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Large screen approaches to identify novel malaria vaccine candidates☆

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ABSTRACT

Until recently, malaria vaccine development efforts have focused almost exclusively on a handful of well characterized *Plasmodium falciparum* antigens. Despite dedicated work by many researchers on different continents spanning more than half a century, a successful malaria vaccine remains elusive. Sequencing of the *P. falciparum* genome has revealed more than five thousand genes, providing the foundation for systematic approaches to discover candidate vaccine antigens. We are taking advantage of this wealth of information to discover new antigens that may be more effective vaccine targets. Herein, we describe different approaches to large-scale screening of the *P. falciparum* genome to identify targets of either antibody responses or T cell responses using human specimens collected in Controlled Human Malaria Infections (CHMI) or under conditions of natural exposure in the field. These genome, proteome and transcriptome based approaches offer enormous potential for the development of an efficacious malaria vaccine.

1. Introduction

The *Plasmodium falciparum* genome project began in 1997 with optimism that it would lead to a better understanding of the parasite, better disease treatments, and an effective malaria vaccine. The first complete annotated genome sequence was published five years later in 2002 [1], followed by the proteome [2,3] and transcriptome [4,5]. These datasets have become important tools advancing the field of malaria research with specific application for the development of vaccines, drugs and diagnostics.

Prior to the genome sequencing era, only a few dozen *Plasmodium* proteins had been identified. Therefore malaria vaccine development had been largely empirical, evaluating different platforms and formulations that might improve protective efficacy of the few known antigens. Those efforts were largely unsuccessful [6]. The advent of the "genomic era" following the sequencing of the genome of *Haemophilus influenza* in 1995 [7] and associated technological advances has resulted in the availability of large-scale datasets of genomic, proteomic and transcriptomic information which have enabled a switch in focus from vaccine platforms to vaccine antigens. Conceptual changes driven by more refined consideration of the specific target profile have also occurred (http://www.who.int/immunization/topics/malaria/vaccine_roadmap/en/). Because the parasite has several developmental stages with a dynamically changing transcriptome and proteome, each discovery effort must be tailored to the purpose of a specific vaccine. For example, a vaccine might seek to induce antibodies against the surface proteins of infected erythrocytes, or antibodies against the invasion proteins of blood stage merozoites, or T cell responses against proteins expressed by intrahepatic parasites. Likewise, there has been increasing recognition that an effective vaccine will probably need to be multivalent, directed against multiple antigens which may be expressed in different stages of the parasite life cycle.

One of the biggest hurdles in the development of an effective vaccine against complex microorganisms that encode thousands of proteins, such as the *Plasmodium* spp. parasite, is the identification of the subset of key antigens that can be induce a protective immune response. Advances in high throughput sequencing during the last decade have made possible "reverse vaccinology" [8], which takes advantage of genome sequence data, and applies bioinformatics to predict immunogenic vaccine antigen candidates. Annotated proteomic features associated with antigenicity and vaccine efficacy, such as extracellular location, outer

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membrane proteins, signal peptides, and B- and T-cell epitopes can be computationally predicted based on the amino acid sequence with a consequent reduction in the number of candidate antigens. However, although such antigen prediction can lower the priority of some potential candidates, it tends to produce large lists that require additional labor intensive and time consuming laboratory investigations to further narrow down the vaccine candidates. Bioinformatic approaches may also fail to identify *bone fide* antigens that would otherwise appear promising using empirical methods. Additional efficient, high throughput screening approaches are needed that can be accomplished on a whole genome scale to provide more empirical data to inform antigen selection.

A refinement of reverse vaccinology is the discipline of immunomics which integrates genomics and immunology by using biological samples from humans or animals with immunity to the target pathogen to identify the subset of pathogen-derived proteins or their epitopes that are recognized by the host immune system [9,10]. A critical component of these proteome-wide immune screening assays is the use of clinically relevant selection criteria, such as induction of antibodies of a specific subclass or T cell responses characterized by Th1 (or Th2) cytokines.

2. Human models establishing the feasibility of a malaria vaccine

A highly effective malaria vaccine would be a valuable tool to reduce the disease burden and is considered a critical component of global eradication [11]. Optimism that an efficacious malaria vaccine can be developed derives from two independent lines of research. In the first instance, individuals living in areas where *P. falciparum* is endemic and are repeatedly exposed to the parasite throughout childhood and adolescence develop naturally acquired immunity (NAI)[1] which decreases the incidence and severity of clinical malaria episodes and prevalence and density of blood stage parasitemia but does not prevent infection [12,13]. Moreover, passive transfer of immunoglobulin from individuals with NAI can decrease peripheral parasitemia and resolve clinical symptoms in the recipient [14,15]. This suggests that antibody responses against antigens expressed in the blood-stage of the parasite life cycle are important targets of NAI. Thus, profiling the antibody responses associated with NAI and comparing to the profiles of age-matched susceptible cases could inform vaccine antigen discovery.

The second line of evidence comes from studies showing that immunization with *Plasmodium* sporozoites that are attenuated by radiation such that they can invade the hepatocyte but cannot develop beyond late liver stages (Radiation-Attenuated Sporozoites, RAS) can induce sterilizing immunity against challenge with infectious sporozoites in mice and humans [16–19]. Studies in murine models indicate that T cell responses (especially $CD8^+$ T cells) directed against antigens expressed in the liver stage of the parasite life cycle are the critical mediators of this sporozoite-induced immunity [19–22].

