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Plasmodium Immunomics

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Abstract

The *Plasmodium* parasite, the causative agent of malaria, is an excellent model for immunomic-based approaches to vaccine development. The *Plasmodium* parasite has a complex life cycle with multiple stages and stage-specific expression of ~ 5,300 putative proteins. No malaria vaccine has yet been licensed. Many believe that an effective vaccine will need to target several antigens and multiple stages, and will require the generation of both antibody and cellular immune responses. Vaccine efforts to date have been stage-specific and based on only a very limited number of proteins representing < 0.5% of the genome. The recent availability of comprehensive genomic, proteomic and transcriptomic datasets from human and selected non-human primate and rodent malarias provide a foundation to exploit for vaccine development. This information can be mined to identify promising vaccine candidate antigens, by proteome-wide screening of antibody and T cell reactivity using specimens from individuals exposed to malaria and technology platforms such as protein arrays, high throughput protein production and epitope prediction algorithms. Such antigens could be incorporated into a rational vaccine development process that targets specific stages of the *Plasmodium* parasite life cycle with immune responses implicated in parasite elimination and control. Immunomic approaches which enable the selection of the best possible targets by prioritizing antigens according to clinically relevant criteria may overcome the problem of poorly immunogenic, poorly protective vaccines that has plagued malaria vaccine developers for the past 25 years. Herein, current progress and perspectives regarding *Plasmodium* immunomics are reviewed.

Keywords

Plasmodium; Malaria; Immunomics; Immune screening; Antigen discovery; Antigen identification; Vaccine; Diagnostics

Research Highlights

- Genomes to Vaccines strategies
- Genome-wide or proteome-wide identification of novel target antigens
- Mining genomic data of complex pathogens for vaccine development
- Protein microarrays

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- Epitope-based T cell screening

1. Introduction

Malaria, caused by infection with parasites of the genus *Plasmodium*, remains a significant public health problem worldwide and is one of the leading causes of morbidity and mortality in tropical and sub-tropical regions. Half of the world's population is at risk of malaria, with an estimated 250 million cases and one million deaths (mostly of children under 5 years of age) annually amongst the 3.3 billion people at risk (<http://malaria.who.int/wmr2008/malaria2008.pdf>). These figures underestimate the burden of infection as humans may be infected with multiple distinct species and strains. Malaria also exerts a great economic burden on endemic countries (Sachs and Malaney, 2002; Teklehaimanot et al., 2007). *Plasmodium falciparum* is responsible for the majority of malaria-induced deaths and most of the morbidity associated with malaria in sub-Saharan Africa and has therefore been the focus of most research. However, in tropical and subtropical areas, *Plasmodium vivax* can equal *P. falciparum* as a source of community-wide morbidity and *P. vivax* is often the most prevalent malaria infection (Price et al., 2007, 2009). Until recently, the disease caused by *P. vivax* was thought to be clinically less severe than that associated with *P. falciparum* and rarely lethal, but studies in southeast Asia have shown that approximately 25% of patients with severe malaria have *P. vivax* mono-infection, and multi-drug resistant *vivax* has been identified (Tjitra et al., 2008; Price et al., 2009).

The pathogenesis and clinical manifestations of malaria are influenced by many factors, including the genetics of the human host, the age of the host and the transmission dynamics of the parasite (Snow and Marsh, 2002; Schofield and Mueller, 2006). In areas where transmission of *P. falciparum* is most intense, infants are at highest risk of developing severe and fatal malaria. In areas with less intense transmission, older children have a higher incidence of severe and fatal disease than do infants. In malaria-endemic areas, individuals who survive past a certain age will become re-infected and will become clinically ill, but will not develop severe disease or die; that is, they develop naturally acquired immunity, an age-dependant acquisition of non-sterilizing immunity that protects against clinical disease but not parasitemia (anti-disease but not anti-parasite immunity), although anti-parasite immunity does occur to some extent (Baird, 1998; Langhorne et al., 2008; Doolan et al., 2009).

The ability of *Plasmodium* spp. parasites to evade eradication by conventional means highlights the need for new approaches to combat the disease. Primary amongst these are efforts to develop vaccines that prevent or control infection but, despite a relatively intense and systematic research effort conducted since the 1960s, there is still no licensed malaria vaccine (Epstein et al., 2007). Malaria vaccine development has been hindered in part by the complex life cycle of the parasite involving both invertebrate (mosquito) and vertebrate (human) hosts, the numerous intracellular and extracellular environments in which the parasite develops, and a large 23 megabase genome that contains an estimated 5,268 putative proteins, many of which are expressed in different stages of the life cycle and may exhibit allelic or antigenic variation.

2. Vaccines: The challenge and the potential

Vaccines are one of the most cost effective and efficient health care interventions for infectious diseases. Almost all licensed vaccines are based on delivery of a modified whole organism or protein subunit and are not unlike the original smallpox vaccine developed by Dr Edward Jenner in 1796. Effective vaccines have been created almost exclusively for

simple pathogens causing acute illnesses, for example, smallpox, polio, tetanus and yellow fever. Such vaccines were “easy” to develop for two reasons. First, due to the simplicity of the pathogens, only a handful of potential vaccine targets were available. Second, because the diseases were acute rather than chronic, the pathogen had no reason to develop sophisticated immune evasion strategies. Many of the diseases that plague the developing world are chronic infections by complex pathogens adapted to long-term coexistence with the human immune system; for example, malaria, tuberculosis, leishmaniasis and trypanosomiasis. The pathogens causing these diseases express hundreds or thousands of potential antigenic targets, often in distinct phases of their life cycles; moreover they have evolved sophisticated immune evasion strategies. It is perhaps not surprising that preliminary efforts to develop vaccines based on a handful of antigenic targets, chosen without knowledge of the antigenic repertoire of the organisms, have not been particularly successful. Current vaccine targets for complex microorganisms, including malaria, represent less than 1% of the potential targets encoded in pathogen genomes, and this 1% has often been identified for reasons only marginally relevant to vaccine development.

2.1. Human models demonstrating the feasibility of a malaria vaccine

There is increasing recognition that the multiple stages of the *Plasmodium* spp. life cycle, the requirement for distinct immune mechanisms targeting these different stages, and the large allelic variation of parasites in the field, all pose enormous obstacles to the development of an effective malaria vaccine. The feasibility of a malaria vaccine is, nonetheless, supported by experimental data demonstrating protective immunity induced by exposure to intact parasite: (i) sterile infection-blocking immunity can be achieved by experimental immunization with radiation-attenuated *Plasmodium* spp. sporozoites, and (ii) anti-disease immunity can be achieved by long-term natural exposure to the parasite.

With regard to the irradiated sporozoite model: sterile protective immunity against malaria can be induced in animals or human volunteers by immunization with *Plasmodium* sporozoites attenuated by radiation so that they can invade the host hepatocyte but do not fully develop (Nussenzweig and Nussenzweig, 1989; Hoffman et al., 2002). This protection is effective against challenge with high doses of infectious sporozoites, is species-specific but not strain-specific, is efficacious in genetically diverse backgrounds, and is sustained. The primary protective immune mechanism is thought to be cell mediated immunity, with CD8⁺ T cell responses directed against parasite antigens expressed in the liver-stage (specific for parasite-derived peptide/class I major histocompatibility complex (MHC) molecule complexes) implicated as the critical effectors (Doolan and Martinez-Alier, 2006). In rodents, CD8⁺ cytotoxic T lymphocytes can eliminate *Plasmodium*-infected hepatocytes from in vitro culture in an MHC-restricted and antigen-specific manner, and adoptive transfer of CD8⁺ T cells can protect against *Plasmodium yoelii* and *Plasmodium berghei* sporozoite-induced malaria in the absence of other parasite-specific immune responses. CD4⁺ T cells that recognize parasite-derived peptide/class II MHC molecule complexes are thought to also contribute to the induction of optimal protective immunity (Weiss et al., 1993; Carvalho et al., 2002; Doolan and Martinez-Alier, 2006; Oliveira et al., 2008). Evidence in humans is indirect, but antigen-specific CD8⁺ and CD4⁺ T cell responses have been detected against all known pre-erythrocytic stage antigens in individuals experimentally immunized with irradiated sporozoites or naturally exposed to malaria. The importance of antigenic targets and immune mechanisms that are active in this model is highlighted by the fact that infection-blocking immunity in humans rarely, if ever, occurs under natural conditions. Studies have shown that sterile immunity directed against the liver stage and mediated by CD4⁺ and CD8⁺ T cells, but not antibody, can be also induced in mice by immunization with infectious wild type sporozoites under chloroquine treatment (Beaudoin et al., 1977; Orjih et al., 1982; Belnoue et al., 2004) or primaquine treatment

(Putrianti et al., 2009). Recently, it was demonstrated that 10 human subjects exposed to the bites of *P. falciparum*-infected mosquitoes while receiving a prophylactic regimen of chloroquine were protected against subsequent sporozoite challenge (Roestenberg et al., 2009).

