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Finding the sweet spots of inhibition: understanding the targets of a functional antibody against *Plasmodium vivax* Duffy binding protein

Francis B. Ntumngia^a, Christopher L. King^{b,c}, and John H. Adams^{a,*}

^aDepartment of Global Health, University of South Florida, Tampa, FL, USA

^bVeterans Affairs Research Service, Louis B Stokes VA Medical Center, Cleveland, OH, USA

^cCase Western Reserve University, Center for Global Health and Diseases, Cleveland, OH, USA

Abstract

Plasmodium vivax Duffy binding protein region II (DBPII) is an essential ligand for reticulocyte invasion, thereby making this molecule an attractive vaccine candidate against asexual blood-stage *P. vivax*. Similar to other *Plasmodium* blood-stage vaccine candidates, strain-specific immunity due to DBPII allelic variation may complicate vaccine efficacy. Targeting immune responses to more conserved epitopes that are potential targets of strain-transcending neutralizing immunity is necessary to avoid induction of strain-specific responses to dominant variant epitopes. In this article, we focus on different approaches to optimize the design of DBP immunogenicity to target conserved epitopes, which is important for developing a broadly effective vaccine against *P. vivax*.

Keywords

Plasmodium vivax; Malaria; Vaccine; Polymorphism

1. Introduction

1.1. Significance of *Plasmodium vivax* malaria

Plasmodium vivax is the most widely distributed *Plasmodium* sp. causing human disease globally, with over 40% of the world's population at risk of vivax malaria. *Plasmodium vivax* infections account for over 130 million cases of clinical malaria each year (Hay et al., 2004; Baird, 2007), with more than 70% of infections occurring in Asia and the Americas and approximately 12.4% in Africa (Mendis et al., 2001). Despite this widespread prevalence, *P. vivax* malaria has long been overshadowed by the burden caused by *P.*

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*Corresponding author. John H. Adams, 3720 Spectrum Boulevard, Suite 304, Tampa, FL 33612 USA. Tel.: +1-813-974-9916; fax: +1-813-974-0992. jadams3@health.usf.edu.

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falciparum malaria, which is responsible for most malaria-attributed deaths. *Plasmodium vivax* was thought to cause a benign and often self-limiting infection, however with better diagnostic tools, it is now recognized that *P. vivax* often causes more severe disease than previously appreciated (Price et al., 2007). Vivax malaria is known to incapacitate individuals of all ages, resulting in repeated febrile episodes, severe anemia, respiratory distress and occasional compromised birth outcomes in pregnancy (Lacerda et al., 2012). In a non-immune pregnant woman, an acute *P. vivax* infection can result in maternal morbidity and stillbirth. Increasing reports of clinical severity with emerging virulent forms of the parasite (Kochar et al., 2009; Sharma and Khanduri, 2009; Alexandre et al., 2010), widespread drug resistance (Ketema et al., 2009; Mohan and Maithani, 2010; Rijken et al., 2011) and recurrent clinical episodes due to reactivation of the dormant forms (hypnozoites) in the liver (Krotoski et al., 1982), challenge the description of vivax malaria as benign, emphasizing the urgent need to create alternative prophylactic and therapeutic strategies, including the development of a vaccine against this disease.