An alternative sporozoite vaccine modality to that described above is experimental immunization by mosquito bite with sporozoites from *Plasmodium* infected mosquitoes with concurrent chloroquine administration (ChemoProphylaxis and Sporozoites, CPS-immunization; also known as infection–treatment–vaccination, ITV) [23–25]. The RAS and CPS immunization regimens are thought to induce a broad repertoire of T cell specificities, recognizing multiple antigens expressed by the *Plasmodium* spp. parasite during hepatic development [26]. There is evidence that the breadth of response is evolving during the course of immunization [27]. A better understanding of the breadth of the cellular immune response against *Plasmodium* is therefore central to the development of an effective prophylactic vaccine against malaria.

3. Full- and partial-proteome immune-profiling of *Plasmodium* infections in humans

The human vaccine models described above present a unique opportunity to characterize the molecular determinants underpinning the strong immunity they induce. In the case of RAS or CPS regimens, for example, the measure of anamnestic (or recall) responses of T cells from immunized subjects against a panel of *P. falciparum* antigens and their correlation to clinical outcome is possible. For these kinds of differential screenings, the ideal situation is presented when the rate of sterilizing immunity to CHMI is less than 100% (a common outcome), so that decoy responses, immunodominant and/or pre-existing responses can be identified and discounted from future consideration. The main challenges in performing these types of full proteome screens for *Plasmodium spp.* derive from the size and complexity of their genomes. The genome of *P. falciparum* contains about 5300 genes [1]. Many proteins are encoded by complex multi-exons genes sometimes several kilobases in length and contain long stretches of repetitive sequences, a consequence of the organism's low GC content. Consequently, cloning and expression of antigenic targets from *Plasmodium* spp. parasites is particularly difficult and efforts to express *Plasmodium* proteins on a large scale using conventional expression systems have been largely unsuccessful [28–31]. Even at the individual level, expression of full-length proteins has been challenging. For instance, the leading RTS,S vaccine comprises only a fragment of CSP and successful expression of the entire protein in amounts compatible with the development of a full-length CSP vaccine has only recently been achieved in a combinatorial *Pseudomonas fluorescens* expression system [32]. Despite these limitations, expression libraries containing a significant fraction of the *P. falciparum* proteome have been successfully built and screened for the identification of antibody responses [33–35] (see below).

4. Antibody-based proteomic studies to antigen discovery

To inform antibody-based antigen discovery, we and others have been developing proteome-wide approaches that are cost-effective, rapid, and independent of predictive algorithms, with the aim of reducing the laboratory time and cost associated with vaccine and serodiagnostic antigen discovery. In particular, a rapid high throughput approach to efficiently clone and express each individual gene in the entire microorganism's genome using an *E. coli* cell-free transcription/translation expression system was developed at Antigen Discovery, Inc. and the University of California at Irvine [36,37]. The proteome is interrogated with sera from infected and vaccinated individuals to identify immunoreactive vaccine antigen candidates associated with protection. Similarly, antibody response profiles in disease cases and healthy controls can be compared to identify antigens with the most accurate predictive diagnostic value.

This protein microarray platform was originally developed using malaria and vaccinia virus (~220 genes) as the model pathogen [36]. Since then the National Institutes for Health (NIH), the National Institute for Allergy and Infectious Diseases (NIAD) and the Bill and Melinda Gates Foundation (BMGF) have provided support for antigen discovery against more than 30 human pathogens including *M. tuberculosis* [38], *P. falciparum* [33,34,39], *P. vivax*, *B.*

[1] *Abbreviations*: ATLAS, Antigen Lead Acquisition System; CHMI, Controlled Human Malaria Infection; CPS, ChemoProphylaxis and Sporozoites; Hb, hemoglobin; IE, infected erythrocytes; ITV, infection–treatment–vaccination; NAI, naturally acquired immunity; NSR, not-so-random; RAS, Radiation-Attenuated Sporozoites.

melitensis [40], *C. trachomatis* [41], *F. tularensis* [42,43], *B. pseudomallei* [44,45], *C. burnetii* [46–48], *B. burgdorferi* [49], *Salmonella enterica* typhi, *R. prowazekii*, *R. rickettsii*, *O. tsutsugamushi*, *B. henselae* [37], *L. interrogans*, *T. gondii* [50], *C. albicans* [51], *Schistosoma mansoni* [52], *Schistosoma japonicum* [52], and viruses including vaccinia [36,53–55], monkeypox, Herpes 1 & 2, Varicella zoster, HPV [56], HIV, Dengue, influenza, West Nile and Chikungunya. In total, we have cloned more than 60,000 genes from infectious microorganisms, printed the encoded proteins on 25,000 microarrays and probed the arrays with 12,000 serum specimens determining disease associated antibody profiles in people infected with each agent. The individual proteins printed on these arrays capture antibodies present in serum from infected individuals and the amount of captured antibody can be quantified using fluorescent secondary antibodies. In this way, a comprehensive profile of antibodies that result after infection or exposure can be determined that is characteristic of the type of infection and the stage of disease [49,54,55].

This unbiased systems biology approach offers a powerful means of identifying vaccine antigens, surrogate biomarkers associated with protection, and serodiagnostic antigens of pathogen exposure. Arrays can be produced and probed in large numbers (>100 serum specimens/day) while consuming small quantities of individual sera (~2 μl of sera/patient). This permits assessing the repertoire of antibodies created in response to infection or vaccination from large collections of individual patient sera, and can be used to perform large-scale sero-epidemiological, longitudinal and sero-surveillance analyses not possible with other technologies. Immunoglobulin isotypes and IgG subtypes can be independently determined. Moreover, microarrays offer the potential to express all proteins of an infectious agent and may allow for identification of novel antigens, otherwise undetectable by methods like 2-D gels that are highly biased by microbial protein expression patterns.