With regard to naturally acquired immunity: in areas where malaria is endemic, there is a decrease in the incidence of infections, the prevalence and density of parasitemia, and the morbidity and mortality associated with *Plasmodium* spp. infection with age (Baird, 1998; Doolan et al., 2009). Epidemiological data indicate that natural exposure to sporozoites does not induce complete (sterilizing) anti-parasite and anti-disease immunity. For example, 72% (60/83) of adults resident in a malaria-endemic area of Kenya who were cleared of *P. falciparum* parasites by drug treatment subsequently developed clinical malaria within 3 months of drug treatment (Hoffman et al., 1987). Data also indicate that naturally acquired immune mechanisms responsible for the acquisition of clinical protection in endemic areas affect the prevalence of *P. falciparum* parasitemia, limit the density of parasitemia and thereby decrease the malaria-associated morbidity and mortality (Marsh and Kinyanjui, 2006; Langhorne et al., 2008). The widespread persistence of patent parasitemia in asymptomatic individuals resident in malaria-endemic areas, and the ability of hyperimmune serum or purified immunoglobulin from individuals with lifelong exposure to endemic malaria to passively transfer protection, as evidenced by a marked decrease in *P. falciparum* blood-stage parasitemia and resolution of symptoms in the recipients (Cohen et al., 1961; McGregor and Carrington, 1963; Sabchareon et al., 1991), suggest that antibodies directed against parasite antigens exposed on the surface of merozoites or infected erythrocytes, or released from apical organelles at the moment of invasion, are the critical protective mechanism in naturally acquired immunity. However, cellular immunity, in particular that mediated by CD4⁺ T cells, has been implicated with an important role in blood-stage immunity (Good et al., 2005). Nonetheless, data from a number of epidemiological studies show that the pre-erythrocytic stage is targeted by immune responses during the development of naturally acquired immunity as demonstrated by antibody responses as well as CD4⁺ and CD8⁺ T cell responses specific for sporozoite/liver stage proteins (Doolan and Martinez-Alier, 2006; Doolan et al., 2009). Most recently, field studies have shown that the *P. falciparum* circumsporozoite (CSP)-based RTS, S vaccine reduced risk of clinical malaria, delayed time to new infection, and reduced episodes of severe malaria over 6 months in African children and that protection against both infection and clinical disease was sustained for at least 18 months (Cohen et al., 2010). Those data establish that pre-erythrocytic stage-specific immune responses are induced by natural exposure to *P. falciparum*-infected mosquitoes and that such responses can provide protection.

2.2. Problems with historical approaches to target antigen identification

The human and animal models represented by irradiated sporozoite immunization or naturally acquired immunity represent powerful models for the development of a vaccine to completely prevent infection or to prevent death and severe disease, respectively. However, the specific target antigens and epitopes of the protection are largely unknown, and correlates of protection after experimental immunization or natural exposure are unclear. In the pre-genomic era, decades of research had identified no more than a handful of possible *P. falciparum* vaccine targets (http://www.who.int/vaccine_research/links/Rainbow/en/index.html). We now know that the genomes of the *Plasmodium* parasites responsible for human, non-human primate and murine malaria are each 23–27 Mb in size and encode more than 5,000 putative proteins (Table 1), of which ~ 77% have orthologs in the different *Plasmodium* spp. (www.PlasmoDB.org version 6.4). Therefore, less than 0.5% of the *P. falciparum* genome is represented by antigens currently in clinical development. Furthermore, subunit vaccines

currently in development are based on a single or few antigens and may therefore elicit too narrow a breadth of response, providing neither optimal protection nor protection on genetically diverse backgrounds. For instance, some antigens expressed in the *Plasmodium* sporozoite/hepatic stage and recognized by CD4⁺ and CD8⁺ T cells have been characterized (*Pf*CSP, sporozoite surface protein 2 (SSP2/TRAP), liver stage antigen 1 (LSA1), exported protein 1 (EXP1)), but the associated immune reactivity is relatively weak and does not seem able to account for the protective effects associated with irradiated sporozoite immunization (Doolan et al., 2003). In the rodent model, the CSP has been identified as a target of protective immunity (Doolan and Martinez-Alier, 2006) but recent studies in CSP transgenic mice that are tolerised to the CSP showed a reduction but not complete abrogation of protection induced by immunization with irradiated *P. yoelii* sporozoites (Kumar et al., 2006) and mice immunized with irradiated *P. berghei* sporozoites were completely protected against challenge with transgenic *P. berghei* parasites expressing the *P. falciparum* CSP in the absence of functional responses against the CSP (Gruner et al., 2007). In humans, although the *P. falciparum* CSP-based RTS, S vaccine has shown protection against clinical disease following challenge in the field, it does not induce sterile protection (Cohen et al., 2010). Collectively, these studies suggest that other non-CSP antigens play an important role in pre-erythrocytic stage protective immunity. Failure to protect against field challenge has also been noted with candidate vector vaccines based on another pre-erythrocytic stage antigen, SSP2/TRAP (fused to multiple T cell epitopes derived from other pre-erythrocytic stage antigens) even though the vaccines were immunogenic (Moorthy et al., 2004; Bejon et al., 2006) and conferred a delay in onset of blood-stage parasitemia following sporozoite challenge in malaria-naïve adults (McConkey et al., 2003). In the case of the asexual blood-stage, recent clinical trials with the two leading candidate antigens, apical membrane antigen 1 (AMA1) and merozoite surface protein 1 (MSP1) have proved disappointing (Malkin et al., 2007; Ogutu et al., 2009; Sagara et al., 2009; Spring et al., 2009). Laboratory (Doolan et al., 1996) and field studies (John et al., 2005; Gray et al., 2007; Osier et al., 2008) support the concept of a multi-valent malaria vaccine where robust immune responses to multiple antigenic targets may be important for effective protection. It is possible or even likely, therefore, that failure to develop a malaria vaccine despite decades of effort can be attributed to the historic focus by malaria researchers on a limited and arbitrary list of less than 0.5% of potential target antigens.

Of the more than 5,000 proteins expressed during the life cycle of the *Plasmodium* spp. parasite, it is not known which protein antigens mediate the protective immunity induced by whole organism vaccination or whether protection is directed predominantly against one or a limited number of antigens or against a large number of antigens many of which have not yet been identified. Due to various factors related to antigen abundance and immunodominance, not all possible antigens may be recognized by natural immune responses (Sercarz et al., 1993). Preliminary studies suggest that, in the context of host immune recognition of the *P. falciparum* parasite, a large number of antigens are recognized, dispersed amongst a large fraction of the proteome (Doolan et al., 2003; Crompton et al., 2010; Trieu, A and Doolan, DL, unpublished data). These data highlight the importance of identifying the repertoire of antigens and epitopes targeted by sporozoite-induced and naturally acquired immune responses. Recently, genetically attenuated parasite and whole parasite approaches are being explored for malaria vaccine development, in an attempt to mimic the immunity induced in the whole parasite human models (McCarthy and Good, 2010; Vaughan et al., 2010).

The challenge for next generation malaria vaccine development, therefore, is to select optimally protective antigens and to ascertain whether a few or many such antigens underlie the protective immunity induced by immunization with irradiated sporozoites or by repeated natural exposure.

2.3. Plasmodium genomics, proteomics, transcriptomics datasets – foundation for a new approach

The genomic sequences of *P. falciparum* 3D7 strain, *P. vivax* Sal I strain, *P. yoelii* 17XNL strain and *Plasmodium knowlesi* strain H have been now completed (Carlton et al., 2002; Gardner et al., 2002; Carlton et al., 2005, 2008; Pain et al., 2008) and others including that of the *P. falciparum* IT line, a *P. falciparum* Ghanaian clinical isolate, *Plasmodium ovale* (Nigeria I/CDC strain), non-human primate malaria (*Plasmodium reichenowi* Dennis), rodent malarias (*P. berghei* ANKA, *Plasmodium chabaudi* AS) and avian malaria (*Plasmodium gallinaceum*) are in progress (<http://www.genome.gov/Pages/Research/DER/PathogensandVectors/PlasmodiumWhitePaperV8.pdf>; http://www.sanger.ac.uk/Projects/P_falciparum/; <http://www.sanger.ac.uk/sequencing/Plasmodium/ovale/>). In addition, partial genome sequence is available for an additional 16 geographically diverse *Plasmodium* parasites (15 *P. falciparum*, one *P. reichenowi*) as part of an undertaking by the Broad Institute (MA, USA) to survey genetic variation across the *P. falciparum* genome (Volkman et al., 2007) (www.GOLD.org).

The corresponding proteomes of *P. falciparum*, *P. yoelii* and *P. berghei* (Carlton et al., 2002; Florens et al., 2002; Lasonder et al., 2002; Hall et al., 2005; Tarun et al., 2008; Lasonder et al., 2008), and the transcriptomes of *P. falciparum*, *P. vivax*, *P. yoelii* and *P. berghei* (Kappe et al., 2001; Bozdech et al., 2003, 2008; Le Roch et al., 2003, 2004; Llinas and DeRisi, 2004; Wang et al., 2004; Gruner et al., 2005; Hall et al., 2005; Llinas et al., 2006; Rosinski-Chupin et al., 2007; Mikolajczak et al., 2008a; Tarun et al., 2008; Westenberger et al., 2010) have been elucidated. Numerous comparative genomic data sets are also available (Carlton et al., 2005; Hall et al., 2005; Zhou et al., 2008; Mackinnon et al., 2009; Liew et al., 2010).

These genomic, proteomic, transcriptomic and comparative genomic datasets provide the foundation for novel approaches to target antigen selection on a genome-wide or proteome-wide scale. With the appropriate tools to exploit these data, we have an unprecedented opportunity to develop and implement a rational approach to target antigen selection. However, there is currently no algorithm that can be used effectively to identify the target antigens or epitopes of protective immune responses from genomic sequence data. Furthermore, the identification of antigens that will stimulate the most effective immune responses against the target pathogen is problematic, particularly when the genome of the pathogen is large. The enormous opportunity offered by knowledge of pathogen genomic sequences can only be exploited by a rational, systematic approach to vaccine target selection.

3. Immunomics – integrating genomics, proteomics and molecular immunology

The publication of the complete genome sequence of a free-living organism, *Haemophilus influenzae* in 1995 (Fleischmann et al., 1995) marked the beginning of the era of genomics. At the time of this review, there were 1,286 published complete genomes, 4,380 ongoing bacterial genome projects and 1,338 ongoing eukaryotic genome projects listed on the Genomes OnLine Database (GOLD) version 2.0 (<http://www.genomesonline.org/>). The completion of the human genome project in 2000 (Lander et al., 2001; Venter et al., 2001) and its subsequent refinements (Istrail et al., 2004), together with information on the genomes of many important human pathogens including *P. falciparum* (Gardner et al., 2002) and its main vector *Anopheles gambiae* (Holt et al., 2002), offer exciting possibilities for exploring the molecular pathogenesis of infectious diseases, identifying mechanisms and

determinants of virulence, and identifying new targets for therapeutic interventions. With hundreds, thousands or even hundreds of thousands of genes and protein states to identify, correlate and determine their biological significance, it is no longer adequate to study genes, gene products or processes on an individual basis. Rather, we have entered the “omic” era in biology (www.genomicglossaries.com/content/omes.asp). However, there is still no way to efficiently analyse genomic, proteomic and transcriptomic data to identify which antigens among many thousands are appropriate targets for these interventions.

