appear to contain antibodies capable of blocking the activity of AMA-1 specific antibodies (Miura et al., 2008).

4.3 Antigens expressed on the surface of infected erythrocytes

The access of antibodies to merozoite antigens is limited as merozoites quickly invade new erythrocytes. Antibodies have the ability to gain entry to into infected erythrocytes (Bergmann-Leitner et al., 2009), but cannot mediate ADCI and would act independently of phagocytic cells as described above. Therefore, efforts are underway to identify antigens on pRBC which are theoretically always accessible for binding by specific antibodies. The surface localization of the antigens indicates that they are crucial for sequestration of the pRBC in the placenta or post-capillary venules. This warrants their exploration as potential vaccine targets. The variant surface antigens (VSA) (reviewed in (Hviid, 2010)) have been shown to mediate sequestration of the parasite and immune responses towards these antigens confer protection in a strain-specific manner. One of the members, the *P. falciparum* erythrocyte membrane protein (*Pf*EMP)-1, has been reported to be encoded by the *var* gene family and displays varying immunogenicity depending on the variant that is generated (Bull et al., 2005). One of these variants, *var2csa* is expressed by parasites that sequester in the placenta leading to severe malaria attacks in primagravid women often resulting in miscarriage and/or death of the mother (reviewed in (Beeson and Duffy, 2005)). This has led to efforts to develop vaccines that will be administered prior to or early in pregnancy (Avril et al., 2009).

4.4 Multi-antigen responses: Reducing the risk for clinical infection or reducing parasite density

The analysis of naturally acquired antibodies induced by the malaria parasite in an attempt to identify their antigen-specificity is challenging. Studies have frequently focused on a few select antigens, thus ignoring this complexity and the possibility of synergy in the response to multiple antigens. The complexity of these humoral responses was demonstrated using microarray assays in which 18 recombinant antigen fragments spanning various regions and alleles of four leading vaccine candidates (namely, MSP-1, MSP-2, MSP-3 and AMA-1) were tested (Gray et al., 2007). The results clearly demonstrate complex combinations of specific antibodies leading to an association with some form of protection. Reactivity to individual antigens did not correlate with protection, but combinations of antibodies to AMA-1 and allelic variants of MSP-2 were prevalent in individuals protected against clinical malaria.

A field study in Senegal in which factors such as reappearance of parasites, asymptomatic carriage of parasites, time to first clinical episode, and incidence of clinical episodes were considered led to the observation that antibodies to NANP, MSP-1p19, *Pf*EMP-3, *Pf*EB200 were associated with a lower risk for severe disease (Perraut et al., 2003). Another comprehensive study conducted in Senegal (305 children followed over 1 year) showed that

different mechanisms mediate protection: higher levels of IgG1 specific for GLURP and IgG3 for MSP-2 in children correlate with resistance to malaria and high-level parasitemia compared to malaria-susceptible children (Courtin et al., 2009). Higher anti-MSP-1 IgG1 levels were associated with protection against high-density parasitemia. The study also evaluated the *in vitro* anti-parasite activity of the sera from these children and reported an age-dependent decline in the *in vitro* GIA activity of the sera. The GIA activity was dependent on anti-MSP-1, anti-AMA-1 and anti-MSP-2 specific antibody titers.

A recent comprehensive meta-analysis of 33 clinical studies investigated the relationship between anti-merozoite antibodies and the incidence rate of malaria (Fowkes et al., 2010). The closest association between antibody titers and reduced risk was observed with IgG specific for the C-terminus of MSP-3 and MSP-1 (MSP-1p19). In contrast, antibodies directed to the N-terminus of MSP-1 and the presence of antibodies to MSP-2 was not significantly associated with protection. The analysis also revealed a positive association between reduced risk for infection and antibody titers against AMA-1 and GLURP-R0.

5. MSP-1 and its role in immunity and infection

Our laboratory has focused on the major merozoite surface protein -1 (MSP-1). This antigen was identified in immune complexes from merozoite lysates (gp195), which provided the rationale for developing vaccines against it (Lyon et al., 1997). MSP-1 is first produced as a 195kD precursor that undergoes two successive proteolytic cleavage events (Blackman et al., 1994). The second processing event occurs immediately before invasion, resulting in the cleavage of the p42 molecule into a p33 and a p19 fragment. The p19 fragment remains attached to the merozoite surface through a GPI anchor (Gerold et al., 1996) and is comprised of two epidermal growth factor (EGF)-like domains (Morgan et al., 1999), which may have a role in the invading complex. Serological studies have provided significant evidence suggesting that immune responses directed against the C-terminus of MSP-1 (MSP-1p19 and MSP-1p42) are associated with immunity in preclinical models (Long et al., 1994; Egan et al., 1999; Darko et al., 2005; Parkkinen et al., 2006). Moreover, protective immunity as defined by lower mortality and morbidity of individuals residing in endemic areas was also associated with MSP-1p19 (Egan et al., 1999; John et al., 2004).