When we began investigating this approach more than 10 years ago, there was a lack of empirical data to answer basic questions about differences in breadth, intensity, kinetics, and longevity of immune responses induced by antigens derived from different infectious agents. Questions such as: what percentage of the entire microorganism proteome is recognized by the immune system? Are there differences in antibody reactivity profiles between individuals? Do different host species exposed to the same agent differ in reactivity profiles? Can antibody profiles predict disease stage? Are there characteristic antibody profiles that distinguish gram negative and gram positive bacteria, from responses induced by eukaryotic parasites? Can antigenicity be predicted based on the amino acid sequence alone? Today some generalizations about antigen recognition can be made, supported by rapidly expanding empirical data sets.

4.1. Protein microarray studies of naturally acquired immunity

Several published malaria proteome microarray studies have been aimed at understanding the phenomenon of NAI to malaria. A seminal paper by Crompton et al. [34] showed that naturally exposed individuals from Mali produce antibodies against hundreds of *P. falciparum* antigens. Both children and adults have more reactivity after the high transmission season, and adults have more reactivity than children. When adolescent children who survived the malaria season without experiencing symptoms were compared with age-matched children who had a malaria episode, 49 antigens were identified that were associated with protection from clinical malaria [34]. Interestingly, Ab reactivity against several conventional lead vaccine candidates (CSP, LSA-3, MSP-1, MSP-2, and AMA-1) did not discriminate between protected and susceptible children. Similar observations from a low-transmission setting of the Peruvian Amazon showed a limited set of *P. falciparum* protein antigens associated with the development of naturally acquired clinical immunity [57]. Overall this approach is leading the discovery of many novel candidate vaccine antigens.

The high throughput nature of the microarray platform (requiring ~2 μl of serum) lends itself to sero-epidemiological surveys. One conclusion from worldwide epidemiological studies using protein microarray serology is that the breadth and intensity of the antibody response is proportional to the level of parasite exposure in the environment. For example, Baum et al. compared protein microarray seroreactivity from individuals living in Kenya at different elevations [58]. In lowland areas exposure to parasite infected mosquitoes is much more intense than in the highlands, and the breadth and intensity of the antibody response is also elevated in the lowland population. Similar relationships between parasite exposure and the serological response have been observed across Africa, Papua New Guinea and Peru (unpublished). In a different study in Kenya, the influence of HIV infection on immunity to malaria was investigated, since HIV-malaria co-infections are common in many areas but are poorly understood at the immunoproteomic level. In that study, individuals from areas of high and low endemicity had different antibody profiles, as seen elsewhere, but surprisingly HIV infected patients with normal $CD4^+$ counts had the same reactivity against malaria antigens as HIV negative individuals leading to the conclusion that early stage HIV infected patients are not at risk of losing NAI [59]. Individuals with advanced AIDS were not examined in that study.

P. falciparum erythrocyte membrane protein 1 (*Pf*EMP1), which mediates parasite sequestration and host immune evasion, is considered an important antigen in the context of NAI [60,61]. In the first protein microarray based study of variant antigens in malaria, an array was produced containing 123 var domains derived from *P. falciparum* field isolates from Papua New Guinea [60]. The data showed that the anti-*Pf*EMP1-DBLα antibody responses increase in diversity, magnitude, and prevalence with age. In another study, reactivity to 21 *Pf*EMP1 fragments on a protein microarray was measured in serum samples from Malian children aged 1–6 years and adults [61]. Seroreactivity to *Pf*EMP1 fragments was higher in adults than in children. In a different study, differential recognition of terminal extracellular *P. falciparum* VAR2CSA domains were determined by printing a protein microarray which included five overlapping fragments of the 3D7 VAR2CSA extracellular region and probing this with sera from multigravid, malaria-exposed Malian women (manuscript in press). Women with a history of at least one pregnancy had antibody responses against four of these fragments and had stronger reactivity against the two distal fragments than did nulliparous women, children, and men from Mali, suggesting that the C-terminal extracellular VAR2CSA domains are a potential focus of protective immunity.

Protein microarrays have also been applied to study the effect of sickle-cell trait on the *P. falciparum* specific antibody response in a cohort of naturally exposed individuals from Mali [62]. Heterozygous states of hemoglobin (Hb) A and HbS (HbAS, sickle-cell trait) or HbC (HbAC) protect against *P. falciparum* malaria but the mode of protection is unclear. One hypothesis is that HbAS and HbAC accelerate the acquisition of immunity to malaria, possibly by enhancing *Pf*-specific antibody responses. However, in this study, protein microarray data showed there were no significant differences in antibody profiles between individuals with normal or sickle cell traits [62].

The array technology has been also applied to compare antibody profiles between the Fulani and Dogon ethnic groups in Mali, which differ in prevalence of symptomatic clinical malaria (manuscript in submission). Elevated IgG and IgM antibody levels were reported in the genetically resistant Fulani people, compared to the more susceptible sympatric Dogon population.

The collective message from all these studies is that naturally acquired immunity is associated with elevated antibodies against

hundreds of blood stage antigens. A vaccine that boosts antibody responses against a collection of these blood stage antigens may allow children to reach protection from symptomatic malaria at an earlier age. The waning immune response in adults residing in areas undergoing elimination could also be boosted with a vaccine of this kind.