The necessity for more specialised collections of data relating to specific fields, and the need to bridge the gap between previously separated fields, is becoming increasingly obvious. Of particular relevance to the development of vaccines and diagnostics is a field called immunomics that bridges the disciplines of genomics and proteomics by involving the immune system. Immunomics focuses on elucidating the set of antigens that interact with the host immune system and the mechanisms involved in these interactions (Rinaudo et al., 2009). It is distinct from the field of reverse vaccinology, which aims to identify the complete repertoire of antigens that an organism is capable of expressing on its surface (Moriel et al., 2009; Rinaudo et al., 2009; Seib et al., 2009).

The immunome can be defined as the set of antigens or epitopes that interface with the host immune system (De Groot and Berzofsky, 2005; Sette et al., 2005). Just as the proteome of an organism derives from the genome, the immunome derives from the proteome; the nature of the immunome is as dependent on the host as it is on the pathogen. Immunomics allows for a rational approach to target antigen selection for prophylactic or therapeutic interventions on a genome-wide scale, using biological samples from humans or animals with immunity to the disease of interest.

3.1. Immunomics – a promising solution to the challenge of malaria vaccine development

Immunomics provides a potential solution to the challenge of identifying target antigens for an effective malaria vaccine. The availability of genomic, proteomic, transcriptomic and comparative genomic data from *P. falciparum* and other malaria parasites, combined with advances in computational and molecular immunological tools to exploit these data, provide an unprecedented opportunity to develop and implement a rational immunomic-based approach to target antigen selection, using genomic and proteomic data and biological samples from human volunteers with immunity to malaria. A key aspect of the strategy is functional significance as assessed by immunogenicity (governed by the capacity of the antigen to be recognized by recall *Plasmodium*-specific immune responses in protective human models) and biological activity (in vitro). The credentials of each protein will allow the development of a prioritized list of targets for novel next generation vaccines. Immunomics thus offers a systematic and rational approach to vaccine target selection which, until now, has been approached by the field in an ad hoc and haphazard manner.

The *Plasmodium* parasite represents a good model for immunomics because: (i) malaria is a significant public health problem in the developing world; (ii) there are two human models of protective immunity that establish the feasibility of developing a malaria vaccine (immunization by radiation-attenuated *Plasmodium* sporozoites, relying on the generation of protective cellular responses against pre-erythrocytic stage antigens; and naturally acquired immunity, relying on the generation of protective antibody responses against blood-stage antigens); (iii) both cellular and humoral immune responses to several antigens simultaneously will likely be required, in accordance with the two models of protection in humans; (iv) individuals exposed to the parasite develop a multifaceted immune response, including antibodies and CD8⁺ and CD4⁺ T cell components directed against multiple antigens, and reagents (sera/plasma and cells) from immune individuals that presumably reflect the entire repertoire of parasite-induced T cell and antibody specificities represented

in such individuals are available for use; (v) the genomes of the major human pathogens *P. falciparum* and *P. vivax* as well as model rodent (*P. yoelii* 17XNL) and zoonotic (*P. knowlesi*) parasites have been sequenced and the corresponding proteomes and transcriptomes have been elucidated; (vi) sub-optimal protection against parasite challenge has already been achieved using current molecular vaccine technologies, indicating their feasibility and potential for improvement; (vii) candidate antigens can be assessed either in vitro (using an appropriate immune readout), in animal model challenge systems (using the corresponding *Plasmodium* sp.), or in humans by challenging with *P. falciparum* sporozoites or parasitized erythrocytes; (viii) candidate vaccines can be tested for safety and protection against sporozoite challenge in the USA or Europe and transitioned as appropriate to field testing in Africa or southeast Asia; and (ix) malaria, like many other important human pathogens in the developing world, is a parasitic disease and thus has developed mechanisms to avoid or modulate the host immune system, meaning that the technological solutions developed for malaria should potentially be applicable to other chronic infectious agents. It is anticipated, therefore, that a strategy developed using *Plasmodium* as a model could be transitioned to other pathogens causing chronic infectious diseases in the developing world and for which large-scale genomic datasets are already available or are in progress, including tuberculosis, leishmaniasis, trypanosomiasis and schistosomiasis.

3.2. Immunomics – the details

The complexity of the *Plasmodium* spp. parasites, which each express over 5,000 predicted proteins (Table 1), dictates a rational approach to prioritize open reading frames (ORFs) representing putative antigens for evaluation. A logical approach to prioritization is to consider the primary life cycle stage and corresponding immune response(s) targeted for intervention, based on the human models demonstrating the feasibility of a malaria vaccine; namely immunization by radiation-attenuated *Plasmodium* sporozoites, relying on the generation of protective cellular responses against pre-erythrocytic stage antigens; and naturally acquired immunity, relying on the generation of protective antibody responses against blood-stage antigens.

To identify targets of T cell responses directed against the *Plasmodium*-infected hepatocyte, for example, priority could be given to the subset of 1,049 putative *P. falciparum* sporozoite proteins identified by multi-dimensional protein identification technology (MudPIT) of *P. falciparum* sporozoite preparations (Florens et al., 2002) which provides a semi-quantitative analysis of protein levels. This dataset of 1,049 proteins could be ranked according to their relative level of expression in the sporozoite proteome, as indicated by the spectral count (number of MS/MS spectra identified per protein) and percentage of sequence coverage. More recently, the proteomes of *P. falciparum* oocyst-derived sporozoites and salivary gland sporozoites have been elucidated (Lasonder et al., 2008), identifying 477 protein in salivary gland sporozoites of which only 161 were also expressed by oocyst-derived sporozoites. A similar distinction between the transcriptomes of oocyst-derived sporozoites and salivary gland sporozoites was reported in the *P. yoelii* model, where it was noted that ~ 10% of genes exhibited differential expression, with 124 genes up-regulated and 47 genes down-regulated in salivary gland sporozoites compared with oocyst sporozoites (Mikolajczak et al., 2008b); it was suggested that the subset of up-regulated genes may play a role in infection of the mammalian host, as has already been established for UIS3 and UIS4 which are found in that gene list (Kaiser et al., 2004; Mueller et al., 2005a,b).

The identification of genes and proteins expressed first during the hepatic stage (and not expressed in the sporozoite stage) has been problematic due to the very limited availability of liver-stage parasite material and the poor infectivity by *P. falciparum* of primary hepatocytes or hepatoma cell lines in vitro (Blair and Carucci, 2005; Tarun et al., 2006). Recently, however, a proteome and transcriptome survey of the liver stage of the *P. yoelii*

rodent parasite has been published (Tarun et al., 2008). That study identified a total of 654 proteins (92%) in the *P. yoelii* liver-stage proteome that have orthologs in *P. falciparum*; 174 proteins appeared to be unique to the liver stage. Only 66% ($n = 1,305$) of genes identified in the *P. yoelii* liver-stage transcriptome had *P. falciparum* orthologs (Tarun et al., 2008). Another study reported a partial analysis of the *P. berghei* sporozoite transcriptome, identifying 123 gene transcripts in *P. berghei* salivary gland sporozoites, 66 of which had not been previously described in sporozoites (Rosinski-Chupin et al., 2007). A recent study elucidated the differential transcriptome of *P. falciparum* salivary gland sporozoites versus sporozoites co-cultured with primary human hepatocytes and identified 532 *P. falciparum* genes which were up-regulated following co-culture, including some genes known to be implicated in hepatocyte invasion or to be expressed in hepatic stages (Siau et al., 2008). The authors suggested that other genes in that subset are likely involved in hepatocyte invasion and/or liver-stage development, and demonstrated with functional studies that two of their novel genes (Sporozoite Invasion-Associated Proteins 1 and 2; SIAP-1 and SIAP-2) were sporozoite surface proteins involved in hepatocyte invasion and another two were predominantly expressed during liver stage parasite development (Liver-Stage Associated Protein 1 and 2; LSAP-1 and LSAP-2). Interestingly, 20% of the up-regulated *P. falciparum* genes identified in that study have no identified ortholog in any rodent species, indicating specificity for the interaction between the *P. falciparum* parasite and the human liver. Five of the 532 proteins (including SIAP1) were previously shown to be recognized by peripheral blood mononuclear cells (PBMCs) from individuals immunized with irradiated *P. falciparum* sporozoites (Doolan et al., 2003), further supporting their potential as targets of pre-erythrocytic stage immunity. The expression of two genes known to be targets of protective immunity, PfCSP and PfAg2/CelTOS (Doolan, DL, unpublished data), were not affected by co-culture activation.

Data from other *P. yoelii* genome-wide expression datasets including a cDNA library from sporozoites transformed axenically into early exoerythrocytic forms (652 unique transcripts) (Wang et al., 2004), a cDNA library of laser capture microdissected mature liver stages (623 unique transcripts) (Sacci et al., 2005) and the transcriptome of liver stages at three points during their liver stage maturation (1,985 transcripts) (Tarun et al., 2008), as well as other studies of rodent *Plasmodium* parasites (Kappe et al., 2001; Kaiser et al., 2004; Gruner et al., 2005; Kumar et al., 2007; Zhou et al., 2008) could also be leveraged to identify putative *P. falciparum* liver stage proteins.

It should be noted however, that despite advances such as the development of axenic methods for cultivation of *Plasmodium* sporozoites into early liver stages (Kaiser et al., 2003) and establishment of a human hepatocyte cell line that supports in vitro development of the exo-erythrocytic stages of *P. falciparum* and *P. vivax* (Sattabongkot et al., 2006), the transcriptome and proteome of early liver-stage rodent parasites and of early and late liver-stage *P. falciparum* are still to be elucidated. Since the development of radiation-attenuated *Plasmodium* parasites is arrested at early liver-stage (Silvie et al., 2002), it is likely that antigens expressed by the parasite soon after hepatocyte invasion may be important targets for a pre-erythrocytic stage vaccine.