5.1 The role of MSP-1 in natural immunity

Various biological factors influencing the function of MSP-1 specific antibodies have been reported in individuals with natural immunity:

(1) The role of MSP-1 specific antibody titers, isotype and the association with protection and/or reduction in morbidity:

A meta-analysis of 33 clinical studies revealed that the presence of MSP-1p19 specific antibodies is associated with a lower incidence rate of malaria (Fowkes et al., 2010). Moreover, high levels of anti-PfMSP-1p19 immunoglobulin G were associated with reduced malaria in an age-adjusted multivariate analysis (Perraut et al., 2005). In contrast, other reports failed to show any associations between MSP-1p19 (MSP-1) Abs and clinical outcome (Dodoo et al., 1999; Nebie et al., 2008). At this point we can only speculate about the cause of this discrepancy. As outlined above, the differences in the methodology and/or

choice of plate antigen may be responsible for some of these issues. It is, however, interesting to note that full length MSP-1p42 was only used in one study as the plate antigen and this study reported a reduced risk of malaria (Al-Yaman et al., 1996).

(2) The role of antibody isotype and functional activity against the parasite:

Longitudinal studies have demonstrated an association between the IgM and IgG responses to MSP-1p19 and the degree of clinical disease and anemia in infants and pregnant women (Branch et al., 1998). Similarly, high antibody levels of MSP-1 specific IgG1 were associated with reduced morbidity (Riley et al., 1992; Al-Yaman et al., 1996) with protection against high-level parasitemia (Fowkes et al., 2010) and clinical disease (Egan et al., 1996; Cavanagh et al., 2004; Soe et al., 2004).

(3) The role of fine specificity of antibodies and association with protection:

IgG derived from sera obtained from Kenyan residents were tested for their impact on parasite viability and growth. The results demonstrated that the invasion inhibitory antibodies were specific for the C-terminal MSP-1p19 (John et al., 2004). Overall, there was a lack of association of total IgG or IgG subclass Abs to MSP-1p19 measured by ELISA with either invasion-inhibitory activity or protection against infection. In contrast, a study analyzing sera from children in West Africa (Sierra Leone and Gambia) demonstrated a strong association between antibody titers to the C-terminus of MSP-1 (MSP-1p19) and protection against clinical malaria and high level parasitemia (Egan et al., 1996). Thus, the fine specificity (*i.e.*, epitope specificity) of the MSP-1 specific antibodies appears to be important and testing only for antibody titers to the total molecule or a fragment may result in the loss of an association with a clinical response (Corran et al., 2004; Okech et al., 2004). The C-terminus of the MSP-1 is comprised of two EGF-like domains and depending on which of the domains the antibodies recognize results in either growth inhibition or no functional activity against the parasite (Chappel et al., 1994; Darko et al., 2005).

5.2 Efficacy of MSP-1 based vaccines in naïve and malaria-exposed individuals

Several clinical trials have been conducted testing either MSP-1p42 or MSP-1p19 as vaccine candidates. The objective of using the larger subunit, MSP-1p42, was to ensure that potential helper epitopes, which can induce antibodies are present in the immunogen. Moreover, the N-terminus p33 portion of the molecule contains most of the known T cell epitopes. The nature of the C-terminus, *i.e.*, due to several disulfide bridges associated with the EGF-like domains, renders the structure rigid and thus resistant to processing by antigen presenting cells, and therefore does not contain any dominant T cell epitopes. Thus, using the full length p42 fragment, which is expressed on pRBC starting at the trophozoite stage, would allow antibodies to bind to the parasites even inside the pRBC thereby potentially preventing the rupture of schizonts. This fact motivated two institutions (National Institute of Health (NIH) and Walter Reed Army Institute of Research (WRAIR)) to proceed with two independent MSP-1p42 based vaccines. At the NIH, a mixture of MSP-1p42 (FVO) and MSP-1p42 (3D7) was adjuvanted with Alhydrogel together with or without the addition of CpG7909 (Ellis et al., 2010) while the WRAIR vaccine consisted of single-allele vaccines, MSP-1p42 (3D7) (Ockenhouse et al., 2006) or MSP-1p42 (FVO) (Spring et al., manuscript in preparation), adjuvanted with GSK's adjuvant system, $AS02_A$ or $AS01_B$, respectively. Both were tested in US naïve individuals. All studies reported good immunogenicity and - in the

