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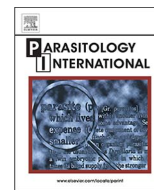

Identification of target proteins of clinical immunity to *Plasmodium falciparum* in a region of low malaria transmission

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ABSTRACT

The target molecules of antibodies against falciparum malaria remain largely unknown. Recently we have identified multiple proteins as targets of immunity against *Plasmodium falciparum* using African serum samples. To investigate whether potential targets of clinical immunity differ with transmission intensity, we assessed immune responses in residents of low malaria transmission region in Thailand. Malaria asymptomatic volunteers (Asy: n = 19) and symptomatic patients (Sym: n = 21) were enrolled into the study. Serum immunoreactivity to 186 wheat germ cell-free system (WGCFS)-synthesized recombinant *P. falciparum* asexual-blood stage proteins were determined by AlphaScreen, and subsequently compared between the study groups. Forty proteins were determined as immunoreactive with antibody responses to 35 proteins being higher in Asy group than in Sym group. Among the 35 proteins, antibodies to MSP3, MSPDBL1, RH2b, and MSP7 were significantly higher in Asy than Sym (unadjusted $p < 0.005$) suggesting these antigens may have a protective role in clinical malaria. MSP3 reactivity remained significantly different between Asy and Sym groups even after multiple comparison adjustments (adjusted $p = 0.033$). Interestingly, while our two preceding studies using African sera were conducted differently (e.g., cross-sectional vs. longitudinal design, observed clinical manifestation vs. functional activity), those studies similarly identified MSP3 and MSPDBL1 as potential targets of protective immunity. This study further provides a strong rationale for the application of WGCFS-based immunoprofiling to malaria vaccine candidate and biomarker discovery even in low or reduced malaria transmission settings.

1. Introduction

Malaria remains a global health problem in the tropical and subtropical region of the world. An estimated 212 million cases of malaria occur annually with 429,000 deaths reported in 2015 [1]. Malaria control efforts, such as long-lasting insecticidal nets, indoor residual spraying and expanding access to the artemisinin-based combination therapies, are undoubtedly effective [1]. However, the recent spread of parasite resistance to artemisinins and mosquito resistance to insecticides may instigate a reversion of the positive trend [1,2]. One

Abbreviations: WGCFS, Wheat germ cell-free system; ASC, AlphaScreen Counts; Asy, asymptomatic malaria Thai volunteers; Sym, symptomatic malaria Thai volunteers; Nor, normal (malaria naïve) Thai volunteers; Asy + Sym, either asymptomatic or symptomatic malaria volunteers; Adjusted p-value, adjusted p-value post multiple comparisons by a Holm method; Non-M pro, malaria unrelated proteins, i.e., flowering locus T (FT), dihydrofolate reductase (DHFR), and wheat germ cell-free translation mixture without mRNA (WGE) serving as negative controls used in this study

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promising complementary measure is vaccination. However, a sole phase 3 clinical trial of a leading malaria vaccine, RTS,S/AS01, showed moderate efficacy [3]. Moreover, malaria vaccine development efforts to date have yielded only a handful of vaccine candidates, with few that progressed to clinical trials having no or marginal efficacies [4][as reviewed in [5]]. The major challenge for the identification of novel vaccine candidates remains to be poor understanding of the *Plasmodium falciparum* antigens that are targets of naturally acquired protective immunity.

Although studies in malaria endemic regions clearly show that protective immunity, at least for clinical malaria, can be developed in individuals exposed to repeated *P. falciparum* infections [6], the mechanism of this protective immunity remains largely unknown. However, antibodies are key in protection against high parasitemia and clinical malaria, as demonstrated by passive-transfer studies in humans [7,8]. Therefore, there is an urgent need to objectively profile antibody responses against *P. falciparum* antigens for deeper understanding of the mechanism of acquired immunity as well as identification of novel vaccine candidates.

To identify the key targets of protective immunity, population-based immuno-epidemiological studies have of recent been employed to establish temporal associations between antibody profiles and subsequent clinical outcome [9–14]. In these studies, recombinant plasmodia proteins expressed using in vitro bacterial translation systems and printed on microarray chip were probed with sera obtained from malaria-exposed individuals [10,11]. Using these approaches, several antigens were identified as potential vaccine candidates. However, the major limitation of this strategy is the possibility of proteins expressed by the bacterial expression systems not attaining natural conformations [15,16]. On the other hand, only few proteins expressed by eukaryotic expression system have been profiled against natural immunity [9,12]. Importantly, we have recently demonstrated that wheat germ cell-free system (WGCFS), a robust eukaryotic alternative to express plasmodial proteins, overcomes these limitations [17,18]. It has been shown that animal antibodies raised against multiple WGCFS-synthesized recombinant proteins recognize their respective native proteins and have parasite growth-inhibition activity in vitro [19]. The recombinant proteins also strongly react with sera obtained from individuals exposed to *P. falciparum* [reviewed in [19]]. These data clearly shows that the proteins are to a great extent correctly folded and can be considered as representative of native proteins [19]. We thus, in our previous study, created a WGCFS-synthesized protein library consisting of 1827 recombinant proteins, representing ~35% of the *P. falciparum* proteome. A 12-month prospective study conducted in a malaria high-endemic area in Uganda, where entomological inoculation rate (EIR) of > 100 infective bites/year, identified antibody responses against 53 proteins, which are natively expressed mainly in asexual-blood and sporozoite stages, significantly associated with protection from clinical malaria [20]. With decreasing malaria transmission and as we gear towards malaria elimination, it is of great importance to understand whether targets of protective immunity against falciparum malaria are similar with those in low transmission areas such as in South East Asia (http://www.aplma.org).