4.2. Protein microarray studies of sporozoite induced immunity

In our initial studies with the protein microarray platform, we reported results from a clinical trial of experimental immunization with radiation attenuated sporozoites in which some of the volunteers were protected from an experimental challenge and others were not [33,35]. Microarrays containing 2320 *P. falciparum* proteins were used to determine the profile of antibody responses in volunteers immunized with sporozoites administered by the bites of irradiated mosquitoes. Immunized volunteers reacted against a collection of *P. falciparum* proteins more strongly than mock infected or naïve individuals. After being challenged with viable sporozoites from infected mosquitoes (Controlled Human Malaria Infection, CHMI), the antibody profile in the protected subjects did not significantly change, indicating that immunization produced a sterilizing infection-blocking immune response (supported by PCR evidence) that blocked maturation of the organism at an early stage post-challenge, and no new antigens were presented to the immune systems as a result of the challenge. In contrast, in unprotected subjects antibody titers increased against dozens of additional proteins after challenge, indicating that new blood stage antigens were presented to the immune system as blood stage parasitemia progressed during the course of clinical malaria. To determine whether antibody responses at the time of challenge could differentiate between protected and unprotected volunteers, the corresponding protein microarrays profiles were compared. A panel of 19 pre-erythrocytic stage antigens was identified as strongly associated with sporozoite-induced protective immunity; 16 of these antigens were novel and have been independently identified in sporozoite and/or liver stage proteomic or transcriptomic datasets [35]. Reactivity to any single antigen did not correlate with protection but there was a highly significant difference in the cumulative signal intensity of multiple antigens between protected and not protected individuals [35]. These data provide the first evidence that sterile protective immunity against malaria is directed against a panel of protective *P. falciparum* antigens rather than a single dominant antigen. These results also have important implications for vaccine development, suggesting that an efficacious malaria vaccine should be multivalent and targeted at a select panel of key antigens, many of which have not been previously characterized.

This vaccination modality which uses the bites of irradiated *P. falciparum*-infected mosquitoes to administer the irradiated sporozoites is not a practical pharmaceutical product. Taking on this translational research challenge, Sanaria, Inc. (Gaithersburg, MD) has developed the capacity to produce vialed aseptic, purified, and cryopreserved radiation-attenuated and non-attenuated *P. falciparum* sporozoites in a highly regulated, cGMP compliant, industrial setting [63]. Several CHMI clinical trials have been conducted which show the vaccine to be safe and capable of inducing protective immunity after intravenous administration [64–68]. Protein microarray analysis of some of these specimens is in progress.

Analysis of specimens collected from individuals experimentally immunized by CPS-immunization (ChemoProphylaxis and Sporozoites) has also been conducted. In those studies, a down-selected proteome array containing 809 of the reactive antigens recognized in other cohorts [33–35] has been probed with plasma specimens from CPS-immunized individuals and the responses compared with that of specimens from adult individuals from Kenya with NAI. CPS and NAI both induce potent antibody responses against a large number of *P. falciparum* antigens, but while many antigens overlap in the two profiles, other antigens are distinctly different [69]. Thus, NAI induces antibodies against hundreds of antigens derived from blood stage parasites, but since chloroquine prevents replication of blood stage parasites there are many antigens recognized in the NAI profile that are not recognized by CPS immunized individuals. Conversely, CPS immunized subjects have antibodies against hundreds of antigens that are lacking in individuals with NAI, and are postulated to represent antigens associated with pre-erythrocytic stages of infection [69].

4.3. Protein microarray studies of pre-erythrocytic vaccine induced immunity

The leading malaria vaccine candidate is the GlaxoSmithKline vaccine RTS,S/AS01, a hybrid virus-like particle formulation of recombinant partial-length circumsporozoite protein and hepatitis B surface antigen expressed in *Saccharomyces cerivisiae*, adjuvanted in liposomes with MPL® and QS21 immunostimulants [70]. This vaccine protected ~50% of volunteers against experimental challenge 2 weeks after the last immunization, and 22% of volunteers when challenged at 6 months [71]. Recently, results of the Phase 3 study of RTS,S in sub-Saharan Africa have been reported, with a demonstrated reduction in clinical malaria in the target age group of infants aged 6–12 weeks at first vaccination of only 18% following the 3-dose primary schedule or 26% following a booster dose with RTS,S at 18 months; efficacy in children aged 5–17 months was slightly higher (28% and 36%, respectively) [72]. Protein microarray analysis of specimens from this field trial showed that the breadth and magnitude of the antibody response to both liver and asexual blood stage antigens was significantly lower in RTS,S vaccines than in controls [73,74] consistent with a partial effect associated with reduction of blood stage parasitemia in the vaccinated population [72].

5. T cell-based proteomic studies to antigen discovery

Methods for the identification of T cells antigens have been reviewed in detail recently [75]. In the case of malaria, two main approaches have been explored to identify *P. falciparum* antigenic targets of T cell responses induced by experimental immunization or natural exposure to malaria.

5.1. Epitope-based prediction algorithms

One approach to identifying targets of T cell responses has been the use of computerized algorithms that predict putative $CD8^+$ or $CD4^+$ T cell epitopes from proteins translated from genomic sequence [76,77]. A key component of this approach is the identification of HLA supertypes that account for the majority of HLA polymorphisms such that a limited number of HLA supertypes are representative of all racial and ethnic populations [78]. These supertypes share largely overlapping peptide binding specificities, allowing the definition of motifs that predict the binding affinity of a given peptide sequence to specific HLA class I and class II molecules [79,80]. Synthetic peptides representing the predicted $CD8^+$ and $CD4^+$ T cell epitopes can be then synthesized and screened for immune recognition using specimens from individuals experimentally immunized or naturally exposed to the pathogen of interest. The accuracy of these epitope predictions was validated in the malaria model for both $CD8^+$ and $CD4^+$ T cell responses, using specimens from RAS immunized and naturally exposed individuals [81,82].