In general, it is logical to focus on those putative proteins identified by proteomic analyses, rather than the larger set of putative proteins identified by transcript analyses because T cell responses would be directed against protein products, not transcripts. In recent global analyses of transcript and protein levels, proteins were identified for only 57% (1,206/2,111) of transcripts from *P. falciparum* salivary gland sporozoites detected over background (Le Roch et al., 2004) and for 41% (816/1,985) of *P. yoelii* transcripts up-regulated more than two-fold in liver stages relative to blood stages (Tarun et al., 2008). Looking across the *P. falciparum* life cycle, on average 2.7 times as many transcripts as proteins were detected at a

particular stage (Le Roch et al., 2004). Furthermore, it is possible that abundant transcripts in salivary gland sporozoites might only be translated in liver stages, analogous to the situation of translational repression of mRNAs where sexual stage proteins are transcribed and stored in the blood stages and only translated after the parasite transitions into the insect vector (Mair et al., 2006; Kooij and Matuschewski, 2007). However, since T cell responses are not only directed against surface or high abundant proteins, those genes for which transcripts are detected in the sporozoite stage or hepatic stage but which are not detectable by MudPIT may nonetheless represent good targets of T cell responses. A more comprehensive approach to identifying T cell targets, therefore, would be to screen all genes identified by either proteomic or transcript analyses as present in the pre-erythrocytic (sporozoite or hepatic) stage of the *P. falciparum* parasite. To date, 2,111 sporozoite-associated *P. falciparum* genes have been identified (Le Roch et al., 2004). Antigen targets of CD4⁺ T cell responses may also represent good components of a blood-stage malaria vaccine.

Conversely, to identify targets of antibody responses directed against parasite antigens exposed on the surface of merozoites or infected erythrocytes, after release from the apical organelles or parasitophorous vacuole, priority could be given to the subset of putative *P. falciparum* proteins identified by MudPIT of *P. falciparum* blood-stage parasite preparations. It has been suggested that expression peaking in trophozoite or schizont stages may be desirable since secreted and surface proteins, and those in secretory organelles generally, are expressed during mature stages (Coppel, 2009). The initial report by Florens and colleagues (2002) identified 839 putative *P. falciparum* merozoite proteins and 1,036 putative trophozoite proteins. Another study (Lasonder et al., 2002) identified 714 proteins in asexual blood stages (trophozoites and schizonts). Subsequent transcriptomic studies identified 1,474 merozoite, 3,217 schizont, 3,295 trophozoite, 2,533 ring and 3,363 gametocyte genes (Le Roch et al., 2003, 2004). It should be noted that a number of proteins may be expressed in more than one stage of the parasite's life cycle.

Information from other datasets, such as *Plasmodium* whole-genome synteny maps which may provide information on species-specific genes with a predicted role in host-parasite interactions (Kooij et al., 2005), in silico gene function prediction using ontology-based pattern identification (Zhou et al., 2005), and correlations between protein expression and gene expression datasets (Hall et al., 2005), could also be considered in the ranking schema, as well as other characteristics that may be indicative of accessible immune targets, such as presence of signal sequences and/or transmembrane domains or glycosylated elements, although those criteria may be of limited value for antigens targeted by T cells. Among the 712 *P. yoelii* proteins detected in the *P. yoelii* liver-stage (and therefore potential targets of T cell responses), only 93 were annotated to contain a signal peptide and only 76 proteins were predicted to be membrane-associated (Tarun et al., 2008). Prediction of the set of parasite proteins exported into the erythrocyte using the vacuolar transport signal (VTS) (also known as the Host-Targeting (HT) motif) (Hiller et al., 2004) or the *Plasmodium* Export Element (PEXEL) motif (Marti et al., 2004), discovery of the *Plasmodium* translocon of exported proteins (PTEx) trafficking pathway that is responsible for the passage of proteins across the vacuolar membrane (de Koning-Ward et al., 2009), and elucidation of the malaria "exportome" (Sargeant et al., 2006) and malaria "secretome" (van Ooij et al., 2008) may also provide useful information for blood-stage vaccine target identification. A recent opinion on criteria for selecting and ranking potential asexual blood-stage vaccine candidates highlighted cell biological properties, immunological properties and manufacturability of the protein as important criteria (Coppel, 2009). That author suggested that PEXEL-containing proteins would not be good vaccine candidates since they would be present in the red blood cell cytoplasm and therefore not accessible to antibody. A similar exclusion criterion would not apply to the pre-erythrocytic stage where T cells rather than

antibodies are considered the primary effectors; indeed, PEXEL motifs have been identified in known pre-erythrocytic stage vaccine candidate antigens, including CSP (Singh et al., 2007).

Another bioinformatic-based strategy which has proved potentially useful for malaria vaccine development is the identification and selection of alpha-helical coiled coil domains of proteins predicted to be present in the erythrocytic stage of the parasite, where the corresponding synthetic peptides are predicted to mimic structurally native epitopes (Villard et al., 2007; Corradin and Kajava, 2010).

3.3. Proteome microarray chips for serological analysis

RNA and protein expression do not always correlate (Gygi et al., 1999), and host immune responses are generated against proteins, emphasizing the importance of genome-wide screening based on the translated products of gene sequences. However, despite the increasing availability of genome sequences from many human pathogens, the production of complete proteomes remains a bottleneck. To address this, a high throughput PCR recombination cloning and expression platform has been developed by Philip Felgner and colleagues at the University of California, Irvine, USA (Davies et al., 2005a; Vigil et al., 2010a) (Fig. 1). This platform offers a lot of potential for elucidating the profile of antibodies that develop after natural or experimental infection or after vaccination with attenuated organisms and identifying immunoreactive antigens of interest for vaccine development or diagnostics. The PCR cloning recombination method relies on the principle that *Escherichia coli* has an efficient and well-characterized homologous recombination and gap-repair system (Oliner et al., 1993) so *E. coli* cells are able to recombine homologous sequences with high fidelity and speed. The protein expression platform takes advantage of the fact that cell-free in vitro transcription and translation (IVTT) systems for gene transcription and translation based on plasmid expression vectors incorporating bacterial phage T7 promoters to couple T7 RNA polymerase driven transcription with translation offer standardized rapid and high throughput (robust and efficient) generation of recombinant proteins with a high protein yield in a relatively small reaction volume (Klammt et al., 2004; Ozawa et al., 2004); e.g., 25–100 µg protein per 50-µl reaction volume using the reagent kits commercially available from Roche (rapid translation system (RTS) 100 *E. coli* HY kits) or Invitrogen (Expressway™ Mini Cell-Free Expression System). The Roche RTS 100 *E. coli* Disulfide Kits where the cell-free *E. coli* IVTT reactions are conducted under non-denaturing conditions provide an alternative expression option for proteins with disulphide bonds; the absolute requirement for disulfide bonding for serological detection of the intracellular mature virion membrane protein of vaccinia virus has been recently documented (Davies et al., 2008). Inclusion of N-terminal histidine or C-terminal hemagglutinin epitope tags on the protein product allows for quality control assays to determine whether the IVTT produced proteins are full-length or truncated. Issues of protein integrity and breakdown products, and solubility of the generated protein, remain a potential problem for IVTT systems, as with conventional protein expression systems. However, these concerns are minimized by the use of unpurified products since antibody responses would be directed against peptide fragments, at least in the case of linear B cell epitopes. Another disadvantage is the cost of the system which precludes its use for generating large quantities of individual proteins.

The IVTT protein expression systems are particularly suited for application to *Plasmodium* since efforts to date to express *P. falciparum* proteins on a large scale have been largely unsuccessful (Aguiar et al., 2004; Mehlin et al., 2006; Vedadi et al., 2007; Birkholtz et al., 2008). Even on a small scale, *P. falciparum* proteins have proven particularly difficult to express in bacteria, yeast or insect cells using conventional methodologies compared with other organisms, possibly due to their high A:T content (82%, the highest A:T content of

any known organism (Gardner et al., 2002) and rare codon usage (Weber, 1988). In the Felgner laboratory, 93% expression efficiency of *Plasmodium* proteins is routinely obtained using the *E. coli* IVTT protein expression platform (Doolan et al., 2008), contrasting with rates reported in other large-scale *P. falciparum* studies in conventional *E. coli* expression systems ((7–16% (Aguilar et al., 2004), 21% (Vedadi, 2007) or 6% (Mehlin et al., 2006)), or the wheat germ cell-free system (75% (Tsuboi et al., 2008) 75% (Tsuboi et al., 2010)). The actual frequency of *P. falciparum* expression in the *E. coli*-based cell-free system may be greater than 93% since in some cases the N-terminal histidine or C-terminal hemagglutinin tags used to confirm expression may be present but conformationally obscured and not accessible to antibody on dot blots. The high success rate seen with the cell-free transcription/translation system may be attributed, at least in part, to the fact that the system is supplemented with rare t-RNAs to help translate A:T rich genes, that there is no cell to be killed by a potentially lethal expressed product, and that the proteins do not have to be purified prior to printing.

