Association between Antibody Responses to Blood Stage Parasitic Antigens and Protection from *Plasmodium falciparum* and *Plasmodium vivax* Malaria in Timika, Indonesia

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Abstract

Malaria is a major health problem causing over 212 million clinical cases every year around the world. In Indonesia, Timika is one of the highest malaria-endemic areas with a high annual parasite incidence (876 per 1,000 people per year) and high co-endemicity of *P. falciparum* as well as *P. vivax*. Moreover, a high rate of antimalarial drug resistance has also been reported in Timika, illustrating the need for developing other therapeutic tools such as vaccines to prevent disease.

Clinical immunity to malaria only develops after many years of constant exposure to the parasites. This form of protection does not result in sterilising immunity but prevents clinical cases by significantly reducing parasite density. Naturally acquired immunity predominantly targets blood-stage parasites and is known to require antibody responses, but despite the key role that antibodies play in protection the antigenic targets of immunity are not completely defined.

The aim of this study was to investigate associations between antibody responses and protection from symptomatic malaria in a field study conducted in Timika to identify targets of naturally acquired immunity to malaria. Naturally acquired immunity to seven *Plasmodium falciparum* and one *Plasmodium vivax* merozoite-stage vaccine candidate antigens were analyzed by ELISA, parasite growth inhibition functional assays (GIA) and ELISPOT. The main findings revealed that high antibody levels to *P. vivax* Duffy binding protein (PvDBP) were associated with high parasitemia. This finding suggests that PvDBP could be used as a serological marker for recent exposure. In contrast, high antibody responses to *P. falciparum* erythrocyte antigen 175 (PfEBA-175) Region IV-V, *P. falciparum* reticulocyte binding protein-like homologue 4 (PfRh4), PfRh5 and *P. falciparum* Rh5 interacting protein (PfRipr) predict protection from symptomatic malaria. Furthermore, PfRh5-specific memory B cells could be detected among protected malaria-exposed healthy controls as well as asymptomatic individuals, suggesting a role for these cells in
sustaining long-term immunity. Together, these findings highlight that PfEBA-175, PfRh4, PfRh5, and PfRipr are important targets of naturally acquired immunity to malaria and promising vaccine candidates.
Declaration

This is to certify that:

- The thesis comprises only my original work towards the Master of Philosophy-Medicine except where indicated in the Preface
- Due acknowledgement has been made in the text to all material used
- The thesis is fewer than 50,000 words in length, exclusive of table, maps, bibliographies and appendices

Retno Ayu Setya Utami
Preface

The work described in this thesis was conducted at The Walter and Eliza Hall Institute of Medical Research under the supervision of Dr. Diana Hansen and Prof. Alan Cowman. I was supported by the Australia Awards Scholarship offered by the Department of Foreign Affairs and Trade (DFAT).

I have assessed my contribution to this thesis as:
Chapter 3: 80%
Chapter 4: 85%

These studies could not have been accomplished without the collaboration and support from the individuals stated below. For that, I accordingly acknowledge the important contributions made by the following individuals to work presented within this thesis.

**Chapter 3. Antibody Responses to Plasmodium falciparum and Plasmodium vivax Invasion Ligands**

**Dr. Rintis Noviyanti** provided the plasma samples, clinical information of the Timika cohort, as well as intellectual input to the study.

**Dr. Julie Healer** and **Ms Jenny Thompson** expressed and provided recombinant PfEBA-175, PfEBA-140, PfRh2, PvDBP proteins, as well as full-length version of PfRh5, PfRipr, and CyRPA proteins used in this study.

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**Dr. Chris Chiu** provided statistical assistance.

**Dr. Lisa Ioannidis** and **Miss Ann Ly** performed the ELISPOT experiments and analysis for Figure 3.5.

**Prof. Alan Cowman** provided intellectual input and direction to the study.
Dr. Diana Hansen provided intellectual input to the direction of the study design and edited the manuscript.

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Dr. Lisa Ioannidis provided technical assistance in analysing flow cytometry results.

