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Immunological Characterization Of Duffy Binding Protein Of Plasmodium vivax

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Immuneological Characterization of Duffy Binding Protein of *Plasmodium vivax*

by

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A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy
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DEDICATION

To my parents, family, friends and colleagues all over the world who have shown me that this is not just a quest to solve a problem or even doing what is morally right but a gentle voice glorifying God.
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ABSTRACT

*Plasmodium vivax* Duffy binding protein (DBP) is an essential ligand for reticulocyte invasion making it a premier asexual blood stage vaccine candidate. However, strain-specific immunity due to DBPII allelic variation may complicate vaccine efficacy, suggesting that an effective DBPII vaccine needs to target immune responses to conserved epitopes that are potential targets of strain-transcending neutralizing immunity. Anti DBPII monoclonal antibodies, which were previously characterized by COS7 cell binding assay as inhibitory and non-inhibitory to DBPII-erythrocyte binding, were mapped to DBPII gene fragment libraries using phage display. Inhibitory mAb 3C9 binds to a conserved conformation-dependent epitope in subdomain 3 while non-inhibitory mAb 3D10 binds to a linear epitope in subdomain 1 of DBPII.

More definitive epitope mapping of mAb 3D10 was achieved using a random peptide library displayed on phage. Since DBP region II is mostly made up of alpha-helices, we used a randomized helical scaffold library, the Affibody library, displayed on phage, to determine epitope of conformation-dependent antibodies.

The immunogenicity of the identified epitopes was evaluated in mice and the immune sera evaluated for binding to DBPII by ELISA and inhibition of DBPII-erythrocyte binding by the COS7 cell assay. Immune serum from the mAb3C9
epitope blocked DBPII-erythrocyte, suggesting this epitope could be a good subunit vaccine target.
CHAPTER ONE:
INTRODUCTION

WORLDWIDE MALARIA

Malaria, a preventable and curable infectious disease, has plagued mankind for the centuries and is cumulatively the deadliest disease. The unmistakable overlap of the prevalence of malaria with certain human erythrocytic polymorphisms, hemoglobinopathies, enzymopathies and immunogenetic variants are visible marks of the patterns of selection, where the homozygote's disadvantage (if any) is balanced by the attenuated virulence of the malaria parasite to the heterozygote host (Haldane, 1949). The phylum Apicomplexa of the obligate intracellular parasite, Plasmodium, is known for its flagship apical organelles (rhoptries, micronemes and dense granules) seen in the invasive stage. Most species in this phylum simultaneously use three distinct genomes; housed in the nucleus, mitochondrion and apicoplast (a non-photosynthetic chloroplast remnant) enabling a complex life cycle as it effortlessly invades and takes over a myriad of sundry cells. Quinine followed by Chloroquine, a less toxic derivative, was the drug of choice until resistance was seen in the 1960's. Artemisinin in combination with at least one other drug is the accepted treatment today to prevent speedy development of drug resistance by the parasite.
In humans, malaria is caused by *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. knowlesi*; and spread by 70 sub-species of the female *Anopheles* mosquito. An estimated 198 million cases of malaria and 584,000 deaths were left in its wake in 2013. Although the true global burden of the disease is unknown, due to wide-spread under-reporting, globally, 3.3 billion people, are at risk of being infected ("World Malaria Report 2014," 2014)

**Malaria caused by *P. vivax***

*Plasmodium vivax* has the widest geographical distribution and is a major cause of morbidity (Gething et al., 2012). Although vivax malaria is historically called 'benign tertian malaria', when trying to eliminate malaria it is often the ‘last parasite standing’ (Garnham, 1951; Tatem et al., 2010).

During the acute vivax malaria stage, paroxysms are accompanied with headache (87%), cough (42%), muscle pain (35%), nausea (29%), vomiting (24.5%), abdominal pain (25%), diarrhea (4%), clinical anemia (5%), a palpable spleen (8%) and palpable liver (3%) (Luxemburger et al., 1998). In recent years there has been an increase in the number of cases of severe and fatal vivax malaria in both children and adults particularly with severe anemia and low birth weight (Genton et al., 2008; Kochar et al., 2005; Lacerda et al., 2012; Rijken et al., 2012; Tjitra et al., 2008). Strains with low sensitivity to primaquine (the only drug to prevent relapse) are also increasing (Baird & Hoffman, 2004) along with widespread resistance to existing antimalarial drugs (Mohan & Maithani, 2010; Rijken et al., 2011).


*Plasmodium vivax* preferentially invades reticulocytes (Butcher, Mitchell, & Cohen, 1973; Mons, 1990) with Duffy blood group antigen otherwise known as Duffy antigen receptor for chemokines (DARC) (Miller, Mason, Clyde, & McGinniss, 1976). DARC, a receptor on the surface of red blood cells (RBCs), is encoded by the Fy gene with codominant alleles FY*A and FY*B. A point mutation at the binding site of the transcriptional factor GATA-1 in the Duffy gene promoter renders the phenotype Duffy antigen negative erythrocytes (Tournamille, Colin, Cartron, & Le Van Kim, 1995). Interestingly, though the GATA-1 mutation appears on both alleles, Duffy negative Africans are more likely to have FY*B while a Duffy negative South East Asian would have FY*A (Zimmerman et al., 1999). The Fy(a)/Fy(b) phenotype is defined by a nucleotide transition, giving Gly(Fya) or Asp(Fyb) at position 42 in the extracellular amino-terminal domain (Tournamille, Le Van Kim, Gane, Cartron, & Colin, 1995). FY*A allele seems to confer some degree of resistance to vivax malaria as compared to FY*B as antibodies that are either naturally acquired or artificially induced block rPvDBPII from binding to Fy(a) erythrocyte better than to Fy(b) erythrocytes (Chittoria, Mohanty, Jaiswal, & Das, 2012; King et al., 2011; Maestre et al., 2010; Souza-Silva et al., 2014).

**MALARIA LIFE CYCLE**

Although symptoms of malaria have been described in Chinese literature dating back to 2700 BC, it was not until 1880 that Charles Louis Alphonse Laveran “follow(ed) the pigment” and disproved the theory of the day that malaria
was caused by a bacterium. He noticed the parasitic protozoan in the blood of patients with malaria and that quinine treated them. By 1897, Patrick Manson and Ronald Ross working together to “follow the flagellum” (in reference to the male gamete) demonstrated that malaria parasites could be transmitted from infected patients to mosquitoes which were identified as the definite host.

The life cycle of *Plasmodium* starts with an infected female *Anopheles* mosquito that bites a human for a blood meal and transmits a few sporozoites from its saliva. These sporozoites invoke an antibody response to sporozoites proteins. Following the infectious bite, MacCallum (1898) showed that the parasite travels through the blood to the liver where it develops silently undergoing clonal expansion for the first 10 days or so in the liver. This is the exoerythrocytic stage. Within the liver, these sporozoites grow and undergo several rounds of mitotic division to release tens of thousand merozoites. These merozoites seem to act as a ‘stealth shuttle’ (Alan F. Cowman & Kappe, 2006) as it dodges phagocytes like Küpffer cells and dendritic cells and makes its way into the bloodstream. In *P. vivax*, sporozoites can differentiate into a dormant stage called a hypnozoite that, upon activation after months or years, causes clinical relapse (Krotoski, 1985). Cell-mediated T-cell response is the most important immune response, destroying infected hepatocytes.

The released merozoites then invade erythrocytes initiating the pathogenic erythrocytic stage. Inside the erythrocyte, the merozoite grows from a ring form to a trophozoite, which remains uninucleated. The trophozoite’s nucleus divides asexually to form schizonts, which further divide to form mononeucleated
merozoites. When the merozoites are mature, the erythrocyte ruptures releasing approximately 20 merozoites and some parasitic waste (toxins). These released merozoites invade new erythrocytes and the cycle of replication and rupture continues, bringing about the characteristic fever and chills cycle of the disease. Fever also occurs 2 – 3 days before parasite load is high enough to be detected in blood. *P. vivax* has a lower pyrogenic threshold than *P. falciparum*. Under drug pressure, sometimes *P. falciparum* ring-stages can become dormant and hide in the RBCs until conditions for replication becomes more favorable, although it is uncertain if the same phenomenon occurs for *P. vivax*. Naturally acquired immunity (NAI) is mainly associated with the blood stage of the disease with the production of antibodies specific to the merozoite proteins. Occasionally, once immunity to blood-stage is acquired, infections can fall to below limits detectable by standard microscopic methods, but parasite-infected blood cells are not completely cleared. When these infections re-emerge to a detectable level, even causing a clinical episode, it is called recrudescence. These infections are distinct from relapse infections, which are initiated by new merozoites emerging from dormant liver stage parasites.

Some merozoites form male or female gametocytes that do not rupture erythrocytes. In *P. vivax*, gametocytes develop in 56 hours after infecting a reticulocyte and can be seen in the blood before the onset of clinical symptoms. When a female anopheline mosquito feeds on an infected human, it ingests the parasites in the blood, beginning the sporogonic cycle. In the mosquito, gametocytes develop into male and female gametes capable of forming a zygote.
The zygote becomes a motile and elongated oökinate that invades the mosquito’s midgut wall forming an oocyst. Over several days an oocyst undergoes 10-11 rounds of mitosis to form a syncytial cell sporoblast that will release thousands of sporozoites. These sporozoites then migrate to the salivary gland ready to continue the cycle when the mosquito takes its next blood meal. Thus the parasite undergoes more than ten stages of cellular differentiation and invades at least four types of cells within two different hosts.

**Immune response to malaria**

Historically, malaria immune naïve humans were protected or had a mild form of the disease after adoptive transfer of serum from an infected human (Cohen, Mc, & Carrington, 1961; McGregor, 1964). In the mid 1960s, experiments on neurosyphilitic patients immune naïve to malaria, proved that immunity to malaria is species and strain specific (Jeffery, 1966). Increased frequencies of CD4+ T cells and memory CD4+ T cells have been correlated with protection from *P. falciparum* and *P. vivax* infection in humans, respectively (Jangpatarapongsa et al., 2006; Reece et al., 2004). T cells help produce high affinity antigen specific B cells, regulate inflammatory response and activate natural killer (NK) cells and monocytes. High affinity inhibitory antibodies and memory T cells targeting critical regions of molecules may both be essential for an effective immune response (Mueller et al., 2013).

The first humoral response is against sporozoites proteins like circumsporozoite protein (CSP) and thrombospondin related anonymous protein
(TRAP) (Cerami, Kwakye-Berko, & Nussenzweig, 1992; Hedstrom et al., 1990; Sultan et al., 1997; Vanderberg, Nussenzweig, & Most, 1969). Several pre-erythrocytic antigen specific CD4+ and CD8+ T cell epitopes have been identified (Cockburn et al., 2010; Joshi, Bharadwaj, Chatterjee, & Chauhan, 2000; Overstreet, Cockburn, Chen, & Zavala, 2008).

Antibodies to merozoite proteins and ligands that are critical for junction formation and invasion play a crucial role in preventing merozoite invasion of RBC. In *P. falciparum* antigenic variation and cytoadherence of infected RBCs, caused by approximately 60 var genes expressed one at a time that encode for erythrocyte membrane protein 1 (PfEMP1), is a key contributor to complicated malaria (Biggs et al., 1991; Roberts et al., 1992; Scherf et al., 1998). While sequestration enables the parasite to avoid spleen-dependent killing, antigenic switching is a challenge for antibody production. *P. vivax* does not have var genes and because of the flexibility of its host cell it does not need to sequester. Antibodies also enhance phagocytic activity of monocytes and macrophages.

During the acute phase of the disease, a strong Th1 pro-inflammatory response is typical for malaria with the production of inflammatory cytokines and chemokines like tumor necrosis factor (TNF)-alpha, IFN-gamma, IL-1, IL-6, and IL-12 to help prevent hyperparasitemia (Ockenhouse et al., 2006). Although inflammatory cytokines help clear parasitemia, it can cause severe disease due to endothelial dysfunction, sequestration and anemia. Hepcidin, the iron regulatory hormone secreted from macrophages and hepatocytes, is up-regulated in the presence of pro-inflammatory cytokines and may play a key role
in anemia related complications of *P. falciparum* infection (Nemeth et al., 2004; Nweneka, Doherty, Cox, & Prentice, 2010). However, a strong regulatory response determined by plasma IL-10 levels, have been found to be more elevated in *P. vivax* compared to *P. falciparum* infection (Goncalves, Scopel, Bastos, & Ferreira, 2012; Han et al., 2010). Antigen-specific CD4+ T cell expansion is inversely regulated by increase in CD4+(FoxP3+) natural regulatory T cells (Jangpatarapongsa et al., 2008). CD4+ T cells in turn activate NK and YδT cells (Artavanis-Tsakonas et al., 2003; D'Ombrain et al., 2008), which are thought to act as a bridge between innate and acquired immunity.