The protein microarrays are constructed in four main steps (Fig. 1): (1) PCR amplification of each predicted ORF (complete or partial if > 3,000 bp) using custom PCR primers comprising 20 bp of gene-specific sequence with 33 bp of “adapter” sequences which are homologous to the cloning site of the linearized T7 expression vector and allow the PCR products to be cloned by in vivo homologous recombination in competent *E. coli* cells; typically, genomic DNA is used as the PCR template and genes lacking introns are amplified as full-length products; for genes with introns, the exons are amplified; genes or exons longer than 3,000 bp are amplified as large overlapping fragments; (2) in vivo recombination cloning (directionally and in-frame) into a linearized T7 expression vector pXT7 (Davies et al., 2005a) encoding an N-terminal 6x-histidine tag and a C-terminal hemagglutinin tag, by mixing the PCR fragments reaction with linearized recombination cloning vector and co-transforming into competent *E. coli* cells; the resulting bacterial clones are grown up as minipreps and plasmid DNAs isolated and sequenced using common 5' and 3' primers to verify fidelity with the expected gene product; (3) protein expression of sequence-confirmed plasmids in an *E. coli* cell-free in vitro transcription/translation system supplemented with T7 RNA polymerase (5 hour reaction according to the manufacturer's instructions using Roche RTS 100 *E. coli* HY kits or Invitrogen Expressway); and (4) microarray chip printing, directly onto nitrocellulose coated glass FAST™ slides (Whatman) using the crude reactions without further purification in order to maintain a high efficiency of protein expression by eliminating loss associated with manipulation, handling and purification; the microarrays are printed within a few hours after the expression reaction is complete to reduce protein instability problems. The recombination cloning method enables hundreds of genes from the microorganism of choice to be cloned into expression vectors within a few days; a recent modification allows the recombination to occur in vitro with much lower amounts of recombination product to be transformed into *E. coli* (Beare et al., 2008). Protein expression is monitored either by dot blot or microarray using monoclonal anti-polyhistidine and anti-hemagglutinin antibodies. Chips can be fabricated with a large number of proteins to profile immunoreactivity on a large scale. Currently, optimal spotting densities are in the order of approximately 2,500 proteins per three-pad array or 1,150 proteins per eight-pad array or 250 proteins per 16-pad array but theoretically numbers could be expanded to 10,000 proteins per array. Chips are stored at room temperature (18–20°C) in a desiccator with a shelf-life of at least several months.

Each microarray chip contains the following controls: (i) a ‘no DNA’ negative control in which an empty plasmid vector is placed in the RTS reaction to provide a baseline signal for fluorescence readout, as a measure on non-malaria induced reactivity to the spots; (ii) serially diluted human IgG as a positive control to confirm reactivity of secondary antibodies and account for potentially non-viable hybridization steps (secondary and tertiary

antibody binding, washing, etc) and to generate a standard curve to normalize data from arrays probed at different times; and (iii) serially diluted Epstein-Barr Virus nuclear antigen-1 as a methodological control given the high prevalence of latent Epstein Barr Virus infection in human populations but which vary in reactivity between subjects.

Chips are blocked in protein array blocking buffer, probed with sera or plasma specimens pre-incubated with *E. coli* lysate to reduce background (typically diluted 1:100 in blocking buffer), stained with Cy3 conjugated anti-human/mouse/rat IgG or subtype secondary antibody, and scanned using a laser confocal scanner. Spot intensities are quantified using specialized software which automatically indexes the signal intensity of each spot on the array to its identity and database location. The signal intensity of each antigen can be presented as a heatmap (rainbow palette) by increasing signal density, analogous to the typical presentation of DNA microarray data. Since sera or plasma specimens for microarray screening are readily obtained from individuals of all ages and in any laboratory or field setting, and as little as 1–2 µl of undiluted sera are required (typically diluted 1:100 in blocking buffer containing 1/10 volume *E. coli* lysate), large number of samples can be screened to enable screening on a seroepidemiological scale and to ensure statistical power sufficient to identify population-specific responses to single antigens. A single individual can clone more than 300 genes in a day and probe microarray chips with 100 different sera samples per day (Vigil et al., 2010a).

A National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH/ NIAID, USA) grant supported the initial generation of *P. falciparum* protein microarrays which established proof-of-concept for proteome scale protein microarrays (Sundaresh et al., 2006; Doolan et al., 2008). The Felgner laboratory has also now completed the proteomes for Vaccinia virus (Davies et al., 2005a, 2007, 2008), *Francisella tularensis* (Eyles et al., 2007; Sundaresh et al., 2007), *Burkholderia pseudomallei* (Felgner et al., 2009; Yang et al., 2009), *Coxiella burnetii* (Beare et al., 2008; Chen et al., 2009), *Chlamydia trachomatis* (Molina et al., 2010) and *Borrelia burgdorferi* (Barbour et al., 2008) and probed a number of other viral (HIV 1 and 2, HPV, HSV-1 and 2, Dengue, Monkeypox, Varicella zoster virus, West Nile Virus, Chikungunya virus,), bacterial (*Mycobacterium tuberculosis*, *B. burgdorferi*, *Brucella melitensis*, *Chlamydia muridarum*, *Salmonella typhi*, *Rickettsia prowazekii*, *Orientia tsutsugamushi*, *Bartonella henselae* and *Leptospira interrogans*) and parasitic (*Schistosomiasis japonicum* and *Schistosoma mansoni*; (Driguez et al., 2010) proteomes. To date, a total of 31,000 cloned genes derived from 25 infectious microorganisms have been probed against an inventory of more than 8,000 sera from infected, vaccinated and healthy people worldwide (Vigil et al., 2010a) and immunodominant antigens from each agent (see references above) as well as serodiagnostic antigens (Sundaresh et al., 2007; Beare et al., 2008; Felgner et al., 2009; Yang et al., 2009) have been identified.

Data from these studies show a good correlation between independent print runs (Benhnia et al., 2008) (as shown by a good correlation between the signal intensities for any given antigen when probed on different chips) and establish that chip signal intensity is proportional to antibody titer and plotting signal intensities for a given antigen against the serial dilution of sera produces a sigmoid 'titration curve' identical to traditional ELISA (Davies et al., 2008). Therefore, signal intensities of a given antigen can be compared between sera. Data also show microarrays and traditional ELISA assays give very similar results, with no marked differences in the immunoreactivity of proteins produced by in vitro expression or by more traditional methods, as evaluated in the malaria and vaccinia models, although some variability has been noted for some proteins (Davies et al., 2008; Doolan et al., 2008; Crompton et al., 2010). Moreover, microarrays can be probed with different secondary antibodies to determine subtype and isotype profiles (Eyles et al., 2007; Molina et al., 2010). Finally, a good correlation between chip serology, traditional serology and

clinical disease stage classification was observed in the Lyme disease model (Barbour et al., 2008), protein microarray activity was correlated with in vitro virus neutralizing activity in the vaccinia model (Davies et al., 2007), serodiagnostic antigens were identified for tuberculosis, *F. tularensis* (Sundaresh et al., 2007), Q fever (Beare et al., 2008; Vigil et al., 2010b), melioidosis (Felgner et al., 2009; Yang et al., 2009), and candidemia (Mochon et al., 2010), and potential vaccine candidate antigens were identified for vaccinia (Davies et al., 2005b), malaria (Doolan et al., 2008; Crompton et al., 2010; Trieu, A and Doolan, DL, unpublished data), *F. tularensis* (Eyles et al., 2007), and Chlamydia (Molina et al., 2010) models. Those proteins that are most frequently recognized in a population are termed “serodominant” (in contrast to “immunodominant” which usually refers to antigens recognized in the context of a single individual). An overlap in the antibody profiles revealed by protein microarray and the immunodominant antigens recognized by proliferative CD4⁺ T cells has been also noted (Jing et al., 2008), as would be predicted for cognate T-B cell collaboration. Overall, results in multiple disease models show that the protein microarray platform is a powerful tool for identifying potential vaccine candidate antigens and serodiagnostic antigens.

In studies to date in the malaria model, we have demonstrated distinct profiles of antigen reactivity for populations with different histories of malaria exposure, with specificities varying between individuals within a given population, and have identified a number of antigens which are recognized as serodominant by individuals residing in areas of distinct malaria transmission and that may represent excellent vaccine candidates. In the initial proof-of-concept studies for protein microarrays (Sundaresh et al., 2006; Doolan et al., 2008), 250 putative proteins were selected from the *P. falciparum* genomic sequence database (www.plasmoDB.org) based on stage-specific transcription or protein expression, subcellular localization, secondary protein structure and documented immunogenicity in humans or animal models. Protein microarray chips were generated as described above, using *P. falciparum* clone 3D7 genomic DNA template, at $\geq 90\%$ efficiency for each of the PCR, recombinatorial cloning and protein expression steps. PCR conditions were optimized to increase the efficiency of target sequence amplification using low temperature annealing and elongation protocols. Arrays were probed with human sera from one of four groups which differed in immune status: sterile immunity or no immunity against experimental challenge following vaccination with radiation-attenuated *P. falciparum* sporozoites, partial immunity acquired by natural exposure, and no previous exposure to *P. falciparum*. Overall, 72 highly reactive *P. falciparum* antigens were recognized by sera from naturally exposed individuals and/or sporozoite immunized subjects; 16 of the 23 most immunoreactive proteins were well-characterized *P. falciparum* antigens, many of which are under clinical development and evaluation. Of the 56 other (novel) proteins, three have been identified previously by MudPIT analysis of erythrocyte ghosts (Wu et al., 2004), three have been identified previously by an epitope-based T cell screening approach (Doolan et al., 2003) and 50 have not previously been described as immunologically reactive. Of the known antigens, naturally exposed individuals preferentially recognized blood-stage antigens (e.g. MSP1), whereas sporozoite immunized volunteers responded predominantly to CSP. In the sporozoite-immunized groups, the pre-challenge antigen repertoire was broader in protected versus unprotected subjects; this repertoire was unchanged after challenge in protected subjects, but unprotected subjects experiencing clinical malaria developed an additional subset of antibodies broadly similar to naturally exposed individuals. Specific genomic/proteomic features were associated with high immunoreactivity.