1.2. Functional assays for *P. vivax*

In contrast to *falciparum* malaria, there has been no coherent vaccine program for *P. vivax*. Comparatively few blood-stage antigens have been expressed for *P. vivax* and the inability to culture this parasite in vitro has hampered the development of functional assays to study the biology of the parasite. *Plasmodium vivax* only infects immature erythrocytes, or reticulocytes, that normally account for 1–2% of erythrocytes in the peripheral blood circulation that rapidly mature (~24 h) into erythrocytes unable to support *P. vivax* invasion and growth. This complicates the use of ‘normal’ human blood for long-term culture of *P. vivax*. Hence parasites are usually obtained directly from *P. vivax*-infected patients or non-human primates to support short-term cultures. As a consequence, such studies are costly and often restricted to programs with access to infected subjects or animals and good culture facilities. Progress towards development of a robust, long-term *P. vivax* culture has been slow. Application of the short-term ex vivo invasion assays initially developed (Grimberg et al., 2007; Udomsangpetch et al., 2008) and recently refined (Russell et al., 2011) have shown that naturally acquired and artificially induced antibodies to *P. vivax* Duffy binding protein region II (PvDBPII) can partially inhibit *P. vivax* invasion of human erythrocytes. Given the limitations of directly measuring inhibition of *P. vivax* merozoite invasion, several biological assays have been developed that serve as useful surrogates to evaluate the potential inhibitory effects of antibodies against *P. vivax* invasion ligands. Two of these assays measure binding of soluble recombinant DBPII to the Duffy antigen receptor for chemokine (DARC) on the erythrocyte surface by flow cytometry (Tran et al., 2005; Grimberg et al., 2007), or directly to a chimeric recombinant protein that includes the N-terminal binding domain of DARC (Choe et al., 2005; Hans et al., 2005), while a third assay, the COS 7 cell assay, involves DARC-positive erythrocytes binding to transiently expressed recombinant DBPII on the surface COS 7 cells (Chitnis and Miller, 1994; Michon et al., 2000). The latter assay has the advantage of not requiring the difficulty of purifying and refolding recombinant DBPII. Overall, these assays have helped in defining the structural determinants for receptor recognition (VanBuskirk et al., 2004b), identified dominant epitope targets for immune antibody neutralization (Chootong et al., 2010; Ntumngia and Adams, 2012) and confirmed that natural immunity to DBP is biased towards

strain-specific immune responses (King et al., 2008; Cole-Tobian et al., 2009; Ntumngia et al., 2012). In addition to these in vitro assays and short-term invasion assays, several primates offer the potential for candidate vaccine validation (Collins et al., 1999; Herrera et al., 2002). Although these in vitro biological assays have helped advance the search for new targets and preclinical vaccine studies in *P. vivax*, immunization challenge studies in primates remain a more direct measure of vaccine efficacy in vivo.

Efforts are recently underway to establish a continuous in vitro culture system for *P. vivax* with more facile methods to reliably (and more cheaply) obtain reticulocytes derived from hematopoietic stem cells (HSCs). These projects will optimize the capacity to utilize short-term ex vivo invasion assays while developing a long-term culture system for *P. vivax* blood stages. This effort will be complemented by the recent application of flow cytometry for detecting different stages of malaria-infected erythrocytes, which has increased the ability to accurately measure the low frequencies of *P. vivax* merozoite invasion events into reticulocytes typical of the short-term in vitro cultures (Grimberg et al., 2007). It is expected that successful implementation of these culture systems will enhance vivax malaria research by providing the much-needed tools required to study the biology of the parasite.

1.3. Naturally acquired immunity to *P. vivax* infection

Naturally acquired immunity (NAI) is the capacity of the host to respond more effectively during the second and subsequent exposures to a pathogen compared with the primary exposure. The quality and longevity of this adaptive response is highly variable, with some individuals acquiring long-term protection following a limited number of exposures, whereas in other cases repeated or ongoing exposure is needed to generate and sustain protective immunity. Both situations occur in malarial infection depending on the host, parasite, age, type of prior exposure and transmission intensity (related to parasite diversity). An emerging consensus indicates that the essential features of NAI are multifactorial, being primarily directed to blood-stage infection. In *P. vivax* malaria, the development of NAI is achieved by, or dependent upon, exposure to both primary blood-stage infections as well as relapse infections in the blood. NAI appears to involve two basic phenotypes. The first demonstrates resistance to severe malaria-related illness (severe anemia; hemoglobin < 5 g/dL, organ failure, acidosis), which appears to be acquired quickly and persists for a long period of time. The second is manifested as protection against high-density parasitemia and uncomplicated clinical disease (fever, malaise and anemia).