The initial proof-of-concept study for this epitope-based approach to T cell antigen discovery, also conducted in the malaria model, identified a number of proteins recognized by T cells from

RAS immunized volunteers [83]. One of those proteins has been shown to be a target of cross-species protection in murine models ([84], in preparation). Subsequently, a more comprehensive study has been undertaken. A subset of ~1500 *P. falciparum* proteins representing the complete set of putative proteins thought to be expressed in the pre-erythrocytic stage of the *P. falciparum* parasite and representing approximately 30% of the *P. falciparum* genome were identified. For each putative protein, class I and class II epitopes restricted by multiple HLA supertype alleles were predicted by computerized algorithms. A set of 20 peptide sequences which bound with high affinity to three MHC class I supertypes and the degenerate HLA-DR class II supertype were synthesized and screened for immune reactivity with specimens from immune or semi-immune individuals ([83,85]; manuscript in preparation). Proteome-wide or sub-proteome screens of T cell targets of CPS or RAS are ongoing or pending. Results to date show that T cell responses to the *Plasmodium* parasite are broadly distributed in the proteome, rather than narrowly focused on a few immunodominant antigens and epitopes, providing experimental validation of the concept that malaria vaccines should be designed to target multiple antigens rather than one or a few antigens. Studies also identified a prioritized set of antigens which are highly T cell reactive and therefore would be predicted to be excellent target antigens for inclusion in a vaccine designed to prevent *Plasmodium* infection.

5.2. Antigen Lead Acquisition System (ATLAS)

An alternative T cell antigen discovery platform has been developed in recent years to identify potential antigenic targets using a pan-proteomic screening approach [86] (Fig. 1). With this technology, dubbed ATLAS (Antigen Lead Acquisition System), the full proteome of a pathogen is expressed as individual clones in bacterial hosts (*E. coli*) which are co-cultured with professional antigen presenting cells (APC) derived from the blood of selected human donors. As the APC ingest and process the *E. coli*-enclosed proteomic library, they process and present peptide epitopes in the context of MHC class I or class II molecules that can be recognized by autologous T cells derived from the same patient. For MHC class I presentation, the *E. coli* co-express a cytoplasmic variant of *Listeria monocytogenes* listeriolysin O which forms pores in the acidified endosome which provides access for the expressed protein to the cytosol for processing and presentation though the proteasome/TAP pathway [87,88]. If recognition events occur (hit), a phenotypic readout of T cell activation can be measured (e.g. production of IFN-γ). This approach offers a marked benefit over the use of overlapping peptide pools in that it decreases the number of determinants that need to be screened against (e.g. antigens) while at the same time ensuring that the epitopes are representative of the subject's antigen processing machinery and are always presented in the optimal MHC restriction context. When this platform is used to analyze blood samples from suitably stratified patient cohorts for their responses to a given pathogen, the pattern of responses can be used to infer the identity of pathogen proteins associated with productive, non-productive or even deleterious immune responses. To date, this platform has been applied successfully to identify T cell antigens from several microbial proteomes, with sizes ranging from a few dozen (HSV-2) to a few thousand expressed genes (*S. pneumoniae*, *C. trachomatis*) [86,89,90]. Phase 2 clinical trials are currently evaluating a candidate vaccine against *Pneumococcus* comprised of antigens identified using this technology.

Genocea Biosciences Inc. (Cambridge, MA) has built a full-length expressed protein library encompassing a significant fraction of the proteome of *P. falciparum* (currently about 1500 expressed proteins). In order to minimize the requirements for blood samples, the library was tailored to genes that have been associated with the pathogen's hepatic development stage, through analysis of proteomic and/or transcriptomic data for the murine strain *P. yoelii* and for which clearly identified orthologs existed in the published genome of *P. falciparum* [91,92]. This library has been further refined using a high quality proteome analysis of *P. falciparum* sporozoites as well as liver stage parasites differentiated in humanized mice (S. Kappe, personal communication). Preliminary screens with retained samples from RAS and CPS-immunized subjects demonstrate that this technology is capable of identifying effector memory (T_{EM}) responses against specific *P. falciparum* antigens (data unpublished). The nature of these types of screens, combined with the low frequencies of circulating *P. falciparum*-specific T_{EM} cells requires a fairly large number of samples to be analyzed before antigenic hits can be confirmed with statistical confidence. The completion of upcoming clinical trials involving RAS and CPS regimens and the availability of additional samples in late 2015 hopefully will confirm initial trends observed with the ATLAS technology. Finally, building a more complete library for this screening platform may ultimately be needed given the evidence that cross-stage antigens (e.g. blood stage) could be important for protection. This should prompt further technological developments to decrease the blood volume requirements while at the same time maintain the ability to detect low frequency antigen-specific T_{EM} cells. These further developments may prove timely and useful for a wide range of immune-profiling projects.

6. Transcriptomic approaches to antigen discovery

The premise for transcriptome-based discovery is that pathogen life cycle stages or phenotypes that are targets of vaccines should upregulate a specific subset of genes encoding protective antigens. As an example, increased transcription of genes encoding antigenic proteins has been shown for *Neisseria meningitidis* serogoup B and led to the identification of new vaccine candidates that protected mice against meningococcus and have been included in the human meningococcal vaccine [93,94].

In the case of malaria, parasite transcriptomes can be exploited in two ways for vaccine antigen discovery [95]. First, where a specific parasite stage or parasite phenotype is the target for a vaccine strategy, then differential transcriptional profiling can be used to define its discrete antigens. Second, where an immune response has been associated with protection, expression libraries that represent the translated products of the parasite transcriptome can be screened for recognition by protective antibodies or T cells.