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Dr. Diana Hansen provided intellectual input to the direction of the study design and edited the manuscript.
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I love you to the moon and back.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>Ab</td>
<td>Antibody</td>
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<tr>
<td>Ab-C’</td>
<td>Antibody-mediated complement-dependent</td>
</tr>
<tr>
<td>ACT</td>
<td>Artemisinin combination therapy</td>
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<td>ADCI</td>
<td>Antibody-dependent cellular inhibition</td>
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<td>ADRB</td>
<td>Antibody-dependent respiratory burst</td>
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<td>AMA</td>
<td>Apical membrane antigen</td>
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<tr>
<td>API</td>
<td>Annual parasite incidence</td>
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<td>ASC</td>
<td>Antibody secreting cell</td>
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<tr>
<td>BCIP</td>
<td>5-bromo-4-chloro-3-indolyl phosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>Ca(^{2+})</td>
<td>Calcium</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CM</td>
<td>Cerebral malaria</td>
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<tr>
<td>Coef</td>
<td>Regression coefficient</td>
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<tr>
<td>CO(_2)</td>
<td>Carbon dioxide</td>
</tr>
<tr>
<td>CR</td>
<td>Complement receptor</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite protein</td>
</tr>
<tr>
<td>CyRPA</td>
<td>Cysteine-rich protective antigen</td>
</tr>
<tr>
<td>DARC</td>
<td>Duffy antigen receptor for chemokine</td>
</tr>
<tr>
<td>DBL</td>
<td>Duffy binding-like protein</td>
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<tr>
<td>DHP</td>
<td>Dihydroartemisin-piperaquine</td>
</tr>
<tr>
<td>dl</td>
<td>Decilitre</td>
</tr>
<tr>
<td>D-PBST</td>
<td>Dulbecco's phosphate buffered saline-Tween20</td>
</tr>
<tr>
<td>EBL</td>
<td>Erythrocyte-binding ligand</td>
</tr>
<tr>
<td>EGF-like</td>
<td>Epidermal growth factor-like</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
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<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot</td>
</tr>
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<td>EPA</td>
<td>ExoProtein A</td>
</tr>
<tr>
<td>FcR</td>
<td>Fragment crystallizable receptor</td>
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<tr>
<td>FH</td>
<td>Factor H</td>
</tr>
<tr>
<td>FVO</td>
<td><em>P. falciparum</em> vietnam oak-knoll</td>
</tr>
<tr>
<td>GIA</td>
<td>Growth inhibition assay</td>
</tr>
<tr>
<td>GLURP</td>
<td>Glutamate-rich protein</td>
</tr>
<tr>
<td>GTS</td>
<td>Global technical strategy</td>
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<tr>
<td>HBs</td>
<td>Hepatitis B virus S antigen</td>
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<tr>
<td>HEPES</td>
<td>Hydroxyethyl-piperazineethane-sulfonic acid</td>
</tr>
<tr>
<td>HI-FCS</td>
<td>Heat-inactivated fetal calf serum</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
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<tr>
<td>IFN</td>
<td>Interferon</td>
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<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
<td>kDa</td>
<td>KiloDalton</td>
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<tr>
<td>LLPC</td>
<td>Long-lived plasma cell</td>
</tr>
<tr>
<td>LM</td>
<td>Light microscopy</td>
</tr>
<tr>
<td>MAC</td>
<td>Membrane attack complex</td>
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Chapter 1. Introduction

1.1. Overview of Malaria Disease

The World Health Organization estimated that there were approximately 212 million cases (range: 148-304 million) and 429,000 deaths caused by malaria parasites (range: 235,000-639,000) in 2015. Malaria is an ancient disease that is caused by infection with protozoan parasites from genus *Plasmodium* spp. Five *Plasmodium* species can cause malaria in humans; *Plasmodium falciparum, Plasmodium vivax, Plasmodium malariae, Plasmodium ovale*, and the recently reported-*Plasmodium knowlesi*. Of the five *Plasmodium* species that infect humans, *P. falciparum* is the most virulent, causing 99% malaria death cases in 2015, while *P. vivax* is the most widely distributed species (WHO, 2016).

Malaria is transmitted in 91 countries and territories. Most of the malaria cases are found in sub-Saharan Africa (90%), and around 7% of world's clinical attacks occur in the South-East Asian Region, then followed by Mediterranean Region with 2% clinical attacks. Almost 70% of the fatalities are children under five years. This makes malaria a lead killer of children by claiming the life of a child every two minutes. Malaria is not only an important health issue but also a socio-economic burden for governments and communities. Countries with high malaria prevalence usually have inadequate health facilities, low gross national income, and low total domestic government spending per capita (WHO, 2016).

With the deployment of an effective vaccine still years away, there has been an increased focus on interventions aimed at reducing malaria transmission at the population level. Indeed, the 2016 World Malaria Report has stated that the number of malaria cases and mortality rates declined by 41% and 62% globally from the year 2000, respectively. Some of the implementations that facilitated this decrease of malaria cases and mortality include rapid diagnostic tests, artemisinin combined therapies,
long-lasting insecticide-treated mosquito nets, increased donor funding and an increased awareness of people about the risks of malaria disease. Although interventions to control malaria transmission alleviated the burden of disease, it is being increasingly acknowledged that these strategies alone will not be sufficient to achieve eradication and million clinical cases continue to occur annually. Furthermore, resistance to novel drugs such as artemisinin is starting to emerge and it is spreading in the South-East Asian Region (Ashley et al., 2014; WHO, 2016), which poses a significant problem and highlights the need to develop novel therapeutic tools such as vaccines to reduce infection and prevent disease.