NAI in *P. vivax* infection increases with age in endemic populations as a result of a boosting effect due to repeated exposure to infection (Cattani et al., 1986; Xainli et al., 2002). NAI does not prevent infection, but decreases parasite densities and reduces the frequency of clinical symptoms and disease severity. It is unlikely that naturally acquired sterile immunity is ever achieved. In highly endemic areas such as in Papua New Guinea, immunity to vivax malaria becomes prevalent early in childhood and corresponds to suppression of blood-stage parasite growth and few or sometimes complete absence of clinical disease in older children and adults (Lin et al., 2010). Interestingly, under similar transmission conditions, blood-stage immunity to *P. vivax* occurs more quickly than to *P. falciparum* (Michon et al., 2007), suggesting more restricted pathways for parasite invasion. Alternatively, frequent relapses of

P. vivax may boost blood-stage immunity. In most *P. vivax* endemic areas, parasite transmission is low or periodic and clinical disease is frequently observed in adults because robust clinical immunity fails to develop during childhood. However, in some low *P. vivax* transmission areas such as in the Amazon Basin or south Pacific, individuals with asymptomatic parasitemia are often encountered (Alves et al., 2002; Harris et al., 2010), suggesting that NAI can occur with relatively few infections or host genetic factors (e.g. Fy^a allele) may play a protective role against clinical vivax malaria (King et al., 2011). Early studies have shown that as few as three or four infections with the same or similar strains of *P. vivax* in subjects with neurosyphilis induced robust clinical immunity (Boyd, 1947). This suggests that in areas with little parasite diversity, clinical immunity may develop fairly rapidly, supporting the possibility of a successful *P. vivax* vaccine.

1.4. Plasmodium vivax asexual blood-stage vaccine candidates

Asexual blood-stage vaccines are aimed at inducing neutralizing antibodies that are capable of inhibiting parasite invasion and development within the host erythrocytes, preventing clinical manifestation of disease symptoms and reducing transmission to mosquitoes. The humoral immune response to blood-stage antigens is believed to be an important component of naturally acquired immunity to malaria (Marsh and Kinyanjui, 2006; Yazdani et al., 2006). During asexual blood-stage development, a series of parasite proteins are secreted that play critical roles during parasite invasion of host erythrocytes (Chitnis, 2001; Gaur et al., 2004; Cowman and Crabb, 2006). These merozoite invasion-related molecules represent ideal targets for vaccine-mediated immunity against blood-stage infections of the parasite. A series of *P. vivax* merozoite antigens have been identified and immunologically characterized in preclinical studies, and offer great potential as candidates for vivax malaria vaccines, including the apical membrane Antigen-1 (AMA-1) (Kocken et al., 1999; Rogers et al., 1999), DBP (Michon et al., 2000; Singh et al., 2001; Xainli et al., 2003; Ntumngia et al., 2012) and reticulocyte binding protein (RBP), which play a crucial role in the targeting of reticulocytes for invasion (Cattani et al., 1986; Galinski et al., 1992; Galinski et al., 2000), and the merozoite surface proteins (MSPs), which include MSP-1 (Soares et al., 1997; Yang et al., 1999; Dutta et al., 2001; Storti-Melo et al., 2011), the MSP-3 family (Galinski et al., 1999; Galinski et al., 2001; Audran et al., 2005; Sirima et al., 2007), and MSP-9 (Barnwell et al., 1999; Vargas-Serrato et al., 2002; Lima-Junior et al., 2008). The potential vaccine properties of these antigens is evident by their immunogenicity in animal models, recognition by antibodies from individuals exposed to *P. vivax* and most importantly, the ability to elicit parasite invasion-inhibitory antibodies. Of these antigens, MSP-1 and the DBP have received the most attention but none have yet advanced to phase I clinical trials. The current status of these antigens as vaccines has been reviewed (Arevalo-Herrera et al., 2010; Valencia et al., 2011).

2. PvDBP: status as a vaccine

The PvDBP is the best-characterized and leading vaccine candidate for blood-stage *P. vivax* malaria.