6.1. Deciphering differential transcriptomes to identify vaccine targets

P. falciparum gene expression during different parasite life cycle stages was first profiled by microarrays [4,5]. In one study of cDNA from nine stages (sporozoite stage, seven asexual erythrocytic stages, two gametocyte stages), 4557 of 5159 predicted genes were detected [4]. Genes with similar or related functions clustered in expression profiles, and cross-hybridization studies indicated that most genes are well conserved. Subsequent studies showed that field isolates could be reliably studied by microarray [96] or by RNA sequencing studies [97]. While most genes are largely invariant across strains, some highly variable genes (e.g. *var*, *rif*, *stevor*, and *msp2* genes) have generally reduced signals by microarrays [98,99]. Because the host immune system drives sequence variation, these variable genes may include important vaccine candidates. In particular, *var* genes encode large variant antigens (200- to 400-kDa) implicated in adhesion to vascular receptors [100], and therefore believed to impart parasite virulence. There are 59 *var* genes in

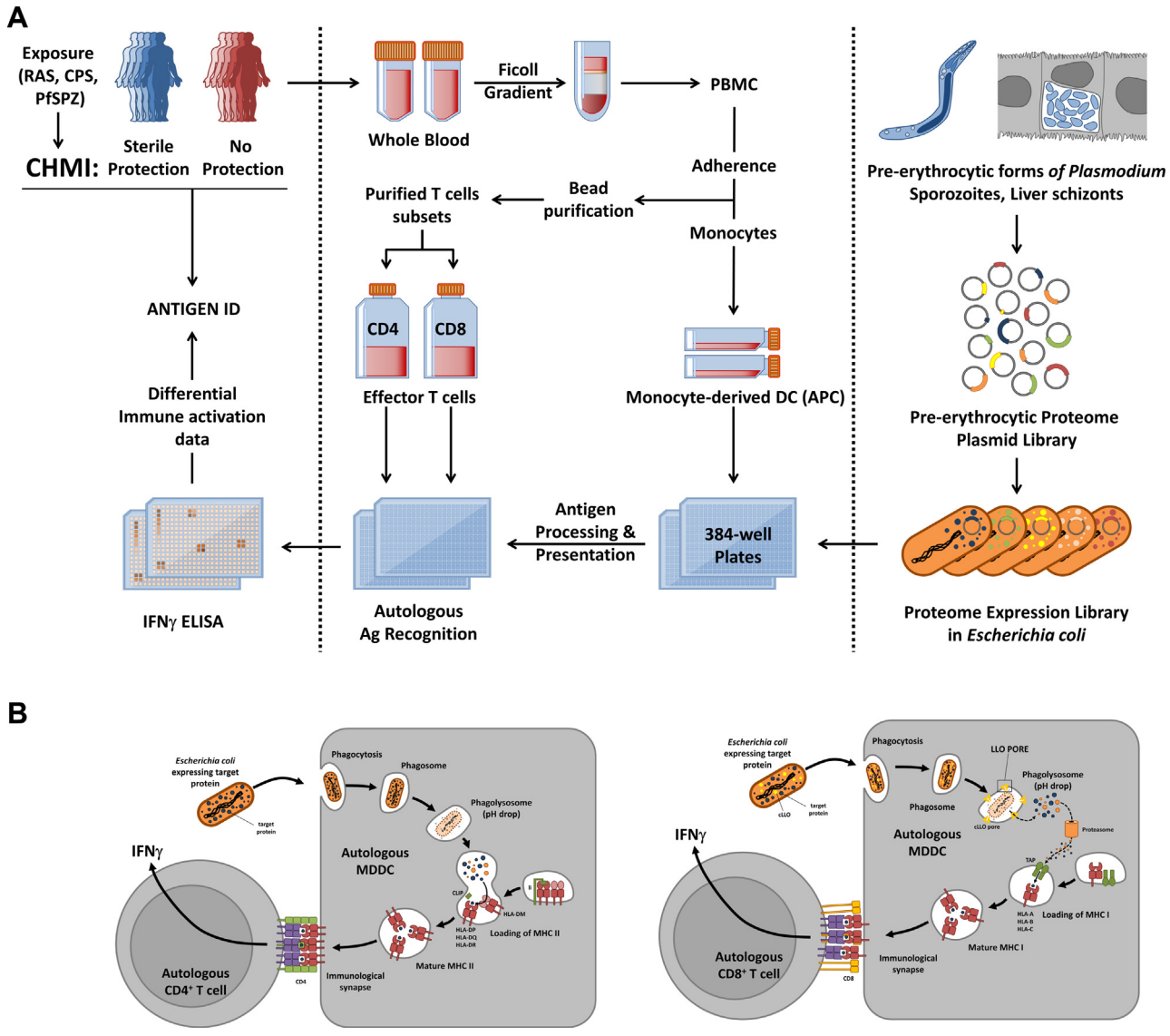

Fig. 1. T cell antigen discovery using ATLAS™ for *Plasmodium falciparum*. (A) ATLAS™ screening in the context of subjects immunized with live-attenuated malaria vaccination regimen. Blood from subjects immunized with RAS or CPS is collected prior to CHMI and Peripheral Blood Mononuclear Cells (PBMC) are isolated using conventional methods (Ficoll gradient). Adherent cells are recovered for differentiation into Monocyte – Derived Dendritic Cells (MDDC) which will serve as antigen presenting cells (APC) in the ATLAS assay, while CD4 and CD8 T cells subsets are amplified separately following bead purification. At completion of differentiation, MDDC are plated on libraries of *E. coli* bacteria expressing pre-erythrocytic *Plasmodium falciparum* antigen identified from sporozoites and/or liver stages. APC are allowed to process the bacteria and their *Plasmodium* cargo for autologous presentation in the context of MHC Class I or II to T cells derived from the same subject. After incubation, culture supernatants are recovered and analyzed for the presence of secreted cytokines (IFN-γ) which act as biochemical marker of antigen recognition and T cell activation. Patterns of T cell activation are compared across subjects and immunization cohorts allowing differential identification of T cell antigens associated with protection from CHMI. (B) Detail of antigen presentation to $CD4^+$ (left panel) and $CD8^+$ (right panel) T cells in the ATLAS™ assay. *Plasmodium* protein expressing *E. coli* clones are phagocytized by differentiated MDDC, which will lead to their degradation in late phagolysosomes and presentation of degradation peptides to autologous CD4 T cells on MHC Class II molecules (left panel). When bacteria contain a cytoplasmic variant of *Listeria monocytogenes* listeriolysin O (cLLO), acidification of the late phagolysosome leads to formation of pores in the vesicles' membrane, allowing cytoplasmic escape of partly degraded *E. coli* and *Plasmodium* cargo content, followed by processing by the proteasome and presentation to autologous $CD8^+$ T cells in the context of MHC Class II molecules (right panel).

the 3D7 *P. falciparum* genome and similar numbers in other strains [101].

Placental malaria vaccines provide a good example for blood stage antigen discovery by transcriptomic approaches. Infected erythrocytes (IE) bind CSA in the placenta, but do not bind CD36, ICAM-1, or other endothelial receptors that commonly support binding of other parasites [102]. Women become resistant to placental malaria as they acquire antibodies against placental IE [103]. This suggests that IE express a finite number of antigens or epitopes during pregnancy that are not displayed during childhood infections. qPCR of all known *var* genes in CSA-selected laboratory parasites revealed that *PFL0030c*, now known as *var2csa*, was transcribed at higher levels than in non-selected parasites [104]; *var2csa* is also upregulated in isolates from pregnant women [105,106].