case of the single allele vaccine - moderate growth inhibitory activity was induced. The MSP-1p42 vaccines generated at WRAIR underwent further clinical evaluation in Phase Ib studies conducted in Kenya (Stoute et al., 2007) and Mali (Thera et al., 2006), where adults were immunized with the MSP-1p42 (3D7) adjuvanted in $AS02_A$. In Kenya, although pre-existing MSP-1p42 antibody titers in the participants were high, they could be boosted by the vaccine. In addition, Phase Ib (Withers et al., 2006) and Phase IIb (Ogutu et al., 2009) studies were conducted in Kenyan children (1-4 years old) using the same vaccine formulation. Results from this study indicated that younger children mounted stronger vaccine responses in terms of the magnitude of the antibody response. Sera from the Phase IIb study displayed strong growth inhibitory activity against the heterologous FVO parasites indicating that the predominant strain circulating at the time of natural exposure was different from the vaccine strain 3D7 (Angov et al., manuscript in preparation). Growth inhibitory activity to the 3D7 parasites was only observed in a small proportion of the study population with no significant difference between the rabies control group (Rabipur) and the malaria vaccine group. Some 3D7 specific GIA activity was reversible by antigen add-back confirming that some of the activity in the sera was due to MSP-1 specific antibodies. The major conclusion from this trial was that although the vaccine was safe and immunogenic, in the context of the heterologous exposure, the vaccine did not induce sufficiently cross-reactive responses. Future studies should include MSP-1p42 allele(s) that are better matched to the dominant circulating parasites. Another important consideration is to clear parasitemia in the study participants prior to and during the course of the vaccination. The presence of parasites during vaccination could lead to competitive immune responses thus curtailing the vaccine's potential.

Some vaccine approaches have focused on the MSP-1p19 fragment rather than the full-length MSP-1p42 since this region of the molecule contains the highly conserved functionally important B cell dominant epitopes. In one particular construct, helper T cell epitopes from tetanus toxoid were used to generate a chimeric P30P2-MSP-1p19 protein (Keitel et al., 1999). Others approached the lack of T cell help by generating a chimeric AMA-1/MSP-1p19 vaccine, which was tested in a Phase Ia trial (Malkin et al., 2008). This vaccine induced antibodies to both antigens; however the antibodies reacted primarily to the recombinant antigens by ELISA, less well by immunofluorescence assay (IFA) on whole parasites, and showed no activity in growth inhibition assays.

Therefore, with regards to further characterizing of MSP-1-based vaccine approaches, there is a need for reliable readout preclinical methods to enable prediction of protection and to facilitate the down-selection of vaccine candidates. One such attempt was the development of a transgenic rodent malaria parasite (*P. berghei*) expressing the *P. falciparum* MSP-1p19 transgene (De Koning-Ward et al., 2003). These parasites may be useful in the down-selection of vaccine candidates by either immunization strategies in the murine model (which does not necessarily address immunogenicity in humans) or passive transfer strategies to characterize the immune potential of antibodies induced in vaccinated individuals. Moreover, a recent report suggests that some anti-MSP-1 specific antibodies may mediate ADCI. Due to the incomplete compatibility between human Abs and murine Fc receptors, current mouse models are unable to use this pathway and may lead to false negative results. There are some experimental alternatives such as transgenic mice that express one of the human Fc receptors (CD32 or CD64) or humanized mice. Humanization refers to irradiated mice that have been reconstituted with human leukocytes (Badell et al., 2000; Pleass et al., 2003) or hematopoietic stem cells which leads to the production of an array of human blood cells in the mice.