2.1. PvDBP is essential for host erythrocyte invasion

Malaria parasites depend on specific receptor-ligand interactions for successful invasion of the host erythrocytes (Gaur et al., 2004; Cowman and Crabb, 2006). The PvDBP is an apical organelle protein (microneme) associated with the decisive and irreversible step of junction formation between the merozoite and the host surface receptors during invasion, giving PvDBP priority as a prime target for vaccine-induced, antibody-mediated immunity against asexual stages of the parasite (Miller et al., 1975; Adams et al., 1990; Chitnis, 2001; Gaur et al., 2004; Choe et al., 2005; Singh et al., 2005). In *P. vivax* the interaction between the DBP and its cognate receptor, DARC, also known as the Duffy blood group antigen (Fy) on human erythrocytes, is central to blood-stage infection (Miller et al., 1976; Barnwell et al., 1989; Wertheimer and Barnwell, 1989). Fy is a minor blood group antigen that has two immunologically distinct alleles, referred to as Fy^a or Fy^b resulting from a single point mutation. Historically, the vital need of the DBP-Fy interaction for erythrocyte invasion is evident from the virtual absence of *P. vivax* malaria in regions with a high prevalence of Fy negativity (Miller et al., 1976; Guerra et al., 2010). A recent study has provided additional compelling evidence of the importance of the DBP-Fy interaction by demonstrating the protective effect against clinical vivax malaria by the Fy^a allele (King et al., 2011). Individuals with the Fy^{a+b-} phenotype demonstrated a 30 to 80% reduced risk of clinical vivax, but not falciparum, malaria in a prospective cohort study in the Brazilian Amazon. The Fy^a allele has reached fixation in southeast Asian populations, areas thought to be the wellspring of *P. vivax* malaria (Howes et al., 2011). Conversely, Fy^b is present in north and northern central European populations and admixed in many populations with strong northern European influence. This distribution of Fy alleles suggests that it may play a protective role against *P. vivax* malaria (King et al., 2011). Importantly, inhibitory antibodies to DBP-II were much more effective in blocking binding of DBP-II to erythrocytes expressing Fy^a compared with Fy^b, suggesting that the relative frequencies of Fy^a and Fy^b alleles in populations may effect DBP-II vaccine efficacy.

Recent studies have reported evidence of *P. vivax* transmission among Duffy blood-group negative individuals in western Kenya (Ryan et al., 2006), the Brazilian Amazon region (Cavasini et al., 2007), Madagascar (Menard et al., 2010), Mauritania (Wurtz et al., 2011), Angola and Equatorial Guinea on the west coast of Africa (Mendes et al., 2011) and Ethiopia (CL King, personal observations), although the frequency appears low. In Madagascar, with admixture of Fy- and Fy+ populations of diverse ethnic backgrounds, a significant reduction in the prevalence of clinical *P. vivax* malaria among Fy- compared with Fy+ individuals was observed, suggesting a partial impairment of *P. vivax* invasion of Fy- relative to Fy+ erythrocytes (Menard et al., 2010), while in the case of the Brazilian Amazon region, just two cases of *P. vivax* malaria in Fy- individuals was observed in samples collected from the Rondonia State (Cavasini et al., 2007). It is still not clear if these cases are just isolated events or a phenomenon in which *P. vivax* could be evolving and adapting to use receptors other than Duffy for erythrocyte invasion or that a completely independent DBP-Fy pathway may exist for invasion. Moreover, it is possible that PvDBP-II remains the critical invasion ligand, yet uses different receptors on the erythrocytes for invasion. Although the DBP-Fy pathway remains the only known and characterized invasion pathway for *P. vivax* invasion of host erythrocytes, further analyses are needed to understand

the dynamics of the Duffy gene and its possible contribution as a modulator in host susceptibility to malaria.

DBP belongs to the Duffy binding-like erythrocyte-binding protein (DBL-EBP) family, encoded by erythrocyte binding-like (*eb1*) genes. EBPs are sequestered in the micronemes and released to the merozoite surface to bind receptors on the erythrocyte during invasion (Adams et al., 1990, 1992). Other members of this family include the DBPs of *Plasmodium knowlesi* and the *P. falciparum* ligands EBA-175, JESEBL/EBA-181 and BAEBL/EBA-140 (Sim et al., 1990; Adams et al., 2001; Mayer et al., 2001; Narum et al., 2002; Gilberger et al., 2003). DBL-EBPs are characterized by two conserved cysteine-rich domains and share similar molecular structures and functional characteristics (Adams et al., 1990, 1992, 2001; Sim et al., 1990; Chitnis and Miller, 1994). Some species, such as *P. falciparum* and *P. knowlesi*, have multiple *eb1* genes, which enable them to use alternative receptor pathways for the essential junction formation step of invasion. *Plasmodium vivax*, on the other hand, has a single *eb1* gene and depends on recognition of a single receptor (Fy) for invasion (Dvorak et al., 1975; Miller et al., 1975; Aikawa et al., 1978; Haynes et al., 1988). This dependence on a single receptor for junction formation represents a weakness for *P. vivax* and further strengthens the choice of PvDBP as an ideal vaccine target.