1.2. Malaria in Indonesia

According to the Ministry of Health Republic of Indonesia, the five provinces with the highest Annual Parasite Incidence (API) in 2016 were Papua (45.85), West Papua (6.79), East Nusa Tenggara (5.41), Maluku (3.95), and North Maluku (2.44) (MoHRI, 2017). All of those Provinces are located in the Eastern part of Indonesia. Timika, a city in the Papua province, was reported to have an overall incidence of malaria of 876 per 1,000 people per year (Karyana et al., 2008). This city is located in coastal and mountainous areas surrounded by massive forest. Malaria transmission in this area is limited to the lowland area where it is associated with three mosquito vectors: Anopheles koliensis, Anopheles farauti, and Anopheles punctulatus (Lee et al., 1980; Pribadi et al., 1998). Data from a malaria survey conducted in Timika between April and July 2013 revealed that 968 out of 2,567 participants (37.7%) had peripheral parasitemia. In total 15.4% of patients had the infection with P. falciparum, 18.3% with P. vivax, 0.01% with P. malariae, 0.0004% with P. ovale, and 0.02% had mixed species infections (Pava et al., 2016). The high co-endemicity of P. falciparum and P. vivax in this area poses an important challenge to eliminate malaria.

The mining sites in Timika have attracted a tremendous economic migration to this area. Migration of Papuans from malaria-free areas such
as the highlands (where there is no malaria transmission) and non-Papuans from provinces with malaria low-endemicity has resulted in a high proportion of the population residing in Timika with no previous exposure to the parasite and as such, no pre-existing immunity to malaria. In their adult life, these migrants are particularly vulnerable to symptomatic infection because of their limited levels of immunity (Karyana et al., 2008). According to Pava et al, the most significant overall parasite prevalence in Timika was found among Papuan highlanders (Pava et al., 2016).

Historically, Papua province is characterized not only by a high prevalence of malaria but also by a high level of antimalarial drug resistance cases. The first chloroquine-resistant cases of malaria were reported in Papua and Papua New Guinea (PNG). Moreover, an antimalarial drug resistance study conducted in Southern Papua in 2004, pointed out that 65% of malaria patients showed a risk of drug treatment failure within 28 days after chloroquine monotherapy for *P. vivax* and 48% after chloroquine plus sulphadoxine-pyrimethamine for *P. falciparum* (Ratcliff et al., 2007b). As a consequence, in 2006 the Ministry of Health Republic of Indonesia changed the antimalarial treatment policy from oral quinine to dihydroartemisinin-piperaquine (DHP), an Artemisinin Combination Therapy (ACT).

In recent years, some cases of artemisinin resistance have emerged in several regions in South-East Asia, such as in Western Cambodia, on the Thailand-Myanmar border, in Southern Myanmar, and in Vietnam (Ashley et al., 2014). In Indonesia, artemisinin is still effective and safe to treat multidrug-resistant uncomplicated malaria cases. Ratcliff *et al* stated that DHP is highly efficacious in reducing *P. falciparum* reinfection and *P. vivax* recurrence in the Timika population (Ratcliff et al., 2007a). A recent DHP efficacy study in Timika corroborated that this treatment is maintained efficacy levels, despite its continuous use for almost a decade for the treatment of uncomplicated *P. falciparum* and *P. vivax* malaria in Timika (Poespoprodjo et al., 2018). Nevertheless, the increasing spread of artemisinin resistance across South-East Asia poses
a serious concern to the region and illustrates the need of all sectors working in malaria to take decisive action to control this global disease.

1.3. *Plasmodium* spp. Life Cycle

The life cycle of *Plasmodium* spp. in humans start from the bite of a female *Anopheles* mosquito that injects sporozoites during a blood meal (Figure 1.1). The sporozoites travel via the bloodstream to the liver, pass through Kupffer cells and invade hepatocytes. In the hepatocyte, each sporozoite multiplies into thousands of exoerythrocytic merozoites within 5 to 8 days. These merozoites are then released into the bloodstream and rapidly invade erythrocytes, defining the beginning of the intra-erythrocytic stage of the life cycle. In *P. vivax* and *P. ovale* infections, some of the exoerythrocytic merozoites stay in the hepatocytes in the form of hypnozoites (Cowman et al., 2012; Cowman and Crabb, 2006).

In the erythrocyte, merozoites differentiate into ring, trophozoites, and schizonts. A mature schizont consists of 16-32 daughter merozoites that are released into the bloodstream after erythrocyte rupture. Then, the extracellular form of the parasite re-invades erythrocytes to repeat the erythrocytic stage. Some merozoites, instead of re-invading erythrocytes follow a different path and develop into female and male gametocytes, the sexual form of the parasite. The female and male gametocytes are taken up by a feeding mosquito and continue their life cycle (sexual stage) in the mosquito midgut and salivary glands. The erythrocytic stage of *P. falciparum*, *P. vivax*, and *P. ovale* takes approximately 48 hours, while *P. malariae* takes 72 hours, and 24 hours for *P. knowlesi* (Cowman et al., 2012; Cowman and Crabb, 2006).

The fusion between female and male gametes results in the formation of a zygote. These zygotes then developed into actively moving ookinetes, which penetrate the midgut wall of the mosquito and form oocysts. Inside the oocyst, the ookinete nucleus differentiates, divides, and develops into sporozoites. The ruptured oocyst results in the release of a thousand sporozoites into mosquito cavity. Sporozoites then find their
way into the mosquito’s salivary glands. The mosquito completes the parasites life cycle when it has a blood meal on a human host (Cowman et al., 2012; Cowman and Crabb, 2006).