In the current study, we attempted to identify *P. falciparum* asexual-blood stage proteins that are potential targets of antibodies associated with clinical protection in western Thailand; a region of low malaria endemicity where EIR of 1–3 infective bites/year [21]. Antibody levels to 186 WGCFS-synthesized recombinant proteins were determined by a high-throughput AlphaScreen method [22]. Antibody responses to 35 proteins in asymptomatic (Asy) group were significantly higher than those in symptomatic (Sym) group. This study further provides a strong rationale for the application of WGCFS-based immunoprofiling approach to malaria vaccine candidate discovery even in regions of low malaria endemicity.

2. Materials and methods

2.1. Parasite culture, total RNA isolation, and cDNA preparation

P. falciparum asexual stage parasite (3D7) was maintained in vitro according to Trager-Jensen method [23] using healthy human erythrocytes (blood group O +) obtained from the Japanese Red Cross Society, supplemented with 5% heat-inactivated human plasma and 0.5% AlbuMAX™ I (Thermo Scientific, Waltham, MA) in an atmosphere of 5% O_2, 5% CO_2, and 90% N_2. Total RNA was extracted from frozen infected RBC (iRBC) pellet rich in late-trophozoite and schizont by TRIzol™ reagent (Invitrogen, Carlsbad, CA) and then treated with DNase I (Invitrogen) at 37 °C for 15 min. cDNA was generated with random hexamers by using Superscript III™ (Invitrogen) at 25 °C for 10 min followed by at 50 °C for 50 min and at 85 °C for 5 min.

2.2. In silico gene selection and cDNA cloning

We selected a set of *P. falciparum* genes from the malaria genome database (PlasmoDB; http://plasmodb.org/plasmo/) which are expressed either (1) at least twice as much in schizont than in trophozoite at the level of transcription [24–26], or (2) exclusively at the merozoite stage [27]. The resulting list of ~200 genes was narrowed down to 192 based on ascending molecular size (< 5.9 kbp, Table S1). To create a cDNA library, 5′ primers were designed as 46-mers: 16-mer S1-tag sequence followed by a 30-mer of unique sequence covering each 5′ open reading frame containing the start codon [28]. For the 3′ primers, 30-mer nucleotide sequences covering each unique sequence upstream of the termination codon were prepared. The cDNA PCR amplification was performed with Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific). The PCR products were then cloned into the pCR™2.1 plasmid using a TOPO TA cloning kit (Invitrogen), and their nucleotide sequences were confirmed by sequencing both 5′ and 3′ ends. Finally, we obtained 186 cDNA clones with cloning of the remaining 6 cDNA being unsuccessful.

2.3. Construction of P. falciparum biotinylated protein library using WGCFS

Transcription templates were prepared by amplification of the *P. falciparum* cDNA clones by a two-step split-primer method as previously described [22,28]. Briefly, the first PCR was conducted with 10 nM of the following primers: sense primer corresponding to the S1-tag followed by a start codon: S1-ATG; 5′-CCACCCACCACCACCAATG-3′, and antisense primers corresponding to the vector (pCR™2.1, Invitrogen) sequence: 1931A; 5′-CGGCCACAGTCGATGAATCC-3′ or 2211S; 5′-GCTGGTGAAAGTAAAAGATG-3′ depending on the orientation of each cDNA insert in the plasmid. The second PCR for transcription templates were constituted with 100 nM SPu: 5′-GCGTAGCATTTAGG TGACACT-3′, 1 nM deSP6E02bls-S1: 5′-GGTGACACTATAGAACTCACC TATCTCTCTACACAAAACATTTCCCTACATACAACTTTCAACTTCCTAT TATGGGCCTGAACGACATCTTCGAGGCCCAGAAGATCGAGTGGCACGA ACTCCACCCACCACCACCAATG-3′ and 100 nM 1923A: 5′-GTCGATGA ATCCAGAAAAGC-3′, or 2214S: 5′-GGTGAAAGAAAAAGATGCTG-3′, depending on the orientation of each cDNA insert in the plasmid. With the "split-primer PCR" strategy, mono-biotin ligation site (bls) was inserted upstream of each cDNA for subsequent protein biotinylation. The WGCFS protocol was modified with addition of WGCFS-expressed crude BirA and 500 nM D-biotin (Nacalai Tesque, Kyoto, Japan) to the translation mixture for simultaneous biotinylation as described [22]. Consecutive in vitro transcription and translation by WGCFS was carried out using the GenDecoder1000 robotic protein synthesizer (Cell-Free Sciences, Matsuyama, Japan) as described [29]. Expression of each recombinant protein in the protein library was confirmed by separation on 12.5% SDS-PAGE, stained with fluorescein avidin D (Vector Laboratories, Peterborough, U.K.) and scanned with Typhoon9400 imager at 488 nm laser excitation (GE Healthcare, Piscataway, NJ) [22]. The

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