2.2. DBP ligand domain is polymorphic

The DBP ligand domain, also known as DBPII, is a 330 amino acid region within the extracellular portion of the DBP, which mediates erythrocyte-binding activity with the Duffy blood group antigen (Fy) (Chitnis and Miller, 1994). This region is characterized by 12 conserved cysteine residues, with the principal determinants for receptor recognition mapped between cysteines 4–7 (Chitnis et al., 1996; Ranjan and Chitnis, 1999; Singh et al., 2003; VanBuskirk et al., 2004b) (Fig. 1). Despite the conserved nature of this region, many residues are variable with a substitution rate four times higher than that of the rest of the DBP and a high ratio of non-synonymous to synonymous mutations clustered within the critical binding segment (Tsuboi et al., 1994; Ampudia et al., 1996; Xainli et al., 2000; Cole-Tobian and King, 2003). These naturally occurring polymorphisms for the most part do not appear to be functionally important for receptor recognition but flank critical residues, which are important for binding (Xainli et al., 2000; VanBuskirk et al., 2004b; Batchelor et al., 2011) (Fig. 1). This pattern is consistent with a high immune selection pressure on DBPII (Tsuboi et al., 1994) and suggests that allelic variation is an important mechanism of immune evasion. Current reports demonstrate that naturally occurring polymorphisms in DBPII confer significant differences in sensitivity to inhibition by immune antibodies (VanBuskirk et al., 2004a; McHenry et al., 2011; Ntumngia et al., 2012) and evidence of DBPII variant-specific antibody responses that correlate with homologous and not heterologous protection (Cole-Tobian et al., 2009). Similar effects of polymorphisms on vaccine efficacy were recently reported with the *P. falciparum* blood-stage vaccine, PfAMA-1, in a phase IIb trial in Malian children (Thera et al., 2011) and laboratory-based studies by immunizations with refolded recombinant proteins (Crewther et al., 1996; Hodder et al., 2001; Healer et al., 2004). These observations indicate that a single amino acid substitution can alter the antigenic character of a malarial antigen and provide compelling evidence that immune selection is a driving force for allelic variation in malaria parasites.

This phenomenon, where polymorphisms cluster around residues critical for binding and variation is driven by natural positive immune selection, is evident in ligands of other microbial pathogens such as the influenza hemagglutinin (HA) (Wilson and Cox, 1990; Fitch et al., 1991), the HIV ligand (gp120) (Barouch, 2007; Walker and Burton, 2008) and the *P. falciparum* vaccine candidate, PfAMA1 (Verra and Hughes, 1999; Polley and Conway, 2001; Healer et al., 2004).

2.3. Immunity to PvDBP

Naturally acquired inhibitory antibodies to DBP-Fy binding and merozoite invasion of human erythrocytes are prevalent in residents of *P. vivax* malaria endemic regions (Michon et al., 2000; Chootong et al., 2010). Individuals tend to show significant quantitative and qualitative differences in their anti-DBP serological responses (Cole-Tobian et al., 2002; Xainli et al., 2002, 2003; Chootong et al., 2010). Generally, the serological response to DBP and inhibition of DBP-erythrocyte binding activity increases with age, suggesting a boosting effect due to repeated exposure through recurrent infection (Michon et al., 2000; Cole-Tobian et al., 2002; Xainli et al., 2002; Grimberg et al., 2007; King et al., 2008). Similar to most blood-stage malaria vaccine candidates, DBP appears to be a relatively poor immunogen. This is evident in the fact that naturally acquired anti-DBP antibodies fail to develop in some individuals with repeated exposure to *P. vivax* and high titer strain-transcending neutralizing antibodies are uncommon (Chootong et al., 2010). Anti-DBP antibodies tend to be weak (Chootong et al., 2010) or short-lived and allele-specific (Xainli et al., 2003; King et al., 2008; Ceravolo et al., 2009). Usually, the initial antibody response is not broadly protective, while strain-transcending immunity develops only after repeated exposure (Cole-Tobian et al., 2002; Xainli et al., 2003). Some individuals, however, acquire high titer, strain-transcending inhibitory antibodies and retain these functional antibodies for years (King et al., 2008; Cole-Tobian et al., 2009), suggesting that a PvDBP vaccine can develop persisting immunological memory that is necessary for a successful vaccine. Similar to the naturally acquired human antibodies, vaccine-induced anti-DBP antibodies also demonstrate a strain-specific inhibition of DBP-erythrocyte binding and invasion of human erythrocytes (VanBuskirk et al., 2004a; Grimberg et al., 2007; King et al., 2008; Ntumngia et al., 2012). Together, these data are consistent with the hypothetical framework that variation is an immune evasion mechanism responsible for strain-specific immunity and stable, broadly inhibitory immunity is achieved when antibodies target functionally conserved epitopes.