1.4. Malaria Clinical Manifestations

The clinical manifestations of malaria are mainly attributed to the rapture of a mature schizont during the erythrocytic stage. The symptoms of uncomplicated malaria are usually nonspecific, including fever, vomiting, nausea, muscle ache and headache. A minority of the cases might progress to life-threatening manifestations such as cerebral malaria (CM), severe anaemia, respiratory distress, acute renal failure, and thrombocytopenia (Mackintosh et al., 2004). CM is the most common reason for death in adults with severe malaria. Although the precise mechanisms leading to CM are not completely understood, it is accepted that high parasite densities are a major determinant of disease development. Mature forms of blood-stage malaria express parasitic proteins on the surface of the erythrocyte, which allow them to bind to vascular endothelial cells and avoid clearance in the spleen. This process called parasite sequestration induces obstructions in the blood flow resulting in hypoxia and haemorrhages (Miller et al., 2002) that are associated with the development of organ-specific syndromes. A large body of literature indicates that in addition to parasite sequestration, inflammatory responses also contribute to severe disease. High tumor necrosis factor α (TNF-α) levels (Molyneux et al., 1993), as well as increased production of interferon γ (IFN-γ) and interleukin 1β (IL-1β) (Pongponratn et al., 2003), have been associated with disease severity to human malaria. Consistently, decreased the production of anti-inflammatory cytokines such as IL-10 and transforming growth factor β (TGF-β) has been found to be associated with disease (Kurtzhals et al., 1998; Wenisch et al., 1995). Similar to young children, pregnant women are more susceptible to malaria infection and might develop placental malaria (PM). This serious complication has been found to be associated
with adverse pregnancy outcomes including premature labour, intrauterine growth retardation, low birth weight delivery and is responsible for more than 75,000 infant deaths each year (Desai et al., 2007; McGregor, 1984; Rogerson et al., 2007; Steketee et al., 2001).

1.5. Malaria Parasite Invasion into the Erythrocyte

Malaria parasites invade erythrocytes in their asexual stage of the life cycle. Invasion is a rapid, complex, and multistage process. It starts with the attachment of a merozoite to the surface of an erythrocyte through the interaction of parasite ligands and erythrocyte surface receptors. Second, parasites undergo apical reorientation towards erythrocytes membrane and then form a tight junction with the erythrocytes membrane. Lastly, the merozoite gains access into the erythrocyte, creating a protective parasitophorous vacuole (Cowman et al., 2012; Cowman and Crabb, 2006).

Parasite invasion is facilitated by the specific interaction of parasitic ligands with receptors on the erythrocytes. These parasite invasion ligands can be divided into two families: the erythrocyte-binding ligand (EBL), also known as Duffy binding-like protein (DBL) and the binding protein-like homologue (Rh) (Cowman and Crabb, 2006). The EBLs include EBA-175, EBA-140, EBA-181, and EBL-1. The specific receptors on erythrocyte surface for all EBL proteins have been identified, except for EBA-181. *Plasmodium falciparum* reticulocyte binding protein-like homologues consist of five proteins: PfRh1, PfRh2a, PfRh2b, PfRh4, and PfRh5. To date, only the erythrocyte receptors for PfRh4 (complement receptor 1) and PfRh5 (basigin) have been discovered (Tham et al., 2012).

Human *P. vivax* malaria parasites and the monkey *P. knowlesi* malaria parasites require interactions with the Duffy blood group antigen to be able to invade erythrocytes (Miller et al., 1976; Miller et al., 1975). A 140-kDa *P. vivax* protein and 135-kDa *P. knowlesi* protein bind to Duffy-positive but not Duffy-negative human erythrocytes (Haynes et al., 1988; Wertheimer and Barnwell, 1989). However, a recent finding from Menard
et al. revealed that Duffy-negative erythrocytes could be infected with *P. vivax*, suggesting that *P. vivax* might use other invasion pathways that do not involve Duffy antigen (Menard et al., 2010).

1.6. Naturally Acquired Immunity

Millions of individuals living in malaria-endemic countries are protected from severe and life-threatening symptoms of malaria disease due to naturally acquired immunity to the parasites. However, this naturally acquired immunity (Marsh and Kinyanjui, 2006) only develops after years of natural exposure to *Plasmodium* parasites (Figure 1.2). This form of protection does not result in sterilizing immunity but prevents clinical disease by controlling parasite density below the threshold required for the induction of clinical symptoms. In general, immunity to severe malaria develops within the first five years of life, while immunity to mild disease takes longer (at 30 years of age) (Marsh and Kinyanjui, 2006). Clinical immunity to *P. vivax* is acquired faster than *P. falciparum*, with young children developing a protective response that controls *P. vivax* density below the pyrogenic threshold only after two years of exposure. In contrast, clinical immunity to *P. falciparum* disease symptoms appears to require more than 3-4 years (Lin et al., 2010). The reason for this different kinetics in the acquisition of clinical immunity between *P. vivax* and *P. falciparum* are no known, but the might be associated to the biological differences between the two parasite species, which could influence the development of antibody responses to infection.