5.3 Biological effect of MSP-1 specific antibodies on parasite growth and function

Characterizing immune responses induced by MSP-1 vaccines revealed that several factors impact the immunogenicity and functional activity of the induced antibodies (vaccine platforms will be discussed in detail in Section 6): (1) the expression system used for the production of the recombinant protein (*i.e., E. coli*, baculovirus, or yeast) (Arnot et al., 2008; Reed et al., 2009), (2) the amino-acid sequence used for vaccine development (*i.e.* full length gp195, MSP-1p42 or MSP-1p19) (Stowers et al., 2001; Woehlbier et al., 2006) and (3) the vaccine platform used to deliver the MSP vaccine (*i.e*, recombinant protein, recombinant viral vectors or DNA vaccines).

Our laboratory has intensively studied the anti-parasite effects induced by MSP-1p42 specific antibodies. Early observations indicated that MSP-1 specific antibodies impacted the various parasite strains differently depending on their classification as MAD20 or Wellcome/K1-like. These two alleles differ markedly in their p33 fragments while only by four amino acids (E-TSR *vs.* Q-KNG, respectively) in the p19 portion of the molecule. To this end, MSP-1 specific antibodies were able to significantly delay the intra-erythrocytic development of the 3D7, but not the FVO parasite clone (Bergmann-Leitner et al. 2009). In the case of FVO parasites, anti-MSP-1p42 antibodies prevented schizont rupturing by stalling or arresting intra-erythrocytic parasite development likely through direct interactions with intra-erythrocytic parasites within the parasitophorous vacuole, which is putatively connected to the surface of the pRBC by the parasitophorous duct. This duct gives antibodies, but not larger immune components access to the parasite inside the vacuole (Bergmann-Leitner et al., 2009). In contrast, the same antisera tested on the 3D7 parasite clone were unable to interfere with the release of the merozoites. These antibodies were still able to agglutinate merozoites and interfere with invasion. We expanded our analysis to the parasite clone CAMP/FUP that has a p33 and a p19 EGF-like domain 1 identical to the sequence of the 3D7 parasite clone, and an EGF-like domain 2 identical to the FVO parasite clone. We observed the same response pattern as reported for the FVO parasite indicating that antibodies within the EGF-like domain might be responsible for stalling the rupture of the schizonts. In contrast, no significant growth inhibition was observed following successful invasion indicating that EGF-like domain 1 specific antibodies may be mediating this particular biological effect. To test this working hypothesis, the activity of affinity purified antibodies specific to the entire p19 or each of the EGF-like domains were compared to the source material (antibodies induced by immunization with the MSP-1p42 vaccine representing either the FVO or the 3D7 allele). We concluded that only antibodies that bound to regions within the p42 or to the p19 subunit, but not the EGF-like domain 1 or 2 subunits, displayed growth inhibitory activities. This was surprising given previous observations suggesting that responses directed to the p19 were associated with reduced parasite density or clinical disease (see above). However, the proper folding of the recombinant fragments used, which represent the two EGF-like domains has not yet been confirmed. This result may indicate that protective epitopes may depend on proper tertiary structure of the molecule. In support of this theory, it has been reported that inhibitory anti-MSP-1 specific antibodies map to epitopes formed through the "properly" folded p19 subunit and not to its sub-domains (McBride and Heidrich, 1987; Uthaipibull et al., 2001).

One biological function known to be displayed by some MSP-1 specific antibodies is the inhibition of the secondary processing of the MSP-1 molecule into the p33 and the p19 portion (Blackman et al., 1994). This activity was found in sera from individuals with acquired natural immunity (Patino et al., 1997). In this assay, merozoites are prepared from synchronized blood stage cultures and their ability to process MSP-1p42 into p33 and p19 in the presence or absence of immune antibodies is evaluated. This method is qualitative at best and due to its nature not designed for high-throughput testing. Thus, very few laboratories use the technique for the evaluation of functional activity in immune sera.

6. Future direction

The current body of literature clearly supports the development of a blood stage vaccine. Although such a vaccine would not prevent infection, it can reduce morbidity and mortality associated with malaria infection and therefore such a vaccine would save the lives of many residents of malaria-endemic areas. The experience and knowledge gained from these studies should be used to rationally design new vaccine formulations and future clinical trials. Factors that need to be considered for their success are:

(1) Vaccine platform. Most erythrocytic vaccines tested so far are based on recombinant, soluble proteins. When using recombinant proteins it is paramount to assure proper, thus native-like, protein folding. Sera from malaria-endemic areas are a useful tool to establish the degree of cross-reactivity between the recombinant vaccine and the "native" antigen. Alternative vaccine platforms to those primarily described here, soluble proteins plus adjuvant, are particle-based approaches. When using this approach it is important to assure proper orientation of the protein on the particle. Some proteins that are inherently unstructured such as MSP-2 could benefit from particle formation because the particle provides a stabilizing scaffold. Moreover, the distance between the epitopes and the density may be crucial in order to induce proper immune responses. Such particle presentation could be achieved by using either recombinantly expressed antigen on whole-killed bacteria or viruses. For example, mouse studies using recombinant adenovirus encoding MSP-1 demonstrated "protection" (defined by the authors as delayed and lower parasitemia) (Draper et al., 2009).