Subsequently, microarray and RNA sequencing studies have extended our knowledge of the placental IE transcriptome. Microarray studies of parasites collected from pregnant Tanzanian women identified a suite of eight upregulated invariant genes, and confirmed upregulation of *var2csa* [96]. The genes included in this suite predict proteins with transmembrane and/or signal sequences as well as putative export (PEXEL or VTS) motifs [107,108], as might be expected for candidate vaccine antigens against placental IE. Among these, *PFD1140w* has a PEXEL sequence,

while *PFL0050c* and *PFI1785w* each have a VTS motif. *PFI1785w* belongs to a *P. falciparum* family composed of 16 uncharacterized hypothetical genes, most of which have predicted export sequences (www.PlasmoDB.org). Two genes are members of the HISTa gene family, consisting of 42 paralogs in *P. falciparum* that encode proteins with putative PEXEL sequences [109]. Finally, *PF10_0013* has an atypical PEXEL motif with isoleucine rather than leucine at residue 3, and has been localized to the Maurer's cleft by proteomic analysis [110].

Results obtained by microarray have been confirmed by next-generation sequencing of RNA (RNAseq). Because it yields digital signal, RNAseq displays a much broader dynamic range than microarrays, without the problems of saturation or cross-hybridization [111]. Further, RNAseq can sometimes provide information on transcription even where underlying genomic sequence is not fully defined. Therefore, RNAseq is better suited for studies of *P. falciparum* field isolates, including studies of their variable surface antigens where full sequences are missing. The robustness of the RNAseq technique has been demonstrated by its ability to identify the known placental IE transcriptome [97]. This includes the variant antigen *var2csa*. Four other invariant genes previously identified by microarray studies [96] were also amongst the most upregulated genes in RNAseq studies of IE from pregnant women (*PF*B0115w and 3 members of the variant gene family of exported pHIST proteins (*PF*D1140w, *PF*L0050c, and MAL13P1.470)).

6.2. Serologic screening of transcriptome expression libraries to identify vaccine targets

In contrast to placental malaria where the parasite phenotype is clear, the pathogenesis of severe malaria in children remains largely unknown, and differential transcriptome studies have been less successful for identifying vaccine candidates. Two DNA microarray studies failed to identify parasite genes whose expression was related to disease severity [112,113]. RNAseq of clinical *P. falciparum* isolates using not-so-random (NSR) primers identified a subset of 4 parasite transcripts that distinguished parasites infecting children from those infecting pregnant women, but these RNAseq studies did not assess whether these genes played a role in severe malaria syndromes [97].

In place of differential transcriptomic approaches, severe malaria vaccine antigen discovery has recently employed serologic screening to survey the transcriptome. Human residents of endemic areas develop protective immunity that limits parasitemia and disease. Using a cDNA library–based differential screening method [114] and plasma and epidemiologic data from a Tanzanian birth cohort [115], the protein products of the *P. falciparum* blood-stage transcriptome were surveyed. Plasma from "resistant" and "susceptible" 2-year-old children, defined by their control of blood stage parasite density, were used to identify targets of protective antibody responses [116]. This approach identified two previously unknown hypothetical genes as well as MSP-7, a known vaccine candidate [117].