Naturally acquired immunity predominantly targets blood-stage antigens and appears to require antibody responses since a passive transfer of purified immunoglobulin G (IgG) from clinically immune individuals protects non-immune recipients from high-level parasitemia and disease symptoms (Cohen et al., 1961). Nonetheless, evidence from the field demonstrates that acquired antibodies to malaria antigens rapidly vanish without ongoing exposure (Langhorne et al., 2008). These observations suggested that human malaria-specific antibodies might be
produced by short-lived plasma cells and/or infection might impair the development of memory B cells (MBCs). Despite the key role that antibodies play in protection against malaria, the cellular mechanisms underlying the slow acquisition of immunity have only recently begun to be investigated. A study of children and adults in an area in Mali with seasonal and intense *P. falciparum* transmission illustrates that individuals living in that area gradually develop MBCs, as the frequency of *P. falciparum*-specific MBCs increased with age. Long-lived antibodies specific for *P. falciparum* were also gradually developed over the years despite intense exposure to the parasite (Weiss et al., 2010). On the other hand, individuals living in the extremely low malaria transmission areas enable to stably maintain the generation of MBCs and antibodies over time in the lack of malaria exposure (Wipasa et al., 2010). Together these pieces of evidence suggest that acute infection might have an adverse effect on the MBCs development, which contributes to the slow acquisition of clinical immunity to malaria. In support to this view, recent studies both in humans and mouse infection models revealed that inflammatory responses associated with the induction of malaria clinical episodes prevent the induction of humoral immunity by inhibiting T follicular helper (Tfh) cell differentiation required for efficient B cell help for antibody formation (Obeng-Adjei et al., 2015; Ryg-Cornejo et al., 2016).

1.7. Antibody Mediated-Immunity to Malaria

Despite the key role that antibodies play in immunity against malaria the effector mechanisms responsible for protection are only partly understood (Figure 1.3). The main findings on antibody-mediated protective responses to malaria are summarized below.

1.7.1. Direct Inhibition

Antibodies targeting merozoites may mediate protection by several different mechanisms, including direct growth inhibition. A large number of studies have suggested that antibodies targeting merozoite are capable of
inhibiting the erythrocytes invasion process. Moreover, antibodies to merozoite have been also shown to contribute to the retardation of parasite development, giving rise to a crisis form of the parasite and inhibiting schizont rapture (Beeson et al., 2016; Jensen et al., 1982). Although the precise mechanism by which antibodies interfere with different processes of the intra-erythrocytic life cycle is undefined, it is clear that the binding of antibodies with specific parasite invasion ligands blocks merozoite invasion and subsequent growth into the erythrocyte (Beeson et al., 2016). An experimental tool that has been widely implemented to evaluate the functional activity of antibodies to merozoites is the in vitro growth inhibition assay (GIA). Naturally acquired antibodies have been shown to inhibit the binding between P. falciparum and P. vivax invasion ligands to their erythrocyte receptors, and these inhibitory antibodies have been associated with protection from malaria (Bustamante et al., 2013; Irani et al., 2015; John et al., 2004; King et al., 2008). Moreover, affinity-purified human antibodies to a number of P. falciparum merozoite antigens, including merozoite surface protein 1-19 (MSP1-19), apical membrane antigen 1 (AMA1), PfRh4, PfEBA-175, PfRh5 and merozoite surface protein Duffy binding-like (MSPDBL) 1, and MSPDBL2 have also been shown inhibitory activity in vitro (Badiane et al., 2013; Chiu et al., 2015; Egan et al., 1999; Hodder et al., 2001; Reiling et al., 2012; Tran et al., 2014). Genetically modified P. falciparum laboratory strains have been using in growth assays to assess inhibitory antibodies directed to the individual merozoite antigens, such as to MSP1-19 and the EBA antigens (O'Donnell et al., 2001; Persson et al., 2013; Persson et al., 2008; Tijani et al., 2017; Wilson et al., 2011). However, growth-inhibitory antibodies have not been consistently predictive protection against clinical malaria in longitudinal studies (Marsh et al., 1989; McCallum et al., 2008; Perraut et al., 2005), highlighting the importance of studying other effector mechanisms that could be involved in protection.
1.7.2. Complement Fixation

Soon after schizont rupture, extracellular merozoites are exposed to the human immune system. Antibodies targeting merozoites can recruit the complement system to enhance opsonizing phagocytosis, chemotaxis, and cell lysis. A recent study revealed that naturally acquired and vaccine-induced human antibodies promote complement deposition on the merozoites surface through C1q fixation. This process leads to inhibition of merozoites invasion through activation of the classical complement pathway and results in the formation of a membrane attack complex (MAC) that can lyse merozoites. Moreover, complement fixation by antibodies was found to be associated with protection from symptomatic malaria and high parasitemia (Boyle et al., 2015). Further, antibody-mediated complement-dependent (Ab-C’) inhibition was found to be an important mechanism of inhibitory antibodies and appeared to be required for successful blockade of parasite invasion into the erythrocyte. The major targets of Ab-C’ inhibition were found to be MSP1 and MSP2 (Boyle et al., 2015). On the other hand, *P. falciparum* merozoites were found actively recruit Factor H (FH), a negative regulator of complement activation, to escape from complement-mediated lysis. FH interacts directly with Pf92, a member of the six-cysteine family on the surface of *P. falciparum* merozoites. Recruitment of FH enables the merozoite to downregulate complement activation, resulting in protection from cell lysis (Kennedy et al., 2016).