Naturally acquired immunity (NAI), first described by Robert Koch in 1899, is the collective parasitological and clinical immune response to second and subsequent infections. NAI is largely associated with antibody response to blood stage infection but can also result in reduction of transmission of gametes to the mosquito or interfere with development of gametes in mosquito. NAI is considered to be cumulatively dependent on exposure to multiple parasitic infections (premunition), is relatively species and strain specific, and is modulated by age at first exposure. NAI to *P. vivax* is manifested as protection against high-density parasitemia and uncomplicated malaria at a younger age as compared to *P. falciparum* (Mueller et al., 2009). Unlike NAI to *P. falciparum*, immune naïve adults do no acquire clinical immunity with relatively few exposures than children (Baird, Jones, et al., 1991; Baird et al., 2003). NAI is one of the main rationales behind pursuing a multivalent blood stage vaccine.
MALARIA VACCINES

Vaccination is historically the most effective way to prevent infectious diseases but the malaria parasite is considerably more complex than bacteria and viruses that most vaccines target. With over 5000 genes the malaria parasite is very good at evading the immune system as it uses multiple pathways to invade host cells with polymorphic stage specific ligands. Hence developing a vaccine against malaria has intrigued scientists globally across time. The first success made towards development of a vaccine for malaria was in 1967 using radiation attenuated sporozoites to protect against infection with malaria in mice (Nussenzweig, Vanderberg, Most, & Orton, 1967). Today the RTS,S a pre-erythrocytic, *P. falciparum* subunit vaccine of the repeat region and Tc epitope of a CSP, is the only vaccine candidate that has under gone phase 3 clinical trials. As the efficacy of the vaccine varied greatly with transmission intensity (Bejon et al., 2013) the need for a more efficacious second-generation anti-infection vaccine (against pre-erythrocytic stage) and anti-disease vaccine (against asexual blood stage) is still very urgent. In addition, transmission-blocking vaccines that target pre-erythrocytic and/or sexual sporogonic stage is also being studied. Phase I clinical trials for vaccine candidates against *P. vivax* pre-erythrocytic stage and for transmission blocking have been carried out (reviewed in (Reyes-Sandoval & Bachmann, 2013). However, none of the blood stage vaccine candidates have reached that mile-marker.
**P. vivax asexual blood stage vaccines**

*Plasmodium vivax* merozoite proteins that have a role in reticulocyte invasion and can elicit a significant NAI are being studied such as the apical membrane antigen-1 (PvAMA1), merozoite surface proteins (PvMSPs), reticulocyte binding proteins (PvRBPs) and Duffy binding protein (PvDBP). All of these proteins except for PvMSPs have highly polymorphic immunodominant regions that are suggestive of immune pressure. PvMSP1 and PvDBP have been studied the most.

Though PvRBPs are among the first apically located protein to bind irreversibly to reticulocytes (Galinski & Barnwell, 1996; Galinski, Medina, Ingravallo, & Barnwell, 1992), triggering the release of PvDBP from the mironemes for junction formation (Singh et al., 2005), very few studies have been done to understand NAI to PvRBPs. One study in Brazil showed antibodies correlated significantly to exposure and were directed towards the N-terminal polymorphic putative binding region (Tran et al., 2005).

The apical membrane protein1 is localized in the microneme and AMA1 interacts with rhoptry neck protein-2 (RON2) to form the moving junction complex during merozoite invasion (Srinivasan et al., 2011). PvAMA1 is pro-inflammatory and produces an IgG1 and IgG3 response (Wickramarachchi et al., 2006). Immune response had been detected to linear and conformational epitopes of the polymorphic Domain II and specifically the conserved loop region (Chesne-Seck et al., 2005; Lal et al., 1996; Pizarro et al., 2005).
The merozoite surface proteins family has several potential vaccine candidates; P.vMSP-1 fragments, P.vMSP-3 family (P.vMSP-3.1, P.vMSP-3.3 and P.vMSP-3.10) P.vMSP-4, P.vMSP-5 and P.vRBP-5 1/2 and P.vMSP-9. P.vMSP1 is the most abundant blood stage antigen that undergoes two successive proteolytic cleavages to form MSP1_142 and then MSP1_19 (Blackman, Scott-Finnigan, Shai, & Holder, 1994). Although MSP1 shows humoral and cellular reactivity; and human antibodies to MSP1 correlated with protection against *P. vivax* infection, yet vaccination of non-human primates with MSP1_19 failed to consistently induce antibody production (Valderrama-Aguirre et al., 2005). Of the 11-member *msp3* gene family, B cell epitopes to P.vMSP3.10’s α-helical region has been reported with a predominance of IgG1 antibodies followed by IgG2 (Lima-Junior et al., 2011). Antibody response to P.vMSP9 is high among individuals that carry HLA-DRB1*04 (Lima-Junior et al., 2010). IgG1 and IgG2 response is correlated with age, IFN-γ and IL-4 responses were also generated to the peptide (Lima-Junior et al., 2008).

**The Duffy Binding Protein**

Specific receptor ligand interactions are required for the malaria parasite to invade erythrocytes. The Duffy binding protein (DBP) was first identified in the model parasite *P. knowlesi* (PkDBP) (Adams et al., 1990; Haynes et al., 1988) and then in *P. vivax* (PvDBP) (Fang, Kaslow, Adams, & Miller, 1991; Wertheimer & Barnwell, 1989). The approximately 140 kDa type I membrane DBP is sequestered in the micronemes and expressed during late schizont development.
and then released on the surface of the merozoite at or around the time of invasion (Adams et al., 1990; Fang et al., 1991; Haynes et al., 1988; Wertheimer & Barnwell, 1989).

Unlike *P. falciparum*, *P. vivax* merozoites almost exclusively use the Duffy (Fy) antigen receptor for chemokines (DARC) to invade erythrocytes (Miller et al., 1976). DARC is a glycosylated membrane protein more abundant on the surface of reticulocytes than on mature erythrocytes and is also expressed on the endothelial surfaces of some organs. *In vitro* invasion studies using *P. knowlesi* demonstrated that initial interaction and apical reorientation can take place normally on most human erythrocytes, but junction does not develop and invasion does not progress to junction formation on Duffy-negative human erythrocytes (lack DARC) (Miller et al., 1976). More recently there have been few *P. vivax* cases among Duffy negative individuals (Cavasini et al., 2007; Menard et al., 2013; Ryan et al., 2006) however, these cases seem to be very rare and an alternate reticulocyte receptor and merozoite ligand has not yet been identified. Hence DBP is the leading vaccine candidate.

The invasion of erythrocytes by *Plasmodium* merozoites is a complex, multistep process involving an initial “long-distance” recognition of red blood cell surface receptors and attachment, after which a process of reorientation takes place to align the apical end to face the erythrocyte (Galinski & Barnwell, 1996; Srinivasan et al., 2011), a tight junction is then formed involving high-affinity ligand-receptor interactions. The junction is seen initially as a thickening of the erythrocyte membrane at its point of contact with the merozoite, which then
transforms into a ring that moves around the merozoite, powered by the parasite's actin-myosin motor shedding the surface coat in the process. Upon reaching the posterior pole, the adhesive proteins at the junction are also proteolytically removed, this time by a resident protease facilitating resealing of the membrane. When the invasion is complete, the merozoite finds itself in a vacuole surrounded by a vacuolar membrane called parasitophorous vacuole (A. F. Cowman & Crabb, 2006).

Although this may not be the only mechanism for invasion of reticulocytes by *P. vivax*, PvDBP is the founding member of the family of Duffy-binding-like erythrocyte binding proteins (DBL-EBPs) (Adams et al., 1992) that includes *P. falciparum* EBA175, PfEBL1, *P. knowlesi* DBPα,β and γ among others (Adams, Blair, Kaneko, & Peterson, 2001). The Duffy-binding-like domain is characterized by two cysteine-rich regions (in PvDBP region II and region VI) and conserved exon-intron structures (Adams et al., 1992).

Growing *in vitro* *P. vivax* cultures is a hurdle that is yet to be overcome however, an in vitro functional assay, the COS7-binding assay, was developed to study binding of RBCs to transfecting COS7 cells expressing DBP on the surface of COS7 cells. Using the COS7-binding assay it was determined that the cysteine rich region II of DBP (DBPII) is critical for binding to DARC (Chitnis & Miller, 1994). However, PvDBPII is extremely polymorphic with a nucleotide substitution rate four times higher than the rest of the molecule (Ampudia, Patarroyo, Patarroyo, & Murillo, 1996; J. Cole-Tobian & King, 2003; Xainli, Adams, & King, 2000). Antibody responses to PvDBP are seen in people living in
*P. vivax* endemic areas and the elevated levels of amino acid sequence polymorphisms may be signs that the molecule is under selective pressure (Ampudia et al., 1996; J. L. Cole-Tobian et al., 2002; Fraser et al., 1997; P. A. Michon, Arevalo-Herrera, Fraser, Herrera, & Adams, 1998; Tsuboi et al., 1994).

Using site directed mutagenesis, it was determined that polymorphic residues flanked residues critical for receptor binding suggesting variation in BC epitopes is consistent with an immune evasion mechanism (Hans et al., 2005; VanBuskirk, Sevova, & Adams, 2004). Additional studies confirmed that amino acid substitutions in some of the polymorphic residues alter sensitivity to anti-DBP inhibitory antibodies (VanBuskirk, Cole-Tobian, et al., 2004). In 2011 the crystal structure of *Pv*DBPII was determined and the structure corroborated previous studies indicating that polymorphic residues flanked residues critical for binding, on the surface of *Pv*DBPII (Fig 1.6 A) (Batchelor, Zahm, & Tolia, 2011). Cumulatively, these results suggest that polymorphic residues act as a mechanism to misdirect the immune response away from the conserved residues critical for binding as *Pv*DBPII haplotype-specific antibody response correlates with only homologous and not heterologous protection (Ceravolo et al., 2009; Chootong et al., 2010; J. L. Cole-Tobian et al., 2009; Grimberg et al., 2007; King et al., 2008; VanBuskirk, Cole-Tobian, et al., 2004; Xainli et al., 2002; Xainli et al., 2003).

*Pv*DBPII is largely alpha-helical and may be assigned into three sub-domains delineated by six disulphide bonds. While sub-domain1 of *Pv*DBPII is made up of an antiparallel beta-hairpin, sub-domains 2 & 3 seen as distinct
bundles of three alpha-helixes. NMR studies of PvDBPII in contact with the core region of DARC ectodomain revealed that dimerization of PvDBPII is required for and driven by receptor engagement as DARC ectodomain form a helix that binds to the dimer interphase (Fig 1.7 A) (Batchelor et al., 2014). In addition, the receptor DARC is sulfated at tyrosine residues 30 and 41, right next to residue 42 that expresses the variant Fy(a)/Fy(b) phenotype, and sulfation of tyrosine 41 has been shown to play a critical role in binding to PvDBP (Fig 1.7 B) (Batchelor et al., 2011; Choe et al., 2005).

Although NAI to PvDBPII is present in P. vivax endemic areas, anti-DBPII immune response tends to increase with age and exposure (Chootong et al., 2010; J. L. Cole-Tobian et al., 2002; Grimberg et al., 2007; VanBuskirk, Cole-Tobian, et al., 2004; Xainli et al., 2003). Studies have shown that about 8-10% of P. vivax exposed individuals produce high titer strain-transcending broadly neutralizing antibodies (King et al., 2008), associated with 50% reduction in risk of infection with P. vivax (Chootong et al., 2010; King et al., 2008). Vaccine induced anti-DBP antibodies showed partial erythrocyte binding inhibition in COS7 assay (Devi et al., 2007) as well as partial inhibition of merozoite invasion of erythrocyte (Arevalo-Herrera et al., 2005). Vaccine induced anti-DBP antibodies also induced partial protection in monkeys (Arevalo-Herrera et al., 2005). Crystal structure and site directed mutagenesis studies (Batchelor et al., 2014; VanBuskirk, Sevova, et al., 2004) suggest that strain transcending neutralizing antibodies may be directed to the conserved dimer interface (Fig 1.6 B) or may sterically inhibit dimerization.
In pre-clinical mouse studies, a multi-allele PvDBPII vaccine was more immunogenic and produced a stronger strain neutralizing response than a single-allele vaccine (Ntumngia et al., 2013). To overcome the inherent tendency for anti-DBP immune responses to be strain specific, a novel synthetic PvDBPII called DEKnull was produced. The B-cell immunodominant and polymorphic residues of the DEK epitope (Chootong et al., 2010) were mutated in DEKnull. Compared to the natural parent allele Sal1, DEKnull vaccine was less immunogenic but it produced an immune response that was similar to a single natural allele vaccine (Ntumngia & Adams, 2012; Ntumngia et al., 2013). These studies prove that identifying epitope targets of broadly neutralizing inhibitory antibodies on DBP will be very helpful in designing a vaccine that produces strain-transcending immunity.

PHAGE DISPLAY AND BIOPANNING

In the mid 1980’s George Smith performed a series of seminal experiments that revolutionized protein engineering and the study of protein-protein interactions. Smith demonstrated that a bacteriophage could express an exogenous protein by genetically encoding the gene (here, gene encoding EcoRI endonuclease) as a chimera to protein pIII on the surface of the phage, coining the term phage display (Smith, 1985). These findings meant that proteins could be easily produced in the right conformation and ready to use on the phage by cloning the specific gene into the phage genome; which bypassed the laborious method of producing, selecting, purifying and refolding protein produced within
bacteria. Smith further demonstrated both, that EcoRI-pIII protein chimera retained its ability to bind its specific antibody and that phage displaying the chimera could be selected from a mixture of wild-type phage by affinity enrichment using anti EcoRI antibodies (Smith, 1985). This method of affinity enrichment was termed biopanning (Parmley & Smith, 1988).

A bacteriophage (phage) is a virus that exclusively infects bacteria by attaching to specific bacterial cell wall phage receptors, including sex-pili, flagella, and cell wall lipoproteins, polysaccharides, and lipopolysaccharides. Following infection, the phage uses the host’s machinery to synthesize progeny thereby suppressing the host’s gene expression and metabolic activities. The lytic cycle of the phage usually culminates in the release of viral progeny and bacterial cell content into the medium (Neufeld, Mittelman, Buchner, & Rishpon, 2005).

The filamentous phage (Ff) M13, a single-stranded DNA phage that infects Escherichia coli strain that carries a F- conjugative plasmid, is the most commonly used phage display vector. Surface protein pIII and VIII have been used as chimeras with exogenous protein to be expressed. Major capsid protein VIII has been used to express small peptides in large numbers, to study avidity interactions, while minor capsid protein pIII can be used to display proteins to study affinity interactions (Iannolo, Minenkova, Petruzelli, & Cesareni, 1995; Smith & Scott, 1993). Gene III protein displayed as 5 copies, at one tip of the virion, mediates adsorption to the host sex-pili. The C-terminus of the protein III remains anchored in the phage coat while the N-terminal is responsible for
binding to the F-pilus of the host. Peptides to be displayed are fused to the N-terminal portion of the gene III protein. To maintain infectivity of the phage, a special vector system termed 3+3, was developed that included the wild type pIII gene in a helper phage and the recombinant gene on a phagemid (Bass, Greene, & Wells, 1990; Russel & Model, 1986).