Subsequently, an expanded protein microarray containing 1,200 unique proteins represented by 2,320 whole or partial proteins (because ORFs $>3,000$ bp were cloned as overlapping segments) and constituting $\sim 23\%$ of the *P. falciparum* proteome was generated. This list included all putative *P. falciparum* proteins with evidence of expression at some point

during the parasite life cycle as indicated by mudPIT analysis (Florens et al., 2002); www.plasmoDB.org) at the time of antigen selection as well as ~ 200 other putative proteins selected based on stage-specific transcription or protein expression, subcellular localization and secondary protein structure. This expanded array has been probed with sera from individuals experimentally immunized with irradiated sporozoites and either protected or not protected, and a potential “signature” of antigens associated with irradiated sporozoite immunity identified (Trieu, A and Doolan, DL, unpublished data). This expanded array has also been probed with sera from children and adults naturally exposed to malaria in various regions of Africa as well as Papua New Guinea. Distinct profiles of antigen recognition between adults and children naturally exposed to malaria are apparent, consistent with the proposal that natural immunity to malaria is acquired with age as a result of the sequential acquisition of parasite-specific antibodies (Baird, 1998) and a potential “signature” of antigens associated with naturally acquired immunity (Crompton et al., 2010) as well as a “signature” of antigens associated with irradiated sporozoite induced immunity (Trieu, A and Doolan, DL, unpublished data) have been identified. Data also show differences in immunoreactivity profiles between subgroups of children with different clinical histories (Trieu, A and Doolan, DL, unpublished data). Since a major target of naturally acquired immunity to malaria is the *P. falciparum* Erythrocyte Membrane Protein 1 (PfEMP1), protein arrays representing the repertoires of highly polymorphic *P. falciparum* strain *var* genes from field isolates have also been fabricated and probed with sera from one region of Papua New Guinea (Barry, A, personal communication), with interesting results. Ongoing studies in our laboratory are investigating *P. vivax* protein arrays and cross-species immunity. Overall, data demonstrate the potential for determining antigen-specific serology on a whole proteome scale and comparing immunoreactivity profiles amongst clinically distinct cohorts. Future studies may use protein microarrays to evaluate antigen polymorphism, since each chip can accommodate hundreds of target sequences. This is of particular interest in the case of *Plasmodium* due to data suggesting that sequence polymorphism may have arisen as a result of evolutionary pressure on the parasite to evade the host immune response and that highly polymorphic antigens may represent the best candidates for vaccine development (Barry et al., 2009). Although such polymorphism is an obstacle to vaccine development, some studies suggest that there are constraints in the ability of the parasite to vary (Doolan et al., 1992) and that a limited number of sequences are sufficient to represent the repertoire of sequence polymorphism (Duan et al., 2008).

Translationally active PCR fragments have also been explored for antigen discovery in malaria (Regis et al., 2008) and have been used for IVTT of recombinant proteins for antigen discovery in the *Bacillus anthracis* model (Gat et al., 2006). In the *B. anthracis* model, of the 197 selected ORFs, 129 of the known ORFs (93%) but only 38 (64%) of the unknown ORFs were successfully expressed using a platform distinct from that developed by the Felgner laboratory. Overall, 52 seroreactive immunogens were identified, of which only one was encoded by an unknown ORF (Gat et al., 2006). Protein microarrays fabricated with recombinant protein conventionally expressed in pET vectors, produced in *E. coli* and purified by nickel affinity column have been used to identify disease-specific antibody patterns in clinically diagnosed leprosy patients (Groathouse et al., 2006) and to identify 13 serodominant antigens (from 149 arrayed) in a rabbit model of plague (Li et al., 2005). GST fusion proteins conventionally expressed in pGEX and arrayed on microplates precoated with glutathione have been used to identify seven serodominant antigens (from 156 arrayed) in humans infected with *C. trachomatis* (Sharma et al., 2006).

There are, however, a number of limitations associated with a bacterial cell-free expression system which have recently been reviewed (Birkholtz et al., 2008; Vigil et al., 2010a). The two main disadvantages relate to post-translational modifications such as phosphorylation and glycosylation, and the complexity of protein folding and multimerization. One potential

disadvantage is that there is no glycosylation of protein products; however this may be an advantage for the malaria model since it is thought that native *Plasmodium* proteins are not glycosylated (von Itzstein et al., 2008). However, protein microarray reactivity has been observed to vaccinia extracellular enveloped virion proteins (Davies et al., 2007) as well as *Plasmodium* merozoite surface apical organelle proteins (Doolan et al., 2008; Crompton et al., 2010) that are known to be glycosylated; presumably the polyclonal response generated during infection of eukaryotic cells produces antibodies against the non-glycosylated regions of these proteins. The second main disadvantage is that some protein products might not be folded correctly due to the lack of a proper redox environment in which to form disulfide bonds (Yadava and Ockenhouse, 2003) and immune responses against important conformational epitopes may therefore be missed. Nonetheless, studies have demonstrated high immunoreactivity of proteins for which antibodies directed against conformational B cell epitopes have been associated with protection (e.g. PfMSP1) as well as antigens (e.g. PfAMA1) which are known to be conformationally complex in native structure with multiple disulphide bonds (Doolan et al., 2008; Trieu, A and Doolan, DL, unpublished data). Studies are pending to evaluate the ability of monoclonal antibodies against known conformational epitopes (e.g.: on MSP1 and AMA1) to recognize the proteins produced by either IVTT or by conventional recombinant protein production methods and spotted onto arrays. Other potential limitations of the system include protein degradation/truncation, aggregation and dimerization that could affect immunoreactivity.

The protein microarray platform provides an efficient time and cost-effective method that enables high-throughput and large-scale screening of ORFs from genomic databases for antigen identification for vaccines or diagnostics. It is intended as a screening tool and, once identified, antigens of interest may be expressed in conventional protein expression systems and/or molecular vaccine vectors for further characterization.

3.4. T cell screening using IVTT

Efforts are also underway to use proteins produced by IVTT to screen for targets of T cell responses. Jing et al. (2008, 2009) reported the application of *E coli* IVTT reactions for proteome-wide profiling of CD4⁺ T cell responses to vaccinia virus in humans. In their first study, reported to be the first proteome-wide screen of CD4⁺ T cell responses to a complex pathogen using full-length protein antigens, 180 predicted ORFs in the vaccinia virus genome were expressed in the RTS100 system and unpurified RTS reaction products were tested (at 1:1,000 – 1:10,000 dilutions) for recognition by vaccinia virus-enriched T cell lines derived from 11 Dryvax smallpox vaccinees, using ³H-thymidine proliferation assays (Jing et al., 2008). CD4⁺ responses were detected for 122 ORFs (68%), with a mean of 39 ORFs (range 13–63) recognized per person. CD4 negative cells did not respond, as indicated by intracellular cytokine staining (ICS), but there was a considerable overlap between the CD4⁺ antigens identified in this study and CD8⁺ antigens previously identified. Each of six structural virion proteins were recognized by nine to 11 out of the 11 subjects tested; all six proteins had previously been detected using a vaccinia virus genomic DNA expression library where CD4⁺ T cell responses were documented to only 35 proteins with a diversity of eight to 20 proteins recognized per person (Jing et al., 2007). The authors thus concluded that the whole-proteome RTS method appears to capture a broader within-subject response than that detected with expression library or motif-driven peptide approaches. The same protein set assayed for CD4⁺ T cell responses was probed by protein microarrays, and IgG responses were detected to 45 (25%) of the ORFs. Most ORFs that were immunodominant for antibody responses also frequently elicited CD4⁺ responses, while many other antigens were recognized by CD4⁺ cells only. In a subsequent analysis, arrayed matrices of proteins were used to determine the fine specificity of CD4⁺ T cell clones and bulk CD4⁺ T cell populations, using multi-cytokine ICS, and the CD4⁺ T cell responses were found to be

predominantly restricted by the HLA-DR class II MHC molecule (Jing et al., 2009). Reactivity at the ORF level was confirmed in selected cases with synthetic peptides.

Another variation of IVTT for high throughput identification of T cell antigens has been reported, in the *Anaplasma marginale* model (Lopez et al., 2008). Those authors affinity purified the *E. coli* IVTT reaction products on protein G-conjugated carboxylatemicrospheres beads using monoclonal antibodies to the poly-His tag, to remove inhibitory *E. coli* proteins. The IVTT-expressed bead-bound antigens were tested for immunogenicity in ^3H -thymidine proliferation assays with polyclonal short-term T cell lines from cattle immunized with purified *A. marginale* outer membranes. Additionally, the capacity of IVTT-expressed antigens with or without bead-affinity purification was compared. Of the 50 IVTT-expressed *A. marginale* proteins evaluated, 23 stimulated significant proliferative responses of T cells from one or both animals; five proteins were previously known and 18 were novel. Four immunostimulatory and six non-stimulatory proteins were conventionally expressed in *E. coli* and tested in T-lymphocyte proliferation assays, in parallel with IVTT-expressed proteins; overall, there was strong concordance between IVTT-expressed and conventionally expressed recombinant *A. marginale* proteins to stimulate specific T lymphocyte proliferation. Thus specific T-cell stimulation was achieved even at low antigen concentration and affinity purifying the antigen binding to protein G beads increased the sensitivity of the T-cell proliferation assay.

In our laboratory, we are conducting a series of in vitro and in vivo experiments using unpurified or affinity purified IVTT proteins expressed in a customized vector for high throughput identification of *Plasmodium* antigens recognized by CD4⁺ T cell responses (Caldas Cardoso, F and Doolan, DL, unpublished data). For these studies, individual proteins prepared in 96-well format are delivered to antigen presenting cells for recognition by T cells from individuals experimentally or naturally exposed to malaria using immune readouts such as antigen-specific cytokine production by ELISpot or Cytokine Binding assays.

3.5. T cell epitope-based antigen identification

The IVTT protein-based approaches described above can be used to identify targets of B cell responses (protein microarrays) or CD4⁺ T cell responses (IVTT cellular screening), but are likely not suited to the identification of targets of CD8⁺ T cell responses due to a requirement for target antigen processing and presentation (Yewdell and Haeryfar, 2005; Yewdell, 2007; Vyas et al., 2008).