1.7.3. Opsonic Phagocytosis

Naturally acquired antibodies can coat or opsonize merozoites, apart from directly interfering with the invasion process into the erythrocyte. Opsonization of *P. falciparum* merozoites by antibodies initiates several effector functions, including clearance of opsonized merozoites by phagocytosis (Hill et al., 2013; Osier et al., 2014), antibody-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun et al., 1995), and antibody-dependent respiratory burst (ADRB) (Joos et al., 2010).
Antibodies to *P. falciparum* merozoite antigens are predominantly comprised of the cytophilic IgG1 and IgG3 subclasses that interact with high affinity to fragment crystallizable (Fc) receptor (Jafarshad et al., 2007; Khushmith et al., 1983; Stanisic et al., 2009; Vidarsson et al., 2014). Further, studies on *P. vivax* have also demonstrated that antibodies to *P. vivax* merozoites are predominantly IgG1 and IgG3 (Cutts et al., 2014; Fernandez-Becerra et al., 2010; Franca et al., 2016; Lima-Junior et al., 2011). Together, this evidence suggests that IgG-FcR-mediated antibodies may contribute to *P. vivax* immunity, even though this idea requires further studies.

Opsonization of *P. falciparum* merozoites facilitates phagocytosis by effector cells such as monocytes. Antibody-mediated opsonic phagocytosis induces activation of monocytes with subsequent production of pro-inflammatory cytokines such as TNF-α (Osier et al., 2014). To date several different merozoite surface proteins have been identified as specific targets of opsonizing antibodies, there are MSP2, MSP3, MSPDBL1, MSPDBL2, and glutamate-rich protein (GLURP) (Chiu et al., 2015; Kana et al., 2017; Osier et al., 2014; Sakamoto et al., 2012). Opsonic phagocytosis was found to be significantly associated with reduced risk of clinical malaria in cohort studies of children in PNG and Kenya. Moreover, these antibodies increased with age and appeared to be boosted by recent infections, thereby improving their protective capacity (Hill et al., 2013; Osier et al., 2014).

Unlike merozoite surface proteins, there is a single antigen appears to be the most important target of antibodies to parasitized erythrocytes which is *P. falciparum* erythrocytes membrane protein 1 (PfEMP1) (Bull et al., 1998; Chan et al., 2014). This antigen plays a key role in the pathology of severe malaria by mediating the adhesion of infected erythrocytes and formation of rosettes, which allows the infected erythrocytes to sequester in different organs (Baruch et al., 1995; Chen et al., 1998; Reeder et al., 1999; Smith et al., 1995).
*P. falciparum* expresses highly variant antigens, known as variant surface antigens (VSAs) on the surface of erythrocyte during blood-stage development. Among other VSAs, PfEMP1 is thought to be the dominant target of naturally acquired immunity (Biggs et al., 1991; Leech et al., 1984). Anti-PfEMP1 antibodies are considered to confer protection by opsonizing infected erythrocytes for phagocytic clearance (Celada et al., 1982; Chan et al., 2012).

During ADCI, the interaction between naturally acquired antibodies and monocytes can result in the release of soluble factors from monocytes, which appear to directly inhibit parasite growth or kill parasites (Bouharoun-Tayoun et al., 1995). Antibodies involved in ADCI are acquired through natural exposure to the malaria parasite and induced by vaccines based on a number of merozoite surface proteins. Human vaccine trials based on recombinant MSP1, MSP2, MSP3 and GLURP (Druilhe et al., 2005; Galamo et al., 2009; Jepsen et al., 2013; McCarthy et al., 2011) gave rise to low levels of growth-inhibitory antibodies, but induced a significant level of ADCI responses *in vitro* in the presence of monocytes. Further work is needed to identify associations between ADCI by naturally acquired antibodies and protection from symptomatic malaria.

Respiratory burst against merozoites refers to the mechanism by which antibodies to merozoites interact with neutrophils, thereby inducing cell activation. This interaction leads to the production of reactive oxygen species (ROS), which are highly toxic for malaria parasites (Joos et al., 2010). Nevertheless, the function of high ROS in malaria is ambiguous, because ROS production has also been associated with malarial anaemia in children (Greve et al., 2000). Antibody-dependent respiratory burst has been correlated with parasite clearance (Greve et al., 1999) and it has been recently reported that ADRB is associated with protection from clinical malaria in a longitudinal study (Joos et al., 2010). Additionally, antibodies promoting ADRB are acquired with increasing age and target MSP5 as well as MSP1-19 (Joos et al., 2015; Perraut et al., 2014). These
findings illustrate that ADRB activity may provide protection against malaria disease.