Over the past three decades phage display has influenced many fields in the study of malaria like identifying novel protein-protein interactions, drug design and target validation, diagnostic and therapeutic antibody fragments, vaccine design and epitope mapping. These are briefly discussed below.

A novel interaction between Erythrocyte binding antigen-181 (EBA-181) and human erythrocyte membrane protein 4.1 (4.1R) was identified using *P. falciparum* cDNA library biopanned against 4.1R (Lanzillotti & Coetzer, 2006; Sonja B. Lauterbach, Lanzillotti, & Coetzer, 2003). Novel interaction between the amino-terminus of MSP1 and RBC glycoporphin A (GPA) suggesting an essential role of the MSP1-GPA-band 3 complex during the initial adhesion phase of malaria parasite invasion of RBCs (Baldwin, Li, Hanada, Liu, & Chishti, 2015). Novel mosquito midgut receptor, enolase-binding protein (EBP) that binds to oökinate surface ligand (enolase), is one of the two pathways used by the oökinates to invade the mosquito midgut. The mosquito EBP was identified when SM1, a 12-aa peptide mimotope of enolase, identified after 5 rounds of panning to salivary glands and mosquito midgut was studied (Ghosh, Ribolla, & Jacobs-Lorena, 2001; Vega-Rodriguez et al., 2014). Identification of (novel) enzyme substrates and inhibitors like *P. falciparum* M18 aspartyl aminopeptidase
(PfM18AAP) interaction with the human erythrocyte membrane protein spectrin are being understood using phage display. PfM18AAP seems to have a multifunctional role that utilizes several substrates and digests hemoglobin (S. B. Lauterbach & Coetzer, 2008).

Identifying a possible drug target; a 14-residue long disulfide-bonded peptide selected by phage display screen targeting the catalytic domain of \( P. falciparum \) serine repeat antigen 5 (SERA5) and delays egress in a dose-dependent manner, demonstrating the potential of SERA5 to be targeted by small molecules (Fairlie et al., 2008). As a possible drug or drug design, peptide P1 identified from a 7-mere random peptide library was screened against \( P. falciparum \) infected red blood cells (iRBCs) to identify distinctions on the surface from uninfected erythrocytes. Peptide P1 when conjugated with a peptide having moderate hemolytic activity showed inhibition of parasite growth (Eda, Eda, & Sherman, 2004). A 20-aa peptide (R1) identified from a random peptide library binds to AMA1 and inhibits invasion. During erythrocyte invasion AMA1 binds to RON2 complex to form the moving junction. Crystal structures of AMA-RON2 and AMA-R1 reveal structural mimicry and will help identify key residues (Harris et al., 2005; Vulliez-Le Normand et al., 2012).

As a possible diagnostic antibody fragment; two novel binders to \( P. falciparum \) histidine rich protein 2 (rPfHRP2), identified by screening a human naïve scFv antibody library, have comparable specificities to PfHRP2-specific mouse mAb C1-13 and is more stable, making it a possible tool for point of care diagnostics (Leow, Jones, Cheng, Mahler, & McCarthy, 2014). Fab-phage
display library was made from peripheral blood leukocytes’ mRNA from clinically immune individuals and biopanned against merozoite surface antigen 3 (MSP-3) residues 194 to 257. Of the 3 Fab fragments identified one, RAM1, showed antiparasitic effect (Lundquist et al., 2006).

Subunit vaccine studies have identified a 69-aa peptide F2i that binds to Glycophorin B on RBC and inhibits binding of erythrocyte-binding ligand-1 (EBL-1) on the merozoite. F2i was identified through screening using P. falciparum cDNA phage display library and purified glycophorin and RBCs (Li et al., 2012). Transmission blocking vaccine studies on Female Gamete peptide 1 (FGP1) that binds specifically to the surface of female P. berghei gametes and strongly reduces P. berghei oocyst formation by interfering with fertilization could be considered as a transmission blocking vaccine supplementary target (Vega-Rodriguez, Perez-Barreto, Ruiz-Reyes, & Jacobs-Lorena, 2015). Single-chain Fv fragments (scFv)-phage display libraries derived from malaria immune patients was panned against Pfs48/45, a gamete surface protein of the sexual stages of P. falciparum. Identified human scFv antibodies specific for epitope III of Pfs48/45 could be used to arrest the sporogenic stages inside the mosquito (Roeffen et al., 2001).

Epitope mapping of conformation dependent mAb4G2dc1 that binds to P. falciparum AMA1, critical for erythrocyte invasion (Kocken et al., 1998) was carried out using phage displayed peptide library identifying three peptides that bind to 4G2dc1 and mimic an important epitope of AMA1 (Casey et al., 2004; Sabo et al., 2007). Phage display and crystal structure was also used to map the
epitope of mAb 45B1 to a conserved region of PyAMA1 which is a region that is also recognized by inhibitory mAb 4G2 that is specific for PfAMA1 (David L. Narum, Ogun, Batchelor, & Holder, 2006). Passive immunization with mAb 45B1 specific for P. yoelii AMA1 was protective against a lethal challenge infection with P. yoelii (D. L. Narum, Ogun, Thomas, & Holder, 2000).

Specifically for DBP, three binding inhibitory single-chain variable fragment (scFv) antibodies were identified from a library constructed from 3 human patients infected with P. vivax panned on DBPII (Kim et al., 2007).

Antibody surrogates as randomized fragments on diverse scaffold proteins and designing catalytic antibodies (abzymes) or enzymes with novel specificities are fields that phage display has not yet been used to analyze and overcome the various challenges posed by the malarial parasite.

**Phage display library**

In 1997, Fack and co-workers compared the efficiencies of epitope mapping using phage display by random peptide phage display libraries and by gene-fragment libraries in combination with peptide scan. A single round of panning using the gene-fragment library was enough to affinity select for the peptides corresponding to the respective epitopes of the mAbs. Random peptide library however was successful for only two of the mAbs after three to four rounds of panning (Fack et al., 1997).

In 2001, Coley et al. studied epitope mapping in the malaria antigen, AMA1. They used a combination of techniques, first the antigen fragments
identifies the location of the epitopes then random peptide libraries used to
accurately identify amino acids involved in the epitope and finally, phage display
of mutant fragments confirms the role of each residue in the epitope. A panel of
mAbs was generated to obtain binding reagents to the various domains within
the molecule. It was determined that MAb 5G8 recognizes a short linear epitope
within the pro-domain of AMA1 while the epitope recognized by MAb 1F9 is
reduction sensitive and resides in domain-1, within a disulphide-bonded 57
amino acid sub-domain (Coley et al., 2001). This study is worth emulating for P.
vivax DBP.

Epitope mapping using phage display has been mainly focused on the use
of random peptide libraries and gene fragments libraries derived from selected P.
falciparum proteins. This study, aims to use P. vivax DBPII gene fragment
libraries to determine the epitopes of various inhibitory and non-inhibitory mAbs
by biopanning. In addition, a mAb with linear epitope was panned against a
random 20-aa peptide library while antibodies with conformational dependent
epitopes were biopanned against a random helical scaffold, Affibody library.
The World malaria report 2014 summarizes the status of global efforts to control and eliminate malaria. The report is produced every year by the WHO Global Malaria Programme, with the help of WHO regional and country offices, ministries of health in endemic countries, and a broad range of other partners. Data for this year’s report were assembled from 97 countries and territories with ongoing malaria transmission, and an additional six countries that are working to prevent reintroduction.

This section outlines the public health burden posed by malaria, the strategies that can be used to reduce that burden, and the goals, targets and indicators that have been set for 2015. The report then reviews global progress towards the goals and targets in relation to funding (Section 2), intervention coverage (Sections 3–7), and malaria cases and deaths (Section 8). The review is followed by Regional profiles that summarize trends in each WHO region. Country profiles are provided both for countries with ongoing malaria transmission and for those recently achieving zero local cases. Finally, annexes provide sources of data, details of the methodology used in the analysis, and tables containing country and regional data.

1.1 The public health challenge posed by malaria
Malaria transmission occurs in all six WHO regions. Globally, an estimated 3.3 billion people in 97 countries and territories are at risk of being infected with malaria and developing disease (Figure 1.1), and 1.2 billion are at high risk (>1 in 1000 chance of getting malaria in a year). According to the latest estimates, 198 million cases of malaria occurred globally in 2013 (uncertainty range 124–283 million) and the disease led to 584,000 deaths (uncertainty range 367,000–755,000), representing a decrease in malaria case incidence and mortality rates of 30% and 47% since 2000, respectively. The burden is heaviest in the WHO African Region, where an estimated 90% of all malaria deaths occur, and in children aged under 5 years, who account for 78% of all deaths.

Figure 1.1: Countries with ongoing transmission of malaria in 2013. Reprinted from Publication World Malaria Report 2014, World Health Organization, Introduction, Pages No. 2, Copyright (2014)

Figure 1.2: Spatial distribution of P.vivax malaria endemicity in 2010 (Gething et al., 2012)
Figure 1.3: Malaria life cycle (Arama & Troye-Blomberg, 2014) Journal of internal medicine by BLACKWELL PUBLISHING LTD. Reproduced with permission of BLACKWELL PUBLISHING LTD.
Figure 1.4: Merozoite Invasion of Erythrocytes (Zimmerman, Ferreira, Howes, & Mercereau-Puijalon, 2013) ADVANCES IN PARASITOLOGY by ACADEMIC PRESS. Reproduced with permission of ACADEMIC PRESS.
Figure 1.5: Schematic drawing of the important structural domains of DBP. Where TM, transmembrane domain and Cyto, cytoplasmic domain (VanBuskirk, Sevova, et al., 2004) Copyright (2004) National Academy of Sciences, U.S.A.

Figure 1.6: Crystal structure of PvDBPII. Monomers of DBPII are colored green and yellow (A) overlay of polymorphic residues (blue) and critical receptor-binding residues (purple) (B) epitopes recognized by blocking antibodies (red, most significant; brown, significant), which include the dimer interface and DARC-binding groove. Reprinted by permission from Macmillan Publishers Ltd: Nature Structural & Molecular Biology (Batchelor et al., 2011), copyright (2011)
The functional elements defined in this study are critical for DARC binding to the asymmetric flap. These residues are not part of the putative DARC tight complex. VAR is predominant for placental malaria and is associated with an estimated binding to the surface of infected erythrocytes and binds specifically to chondroitin sulfate A. DBL6 is proposed to function as monomer, analogously to PkDBP.

We examined the crystal symmetry for the DBL6 crystal structure. pvDBP crystallized with two copies in the asymmetric unit but was not observed in DARC structures. Variable epitopes reveal targets of selective pressure. Supplementary Fig. 7: the sixth DBL domain of VAR2CSA.

Mutagenesis of several basic residues in DBL6 cannot account for all mutations that affect binding to DARC reported in the literature. The authors also proposed a 'just-in-time release' strategy.

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CHAPTER TWO:
DEFINING THE IMMUNOREACTIVE SURFACE OF *PLASMODIUM VIVAX*

**DUFFY BINDING PROTEIN REGION II**

**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*. However, strain-specific immunity due to DBPII allelic variation may complicate vaccine efficacy, suggesting that an effective DBPII vaccine needs to target immune response to conserved epitopes that are potential targets of strain-transcending neutralizing immunity. Anti DBPII mAbs, which were previously functionally characterized by *in vitro* COS7 cell binding assay as inhibitory and non-inhibitory to DBPII-erythrocyte binding, were mapped to DBPII gene fragment libraries using phage display. Inhibitory mAb 3C9 binds to a conserved conformation-dependent epitope in subdomain 3 while non-inhibitory mAb 3D10 binds to a linear epitope in subdomain 1 of DBPII. In addition, more definitive epitope mapping of mAb 3D10 was achieved using a random peptide library displayed on phage. To determine the potential of these minimal peptide epitopes to function as an epitope-specific vaccine, their immunogenicity to induce functionally inhibitory
antibodies were evaluated. The mAb 3C9 epitope elicited antibodies that inhibited DBP binding to erythrocytes whereas mAb 3D10 epitope did not.

INTRODUCTION

*Plasmodium vivax* is the second most prevalent cause of human malaria and is globally the most widely distributed leaving about 40% of the world’s population at risk (Gething et al., 2012). Although vivax malaria is historically called ‘benign tertian malaria’ (Garnham, 1951) there have been increasing reports of clinical severity with emerging virulent forms of the parasite (Alexandre et al., 2010; Kochar et al., 2009), recurrent clinical episodes due to reactivation of the dormant forms in the liver (Krotoski et al., 1982) and widespread drug resistance (Baird, Basri, et al., 1991; Price et al., 2014; Rieckmann, Davis, & Hutton, 1989) (Baird, Basri, et al., 1991; Price et al., 2014; Rieckmann et al., 1989), which potentially includes strains with low sensitivity to primaquine, the only drug against relapse, or in people with a poor ability to metabolize the drug to its active form (Baird & Hoffman, 2004; Bennett et al., 2013; Pybus et al., 2013). There is an urgent need to develop new therapies, especially a vaccine to control and prevent vivax malaria. Immunity to asexual blood stage antigens is especially to those that play a role in merozoite invasion of erythrocyte. Merozoite antigens are believed to be an important component of naturally acquired immunity to malaria (Marsh & Kinyanjui, 2006; Yazdani, Mukherjee, Chauhan, & Chitnis, 2006), and represents ideal candidates for vaccine mediated-immunity against blood stage infection (Mueller et al., 2013).
Plasmodium vivax Duffy binding protein (PvDBP) binds to its cognate receptor Duffy antigen receptor for chemokines (DARC) on reticulocytes forming an irreversible junction, critical for merozoite invasion (Adams et al., 1990; Adams et al., 1992; Chitnis, Chaudhuri, Horuk, Pogo, & Miller, 1996; Miller et al., 1976; Miller, Mason, Dvorak, McGinniss, & Rothman, 1975; Wertheimer & Barnwell, 1989). In most circumstances *P. vivax* merozoites almost exclusively use DARC as a receptor to invade reticulocytes (Miller et al., 1976). However, more recently, there have been a few *P. vivax* cases reported among Duffy negative individuals (Cavasini et al., 2007; Menard et al., 2013; Ryan et al., 2006), although an alternate receptor-ligand invasion pathway is unknown. Hence PvDBP remains a prime candidate for vaccine-induced immunity against blood stage *P. vivax* infection.