Among the hypothetical genes, *Pf*SEA-1 was shown to localize to the schizont/parasitophorous vacuole membrane, Maurer's clefts, and the inner leaflet of the RBC membrane in schizont-infected RBCs. Antibodies to *Pf*SEA-1 significantly attenuated parasite growth by arresting schizont egress from infected RBCs, and immunization with the *Pb*SEA-1 ortholog conferred protection to mice against a lethal *P. berghei* ANKA challenge. Naturally acquired antibodies to *Pf*SEA-1 were associated with significant protection from severe malaria in young Tanzanian children, and with significant protection against parasitemia in adolescents and young adults in Kenya [116].

These studies strongly support *Pf*SEA-1 as a candidate for pediatric *falciparum* malaria, as well as the general approach of differential screening of cDNA libraries with carefully curated serum sets. The second hypothetical protein identified by serologic screening was GARP (glutamic acid rich protein). Interestingly, GARP was earlier identified by RNAseq as one of four parasite transcripts specifically associated with parasites infecting children [97]. Because GARP is only encoded in the *P. falciparum* and *P. reichenowi* genomes, future characterization of its potential as a vaccine target will await studies that use the human parasite, possibly in monkey models that support *P. falciparum* infection.

7. Antigen targets of protective immunity

It should be noted that only a fraction of the reactive antigens discovered using high throughput screening approaches are likely to be protective vaccine candidates. Identifying such candidates requires both the careful choice of input samples used for high throughput antibody or T cell based screening, as well as down-selection filtering of the output list of antigens discovered. A simple comparison of disease cases (exposed) with disease-free (unexposed) controls could reveal both protective antigens and other antigens that play no role in protection. In the context of vaccine design for malaria, for example, it is desirable to directly compare the response of individuals who are protected versus those who are unprotected, as in for example, profiling *P. falciparum* reactivity in sera from children in Mali [34] or controlled human malaria infection following immunization with radiation attenuated sporozoites [35]. Those studies identified 46 antigens that were preferentially recognized by asymptomatic children as compared with age-matched children who suffered from malaria, and 16 antigens that were associated with infection-blocking immunity against sporozoite challenge, respectively. After high throughput screening, protection can be assessed in animal models, such as the stringent *P. yoelii* rodent model of malaria. Usually, however, the number of candidates identified by in vitro high throughput screening is large and additional filtering is required prior to in vivo assessment, since immunizing animals with selected antigens, peptides or peptide pools and evaluating protection is only possible for limited numbers of targets. Computational filters can be used to rank-order candidate antigens from large lists of potential antigens of interest. In the case of antibody targets, predicted surface-location or presence of signal peptides, transmembrane domains or PEXEL motifs may qualify a novel candidate for further investigation. Unlike antibodies which need to access surface structures, protective T cell epitopes can be located anywhere in the proteome of the pathogen, and there are currently no validated in silico algorithms which can predict T cell reactivity from sequence data. However, factors such as the absence of human homology determined by blasting the pathogen sequence against the human proteome database can be very informative. Also, for both antibodies and T cells, conservation of the antigen target between *P. falciparum* strains and even among all *Plasmodium* species are would be desirable characteristics for a vaccine with widespread coverage.

8. Future directions for malaria antigen discovery

While more work remains to be done on both asexual blood stages and sporozoite stages for vaccine antigen discovery, other life cycle stages also provide opportunities. Liver stage parasites are known to be a target of protective immunity in animal and human studies, but the relative inaccessibility of these parasites in the large mass of the liver has allowed only incomplete success to define transcriptomes and proteomes [92]. Further, the

protective immune response to pre-erythrocytic parasites is complex, and may involve antibody, $CD4^+$ T cells, and $CD8^+$ T cells [20,21]. This complicates the effort to discern candidate antigens from the liver-stage of the parasite life cycle, especially those not also expressed in sporozoite or asexual blood stages, or to use antibody to screen for protective antigens. Overcoming this challenge will aid in the identification of specific target antigens recognized by RAS-induced protective immune responses, which are thought to be mediated predominantly by $CD8^+$ T cell responses directed at antigens expressed in the liver stage [19,21,22,65].

Another area of increasing interest over the past few years is the development of a malaria vaccine that can block transmission, in response to the challenge by Bill and Melinda Gates in 2007 for global malaria eradication [118] and the subsequent Malaria Eradication Agenda (http://malera.tropika.net). Transmission-blocking vaccines that target parasite antigens expressed during mosquito stages represent a more tractable target for antigen discovery efforts, owing to the greater accessibility of these parasite stages. Because antibody mediates the protective effect of these vaccines, serologic screening of sexual stage cDNA libraries offers a promising approach to new antigen discovery. Serum sets for such studies may come from animals vaccinated with whole parasite preparations from the relevant stages (gametocytes, gametes, zygotes), or may come from naturally exposed individuals who acquire antibodies with transmission-blocking activity.

9. Summary

Herein, we have described a series of distinct large-scale screening approaches to malaria vaccine antigen discovery which take advantage of the rich information in genomic, proteomic and transcriptomic datasets. Such approaches enable the discovery of new antigenic targets of antibody or T cell responses which can be prioritized for clinical evaluation. In our opinion, such genome-based approaches offer enormous potential for the development of a vaccine against a parasite which has co-evolved with the human host for thousands of years and which remains a major problem for global public health.

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Conflict of interest statement

JLB discloses a financial interest in Genocea Biosciences, Inc. DLD and PD have no financial interests to disclose. DHD and PLF disclose a financial interest in Antigen Discovery Inc., which has licensed a protein microarray technology. DHD, PLF and the University of California, Irvine may financially benefit from this interest if the company is successful in marketing its products that are related to this research. The terms of this arrangement have been reviewed and approved by the University of California, Irvine, in accordance with its conflict of interest policies.

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