1.8. Antigenic Targets of Immunity

Due to the extraordinary complexity of *Plasmodium* parasites, the identification of the antigenic target of protective immunity to malaria is not an easy task. Several studies performed to date have revealed the different level of associations between protective immune responses and various malarial antigens. For this thesis, attention was focused on a panel of merozoite-stage invasion ligands. The main properties of these selected antigens are summarized below.

1.8.1. Erythrocyte-Binding Antigen 175

*Plasmodium falciparum* Erythrocyte-Binding Antigen 175 (PfEBA-175) is an important invasion ligand that interacts with glycophorin A on the erythrocyte surface in a sialic acid-dependent manner (Orlandi et al., 1992; Sim et al., 1994). The protein structure of PfEBA-175 is characterized by two cysteine-rich domains in the N- and C-terminal domains (Region II & VI, respectively), a highly conserved domain (Region III-V), followed by a transmembrane domain, and a cytoplasmic tail (Adams et al., 1992). The cysteine-rich domain region II is comprised of two motifs (F1 & F2) (Sim et al., 1992). Further, 12 cysteine residues that are present in F1 and F2 motifs are conserved (Adams et al., 1992). Region III is dimorphic, with either C or F alleles conserved in all *P. falciparum* isolates (Ware et al., 1993). Glycophorin A binds specifically to region II of PfEBA-175 (Sim et al., 1994). Region II was found to be highly polymorphic, suggesting that this region might be under strong immune pressure (Ozwara et al., 2001). A large body of evidence suggests that antibodies specific to region II are associated with protection from malaria (El Sahly et al., 2010; Irani et al., 2015; Jiang et al., 2011; Okenu et al., 2000; Richards et al., 2010). However, recent studies on PfEBA-175 proposed the idea that antibodies to region III–V are also associated with
protection from symptomatic malaria (Richards et al., 2010). Moreover, low circulating antibody concentrations specific to the highly conserved domain (Region III-V) were recently found to be stronger predictors of protection from clinical disease in children compared to antibodies against the polymorphic region II of the molecule (Chiu et al., 2016).

1.8.2. Erythrocyte-Binding Antigen 140

Being a member of the DBL family, PfEBA-140 is characterized by having two cysteine-rich domains, a transmembrane region, and a cytoplasmic tail. A large cysteine-rich domain can be found in the N-terminal region and consists of F1 and F2 regions. On the other hand, a small cysteine-rich domain is located at the 3’ end. All cysteine-rich domains are encoded within the large first exon of the PfEBA-140 gene (Mayer et al., 2001; Thompson et al., 2001). The protein structure of PfEBA-140 is indistinguishable from PfEBA-175. In fact, they are 30% identical in the protein sequence (Thompson et al., 2001). Similar to PfEBA-175, PfEBA-140 is also present in the micronemes and expressed by late-schizonts (Mayer et al., 2001; Thompson et al., 2001). This protein is involved in a sialic acid-dependent invasion pathway by interaction with glycophorin C on the merozoite surface (Maier et al., 2003; Mayer et al., 2006). According to Lobo et al, the location of the PfEBA-140 binding domain is between residues 14 and 22 (Lobo et al., 2003). In contrast to the high polymorphisms in region II of PfEBA-175, a small number of polymorphisms were identified in PfEBA-140 region II (Baum et al., 2003; Mayer et al., 2002). Individuals living in malaria-endemic areas produce antibodies against PfEBA-140 and a high level of anti-PfEBA-140 antibodies has been found to be associated with protection from clinical malaria (Ahmed Ismail et al., 2013; Persson et al., 2013; Richards et al., 2013; Richards et al., 2010; Tijani et al., 2017). Thus, PfEBA-140 is a target of naturally acquired immunity and possibly is a candidate for a malaria vaccine.
1.8.3. Reticulocyte binding protein-like homologue 2

The PfRh2 protein is expressed by mature schizont and localises within the body of the rhoptries (Triglia et al., 2001). The genes encoding PfRh2a and PfRh2b are nearly identical and are located on chromosome 13, suggesting that they have emerged from a gene duplication event. PfRh2 gene consists of a short Exon 1, a short Intron, and a large Exon 2. The short Exon at the 5’ end encodes a putative signal region; meanwhile, the second large Exon encodes the rest of the protein. This big portion of PfRh2 protein is highly hydrophilic and it is comprised of an extracellular domain, a transmembrane domain and a short cytoplasmic tail (Rayner et al., 2000). Almost all amino acids at the N-terminal region of PfRh2a and b are identical and differ significantly only from amino acid 2776 onward at the C-terminus region (Triglia et al., 2001). The estimated molecular mass of PfRh2a is 370 kDa and 383 kDa for PfRh2b (Rayner et al., 2000). The erythrocyte receptor for the PfRh2 proteins are unidentified, but these antigen bind to the erythrocyte through a sialic acid-independent pathway (Duraisingh et al., 2003b). Sahar et al suggested that a 40 kDa region, which is located at the N-terminal domain of PfRh2, is the one responsible for erythrocyte-binding (Sahar et al., 2011). On the other hand, Triglia et al claimed that the binding region of PfRh2 is a 15 kDa region at the N-terminal domain (Triglia et al., 2011). Sequence analysis indicates that PfRh2 polymorphisms are mainly found in the N-terminal region, suggesting that this region is under immune pressure (Reiling et al., 2010). Immunology studies conducted in PNG and Kenya showed that acquired antibodies to PfRh2a and b are correlated with recent exposure and acquired in an age-related manner (Persson et al., 2008; Reiling et al., 2010).