*Plasmodium vivax* DBP is a type I membrane protein localized to the merozoite micronemes and is a member of the Duffy binding-like erythrocyte-binding proteins (DBL-EBP) family characterized by two conserved cysteine rich domains (Adams et al., 1990; Adams et al., 1992) The DBL-EBP and share similar molecular structures and functional characteristics (Adams et al., 2001; Adams et al., 1990; Adams et al., 1992; Mayer, Kaneko, Hudson-Taylor, Reid, & Miller, 2001; D. L. Narum, Fuhrmann, Luu, & Sim, 2002). A 330 amino acid region within the extracellular portion of DBP, also known as DBP region II (DBPII) constitutes the principal determinants for receptor recognition (Adams et al., 1992; Chitnis & Miller, 1994; Ranjan & Chitnis, 1999; VanBuskirk, Sevova, et al., 2004). The central region of DBPII is highly polymorphic (Ampudia et al.,...
1996; Tsuboi et al., 1994; Xainli et al., 2000), in a pattern consistent with high immune selection pressure on DBPII (Baum, Thomas, & Conway, 2003; Baum, Ward, & Conway, 2002). Residues critical for receptor recognition are flanked by polymorphic residues that are not important for binding to the receptor (Batchelor et al., 2011; VanBuskirk, Sevova, et al., 2004; Xainli et al., 2000). These polymorphisms represent a potential challenge to developing a vaccine since they contribute to strain-specificity in naturally-acquired immunity (J. L. Cole-Tobian et al., 2009; McHenry, Barnes, Ntumngia, King, & Adams, 2011; Ntumngia et al., 2012b; VanBuskirk, Cole-Tobian, et al., 2004).

In endemic regions, individuals develop anti-DBPII antibodies, which increase with age, suggesting a boosting effect from repeated exposure to infection (King et al., 2008; P. Michon, Fraser, & Adams, 2000). Furthermore, anti-DBP antibodies tend to be weak, short-lived and strain-specific (Ceravolo et al., 2009; King et al., 2008; Xainli et al., 2003) with allelic differences appearing to be driven by immune selection (McHenry et al., 2011; VanBuskirk, Cole-Tobian, et al., 2004). Few individuals develop high titers of broadly reactive, invasion-inhibitory anti-DBPII antibodies (Chootong et al., 2010; J. L. Cole-Tobian et al., 2009; King et al., 2008). These data are all consistent with the hypothesis that variation is an immune evasion mechanism responsible for strain-specific immunity and that stable, broadly inhibitory immunity is achieved when antibodies target functionally conserved epitopes.

Phage display has been a useful tool for epitope mapping with the advantages of rapidly producing refolded protein, fused to phage coat; forming a
traceable link back to the genotype (Smith, 1985; Wilson & Finlay, 1998). In malaria, phage display has been used to map epitopes on apical membrane antigen 1 (AMA1) (Casey et al., 2004; Coley et al., 2001; David L. Narum et al., 2006; Sabo et al., 2007), merozoite surface protein (MSP) (Demangel, Lafaye, & Mazie, 1996) and circumsporozoite protein (CSP) (Greenwood, Willis, & Perham, 1991; Monette, Opella, Greenwood, Willis, & Perham, 2001; Willis, Perham, & Wraith, 1993). These studies have used a combination of phage display of random peptide libraries, gene fragment libraries, crystal structure studies along with peptide immunizations to determine mimotopes and eventually epitopes on these antigens.

Previously, we generated and immunologically characterized a panel of anti-DBPII murine mAbs. Some of these monoclonal antibodies were broadly inhibitory like mAbs 3C9, 2D10, 2C6 and 2H2 while mAb 3D10 was highly reactive but still a poor inhibitor to DBPII-erythrocyte binding in a standard *in vitro* COS 7 cell assay (Ntumngia et al., 2012b). With the exception of mAb 3D10, all the mAbs specifically recognized only conformational dependent epitopes on recombinant DBPII. Crystallography studies reveal that DBPII is largely alpha-helical and may be assigned into three sub-domains delineated by six disulphide bonds (Batchelor et al., 2011). Using phage display we have previously shown that, each of the sub-domains or natural combinations of the sub-domains of DBPII have varying patterns of affinity to these mAbs (Siddiqui et al., 2012). These data suggest that identifying the epitopes of these inhibitory and non-inhibitory mAbs will help design a strain-transcending subunit vaccine.
In this study, we further map the epitopes of these anti-DBPII mAbs by screening DBPII gene fragment libraries expressed on M13 phage surface for minimal reactive peptide fragments. DBPII epitopes identified for the broadly inhibitory anti-DBPII mAb 3C9 and non-inhibitory mAb 3D10, conjugated to carrier protein KLH, were used to produce an antibody immune response in BLAB/c mice. Anti-3C9 epitope but not anti-3D10 epitope antibodies inhibited DBPII-erythrocyte binding. Site directed mutagenesis was further carried out to determine essential residues within the epitope. The information derived from this study contributes to our understanding of the specific targets for vaccine-elicited protective immunity. In particular, this study demonstrates an approach that may be successful in boosting antibody responses targeted against conserved protective epitopes, with functional inhibition against broader allelic variants and diverse *P. vivax* strains.

**MATERIALS AND METHODS**

**Generation of phage displayed DBPII gene fragment libraries**

The full-length PCR product (20 µg) from DBPII Sal1 and 7.18 haplotypes were digested with DNase I at 5 U/ml. The resulting fragments were purified by sodium acetate precipitation and ragged ends blunted with a Vent polymerase (NEB). The pHENH6 vector was digested with PstI (NEB) and also blunted with Vent polymerase. The resulting products from the randomly fragmented DBPII were cloned into the treated pHEN-H6 vector and then into TG1 *E. coli* by
electroporation. The distribution of the fragments in the library was determined by PCR and sequencing of forty random clones. The distribution of the fragments in the library were determined by PCR and sequencing of forty random clones. The library was then grown in 20mls of 2xTryptone-yeast extract media containing 50 µg/ml of ampicillin at 37°C, shaking until OD 600 nm. $1 \times 10^{11}$ PFU of M13K07 helper phage was added and cultured for an extra 1 h at 37°C without shaking to allow infection. The entire culture was then transferred into 400 ml of super broth, containing 70 µg/ml kanamycin and 50 µg/ml ampicillin, and incubated at 37°C with shaking overnight. The bacteria was pelleted by centrifugation at 8,000 X g for 15 min and the phage precipitated from the supernatant with PEG-NaCl as described (Adda, Tilley, Anders, & Foley, 1999; Coley et al., 2006). The phage was resuspended in 1 ml of PBS and used subsequently or stored at -80°C.

**Panning of phage displayed DBPII gene fragment library**

Affinity panning of DBPII Sal1 gene fragment to anti-DBPII mAbs 3D10, 3C9, 2D10, 2C6 and 2D10 was carried out as described previously (Coley et al., 2001). Briefly, 10 wells of Maxisorp-Nunc ELISA plate were coated over night at 4°C with 100 µl per well of test mAb at a final concentration of 2.5 µg/ml diluted in PBS. Plates were washed twice with PBS and unbound surfaces blocked with 200 µl of 5% skimmed milk in PBS for 2 h at room temperature. After another round of washing, approximately $1 \times 10^{11}$ phage/ well in 1% skimmed milk in PBS were added to wells and incubated for 1 h at room temperature. Non-adherent phage particles were washed off twice with PBS/0.05% Tween-20. The bound
phage was eluted with 100 µl of 0.1M Glycine, pH 2.2 and immediately added to 10 ml of log phase *E. coli* TG1 cells and cultured for 30 mins at 37°C for infection to occur. Ampicillin was added to 50 µg/ml and the culture and incubated for another 30 mins at 37°C before addition of the M13 helper phage and then incubated for another 1 h at 37°C. The culture was then transferred to a 200 ml super broth containing 50 µg/ml of ampicillin and 70 µg/ml of kanamycin and incubated at 37°C overnight with shaking at 200 rpm. The phage was harvested as described above and another round of panning carried out for a total of up to four panning cycles per antibody. Ten clones from each round of panning were selected, their inserts amplified by PCR and sequenced to determine the identity of the fragments of DBPII that binds to each mAb.

**Panning a random peptide library on mAb-3D10**

Panning on mAb 3D10 was performed using a random 20mer peptide library (Courtesy of Foley M and Anders RF, La Trobe University, Australia) as described (Adda et al., 1999; Coley et al., 2001). Briefly, phage peptide library at 10¹¹ phage/well was incubated with mAb 3D10 coated wells for 1 h at room temperature. Eluted phage was allowed to re-infect K91 *E. coli* grown to log phase in 10 ml Tryptone-yeast extract media at 37°C for 1 h and then grown overnight in 200 mls of super broth with 40 µg/ml of tetracycline. Phage was harvested and further rounds of panning carried out as described above. Clones isolated from round 3 were sequenced to determine the peptide sequences.
ELISA of phage displayed rounds of panning

Enrichment ELISA was performed as described (Coley et al., 2001). Briefly, 100 µl of anti-DBP mAbs antibodies at 2.5 µg/ml in PBS were coated on to wells of 96-well microtiter plates (Maxisorp-Nunc) overnight at 4°C. Anti-PfAMA1 specific, mAb-1F9 or 5G8 (Coley et al., 2001) was coated on each plate as negative control antibody. The plates were washed twice with PBS 0.05%Tween-20 and blocked for 2 h at room temperature with 200 µl/well of 10% skimmed milk diluted in wash buffer. After three washes, phage diluted in PBS/1% milk to 1x10^{11} pfu/ml was added in triplicates and incubated for 2 hours at room temperature. Plates were washed four times and the wells were incubated with HRP-conjugated anti-M13 mAb (GE Life Sciences) for 1 h at room temperature. The plates were then washed five times and bound phage detected by incubating wells with 100 µl of H₂O₂ activated TMB substrate (Sigma). The reaction was stopped with 100 µl 2 N H₂SO₄ and plates were read at 450 nm absorbance.

Immunoblots

Approximately 1X10^{12} phage particles were boiled in SDS-PAGE sample buffer for 3 min and separated by SDS-PAGE and electrophoretically transferred on to nitrocellulose membrane. The membrane was blocked overnight in 10% skimmed milk diluted in PBS/Tween-20. The membrane was probed with anti-DBPII mAb, washed with PBS/Tween-20 before incubating with an HRP-
conjugated anti-mouse secondary antibody. The bound antibody was detected by enhanced chemiluminescence (ECL) substrate (Amersham).

**Immunizations**

Peptides corresponding to sequences of the epitopes identified from panning on the gene fragment library and mimotopes from panning on the random peptide were synthesized and conjugated to keyhole limpet hemocyanin (KLH) using Maleimide-Activated mcKLH (ThermoScientific 77611) according to the manufacturer's specifications. The following 6 peptides were produced commercially (Pacific Immunology): 3C9 epitope peptide (3C9-e1) from DBPII sub-domain 3; 3D10 epitope peptide (3D10-e1) from DBPII sub-domain 1; 3D10 mimotope 1; (3D10-m1); 3D10 mimotope 2: (3D1-m2); 3D10 mimotope 3: (3D10-m3) and a control peptide.

The conjugated peptides were used to immunize 6-8wks old Balb/c mice (Harlan) to raise immune sera. All animals were handled in compliance with approved IACUC protocol and good animal practice. Briefly, mice in all groups, (n=10) were bled for pre-immune sera and each immunized thrice at three week intervals with 5 µg of peptide conjugated to KLH emulsified in Titermax® Gold adjuvant. Each animal received a 50 µl antigen-adjuvant mix administered subcutaneously at the base of the tail. Mice immunized with KLH and adjuvant alone served as control. All mice were bled for final serum four weeks after the second boost.
Measurement of antibody titers

Total anti-peptide IgG titers in the serum of each group was evaluated by end point titration ELISA against and to recombinant DBPII Sal1 and peptide conjugated to bovine serum albumin (BSA). Imject™ Maleimide-Activated BSA Spin Kit (ThermoScientific 77667) was used as specified by manufacturer to conjugate the peptides. ELISAs were carried out as described previously (Ntumngia et al., 2012b), 0.1 µg of peptide conjugated to BSA or 0.25 µg of recombinant antigen were coated per well of a microtiter plate over night and blocked with 5% skimmed milk PBS/0.05% Tween 20 detected by 3-fold dilution of mouse sera was used starting at 1:200. Bound antibodies were detected using alkaline phosphatase-conjugated anti-mouse antibody (Kirkegaard & Perry Laboratories). Pre-immune serum, at the lowest dilution, served as background and was subtracted.

Measurement of functional inhibition of DBP-Erythrocyte binding

Pooled immune serum from each group of mice that recognized rDBPII Sal1 by ELISA was tested further for inhibition of DBPII-erythrocyte binding by a modified version of the standard in vitro COS7 cell assay (Chitnis & Miller, 1994; P. Michon et al., 2000). A panel of naturally occurring DBPII alleles were transiently expressed on the surface of transfected COS7 cells as previously described (Ntumngia et al., 2013). Cells were pre-incubated with triple-fold dilution (starting at 1:50) of pooled group sera 42 h post transfection, followed by incubation with human Duffy positive erythrocytes. Inhibition of binding by each
serum was calculated as a percentage of Duffy positive erythrocyte binding in immune versus corresponding pre-immune serum control incubated on every plate (Ntumngia et al., 2013).