One approach to identifying targets of CD8⁺ as well as CD4⁺ T cell responses is an epitope-based approach to antigen identification based on prediction of high affinity binding class I or class II T cell epitopes using computerized algorithms. Such a process whereby T cell epitope predictions are utilized in “reverse” as a tool to identify new antigens, rather than to identify the minimal CD4⁺ or CD8⁺ epitopes within a protein already considered a target of cellular immunity, was conceived in collaboration with Dr. Alessandro Sette and colleagues (originally at Epimmune Inc, now at La Jolla Institute of Allergy and Immunology, San Diego, CA, USA). Proof-of-concept for this approach was demonstrated in the *P. falciparum* malaria model (Doolan et al., 2003). It is an integrated approach that incorporates bioinformatic predictions, HLA supertype considerations, high-throughput binding assays, and cellular immune assays utilizing pools of potential epitopes to identify new antigens (Fig. 2). A key consideration underlying this strategy was the fact that T cells recognize a complex between a specific MHC type and a particular pathogen-derived epitope, and a given epitope will thus elicit a response only in individuals who express an MHC molecule capable of binding that epitope (Doherty and Zinkernagel, 1975a, b; Zinkernagel and Doherty, 1975). Another consideration was the extreme polymorphism of

MHC molecules and the dramatic differences in frequencies of expression of given MHC molecules in different ethnicities (Sette and Sidney, 1998). One means of circumventing the problem of MHC restriction relies on the selection of epitopes restricted by MHC types that can be grouped in broad families or supertypes; these supertypes are characterized by largely overlapping peptide repertoires and are expressed at high frequencies in all major ethnicities (Sette and Sidney, 1998; Southwood et al., 1998; Sidney et al., 2008). While the frequency of individuals positive for a given allele might vary drastically, the overall frequency of each supertype is remarkably constant across different ethnicities. HLA supertypes thus allow for the identification of peptide epitopes capable of binding multiple HLA molecules and offer the prospect of designing broadly reactive epitope-based vaccines using far fewer epitopes than in the case of similar vaccines relying on allele-specific (non-supertypic) coverage. A panel of the eight major supertypes (HLA-A1, -A2, -A3/A11, -A24, -B7, -B44, -DR and -DR3) will allow coverage of more than 99% of any human population, at the level of both MHC Class I and Class II molecules (Sette and Sidney, 1998; Southwood et al., 1998; Sidney et al., 2008); general population coverage in excess of 90% is achieved when as few as four supertypes (HLA-A2, -A3, -B7 and -B44) are considered. Furthermore, due to the focus on HLA supertypes rather than individual HLA alleles, there is no need for HLA typing as an inclusion criteria for the PBMC donors, thereby avoiding the bias and over-representation of Caucasians and HLA*A0201 individuals present in many studies performed to date. Identification of peptide epitopes that bind with high affinity to multiple HLA supertypes is an integral component of the epitope-based T cell screening strategy.

For antigen identification using this strategy, amino acid sequences translated from genomic sequence data are scanned using allele-specific algorithms for epitopes predicted to bind HLA-supertype molecules with high affinity. Pools of algorithm-identified peptides corresponding to each putative protein are synthesized and tested in HLA peptide binding assays for affinity of binding in vitro to the different HLA supertype family members, and for reactivity with PBMC of exposed/vaccinated individuals in assays such as IFN- γ ELISpot. Each pool is designed to include peptides representative of five to eight common class I and class II HLA-supertypes, and with about 10 epitopes per supertype. Due to the population coverage afforded by the panel of common HLA-supertypes, almost any individual should be capable of binding and recognizing at least 20 different epitopes (10 epitopes for each of class I and class II). Peptide pools which recall strong T cell responses can subsequently be deconvoluted to identify the individual peptide epitopes that are recognized. These antigens and epitopes can be prioritized according to the magnitude of the recall response and their capacity to be preferentially recognized by protected versus non-protected volunteers, as well as by their ability to bind strongly to multiple members of the relevant superfamily.

In the proof-of-concept demonstration of the epitope-based T cell screening approach in the *P. falciparum* model (Doolan et al., 2003), a panel of 27 ORFs thought to be expressed in the sporozoite proteome were evaluated; the well-characterized *P. falciparum* pre-erythrocytic stage antigens CSP, SSP2/TRAP, LSA1 and Exp1 were analyzed in parallel. Of these 27 ORFs, 16 were reproducibly recognized by PBMCs from irradiated sporozoite immunized volunteers but not by mock immunized controls. Nine antigens were highly antigenic (recognized by > 50% volunteers in > 25% assays); three antigens were of intermediate reactivity and four were weakly antigenic. Significantly, a number of antigens identified using this strategy were more antigenic than well-characterized antigens currently considered the best vaccine candidate antigens and some of the novel antigens were preferentially recognized by protected compared with unprotected individuals, suggesting the strategy may be able to differentiate between resistance and susceptibility at the molecular level. Recently, a transcriptome and proteome survey of the *P. yoelii* liver stage found that *P. yoelii* orthologs of two of the proteins identified using the epitope-based T cell

screening approach (PFD0425w/PY00455, PFI0260c/PY04544) were identified in the liver-stage schizont proteome, and two (PFC0700c/PY03459, MAL8P1.78/PY00566) were present in the liver-stage transcriptome (Tarun et al., 2008). In another study (Siau et al., 2008), five of the 16 highly antigenic proteins were implicated with roles in hepatocyte invasion and liver-stage development since they were shown to be encoded by genes up-regulated following *P. falciparum* co-culture with primary human hepatocytes (PFD0425w, PFI0165c, PF14_0372, PF14_0074, PF11_0435). One of these proteins (SIAP-1; PFD0425w) was subsequently shown to be involved in host cell invasion based on functional studies (Siau et al., 2008). The *P. berghei* ortholog of another of the antigens identified as highly antigenic using the epitope-based T cell screening approach (Ag2/PFL0800c), designated cell-traversal protein for ookinetes and sporozoites (CelTOS), was shown to mediate transmission to mosquito and vertebrate hosts; targeted disruption of the CelTOS gene in *P. berghei* reduced parasite infectivity in the mosquito host approximately 200-fold, reduced sporozoite infectivity in the liver and almost abolished its cell-passage ability (Kariu et al., 2006). Other studies with *P. falciparum* Ag2/PFL0800c have identified orthologs in all *Plasmodium* spp. and have established that it is highly conserved relative to most other *P. falciparum* vaccine antigens, recognized in the context of multiple genetic restrictions, more immunogenic than all *P. falciparum* pre-erythrocytic stage antigens currently in clinical evaluation, and protective against infection in the *P. yoelii* rodent model (both homologous and cross-species protection) (Doolan, DL, unpublished data).

In addition to demonstrating the potential of an epitope-based approach for the identification and prioritization of novel vaccine antigens, these data suggest that, in the case of the irradiated-sporozoite vaccine, a large number of antigens are recognized, dispersed amongst a large fraction of the proteome. This highlights the importance of identifying the repertoire of antigens and epitopes that are the targets of sporozoite-induced immunity, and for development of multivalent vaccines that mimic the complexity of the whole organism. The epitope-based T cell screeningTM approach is currently being extended to the entire *P. falciparum* pre-erythrocytic stage proteome, with the support of a NIH/NIAID grant (Doolan, DL and Sette, A, unpublished data).

This epitope-based approach has subsequently been applied to map epitopes in other large pathogens and has been reviewed elsewhere). Data from some of those studies have important implications for malaria vaccine development. For example, a study in the vaccinia virus BALB/c mouse model showed that T cell epitopes were not equally distributed across the proteome since 197 CD8⁺ T cell epitopes were identified from 103 of the 206 ORFs evaluated and some genes encoded five or more discrete epitopes each (Oseroff et al., 2008). The authors concluded that there may be a subset of proteins termed “immunoprevalent” that is frequently immunogenic in the context of multiple MHC alleles, and that immunoprevalent proteins may not be the major source of immunodominant T cell epitopes (Oseroff et al., 2008). Those data suggest that immunoprevalent antigens rather than immunodominant antigens should be selected for vaccine development since the former might yield T cell responses in a much broader range of individuals whereas the latter could result in uneven vaccine performance depending on the MHC background.

3.6. B cell epitope identification

Historically, prediction of antibody epitopes has been technically challenging due to inadequate understanding of antibody recognition and binding characteristics, and complicated influences of three-dimensional structure on conformational B cell epitopes (conformationally sensitive and discontinuous epitopes). However, it is likely that significant advances will be made in this area during the coming years as a result of the development of new technologies, the availability of large data sets to assist in predictive

methods, and the constant advances in the availability and prediction of three-dimensional structures (Sette et al., 2005).

3.7. Immunomics – analysis of the outcomes

Immunomic approaches can be applied to characterize the immune responses in individuals experimentally immunized with radiation attenuated sporozoites, genetically attenuated parasites or whole parasite vaccines, or in different populations of individuals naturally exposed to malaria including those with mild disease, severe disease, cerebral malaria or malarial anemia. Since clinical disease and acquired immunity are influenced by the age of the individual, evaluating the immune responses in infants, children and adults may further our understanding of acquired immunity. Important information may also be obtained by comparison of the responses induced by experimental immunization with irradiated sporozoites, which confers sterile protection against sporozoite challenge, with the responses induced by natural exposure to malaria, which induces anti-disease but typically not anti-parasitic immunity (that is, not infection-blocking immunity). Comparison of the immune targets recognized by these clinically distinct cohorts should allow for the identification of *P. falciparum* antigens targeted by immune responses associated with protection.

The *Plasmodium* parasite is a complex and multi-antigenic immunogen. However, due to various factors related to antigen abundance and immunodominance, not all possible antigens may be recognized by natural immunity. Immunodominance is defined as the phenomenon by which natural immune responses do not target the full range of possible peptide epitopes. Rather, only a small fraction of all possible epitopes from a particular pathogen is actually recognized (Yewdell, 2006). Those antigens or epitopes that do induce a response upon immunization of whole native antigens are defined as dominant (Sercarz et al., 1993; van der Most et al., 1996; Yewdell, 2006). Data generated by proteome-wide or genome-wide immunomics approaches would establish whether immune responses in humans induced by parasite exposure are narrowly focused on a few immunodominant proteins and epitopes or alternatively, whether responses are dispersed on a relatively large numbers of parasite antigens, advancing our understanding of the phenomenon of immunodominance in the context of a complex parasitic pathogen.