3. Rational vaccine design for *P. vivax* based on the DBP

Despite the biological role of DBP in *P. vivax* blood-stage development, which justifies its strong consideration as a vaccine candidate, the polymorphic nature of its ligand domain (DBP-II) and associated strain-specific immunity represent serious challenges that may compromise efficacy of a DBP vaccine. As noted above, DBP-II demonstrates the hallmark of immune evasion properties of other microbial ligands under strong immune selection where the most polymorphic region of DBP-II lies within the central-domain (sub-domain 2), which also contains the residues most critical for receptor binding (VanBuskirk et al., 2004b; Batchelor et al., 2011). Similar to blood-stage vaccine candidates of *P. falciparum*,

some concern is justified about whether vaccine-induced immunity to DBP will be strain-specific, with a concomitant limited effectiveness against allelic variants. Indeed the failure of two leading *P. falciparum* blood-stage vaccine candidates to protect against clinical malaria can be attributed to strain-specific responses elicited by vaccination with a monovalent vaccine (Genton et al., 2002; Thera et al., 2011). Based on this concern, to overcome the inherent bias towards inducing a strain-specific immune response, there is need for a rational vaccine design and immunization strategy to focus immune responses to conserved determinants that are targets of strain-transcending anti-DBP inhibitory antibodies. Several basic approaches have been used to tackle similar problems with ligands of other pathogens, which can be applied to advance a DBPII based vaccine that targets diverse *P. vivax* strains.

3.1. Combination allele vaccines

Previous studies have demonstrated that immunization with a single antigen malaria vaccine confers protection for the homologous parasite strain and little or no protection against heterologous strains (Renia et al., 1997; Kennedy et al., 2002; Kocken et al., 2002; VanBuskirk et al., 2004a). However, immunization with a multiple component vaccine representing major potential haplotypes has proven to be a promising approach to overcoming the problem of strain-specific immunity, a strategy that has been demonstrated for the pneumococcal vaccine (Wilson-Adkins and Adkins, 2001) and clinical testing of the blood-stage *P. falciparum* vaccine candidate, PfAMA-1 (Wertheimer and Barnwell, 1989; Duan et al., 2008; Remarque et al., 2008; Kusi et al., 2009). Theoretically, the bulk of the antibody response against such a vaccine would be directed against epitopes common to the constituent alleles, resulting in a broader specificity. A preliminary study in mice (unpublished data) established that a multi-allele vaccine made up of antigenically-distinct DBPII alleles elicited a higher antibody response to the individual antigens used in the combination compared with the single alleles, suggesting that multiple PvDBPII variant alleles may be required in a vaccine for broader coverage. However, development of such a vaccine has major challenges, including the choice and number of alleles required to achieve the broadest specificity. A related study in PfAMA-1 resolved that a plateau effect could impose a limit to the number of alleles that can be included in such a combination to achieve the broadest specificity (Kusi et al., 2010). Other related issues may include production costs; problems associated with quality assurance, formulation and most importantly the possibility for selection of variants not covered in the vaccine formulation.