**Immunofluorescence assay of COS7-surface expressed DBPII**

A few DBPII constructs with single and multiple residue mutations, in epitope residues, were produced (GenScript) on pEGFP vector used for DBPII expression on COS7 cells. COS7 cells transfected with different mutated DBPII transgenes, were incubated on cover slips in 24 well plates for 42 hr (Chitnis & Miller, 1994). The cells were then fixed with PBS/2% formaldehyde for 15 min, washed with PBS and incubated at room temperature with 5 µg/ml of anti-DBPII mAbs 3C9, 3D10 and 2D10 and anti-AMA1 mAb 1F9, as a negative control, for 90 min. After PBS wash, the cells were incubated with rhodamine-conjugated goat anti-mouse secondary antibody (KPL) in PBS/0.1%BSA for 30 min in the dark. The cells were washed with PBS and nuclear stain Hoechst at 1:1000 dilution for 15 mins in the dark. After the final wash, the cells on the coverslip was fixed onto a glass slide with flurophoreG and stored at -20°C until ready to be observed using a fluorescence microscope. Zeiss microscope was used to capture images of 10 cells at 40X magnification for each DBPII-antibody pair at constant exposure time of 88ms for blue channel, 513ms for green channel and 2049ms for red channel. Relative fluorescence unit (RFU) across a cell from end to end was collected. The highest 50 points for the red channel for each cell was used to normalize for the size of cells. The sum of the highest 50 points for 10
cells was used to determine the mean RFU for each DBPII-antibody pair. The mean RFU of DBPII mutants were compared with wild type DBPII Sal1 for the respective antibody.

RESULTS

The phagemid vector pHENH6 was used to be express DBPII as a chimera, fused with a C-terminal c-myc epitope tag and the pIII minor coat protein on the surface of the engineered pHENH6 phage (Fig.2.1). The variable length DBPII fragments came from a gene fragment library of random length PvDBP coding constructed by DNasel-digesting DNA encoding DBPII sequences (Fig. 2.2 and 2.3). Recombinant phagemid libraries for Sal1 and 7.18 that were estimated to have $7 \times 10^5$ pfu/µg and $4.6 \times 10^6$ pfu/µg of plasmid DNA gene fragments. Sequencing of 40 random clones of each library revealed that the fragments in the library spanned the entire coding sequence and no bias towards any particular region was observed (Fig. 2.4); however, as expected most of the fragments were ligated out of frame or in the incorrect orientation. Phage stocks generated from the gene fragment libraries were biopanned on mAbs 3D10, 3C9, 2D10, 2C6 and 2H2 to enrich for high affinity binders to each of these antibodies.

Affinity selection of reactive phage to the target antibody was carried out through the sequential rounds of panning by standard procedures. ELISAs were carried out with positive control mAb 9E10 to standardize library titer in each round of panning, while an anti-PfAMA1 antibody, mAb 1F9, served as a negative control (Figs. 2.5 – 2.9 A and B). Panning on mAb 3D10 selected for
phage clones that bound well to mAb 3D10, but poorly to mAbs 3C9 and 1F9 for both gene fragment libraries (Fig. 2.5 A and B). In contrast, panning on mAb 3C9 selected for phage clones from both gene fragment libraries that bound well to mAb 3C9 and poorly to mAbs 3D10 and 1F9 (Fig. 2.6 A and B). Likewise panning on mAb 2D10 (Fig. 2.7 A and B), mAb 2C6 (Fig. 2.8 A and B) and mAb 2H2 (Fig. 2.9 A and B) isolated phage clones that bound well to their respective target antibody but bound poorly to mAbs 1F9 and 3D10 for both gene fragment libraries. Phage clones isolated from panning on all the antibodies bound well to mAb 9E10, suggesting that the procedure had enriched for clones possessing DBPII coding sequence in-frame and in the correct-orientation.

Sequence analysis of 10 clones from each round of panning was used to identify the length and specificity of DBPII CDS inserts. Clones from the last rounds of panning tended to have inserts of similar sizes while those of the early rounds tended to be more variable. Sequencing results further showed that only clones in the last round of panning were in frame and in the right orientation, corroborating ELISA results. Ten clones from the last rounds of panning on mAb 3D10, had overlapping fragments of sub-domain 1 of DBPII in-frame and in the correct orientation (Fig. 2.5 C and D). Most clones from the last rounds of panning on mAb 3C9 were comprised of overlapping fragments of sub-domain 3 (Fig. 2.6 C and D). However clones from the last rounds of panning on mAb 2D10 (Fig. 2.7 C and D), mAb 2C6 (Fig. 2.8 C and D) and mAb 2H2 (Fig. 2.9 C and D) had a fragment that comprised of almost all of sub-domain 3.
Phage were cultured from two clones enriched for during round 3 panning on mAb 3D10 and three clones enriched for during round 3 panning on mAb 3C9. Each phage clone was tested for reactivity to the selecting mAb by ELISA and Western Blot (Fig. 2.10). Results from both assays indicated that mAb 3D10 phage clones bound specifically to mAb 3D10 (Fig. 2.10 A and C). Similarly, mAb 3C9 phage clones were found to bind specifically to mAb 3C9 (Fig. 2.10 B and D). The observed specificity suggested that the isolated phage clones displayed the minimal fragments of DBP II needed for the mAbs to bind their respective epitopes.

To further characterize the linear epitope for mAb 3D10, a random 20mer peptide library termed Adlib1 (courtesy of Adalta Pty Ltd.) was also used to pan on the antibody. After 3 rounds of biopanning using the approach outlined in Figure 2.1 B, the level of reactivity enrichment was assessed by ELISA. Figure 2.11 shows that phage from rounds 1, 2 and 3 react well with mAb 3D10 (black) and relatively poorly to the negative isotype control anti AMA1 mAb 5G8 (white). Individual clones from round 3 were sequenced. From 10 clones, 4 sequences were identified, each containing what may be a degenerate sequence motif (highlighted in bold underline). An apparent key feature within the 3D10 epitope is a hydrophilic 3-residue motif YK(R/Y/E). Although the Adlib1 peptide library is unrelated to DBP II, YKR is present in the DBP II sequence in the 22 amino acid region of sub-domain 1 that was selected for by the gene fragment library.

Further characterization to determine the potential immunogenicity of the minimal epitopes was pursued next. The minimal peptides identified from
panning with DBPII gene fragment libraries and the random peptide library were produced synthetically and conjugated to KLH carrier protein to produce immunize mice (Table 1). Following two boosts, ELISAs were carried out to determine reactivity of the anti-peptide sera for the minimal peptide epitopes of 3D10 and 3C9. Serum from each mouse was tested for affinity to its corresponding peptide. All peptides, with the exception of the 3D10 epitope peptide, were immunogenic. Further, the serum from each mouse was tested for affinity to rDBPII, by ELISA. Only mice immunized with 3C9 epitope peptide produced an immune response that recognized rDBPII (Fig 2.13 A).

To assess anti-3C9 epitope peptide sera for its potential to inhibit DBPII-erythrocyte binding, a standard in vitro COS7 binding inhibition assay was carried out (Fig 2.13 B). A concentration-dependent inhibition of binding of DBPII-erythrocytes was observed. Serum dilution that correspond to 50% inhibition (IC$_{50}$) of binding of erythrocytes to the DBPII alleles were 1:313.6 for Sal1, 1:309.1 for 7.18, 1:171.6 for AH and 1:52.73 for P. Binding inhibition curves of each allele was compared to Sal1 using Dunnett’s multiple comparison test. Alleles 7.18 and AH showed no significant difference compared to Sal1, suggesting that 3C9-e1 produced and antibody response that recognizes a conserved region of DBPII within these alleles.

Site directed mutagenesis was performed to substitute residues of the epitopes that were identified through biopanning (Fig 2.14). These residues are predicted to be surface exposed and reactive with the mAb. The purpose of this procedure was to validate the relative importance each residue within the epitope
may have in antibody binding. Immunofluorescence assay were performed to
determine the ability of the antibodies to bind to DBPII mutated at specific
residues expressed on the surface of COS7 cells. The relative fluorescence unit
(RFU) was used to compare binding of antibody to wild type DBPII Sal1 and
mutant.

Mutation of a single residue E538A (M20) within the 3C9 epitope
remarkably reduced binding of mAb 3C9 compared to DBPII Sal1 (Fig 2.15 A).
Mutant 6, Y445A/V446A/F530A/F535A (M6) also showed reduction in binding to
mAb 3C9. Structurally, residues E538 and F535, on the epitope, seem to interact
with residues Y445 upstream to the epitope but still in subdomain3 (Fig 2.16 A
and 2.17 C). It is interesting to note that the single mutation of F535 (Mutant 19)
had almost no difference in binding to mAb 3C9 compared to DBPII Sal1 and yet
this mutation greatly reduced binding to mAb 2D10 (Fig 2.15 B). A similar effect
is observed with the single amino acid substitution E529A (Mutant 14), which
greatly reduced binding to mAb 2D10 but no effect on mAb 3C9 binding.
Structurally, V446 seems to interact with G442 which when mutated also showed
reduction in binding (Mutant 4) to mAb 2D10 and not to mAb 3C9 (Fig 2.16 B and
2.17 D). Multiple mutations to residues Y445A, V446A, F530A and F535A
(Mutant 6) reduced binding to both mAb 3C9 and mAb 2D10.

DISCUSSION

Plasmodium vivax DBP is vital for merozoite invasion of Duffy positive
reticulocytes making it the foremost vaccine candidate against blood stage vivax
malaria (Miller et al., 1976). PvDBP is a member of the Duffy-binding-like erythrocyte binding proteins (DBL-EBPs) family (Adams et al., 1992) that includes *P. falciparum* EBA175, PfEBL1, *P. knowlesi* DBPα,β and γ among others (Adams et al., 2001). Studies of DBP have historically helped to define our understanding of the important biological properties of this important family of malaria parasite ligands. The N-terminal cysteine rich region is the ligand domain known as the DBL domain or DBP region II in *P. vivax* that is essential of receptor recognition (Adams et al., 1992; Chitnis & Miller, 1994). Intergenic variation in this region is important for altering receptor specificity while intragenic or allelic variation of the ligand domain alters antigenic character. DBP allelic variation is mainly constrained to polymorphisms in the DBL domain and varies by geographic region (Hwang, Kim, & Kho, 2009; Ju et al., 2012; Premaratne, Aravinda, Escalante, & Udagama, 2011). Some polymorphic residues on DBPII are unique to a certain geographical region while others are common among global vivax alleles, like residues K371E, D384G, E385K, K386N, N417K, L424I, W437R and I503K (Babaeekho, Zakeri, & Djadid, 2009; Hwang et al., 2009; Ju et al., 2013; Ju et al., 2012). Generally, variation occurs in non essential residues flanking residues critical for receptor recognition while variation in some residues is more important than in others. For example, residues 417, 437 and 503 were identified as critical determinants of antigenic character, since altering these residues conferred a significant change in sensitivity to inhibitory anti-DBP antibodies (VanBuskirk, Cole-Tobian, et al., 2004). Overall evidence indicates that variation plays an important role in strain-specific immunity to DBP.
DBPII is largely alpha-helical and may be assigned into three sub-domains delineated by six disulphide bonds. While sub-domain1 of DBPII is made up of an antiparallel beta-hairpin, sub-domains 2 and 3 are seen as distinct bundles of three alpha-helixes (Batchelor et al., 2011). NMR studies of PvDBPII in contact with the core region of DARC ectodomain revealed that dimerization of DBPII is required for and driven by receptor engagement as DARC ectodomain form a helix that binds to the dimer interface (Batchelor et al., 2014). Human anti-DBP inhibitory antibodies have been shown to bind to sub-domain 2 in a surface region adjacent to the dimer interface (Batchelor et al., 2014; Chootong et al., 2010). Given that these dominant Bc epitopes are at the dimer interface are not part of residues critical for either dimerization of receptor binding, anti-DBP immune efficacy appears to be primarily mediated through steric hindrance of dimerization.

Naturally acquired immunity to DBPII is present in *P. vivax* endemic areas, anti-DBPII immune response tends to increase with age and exposure (Chootong et al., 2010; J. L. Cole-Tobian et al., 2002; Grimberg et al., 2007; VanBuskirk, Cole-Tobian, et al., 2004; Xainli et al., 2003). Studies have shown that about 8-10% of *P. vivax*-exposed individuals produce high titer strain-transcending or broadly neutralizing antibodies (King et al., 2008), associated with 50% reduction in risk of infection with *P. vivax* (Chootong et al., 2010; King et al., 2008).

Concurrently, the antibody responses to DBP seen in people living in *P. vivax* endemic areas and the elevated levels of amino acid sequence polymorphisms are consistent with the hypothesis that the molecule is under immune selective
pressure, especially as the DBP polymorphisms are concentrated in the ligand domain region II, which has a nucleotide substitution rate four times higher than the rest of the molecule (Ampudia et al., 1996; J. L. Cole-Tobian et al., 2002; Fraser et al., 1997; P. A. Michon et al., 1998; Tsuboi et al., 1994). In previous studies we have identified epitopes reactive with polyclonal human immune antibodies using an array of overlapping linear peptides attached to plastic pins. This methodology proved to be surprisingly robust in identifying conformational epitopes, especially the dominant Bc epitope. This epitope termed “DEK” for its first three amino acids was confirmed subsequently to be a major target of naturally occurring human immune antibodies and a major determinant for controlling strain specific immunity elicited by vaccination (Ntumngia & Adams, 2012; Ntumngia et al., 2013). The current study sought to identify specific epitope targets of mAbs induced by vaccination in mice, using phage display technology.