(2) Development of preclinical and clinical models that better predict human anti-malarial responses. When using preclinical animal models, the parasite growth kinetic is frequently different between those *Plasmodium* species that are suitable for the respective animal model and *P. falciparum* thus failing to simulate the clinical situation. Testing vaccine candidates in animals can be challenging when testing *P. falciparum* antigens: except for one confirmed antigen, *Pf*CelTOS, (Bergmann-Leitner et al., 2010), malaria antigens are relatively species-specific, *i.e.,* immunization with *P. falciparum* antigens does not confer protection against a heterologous *Plasmodium* species. Thus, investigators often make their decisions based solely on immunogenicity in the animal model (cellular and/or humoral responses notionally thought to be important). Alternatively, investigators have searched for orthologs of the *P. falciparum* antigen in the respective *Plasmodium* species relevant for their preclinical model to conduct immunization and challenge studies. A caveat of this approach is that the ortholog may have a different function than the *P. falciparum* antigen in human malaria or there simply may not be a valid ortholog (*e.g. P. falciparum* LSA-1 does not have an ortholog in

rodent *Plasmodia*). Another important consideration for the establishment of relevant models of human malaria is the immunization and challenge routes. Challenge routes should take into consideration the natural inoculation route. Our work with pre-erythrocytic antigens has shown that vaccine efficacy can vary significantly if the challenge route is changed (intravenous *vs*. mosquito bite) and thus the efficacy of a vaccine could be over- or underestimated if an artificial challenge method is used (Leitner et al., 2010) which may bypass vaccine-induced effector mechanisms (Vanderberg et al., 2007). For erythrocytic antigens this has been an issue as well because - until recently - it was ethically inconceivable to challenge human volunteers with malaria-infected blood due to the risk of transmitting life threatening blood borne diseases. However, extensive testing of the blood source used for the challenge has allowed a limited challenge study with the understanding that significant improvements are needed before blood challenges can be performed routinely similar to the mosquito bite challenges (Moorthy et al., 2009). A final issue to consider is that blood stage challenge in humans may not fully predict the situation where an individual receives a blood stage vaccine followed by mosquito bite challenge, as the vaccine-induced immune responses may be edited (*i.e.*, altered) by the sporozoite and the liver-stage infection.

(3) Improving surrogate readout assays to down-select vaccine candidates. At this time, different assays are being used as surrogate markers for down-selecting vaccine candidates. Without an immune correlate of protection, the predictive value of these readout methods remains questionable. Therefore, identifying immune correlates for the various target antigens is necessary to 'validate' the readout methods and allow their use for rational down-selection of vaccine candidates

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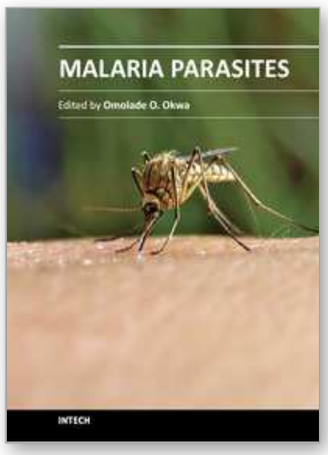

Malaria Parasites

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Malaria is a global disease in the world today but most common in the poorest countries of the world, with 90% of deaths occurring in sub-Saharan Africa. This book provides information on global efforts made by scientist which cuts across the continents of the world. Concerted efforts such as symbiont based malaria control; new applications in avian malaria studies; development of humanized mice to study P.falciparium (the most virulent species of malaria parasite); and current issues in laboratory diagnosis will support the prompt treatment of malaria. Research is ultimately gaining more grounds in the quest to provide vaccine for the prevention of malaria. The book features research aimed to bring a lasting solution to the malaria problem and what we should be doing now to face malaria, which is definitely useful for health policies in the twenty first century.

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