3.2. Immunofocusing

An alternative approach, albeit more challenging than a combination allele vaccine is to focus the immune response to conserved epitope targets of strain-transcending, neutralizing immunity. It has been shown that dominant B-cell epitopes in DBPII are polymorphic surface exposed motifs and generally not important for receptor recognition (VanBuskirk et al., 2004b; Chootong et al., 2010). These dominant polymorphic epitopes tend to create an inherent bias towards a strain-specific immune response and limit the induction of an immune response towards more conserved protective epitopes (Welsh and Fujinami, 2007; Tobin et al., 2008), perhaps by a mechanism similar to that observed in other microbial pathogens (Wilson and Cox, 1990; Smith et al., 2004; Barouch, 2007; Walker and Burton,

2008). In a similar manner, polymorphic dominant B-cell epitopes in DBP II represent an evasion mechanism that misdirects the immune response away from more conserved but less immunogenic epitopes that are potential targets of strain-transcending immunity. Fortunately, this parasite immune evasion mechanism can be overcome since some individuals exposed to *P. vivax* in endemic regions are capable of producing broadly inhibitory antibodies, an indication that conserved epitope targets of strain-transcending immunity are present in DBP II (King et al., 2008). One way to focus immune response to conserved epitopes in DBP II is to eliminate dominant variant epitopes responsible for strain-specific immune responses. Similar to what was previously reported for HIV gp120 (Wu et al., 2010), a recent study demonstrated that a synthetic DBP II allele, termed DEKnull, lacking the immunodominant variant “DEK” epitope on DBP II, retained erythrocyte-binding activity and elicited functional anti-DBP II antibodies to shared epitopes on the native DBP II-Sal 1 allele from which it was derived (Ntumngia and Adams, 2012). Synthetic rDEKnull was reactive with naturally acquired anti-DBP II inhibitory antibodies, indicating that rDEKnull contained conserved epitopes associated with the natural protective immune response despite lacking the dominant epitope. A recombinant DBP II based immunogen that targets immune response to conserved functional epitopes may be necessary to avoid induction of strain-specific responses to dominant variant epitopes.

3.3. Subunit DBP II vaccine

Another approach to avoid strain-specific immunity is to identify minimal conserved epitopes within DBP II that can elicit protective neutralizing antibodies against the intact native ligand domain. Stable strain-transcending immunity in persons naturally exposed to *P. vivax* offers the potential to identify the best target epitopes to guide DBP vaccine development (Michon et al., 2000; Xainli et al., 2003; Grimberg et al., 2007; Chootong et al., 2010). Although most people in endemic areas have weak or non-neutralizing antibodies to DBP, a few “elite responder” individuals are capable of producing broadly reactive high titers of invasion-inhibitory anti-DBP II antibodies (King et al., 2008; Cole-Tobian et al., 2009; Chootong et al., 2010). Phage display technology is a powerful tool for elucidation of the molecular nature of protein-protein domain interactions. This technique makes use of filamentous phage to display epitopes of an antigen of interest on its surface (Smith, 1985; Coley et al., 2001). By screening phage libraries (DBP II gene fragment library or a random peptide library) with broadly inhibitory antibodies, it is possible to obtain peptide mimics or subunits of the native refolded protein that are associated with a protective immune response.

A related and even more dynamic, highly versatile and flexible platform for isolating protective epitopes on DBP II will be to recreate the native conformation of DBP II epitopes in vitro as recombinant proteins expressed by virus-like particles (VLPs) (Chackerian, 2007). A VLP system such as the RNA bacteriophage MS2/PP7 integrates into a single platform the high immunogenicity of VLPs with the affinity-selection capability of phage display to optimize epitope structure and then presents it to the immune system in the same structural context, thus allowing a high-density repetitive display of specific peptides as surface epitopes, which are more stimulatory to B-cell, CD4⁺ cell (Chackerian et al., 2001, 2011) and cytotoxic T-cell (Murata et al., 2003) proliferation. Isolated subunits or phage

clones can be used as vaccine immunogens and have been demonstrated to induce a much higher and broadly reactive antibody response than the antibody used to isolate the phage clone (Casey et al., 2004; Harris et al., 2005) and thus serve as in vitro correlates of protective immunity.