Phage display technology is a great tool for elucidating the molecular nature of protein-protein interactions (Coley et al., 2001; Smith, 1985; Wilson & Finlay, 1998) and we have established the methodology for DBPII. Recombinant filamentous phage was engineered to display DBPII (SalI and 7.18 alleles) on its surface as part of the pIII capsid protein. Selective panning of recombinant phage libraries on antibodies was used to isolate target epitopes or peptide mimics. Positive epitope-containing clones reactive with the anti-DBPII inhibitory antibodies were enriched by successive panning assays. The sequence identity of the selected peptides was determined by DNA sequencing. The identified recombinant fragments and peptides represent epitope-specific reagents that
were used to evaluate reactivity of epitope-specific antibodies. A surprising outcome in this study is the discovery that Bc epitope targets of highly inhibitory anti-DBP are located in subdomain III away from the dimer interface and residues determined to be critical for erythrocyte binding. Only minimal epitope target of an inhibitory antibody could be defined whereas the minimal target of most inhibitory mAbs was the entire subdomain III (Fig 6, 7 and 8). In contrast the minimal epitope of the non-inhibitory mAb 3D10 was located in a surface region of subdomain I closer to the dimer interface.

Site directed mutagenesis studies revealed that binding inhibitory mAbs 3C9 and 2D10 are both sensitive to disruption of Y445, V446, F530 and F535 pocket present on the surface of sub-domain 3. Although F530 and F535 make up part of the 3C9 epitope identified using phage displaying DBPII gene fragment library, single amino acid mutation of these residues did not disrupt binding of 3C9. This suggests that there is some synergistic interaction of these aromatic residues that make up the 3C9 and 2D10 binding pocket on DBPII. E538 seems to have polar interactions with F535 in the epitope and Y445 in the neighboring helix, which seems to be more important for binding of mAb 3C9 than mAb 2D10. However, V446 seems to interact with G442, which is more important for binding of mAb 2D10 than mAb 3C9.

The 3C9 minimal peptide epitope was further characterized by vaccination studies to determine its potential as a subunit synthetic vaccine. The immunogenicity of the ‘protective’ 3C9 epitope was compared to the minimal peptide epitope of 3D10. Although 3D10 represented the highest titer mAb within
the anti-DBP panel created, and was reacted equally to all DBP alleles tested by ELISA, it has virtually no ability to block DBPII to erythrocytes (Ntumngia et al., 2012a).

Various studies have examined the functional efficacy of vaccine-induced anti-DBP antibodies to block DBP erythrocyte binding or inhibition of merozoite invasion of erythrocyte (Arevalo-Herrera et al., 2005; Devi et al., 2007; Grimberg et al., 2007; Ntumngia et al., 2013). As a general rule properly refolded recombinant rDBPII that is capable of erythrocyte binding activity is required for induction of inhibitory anti-DBPII antibodies. Indeed the epitope targets of the protective mAb 3C9 (conformational) and non-protective mAb 3D10 (partially linear) are consistent with this observation. In our approach the DBP peptide immunogens, consisting of the synthetic linear peptides of the 3C9 or 3D10 epitopes were conjugated to carrier KLH, were used to immunize mice. The 3D10 immunogens included mimotope peptides of epitope targets isolated from a random peptide library. Surprisingly, we found that a 3C9 epitope peptide successfully induced an antibody response reactive to the immunizing peptide, the refolded peptide as well as inhibitory to DBPII erythrocyte binding. This result suggests that the 3C9 linear peptide has some inherent structural tendency to assume its 3D conformation displayed on the surface of native DBP and is the target of inhibitory antibody. Peptides like 3C9 epitope peptide, capable of eliciting protective antibodies, may be critical for the design of a sub-unit vaccine against asexual blood stage vivax malaria.
TABLES AND FIGURES

Figure 2.1: Schematic of the different components of phage display system and biopanning for epitope mapping

Figure 2.2: Schematic of the construction of DBPII gene fragment library
Figure 2.3: DNA gels of pHEN-H6 vector and insert DBPII Sal1 and 7.18 digested to make up gene fragment library. (A) pHEN-H6 vector seen in lane 2 was digested with PstI restriction enzyme seen in lane 3 (B) Small scale digest of DBPII Sal1 with DNAseI was carried out to determine DNAseI concentration to obtain the most broad spread of fragments. (C) Lane 3 shows DBPII Sal1 and lane 4 shows DBPII Sal1 DNAseI digested (D) Lane 2 shows DBPII 7.18 and lane 3 shows DBPII 7.18 DNAseI digested. The digested vector and gene fragments were blunt-ended with Vent polymerase and ligated before being electroporated into TG1 *E. coli*.
Figure 2.4: Schematic of sequences generated from DBPII Sal1 & 7.18 gene fragment libraries. Sequencing of PCR products generated by screening 40 individual clones revealed that the gene fragments in the library spanned the entire coding sequence, with no bias towards any particular region. The different colours represent the lengths of the various gene fragments generated.
Figure 2.5: Gene fragments of DBPII Sal1 and 7.18 selected through biopanning on mAb 3D10. ELISA showing reactivity of phage clones enriched by successive panning on mAb-3D10 with gene fragment library (A) DBPII Sal1 and (B) DBPII 7.18. A pool of phage from each round of panning was tested for binding to anti-DBPII mAbs 3D10, 3C9 and the anti-c-myc epitope tag antibody mAb-9E10. The PfAMA1 specific mAb-1F9 served as a negative control antibody. The bars represent mean OD of triplicate wells and error bars indicate ±SD. Individual clones (n=10) from the last round of panning with each of the libraries were PCR amplified and sequenced. The positions of the various peptides identified are indicated in (C) and (D) for mAbs 3D10. The degenerate sequence identified from the phage clones by panning on each on the antibodies is shown.
Figure 2.6: Gene fragments of DBPII Sal1 and 7.18 selected through biopanning on mAb 3C9. ELISA showing reactivity of phage clones enriched by successive panning on mAb-3D10 with gene fragment library (A) DBPII Sal1 and (B) DBPII 7.18. A pool of phage from each round of panning was tested for binding to anti-DBPII mAbs 3C9, 3D10 and the anti-c-myc epitope tag antibody mAb-9E10. The PfAMA1 specific mAb-1F9 served as a negative control antibody. The bars represent mean OD of triplicate wells and error bars indicate ±SD. Individual clones (n=10) from the last round of panning with each of the libraries were PCR amplified and sequenced. The positions of the various peptides identified are indicated in (C) and (D) for mAbs 3C9. The degenerate sequence identified from the phage clones by panning on each on the antibodies is shown.
Figure 2.7: Gene fragments of DBPII Sal1 and 7.18 selected through biopanning on mAb 2D10. ELISA showing reactivity of phage clones enriched by successive panning on mAb-3D10 with gene fragment library (A) DBPII Sal1 and (B) DBPII 7.18. A pool of phage from each round of panning was tested for binding to anti-DBPII mAbs 2D10, 3D10 and the anti-c-myc epitope tag antibody mAb-9E10. The PfAMA1 specific mAb-1F9 served as a negative control antibody. The bars represent mean OD of triplicate wells and error bars indicate ±SD. Individual clones (n=10) from the last round of panning with each of the libraries were PCR amplified and sequenced. The positions of the various peptides identified are indicated in (C) and (D) for mAbs 2D10.
Figure 2.8: Gene fragments of DBPII Sal1 and 7.18 selected through biopanning on mAb 2C6. ELISA showing reactivity of phage clones enriched by successive panning on mAb-3D10 with gene fragment library (A) DBPII Sal1 and (B) DBPII 7.18. A pool of phage from each round of panning was tested for binding to anti-DBPII mAbs 2C6, 3D10 and the anti-c-myc epitope tag antibody mAb-9E10. The PfAMA1 specific mAb-1F9 served as a negative control antibody. The bars represent mean OD of triplicate wells and error bars indicate ±SD. Individual clones (n=10) from the last round of panning with each of the libraries were PCR amplified and sequenced. The positions of the various peptides identified are indicated in (C) and (D) for mAbs 2C6.
Figure 2.9: Gene fragments of DBPII Sal1 and 7.18 selected through biopanning on mAb 2H2. ELISA showing reactivity of phage clones enriched by successive panning on mAb-3D10 with gene fragment library (A) DBPII Sal1 and (B) DBPII 7.18. A pool of phage from each round of panning was tested for binding to anti-DBPII mAbs 2H2, 3D10 and the anti-c-myc epitope tag antibody mAb-9E10. The PfAMA1 specific mAb-1F9 served as a negative control antibody. The bars represent mean OD of triplicate wells and error bars indicate ±SD. Individual clones (n=10) from the last round of panning with each of the libraries were PCR amplified and sequenced. The positions of the various peptides identified are indicated in (C) and (D) for mAbs 2H2.
Figure 2.10: Specificity of isolated phage clones with mAb-3D10 and 3C9. Phage clones identified from round 3 biopanning on mAb 3D10 (C4 and C5) and mAb 3C9 (C4, C6, C9) as well as phage clones expressing sub-domain 1 (sd1) and sub-domain 3 (sd3) fragments of DBPII were tested for cross reactivity with the homologous and heterologous antibodies by ELISA (A and B) and immunoblot analysis (C and D). mAb-3D10 binds specifically to clones C4 and C5 and the sd1 expressing clones, while mAb-3C9 binds only to mAb-3C9 isolated clones and sd3 expressing clones. mAb-1F9 is a non-specific anti-DBPII antibody used as negative control and mAb-9E10 is specific to the c-myc epitope of the phagemid. Each bar represents the mean OD450 of triplicate wells and error bars represent ±SD.
Figure 2.11: Panning a random peptide on mAb-3D10. (a) ELISA showing reactivity of phage clones from rounds of panning on mAb-3D10. mAb-5G8 served as a negative control antibody, while a mAb-5G8 positive binding phage clone (Pos C) served as a positive control. The bars represent mean of triplicate wells while the error bars represent ±SD. (b) Alignment of a sequence of the mAb-3D10 binding epitope on sd1 of DBPII (top) and sequences of three mimotopes (M1, M2 and M3) from random peptide library with affinity for mAb-3D10. The underlined residues show a three amino acid motif common to the DBPII epitope and the sequences from the mimotopes isolated form the random peptide library.
Figure 2.12: Putative epitopes of mAbs 3C9 and 3D10. Crystal structure of DBPII dimer with sub-domains 1 (in green), 2 (in tan) and 3 (in light blue). Putative epitope of inhibitory mAb 3C9 (in red) is on sub-domain 3 while putative epitope of non-inhibitory mAb 3D10 (in navy blue) is on sub-domain 1. The two views represent the front and back of the ligand for the surface model (top) and for secondary structure cartoon (bottom).

Table 2.1: Peptides identified through biopanning used for immunizations.

<table>
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<th>Name</th>
<th>Peptide Sequence</th>
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<tr>
<td></td>
<td></td>
<td>Peptide</td>
</tr>
<tr>
<td>3C9-e1</td>
<td>DILKQELDEFNEVAFENE</td>
<td>+</td>
</tr>
<tr>
<td>3D10-e2</td>
<td>IINHAFLQNTVMKNCNYKRKRR</td>
<td>+</td>
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<td>VKFTDRKYSSMKGYARQGR</td>
<td>+</td>
</tr>
<tr>
<td>3D10-m3</td>
<td>KINMYKEVRTRQLSVRPSPE</td>
<td>+</td>
</tr>
<tr>
<td>Control</td>
<td>TPDERYRELDSHAQNESCV</td>
<td>+</td>
</tr>
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</table>

Peptides were conjugated to KLH and used to immunize groups of ten BALB/c mice. Following two boosts sera was collected. Sera was tested for antibodies to corresponding peptides and to rDBPII Sal1 by ELISA.
Figure 2.13: Anti 3C9-e1 sera characterization. (A) Mice immunized with 3C9-e1 produced an antibody response that recognized rDBPII by ELISA. The numbers for each curve correspond to sera from a mouse. Each point on the curve represent mean OD of triplicate wells and error bars indicate ±SD. (B) Pooled group sera was used to test for erythrocyte-DBPII binding inhibition by *in vitro* COS7 assay. The curves correspond to the different alleles of DBPII that were tested. Each point on the curve represent percent inhibition of two experiments each with triplicate wells and error bars indicate ±SD.

<table>
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<td></td>
<td></td>
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</tr>
<tr>
<td>M2</td>
<td>V446A/L527A</td>
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</tr>
<tr>
<td>M3</td>
<td>Y455A/L527A</td>
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</tr>
<tr>
<td>M6</td>
<td>Y445A/V446A/F530A/F535A</td>
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Figure 2.14: Amino acid sequence alignment of DBPII Sal1 to mutants. Site directed mutagenesis of DBPII Sal1 with alanine substitutions at specific sites depicted in red.
Figure 2.15: Immunofluorescent assay of DBPII mutants expressed on COS7 cells. Fluorescence images of 10 cells for each DBPII-antibody pair was captured. Mean pixel intensity for each DBPII-antibody pair was determined. Each bar represents mean pixel intensity compared to DBPII Sal1 and error bars indicate ±SD.
Figure 2.16: Crystallographic representation of residues on DBPII important for antibody binding. (A) Residues important for binding to mAb 3C9, in red single substitution of mutant 20 and in blue multiple substitutions of mutant 6. (B) Residues important for binding to mAb 2D10, in blue multiple substitutions of mutant 6, in red single substitution of mutant 19 (also a part of mutant 6), single substitution of mutant 4 and 14 in green and purple.
Figure 2.17: Crystallographic representation of putative mAbs 3C9 and 2D10 binding sites on DBPII. (A) Cartoon representation of DBPII monomer in yellow and 3C9 epitope in blue. (B) Cartoon representation of 3C9 epitope with residue side chains as line representation. (C) Polar interactions in green that seem important for mAb 3C9 binding. (D) Polar interactions in green that seem important for mAb 2D10 binding.