In the context of *P. falciparum* vaccine development, the identified targets could be prioritized according to their immune reactivity, presuming that the most immunodominant antigens recognized by individuals with immunity to malaria will be capable of inducing the most robust and protective immune responses when expressed in an appropriate vaccine delivery system. The most promising antigens could then be further characterized in vitro and in vivo using, for example, functional studies. Antigen-specific rabbit polyclonal antibodies could be produced and antigens characterized with regard to stage-specificity, by immunofluorescence antibody tests against the different stages of the parasite life cycle. For those antigens identified based on antibody activity, the biological activity of the antigen-specific antisera could be evaluated in vitro using the inhibition of sporozoite invasion assay (ISI), inhibition of liver stage development assay (ILSDA), growth inhibition assay (GIA), or antibody dependent cellular inhibition (ADCI) assay. However, the most important indication as to the value of a putative antigen for inclusion in a malaria vaccine relates to its protective capacity. Therefore, the capacity of each antigen or its rodent or non-human primate ortholog to protect against *Plasmodium* parasite challenge could be determined in rodent and/or non-human primate models. In the case of sporozoite challenge, the capacity of the antigen to protect against one or more *Plasmodium* spp. parasites could be determined by complete absence of blood-stage parasitemia (sterile immunity) or reduction in liver-stage parasite burden (partial protection). In the case of blood-stage challenge, the capacity of the antigen to protect against one or more *Plasmodium* spp. parasites could be determined

based on peak parasitemia, delay in onset of parasitemia, days from first day to peak day of parasitemia or self-cure.

The identification and characterization of the antigens and epitopes recognized by humans exposed to *P. falciparum* would allow for the design, optimization and evaluation of new candidate malaria vaccines based either on a selected number of antigens and/or epitopes that are more immunogenic than those currently available, or that include many more antigens and/or epitopes and therefore may be more effective (since the response induced by a combination of antigens would be expected to be broader than that elicited by each individual antigen). Reverse vaccinology approaches to vaccine development have recently confirmed that combining multiple antigens in a vaccine provides broader protection against different strains of a pathogen than individual antigens (Sette and Peters, 2007). Such data will allow for the development of a vaccine designed to mimic whole organism induced immunity, by incorporating an unprecedented number of antigens and/or epitopes from those antigens that are targeted by protective immune responses. By eliminating extraneous sequences or antigens that may be detrimental to induction of protective immunity, such vaccines may potentially be more immunogenic and protective than whole organism vaccines.

There is of course no conclusive evidence supporting the hypothesis that protective immunity is a dominant response in anti-malarial immunity nor that immunodominance will equate with vaccine candidacy. However, the immunodominant sporozoite surface antigen, CSP, has been identified as a target of protective immune responses in rodent models (Doolan and Martinez-Alier, 2006; Kumar et al., 2006) and in humans, where it is the central component of the RTS, S vaccine which has been shown to protect malaria-naïve adults against experimental sporozoite challenge and naturally exposed children and adults against field challenge (Cohen et al., 2010). Additionally, in the *P. yoelii* rodent model, the protective immunity induced by PyCSP DNA was shown to be directed predominantly against the only immunodominant CD8⁺ T cell epitope which binds with high affinity to H-2d MHC molecules (Dobano et al., 2007). Although responses could be induced against other subdominant CD8⁺ T cell epitopes, CD4⁺ T cell epitopes and B cell epitopes, those were poor and not capable of conferring significant protection. These data support strategies designed to identify antigens and epitopes that are immunodominant in the context of experimental or natural exposure to malaria parasites.

How to best mimic the complexity of multi-antigenic whole organism vaccines by subunit vaccination is not obvious, however, particularly if protective immunity is mediated by a summation of immune responses directed against multiple antigens and against multiple epitopes on those antigens. One approach that has been explored in malaria (Doolan et al., 1997a) and in other models (Depla et al., 2008) is the design and optimization of a multi-epitope malaria vaccine comprising a defined panel of HLA-degenerate CD8⁺ and CD4⁺ T cell epitopes. A candidate malaria vaccine comprising 33 CD8⁺ T cell epitopes and 13 CD4⁺ T cell epitopes derived from PfCSP, SSP2, LSA1 and EXP1 identified by class I/II algorithm predictions and peptide binding/recognition strategies, and recognized in the context of multiple HLA alleles representative of those most commonly expressed worldwide (Doolan et al., 1997b, 2000), is currently in clinical development with a phase 1/2a study planned for 2010 (Bruder et al., 2010). A multi-epitope vaccine based on this panel of epitopes would be predicted, therefore, to be effective in all target populations and against all *P. falciparum* strains. The efficacy of this multi-epitope vaccine could potentially be compared with that of the whole organism irradiated sporozoite vaccine, genetically attenuated parasite vaccines or other whole parasite vaccines as well as whole antigen subunit vaccines. Such studies would validate the multi-epitope approach, provide important information regarding the potential of multi-epitope-based approaches to mimic whole

organism induced immunity, and the importance and potential of large-scale epitope mining for vaccine development.

4. Conclusion

In the current state of the art, there is as yet no genome-wide approach to vaccine development. Selection of vaccine targets is based on a variety of criteria which, while not irrational, are not systematic. For example, antigens may have come to attention due to historical reasons related to the ease with which murine or rabbit antisera were generated against them. Their selection as candidate vaccine targets may be validated by immunoepidemiological evidence of associations between immune responses and clinical immunity, neutralization or adoptive transfer experiments in animal systems, or induction of protective immunity in animal models with homologous antigens from murine or simian malaria spp. Immunomic-based approaches offer the potential to overcome the deficiencies of the current ad hoc approach to target selection by using biological samples from humans or animals with immunity to malaria and clinically relevant criteria that can be applied to every ORF and thus every potential target within the *P. falciparum* genome. Immunomics offers a rational approach to vaccine design capable of exploiting the wealth of genomic, proteomic and bioinformatic information that has been developed in the genomic and post-genomic eras to identify *Plasmodium* antigens which likely represent excellent candidates for malaria vaccine development or diagnostic applications (Fig. 3). Additionally, it is anticipated that immunomics-based research will further our understanding of the molecular basis of immunity to disease, help elucidate the correlates of protection, and enable the performance of basic studies analysing variables such as the magnitude and kinetics of immune responses and their variation as factors of different vaccine constructs, doses and regimens, and among different individuals.

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Fig. 1. High Throughput Proteomics

Fabrication of protein microarrays for antibody target identification. Protein microarrays are fabricated using a method involving high throughput amplification of each predicted open reading frame from the genomic sequence using gene-specific primers with adapter sequences, followed by homologous recombination into a T7 expression vector that has an N-terminus HIS tag and a C-terminus HA tag. The plasmids are transformed into *Escherichia coli* cells, miniprep, and proteins expressed using in an in vitro cell-free transcription/translation system supplemented with T7 RNA polymerase. The proteins are then printed directly onto microarray chips without further purification. Microarrays are probed with anti-HIS and anti-HA antibodies to confirm expression of full-length proteins in quality control (QC) assays, and with sera from experimentally immunized or naturally exposed subjects. From <http://www.antigendiscovery.com/technology.php>, with permission.

Fig. 2.

Epitope-based T cell screening strategy for T cell target identification. Complete amino acid sequences translated from the genes of interest are scanned using motif identification software for the presence of HLA motif-containing sequences. For each antigen (Ag), peptides representing the top 10 epitopes predicted to bind with highest affinity to each of five common HLA supertypes are identified and synthesized. Peptides are pooled and screened for capacity to induce recall IFN- γ immune responses using peripheral blood mononuclear cells from volunteers immunized with irradiated *Plasmodium falciparum* sporozoites or naturally exposed to malaria. Antigens can be prioritized on the basis of immune reactivity, and the peptide pools deconvoluted to identify the minimal peptide epitopes and their HLA restriction elements.

Fig. 3.

Genomes to vaccines schematic illustration. Methodologies for utilizing genomic sequence data to identify new candidate antigens for use in malaria vaccines require the complex interplay of bioinformatics (denoted by dotted arrows) and molecular manipulations (denoted by solid arrows), many of which involve novel technologies that are amenable to high-throughput processing (see Section 3 for details). Open reading frames (ORF) are denoted by open boxes, and their expressed protein product by solid boxes. There are multiple alternative pathways for many of the steps, only some of which are illustrated. For example, either transcriptionally active polymerase chain reaction (TAP) fragments (linear DNA) or the proteins they encode as expressed in cell-free systems, or circular plasmid DNA generated by recombinatorial cloning, can be used to transfect or transduce antigen presenting cells (APCs) such as dendritic cells (DC); similarly, protein arrays can be used to screen either sera or cells from protected volunteers. This figure is modified from Richie (2004) with permission. MHC, major histocompatibility complex; HLA, human leukocyte antigen; PBMC, peripheral blood mononuclear cell; ISI, inhibition of sporozoite invasion assay; ILSDA, inhibition of liver stage development assay; GIA, growth inhibition assay (blood stage); IFAT, immunofluorescent antibody test; *Pf*, *P. falciparum*.

Table 1

Features of key *Plasmodium* spp. genomes.

<i>Plasmodium</i> species	Strain	Host	Size	# Genes	Mean # exons/gene ^b	Mean gene length (bp) (excluding introns) ²	Mean exon length (bp) ^b
<i>P. falciparum</i>	3D7	Human	23.3 Mb ^a	5,403 ^a	2.4	2283	935
<i>P. vivax</i>	Sal1	Human	26.8 Mb ^b	5,433 ^b	2.5	2164	957
<i>P. knowlesi</i>	H	Primate/Zoonotic	23.5 Mb ^c	5,188 ^c	2.6	2180	837
<i>P. yoelii</i>	17XNL	Rodent	23.1 Mb ^d	5,878 ^d	2.0	1298	641

^aGardner et al. (2002);

^bCarlton et al. (2008);

^cPain et al. (2008);

^dCarlton and Carucci (2002).