To complement and enhance the interaction mapping studies, understanding the specificity of the molecular interactions required for DBP-II-receptor binding is important to best select B-cell epitope targets of neutralizing antibodies. Recently, the three-dimensional crystal structure of DBP-II was resolved, indicating that DBP-II forms a dimer upon receptor binding and identified residues considered functionally important for binding (Batchelor et al., 2011). Epitopes of naturally acquired anti-DBP-II blocking antibodies (Chootong et al., 2010) mapped to the dimer interface and DARC-binding groove suggesting that inhibitory anti-DBP antibodies function by interfering with dimerization (Batchelor et al., 2011). This dimer interface and DARC-binding groove is different from the predicted binding site previously reported (Singh et al., 2006). In another recent study (Siddiqui et al., 2012), inhibitory anti-DBP-II murine monoclonal antibodies (mAbs) mapped to subdomain 3 of DBP-II, which is away from the dimer interphase, thus bringing into question the actual mechanism of action of inhibitory anti-DBP-II antibodies. An important step in defining the targets of neutralizing antibodies on DBP-II will include solving their crystal structure in complex with neutralizing antibodies as described previously for PfAMA-1 (Coley et al., 2001) and HIV gp120 (Zhou et al., 2010). Strongly neutralizing antibodies will obviously target functional regions of the ligand, thereby preventing access to or formation of the receptor-binding pockets. These structural studies will reveal the entire conformational epitope and important structural information regarding potential epitopes interacting with neutralizing antibodies.

In summary, DBP plays a critical role in *P. vivax* merozoite invasion of human reticulocytes and naturally occurring anti-DBP antibodies can inhibit DBP function and block reticulocyte invasion. However, natural immunity to DBP-II is relatively weak, short lived and strain-specific, in part due to variation in dominant epitopes. An effective approach to solve these problems is to design a vaccine that focuses vaccine-elicited immune responses onto conserved epitopes in functional regions within DBP. Each strategy described above has the potential to elicit these types of antibodies that favor boosting responses against conserved protective epitopes, with functional inhibition against broader allelic variants and diverse *P. vivax* strains. In pursuing one or more of these strategies, there is reason to be optimistic that a successful DBP-II-based vaccine may be developed.

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Highlights

- *Plasmodium vivax* Duffy Binding Protein (DBP) is a leading vaccine candidate for blood-stage *P. vivax* malaria.
- Naturally acquired anti-DBP antibodies are associated with protection.
- Polymorphisms in DBP region II (DBPII) may compromise vaccine efficacy.
- A DBPII vaccine needs to focus on an immune response to conserved functional epitopes.
- An ideal vaccine includes a multi-allele, subunit or immunogen devoid of variant epitopes.

TISSAIINHAF^LQNTVMKNCNYKRKR^RERDWD^CNTKKDVCIPDRRYQL^CMKELTNLV^NN
 TDT^NF^HR^DITFRKLY^LK^RK^LIYDAAVEGDLLLKL^NNYR^YN^KD^FCKD^IRWSLGDFGDIIM
 GTDMEGIGYSKVVEN^NLRSIFGT^DEKA^QQ^RRKQW^WNE^SKAQIW^TAMMY^SV^KK^RL^LKGN^FI
W^ICK^LNVAVNIEP^QI^YR^WI^RE^WG^RD^YV^SELPTEV^QKLKEK^CDGKINY^TDKKV^CCKVPP^CQ
 NACKSYDQWITRK^KNQWDVLSNKF^ISVKNAEK^VQTAG^GIIVTPYDILKQELDEFNEVAFEN
 EINKRDGAYIEL^CV^CSV^EEAKKNTQEVVTNVDNAA

Fig. 1.

Amino acid sequence (residues 243 to 573), representing the Duffy Binding Protein (DBP) ligand domain from the Sal1 strain of *Plasmodium vivax*. The polymorphic residues are highlighted (yellow); residues essential for erythrocyte binding (red); residues important but not essential for binding (blue); conserved cysteine residues (bold) and epitopes of inhibitory anti-DBP region II human antibodies (Chootong et al., 2010) are underlined.

Polymorphic residues in most part are not important for receptor recognition. (Adapted from Vanburskirk et al., 2004b).