ACKNOWLEDGMENTS

Jesse L. Schloegel produced DBPII Sal1 library and panning on mAbs 3D10 & 3C9, Joanne L. Casey carried out panning on mAb 3D10 using random peptide library, Francis B. Ntumngia, Samantha B. Jones, Michael Foley and John H. Adams contributed to planning the work carried out.
REFERENCES


CHAPTER THREE:
RANDOMIZED HELICAL SCAFFOLD AFFIBODY LIBRARY TO MAP
CONFORMATIONAL EPITOPES ON *PLASMODIUM VIVAX* DUFFY BINDING PROTEIN

ABSTRACT

*Plasmodium vivax* Duffy binding protein (DBP) is an essential ligand for reticulocyte invasion making it a premier asexual blood stage vaccine candidate. However, as DBP is highly polymorphic in its ligand domain and prone to elicit an immune response that is strain specific. Vaccine design studies have mapped Bc epitope targets of inhibitory and non-inhibitory antibodies to characterize conserved epitopes that could be used to over come the challenges of allelic variation. Since DBP region II is mostly made up of alpha-helices, we used a randomized helical scaffold library, the Affibody library, displayed on M13 phage, to determine conformational epitopes. Affibody libraries, an engineered affinity protein scaffold have been previously used to select for high affinity binders to receptors or enzymes. The novelty of this study is in the use of this randomized helical scaffold library to display conformational epitopes.

INTRODUCTION

*Plasmodium vivax* has the widest geographical distribution and is the
second most prevalent of the five *Plasmodium* species that cause malaria in humans ("World Malaria Report 2014," 2014). Although historically called 'benign tertian malaria' because it is less virulent than *P. falciparum* (Garnham, 1951), when trying to eliminate malaria, *P. vivax* is often the 'last parasite standing' (Tatem et al., 2010). Complicating elimination of vivax malaria is the added obstacle of reactivation or relapse of active blood-stage infections from dormant liver stages, referred to as hypnozoites, that occurs several weeks to years after the first infection by bite of a mosquito (Krotoski, 1985). Clearance of these dormant liver stages requires the use of the 8-aminoquinoline drug primaquine because most drugs effective against blood-stages of *P. vivax* have no effect on hypnozoites. More recently there have also been a rise in the number of severe cases of vivax malaria (Alexandre et al., 2010; Kochar et al., 2009), widespread drug resistance (Baird, Basri, et al., 1991; Price et al., 2014; Rieckmann et al., 1989) including strains with low sensitivity to primaquine (Baird & Hoffman, 2004). There is an urgent need to develop a vaccine to control and prevent vivax malaria.

Naturally acquired immunity to malaria is largely associated with antibody response to blood stage infection, specifically against proteins involved in the invasion of erythrocytes (Marsh & Kinyanjui, 2006; Yazdani et al., 2006). Consistent with this view, many of the parasite antigens considered as targets of this protective immunity display the characteristic patterns of polymorphisms of residues under selective immune pressure. Duffy binding protein is a prime example of such blood-stage target, exhibiting extensive variation in residues of
its functional ligand domain. *Plasmodium vivax* almost exclusively invades Duffy positive red blood cells, although recently there have been a few unexplained sporadic cases of vivax among Duffy negative individuals (Cavasini et al., 2007; Menard et al., 2013; Ryan et al., 2006). The exclusivity of *P. vivax* is attributed to the Duffy binding protein on the merozoite which binds irreversibly to its cognate receptor, the Duffy antigen receptor for chemokines (DARC), on the erythrocyte, forming the junction critical for invasion (Adams et al., 1990; Adams et al., 1992; Chitnis et al., 1996; Miller et al., 1976; Miller et al., 1975; Wertheimer & Barnwell, 1989). Hence DBP region II (DBPII), essential for binding to DARC (Chitnis & Miller, 1994; Ranjan & Chitnis, 1999; VanBuskirk, Sevova, et al., 2004), is a prime asexual blood stage vaccine candidate.

As a result of the highly polymorphic nature of DBPII (Ampudia et al., 1996; Tsuboi et al., 1994; Xainli et al., 2000), naturally acquired immunity is strain specific (J. L. Cole-Tobian et al., 2009; McHenry et al., 2011; Ntumngia et al., 2012b; VanBuskirk, Cole-Tobian, et al., 2004). In pre-clinical mouse studies, a multi-allele DBPII vaccine was more immunogenic and produced a stronger strain neutralizing response than a single-allele vaccine (Ntumngia et al., 2013). To overcome the inherent strain specificity of the immune response to DBPII, a novel synthetic DBPII antigen called DEKnull, was designed. This was derived by mutating the residues of an immunodominant polymorphic B-cell epitope within DBPII, referred to as the “DEK” epitope. Compared to its natural parent allele Sal1, DEKnull vaccine was less immunogenic but produced an immune response that was similar to a single natural allele vaccine (Ntumngia & Adams, 2012;
Ntumngia et al., 2013), suggesting that the immune response was to one or more conserved epitopes. These studies prove that identifying epitopes on DBPII will be very helpful in designing a vaccine that produces strain-transcending immunity.

Almost since the discovery of phage display as a tool, epitope mapping studies have been conducted to help design a vaccine against malaria (de la Cruz, Lal, & McCutchan, 1988; Parmley & Smith, 1989). Although an epitope may only be a few residues, many epitopes are composed of discontinuous residues that come together to create a 3D conformation-dependent epitope of the protein that is crucial for optimal antibody binding, rendering gene fragment libraries and random peptide libraries less than ideal to elucidate all critical residues that make up an epitope. Nonetheless as demonstrated by the studies presented in Chapter 2 it is possible for even linear synthetic peptides to induce some level of inhibition to a native protein dependent on its 3D structure for function. Therefore, providing some additional structural support via a protein scaffold for presentation of DBPII epitopes may enhance immunogenicity and functional efficacy of vaccine-elicited antibodies to inhibit DBP function.

Non-immunoglobulin affinity protein scaffolds is a growing field marked with varied polypeptide folds that are highly stable, easily produced and dotted with various residue positions that can be randomized. One type of helical scaffold library, Affibodies, engineered from Staphylococcal protein A (Fig 3.1), is similar to Monobodies, a bête scaffold engineered from human fibronectin. Affibodies have been used to select for receptors or enzymes with affinities
(dissociation constant $K_D$) in the 10µM to 3nM range (Gunneriusson et al., 1999; Hansson et al., 1999; Nord et al., 1997; Nord et al., 2001). Here we propose that these protein scaffold libraries could also be used to identify conformational epitopes reactive with inhibitory anti-DBPII mAbs.

Crystallography studies reveal that DBPII is largely alpha-helical and may be assigned into three sub-domains delineated by six disulphide bonds (Batchelor et al., 2011). While sub-domain 1 of DBPII is made up of an antiparallel beta-hairpin, sub-domains 2 and 3 are seen as distinct bundles of three alpha-helixes. Inhibitory human and murine mAbs have been shown to bind to sub-domain 2 (Chootong et al., 2010) and sub-domain 3 (presented in Chapter 2), respectively. This study has sought to use an alternate alphahelical scaffold as a tool for isolation of DBPII structural epitopes. An affibody library (Nord et al., 1997), representing a randomized, three alpha-helical bundle structure, was used to screen for conformation-dependent epitope mimics reactive with the murine anti-DBPII mAbs. The purpose of the study was to determine if a rigid scaffold, such as the Affibody, could provide an alternate phage system for identifying and characterizing mimotopes of DBPII.

MATERIALS AND METHODS

Biotinylation of antibodies

Murine mAbs 3D10, 3C9, 2D10 and 1DK of IgG isotype IgG1 and κ light chain were used. Biotinylation of target mAbs 3D10, 3C9 and 2D10 were carried
out using N-Hydroxysuccinimide (NHS) activated biotin with EZ-Link Sulfo-NHS-LC-Biotin (Thermo Scientific 21327) following manufacturer’s instructions at 20 molar fold excess biotin followed by dialysis. The level of biotinylation was determined by evaluation of binding to streptavidin-coated beads as seen on a 10% SDS PAGE gel. Monoclonal antibody 1DK was left unbiotinylated and was used as a control.

**Affibody selections**

The Affibody library (naïve library pAffi100-tryp designed by PÅ 2013) on the phagemid vector pKN1 (Nord, Nilsson, Nilsson, Uhlen, & Nygren, 1995) was grown in *E. coli* strain RR1ΔM15 (supE) (Ruther, 1982). Phage stocks were prepared using M13K07 helper phage. Washed neutravidin magnetic particles (Spherotech NVM-20-5) and Streptavidin beads (Invitrogen Dynabeads M-280) were incubated with excess biotinylated target mAbs for 1 h at room temperature with end-over-end rotation. The beads were then washed and stored at 4°C to be used alternatively through the round of panning. The panning procedure was carried out as described previously (Hansson et al., 1999; Nord et al., 1997) over four rounds with an increasing number of PBST (PBS with 0.05% Tween 20) washes (2, 5 and 6 more washes). Briefly, Affibody library of approximately $10^{11}$ phage forming units in 500 µl was first subjected to negative pre-selection by incubation with neutravidin magnetic particles, 100 nM mAb1DK and 3% BSA for 1 h on a rotator at room temperature. Unbound phage particles were then transferred for selection to target mAb on neutravidin magnetic particles, by
incubating on a rotator for 1 h at room temperature and then washed with PBST. Bound phage particles were eluted for 10 min with 500 µl of Glycine HCl, pH 2.7 and immediately neutralized with 50 µl of TrisHCl, pH 9. The eluted phage was used to infect log phase RR1ΔM15 E. coli grown in 100 ml of Tryptic Soy Broth supplemented with 20% Glucose for 1 h at 37°C. Helper phage was added and incubated for 30 min at 37°C. The cells were pelleted and resuspended in 100 ml Tryptic Soy Broth Yeast Extract media containing with 0.1M IPTG, 100 µg/ml Ampicillin and 25 µg/ml Kanamycin, grown over night at 30°C with shaking. Phage particles were isolated using standard polyethylene glycol precipitation and the concentration was determined for the subsequent round of selection.

**ELISA screen of clones**

Phage clones from the eluate of round four panning were randomly picked and grown in 1 ml of Tryptic Soy Broth Yeast Extract media with 0.1M IPTG and 100 µg/ml Ampicillin for 24 h at 30°C with shaking. Affibody present in the periplasmic membrane was extracted in PBST by repeated freeze-thaw cycles. ELISA plates were coated with 5 µg/ml of human serum albumin over night at 4°C. PBST wash was followed by incubation of 100 µl of periplasmic content from each clone for 2 h at room temperature. Unbound periplasmic content was then washed off with PBST and 5 µg/ml of the primary mAb in 0.5% casein or 1% milk in PBST was added and incubated for 1 h at room temperature. This was followed by addition of secondary goat anti-mouse horseradish peroxidase conjugated antibody at 1:5000 dilution. Absorbance was read at 450nm.
Sequencing

Colonies from round 4 of panning that produced a strong response by ELISA was sequenced. Colony PCR was carried out using primers 5’-CACACAGGAAACAGCTATGACCAT-3’ and 5’-CTATCAGTGCTTTTACACCTTCAAAG-3’. The PCR product was purified by ethanol precipitation and sequenced using BigDye Terminators 3.1 (Applied Biosystems) with cycle sequencing DNA methodology on a MegaBACETM 1000 instrument (Amersham Biosciences).

RESULTS

Anti-DBPII murine mAbs 3C9, 2D10 and 3D10 were biotinylated and then affinity selected on neutravidin and streptavidin beads (Fig 3.2). As a control, a biotinylated human IgG mAbs was also allowed to bind to both kinds of beads. All mAbs bound well to each of the beads and excess is seen in the supernatant, suggesting that the beads were saturated. Gels show two distinct bands for the heavy chain and the light chain. A third bovine serum albumin (BSA) band is seen in wells loaded with streptavidin beads as these beads were stored with BSA (Fig 3.2 B). These purified avidin conjugated-mAbs were used to capture phage displayed affibodies that have an affinity to the mAb.

After four rounds of panning of the Affibody library on each monoclonal antibody we analyzed individual clones. Periplasmic extract from 96 clones from
round four for each mAb panned on, were screened for affinity to the target mAb and another mAb as a control for background (Fig 3.3). In order to account for non-specific binding, only clones that showed greater affinity to the target mAb (in black) compared to the control mAb (in gray) were considered for sequencing. Conformation-independent mAb 3D10 (Fig 3.3 A) has fewer clones that bound with affinity compared to conformation dependent mAb 3C9 (Fig 3.3 B) and mAb 2D10 (Fig 3.3 C). DNA sequencing results form clones identified show that there are few clones that have picked up variant residues at the central Isoleucine 31 residue (Fig 3.4, 3.5 and 3.6).

**DISCUSSION**

The DBPII is primarily composed of alpha-helices with an antiparallel beta-hairpin near the N-terminus (Batchelor et al., 2011). This elongate boomerang shaped structure can be further divided into sub-domains delineated by disulphide bonds. Human inhibitory mAb epitopes have been mapped to the three helix bundle structure of sub-domain 2 (Chootong et al., 2010) while inhibitory murine mAbs bind to sub-domain 3 (presented in chapter 2) which is also made up of a three helix bundle. As a proof of concept experiment, the use of helical scaffold Affibody library to identify epitopes of murine mAbs that vary in both binding inhibition of DBPII-erythrocyte and conformation dependents of DBPII. Similar anti-idiotypic studies on HIV-1 Env-specific antibodies were carried out using the 10th domain of human fibronectin (FNfn10) as an engineered scaffold to generate Monobodies that mimicked both linear epitopes.
(mAbs 2F5 and 4E10) and conformational epitopes (b12 and VRCO1) (Sullivan et al., 2013).

Staphylococcal protein A is widely used in antibody purification and detection (Uhlen, Forsberg, Moks, Hartmanis, & Nilsson, 1992). Protein A has repetitive structures consisting of small individually folded, highly stable alpha-helical domains that specifically binds to the Fc region of IgG from different species (Moks et al., 1986; Samuelsson, Jonasson, Viklund, Nilsson, & Uhlen, 1996; Samuelsson, Moks, Nilsson, & Uhlen, 1994; Stahl & Nygren, 1997). The 58-residue B domain, engineered with a single amino acid mutation for increased chemical stability (Protein Z), was further determined to be tolerant to multiple random amino acid substitutions at the Fc binding surface (Cedergren, Andersson, Jansson, Uhlen, & Nilsson, 1993; Jendeberg et al., 1995; Nilsson et al., 1987; Nord et al., 1995) giving rise to the Z domain libraries (Nord et al., 1997) later to be called Affibodies library (Hansson et al., 1999). Using M13 phage these libraries could be displayed as a fusion protein and be used for affinity selection or biopanning (Nord et al., 1995). This combinatorial library was first randomized at 13 residues distributed across helix 1 and 2 (Fig 3.1). Initially it was thought that Isoleucine at position 31 on helix 2 is important for helix-helix packing and was not included as a residue to be randomized. However, it was later determined that because of its central position on the randomized region, substitution of this residue may provide useful information about binding sites that may use a variant residue at the central position of Ile31.
Selection of Affibodies showing anti-idiotypic characteristic has been well established (Eklund, Axelsson, Uhlen, & Nygren, 2002). Crystal structure of a complex of an in vitro selected Affibody bound to protein Z reveals similar properties to antibody-antigen complex (Hogbom, Eklund, Nygren, & Nordlund, 2003). This study of an artificially evolved protein–protein complex of two globular proteins is similar to Ab-antigen complex marked by the complex burring at 1,665 Å² and the nine H bonds. However the rigidity of the three helix structure compared to the loops that form the complementary determining region of Abs and the lack of a buried water molecule in the Affibody complex, suggests that the interaction is more complementary than the typical Ab-antigen complexes. Hence these studies show that the Affibody can form a complex with a protein similar to that of an antibody, interacting with similar residues, and that the Affibody–protein complex can be selected for in vitro. We use these findings as the bases to study the interaction of anti-DBP antibodies to the helical structure of DBPII using the Affibody library.

Conformation dependent anti DBPII mAbs 3C9 (IC50 = 0.19 and 0.16 µg/ml) and 2D10 (0.35 and 0.18 µg/ml) showed differential inhibitory responses to the DBPII alleles 7.18 and Sal1, respectively, expressed on COS7 cells (Ntumngia et al., 2012b). This suggests that either these antibodies might be binding to different epitopes within the different alleles or that the polymorphisms in DBPII sub-domain 3 has significant influence on functional activity of these antibodies. While both mAbs 2D10 and 3C9 were produced in mice immunized with DBPII 7.18 allele, 2D10 showed almost 2 fold increase in inhibition
compared to Sal1 allele. Conformation independent non-inhibitory mAb 3D10 binds to recombinant refolded DBPII with significantly greater affinity (~5 fold) than denatured rDBPII.

The Affibody library used in this study not only had the original 13 randomized residues, on helix 1 and 2, involved in the Fc-binding activity but also Ile31 was randomized to include Asp, His, Lys and Tyr, each at 10%, at position 31. It is interesting to note that several of the Affibody clones that bound well to the target antibody had one of these substitutes for Isoleucine at position 31.

Amino acid sequence alignment of 3C9 epitope on DBPII (presented in Chapter 2) along with round 4 clones from panning on mAb 3C9 suggests that a good portion of non-randomized region of the Affibody helix is conserved in the 3C9 epitope. Hence making it increasingly difficult to identify residues of importance. MAb 2D10 also seems to bind a region very close to the 3C9 epitope on DBPII (presented in Chapter 2) and clones from round 4 panning also align well to the C terminal region of DBPII subdomain3.

Competition ELISAs and site directed mutagenesis studies need to be carried out to determine essential residues. More in-depth structural alignment studies of DBPII and Affibody clones that were identified may be required to draw conclusions on the residues that are most essential for the mAbs 3C9 and mAb 2D10. Another round of panning may also help identify clones that bind with affinity affinities K_D in the μM to nM range to determine essential residues that make up the helical structure of the epitope.
Figure 3.1: Affibody crystal structure and sequence. Crystal structure of an Affibody (pdb1h0t) with randomized regions shown as sticks in peach and Ile31 in green (Wahlberg et al., 2003).
**Figure 3.2:** SDS-PAGE gel of biotinylated antibodies on neutravidin and streptavidin beads. Biotinylated antibodies incubated with Neutravidin beads (A) and streptavidin beads (B) were run on 10% reducing gel as antibody bound beads (b) and supernatant (s) along with protein ladder (L). Distinct bands for heavy (<66kDa) and light chains (30kDa) can be seen on both gels. A third BSA band (66kDa) is seen in wells with streptavidin beads as BSA was in the storage buffer.

**Figure 3.3:** ELISA on clones from Round 4 panning on murine mAbs. Affibody from periplasmic content of clones from the last round of panning were tested for affinity to its respective antibody as well as a control antibody. Clones that showed higher binding to mAb 3D10 (in A), mAb 3C9 (in B) and mAb 2D10 (in C) compared to the control antibody were studied further.
Figure 3.4: Amino acid sequence alignments for Round 4 panning on mAb 3C9. Clones from round 4 were aligned to 3C9 epitope on DBPII. Highlighted in gray are the amino acid residues that were conserved in the Affibody helix.
Figure 3.5: Amino acid sequence alignments for Round 4 panning on mAb 2D10. Clones from round 4 were aligned. Highlighted in gray are the amino acid residues that were conserved in the Affibody helix.

Figure 3.6: Amino acid sequence alignments for Round 4 panning on mAb 3D10. Clones from round 4 were aligned. Highlighted in gray are the amino acid residues that were conserved in the Affibody helix.
ACKNOWLEDGMENTS

Francis B. Ntumngia, Johan Seijsing, Per-Åke Nygren and John H. Adams contributed to planning the work presented here. Feifan Yu for preparing the Affibody library

REFERENCES


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Malaria causes a huge burden of disease globally, with about 3.3 billion people at risk of being infected ("World Malaria Report 2014," 2014). *Plasmodium vivax* is the second most common cause of human malaria. *Plasmodium vivax* preferentially invades reticulocytes (Butcher et al., 1973; Mons, 1990) with Duffy blood group antigen otherwise known as Duffy antigen receptor for chemokines (DARC) (Miller et al., 1976). *Plasmodium vivax* Duffy binding protein (DBP), expressed on the merozoite surface just in time for invasion, binds to its cognate receptor DARC on the reticulocyte surface forming an irreversible junction, critical for merozoite invasion (Adams et al., 1990; Adams et al., 1992; Chitnis et al., 1996; Miller et al., 1976; Miller et al., 1975; Wertheimer & Barnwell, 1989). *Plasmodium vivax* almost exclusively use DARC as a receptor to invade reticulocytes, however, more recently, there have been a few *P. vivax* cases reported among Duffy negative individuals (Cavasini et al., 2007; Menard et al., 2013; Ryan et al., 2006), this is rare and an alternate receptor-ligand invasion pathway is unknown. Hence PvDBP remains a prime candidate for vaccine-induced immunity against asexual blood stage *P. vivax* infection.

The cysteine rich DBP region II (DBPII) that constitutes the principal determinants for receptor recognition (Adams et al., 1992; Chitnis & Miller, 1994;
Ranjan & Chitnis, 1999; VanBuskirk, Sevova, et al., 2004) is highly polymorphic (Ampudia et al., 1996; Tsuboi et al., 1994; Xainli et al., 2000), in a pattern consistent with high immune selection pressure on DBPII (Baum et al., 2003; Baum et al., 2002). Residues critical for receptor recognition are flanked by polymorphic residues that are not important for binding to the receptor (Batchelor et al., 2011; VanBuskirk, Sevova, et al., 2004; Xainli et al., 2000). These polymorphisms are a challenge to developing a vaccine since they contribute to anti-DBP strain-specific in naturally acquired immunity (J. L. Cole-Tobian et al., 2009; McHenry et al., 2011; Ntumngia et al., 2012b; VanBuskirk, Cole-Tobian, et al., 2004).

To overcome the inherent tendency for anti-DBP immune responses to be strain specific, a novel synthetic DBPII called DEKnull was produced. The B-cell immunodominat and polymorphic residues of the DEK epitope (Chootong et al., 2010) were mutated in DEKnull. Compared to the natural parent allele Sal1, DEKnull vaccine was less immunogenic but it produced an immune response, in mice, that was similar to a single natural allele vaccine (Ntumngia & Adams, 2012; Ntumngia et al., 2013). These studies prove that identifying conserved protective epitopes on DBP will be very helpful in designing a vaccine that produces a broadly neutralizing, strain-transcending immunity.

Previously, we generated and immunologically characterized a panel of anti-DBPII murine mAbs. Some of these mAbs were broadly inhibitory like mAbs 3C9, 2D10, 2C6 and 2H2 while mAb 3D10 was a poor inhibitor to DBPII-erythrocyte binding in a standard in vitro COS 7 cell assay (Ntumngia et al.,
2012b). With the exception of mAb 3D10, all the mAbs specifically recognized only conformational dependent epitopes on recombinant DBPII.

Phage display has been a useful tool for epitope mapping with the advantages of rapidly producing refolded protein, fused to phage coat; forming a traceable link back to the genotype (Smith, 1985; Wilson & Finlay, 1998). Crystallography studies reveal that DBPII is largely alpha-helical and may be assigned into three sub-domains delineated by six disulphide bonds (Batchelor et al., 2011). Using phage display we have previously shown that, each of the sub-domains or natural combinations of the sub-domains of DBPII have varying patterns of affinity to these mAbs (Siddiqui et al., 2012). These data suggest that identifying the epitopes of these inhibitory and non-inhibitory mAbs will help design a strain-transcending subunit vaccine.

In this study, we further map the epitopes of these anti-DBPII mAbs by screening DBPII (Sal1 and 7.18) gene fragment libraries using phage display. After three to four rounds of affinity selection to each of the mAbs, DNA sequence from bacterial clones of the last round the minimal reactive peptide fragments were identified. Interestingly, a region on subdomain 3 was the epitope on DBPII for broadly inhibitory mAbs 3C9, 2D10, 2C6 and 2H2 while non-inhibitory mAb 3D10 epitope was on subdomain 1.

Further, panning using a random peptide library or a random scaffold library and site directed mutagenesis was used to determine the relative importance of residues within the identified epitope region. Panning using a random 20-mer peptide library to define the minimal epitope of conformation
independent mAb 3D10 identified the ‘YKR’ region common between the DPBII epitope and round 3 mimotopes. A random scaffold library was also used to study the epitopes of conformation dependent mAbs 3C9 and 2D10. Further analysis of structural and amino acid sequence alignments needs to be carried out.

Site directed mutagenesis studies revealed that multiple mutations to residues Y445A, V446A, F530A and F535A reduced binding to both mAb 3C9 and mAb 2D10 suggesting that there is some synergistic interaction of these aromatic residues that make up the 3C9 and 2D10 binding pocket on. E538 seems to have polar interactions with F535 in the epitope and Y445 in the neighboring helix, which seems to be more important for binding of mAb 3C9 than mAb 2D10. However, V446 seems to interact with G442, which is more important for binding of mAb 2D10 than mAb 3C9.

Peptides identified from the gene fragment library panning and random peptide panning were conjugated to carrier protein KLH and used to immunize mice. All groups of mice produced an immune response to the respective peptide, determined by ELISA. However, only 3C9 epitope peptide elicited antibodies that recognized rDBPII. Using the standard in vitro COS7 assay a concentration-dependent inhibition of DBPII-erythrocyte binding was observed using anti-3C9 epitope sera. Binding inhibition curves for alleles 7.18 and AH showed no significant difference compared to Sal1. These results show promise that the 3C9 linear peptide may be inherently able to assume its 3D conformation as on native DBP and it is a target of binding inhibitory antibody.
These results from this study contribute to our understanding of the specific targets for vaccine-elicited protective immunity. In particular, this study demonstrates an approach that may be successful in boosting antibody responses targeted against conserved protective epitopes, with functional inhibition against broader allelic variants and diverse *P. vivax* strains.

REFERENCES


APPENDIX 1: IACUC APPROVAL LETTER

MEMORANDUM

TO: John Adams, PhD
FROM: Institutional Animal Care & Use Committee
Division of Research Integrity & Compliance
DATE: 4/2/2013
PROJECT TITLE: Evaluation of PvDBP-RII Immunogen for Immunogenicity and Protective Efficacy toward the Development of an anti-DBP Vaccine against Plasmodium vivax
FUNDING SOURCE: National Institutes of Health; Science Applications International Corporation (SAIC); Bill and Melinda Gates Foundation

IACUC PROTOCOL #: R800000087
PROTOCOL STATUS: APPROVED

The Institutional Animal Care and Use Committee (IACUC) reviewed your application requesting the use of animals in research for the above-entitled study. The IACUC APPROVED your request to use the following animals in your protocol for a one-year period beginning 4/2/2013:

Mouse: BALB/c (6wk/25-28g/female) 300

Please take note of the following:

- IACUC approval is granted for a one-year period at the end of which, an annual renewal form must be submitted for years two (2) and three (3) of the protocol through the eIACUC system. After three years all continuing studies must be completely re-described in a new electronic application and submitted to IACUC for review.

- All Comparative Medicine pre-performance safety and logistic meetings must occur prior to implementation of this protocol. Please contact the program coordinator at compmed@research.usf.edu to schedule a pre-performance meeting.

- All modifications to the IACUC-Approved Protocol must be approved by the IACUC prior to initiating the modification. Modifications can be submitted to the IACUC for review and approval as an Amendment or Procedural Change through the eIACUC system. These changes must be within the scope of the original research hypothesis, involve the original species and justified in writing. Any change in the IACUC-approved protocol that does not meet the latter definition is considered a major protocol change and requires the submission of a new application.

- All costs invoiced to a grant account must be allocable to the purpose of the grant. Costs allocable to one protocol may not be shifted to another in order to meet deficiencies caused by overruns, or for other reasons. Rotation of charges among protocols by month without establishing that the rotation schedule credibly reflects the relative benefit to each protocol is unacceptable.
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