1.8.4. Reticulocyte binding protein-like homologue 4

Enzymatic treatment of erythrocyte defines two major invasion pathways of *P. falciparum*. The one that requires sialic acid moieties on the erythrocyte surface (SA-dependent) and the other one is SA-
independent. Whereas parasite invasion through the interaction of all EBAs and PfRh1 is SA-dependent, PfRh2b, PfRh4 and PfRh5 are important in the SA-independent pathway. Changes in EBAs and PfRh expression and function allow the parasite to alternate invasion pathways. One example is W2mef parasites, these parasites invade in an SA-dependent manner using predominantly PfEBA-175 (Duraisingh et al., 2003a). W2mef does not naturally express PfRh4. However, knockout of PfEBA-175 gene selects for parasites in which PfRh4 expression is activated (Stubbs et al., 2005), allowing parasites to adapt to changes in erythrocyte receptor expression and evade antibody-mediated immunity.

The gene that encodes PfRh4 is found on chromosome 4. Comparable to PfRh2, the PfRh4 gene consists of 1 Intron and 2 Exons that encode a protein of 1716 amino acids with a predicted molecular mass of 220 kDa. The first Exon and a part of the second Exon encode a putative signal sequence region, while the remaining part of second Exon encodes an extracellular domain, a transmembrane domain, and a cytoplasmic tail (Kaneko et al., 2002). PfRh4 binds to erythrocytes through the interaction with complement receptor 1 (CR1), but the binding between them serve as a low-affinity interaction (Tham et al., 2010; Tham et al., 2011). Data from studies in the animal model show that parasite growth inhibitory antibodies could be induced by immunisation with PfRh4 recombinant protein (Lim et al., 2015; Tham et al., 2009). Consistent with these findings, evidence from the field demonstrate that PfRh4 is a target of naturally acquired immunity and anti-PfRh4 antibodies are correlated with malaria protection (Persson et al., 2008; Reiling et al., 2012).

1.8.5. A complex of PfRh5/PfRipr/CyRPA

The latest identified protein member of PfRh family is PfRh5. This protein has a much smaller size compared to other PfRh proteins, with an estimated molecular mass of 63 kDa. It is expressed in the rhoptries during mature schizont stage. A distinct feature of PfRh5 is the lack of a transmembrane and cytoplasmic domain (Rodriguez et al., 2008). PfRh5
binds to its receptor basigin on the erythrocyte surface (Crosnier et al., 2011). The interaction between PfRh5 and basigin is an important step in the invasion process (Baum et al., 2009). Moreover, the gene that encodes PfRh5 cannot be disrupted, suggesting that this protein is essential for parasite survival (Baum et al., 2009; Cowman and Crabb, 2006; Hayton et al., 2008). Sequence analysis of seven *P. falciparum* laboratory strains revealed low-frequency *pfhr5* polymorphisms. Further, affinity purified anti-PfRh5 antibodies raised in rabbits showed significant inhibition of merozoite invasion *in vitro* (Baum et al., 2009).

*Plasmodium falciparum* Rh5 interacting protein (PfRipr) is a micronemes’ protein that forms a complex with PfRh5. Similar to PfRh5, PfRipr also lacks a transmembrane domain and a GPI anchor. The predicted molecular mass of this protein is ~123 kDa. Full-length PfRipr consists of a putative signal sequence and ten epidermal growth factor-like (EGF-like) domains. Two EGF-like domains are located in the N-terminal region, whereas the rest of the EGF-like domains are found toward the C-terminus. PfRipr gene is highly conserved, and antibodies against PfRipr block merozoite attachment and invasion (Chen et al., 2011; Ntege et al., 2016). Additionally, a cohort study in children showed that high IgG3 to PfRipr as well as PfRh5 were strongly associated with protection from malaria (Weaver et al., 2016).

The analysis of the PFD1130w gene conducted by Dreyer *et al* showed that this gene is remarkably conserved. Genetic analysis of twelve *P. falciparum* laboratory strains and six field isolates from Tanzania found only one nonsynonymous single nucleotide polymorphism (SNP) in four *P. falciparum* laboratory strains. This gene is located in the subtelomeric region of chromosome 4. It encodes a ~36 kDa protein called Cysteine-rich Protective Antigen (CyRPA). Just like its name, CyRPA is cysteine-rich and has a putative signal sequence at the N-terminal region (Dreyer et al., 2012). CyRPA is localized in the micronemes and secreted to the surface of merozoite, where it forms a complex with PfRh5 and PfRipr (Reddy et al., 2015). Further, anti-CyRPA monoclonal antibodies have
been found to successfully inhibit parasite invasion in vitro and in vivo (Dreyer et al., 2012).

The complex of PfRh5 and PfRipr lacks a transmembrane domain and a GPI anchor (Chen et al., 2011; Rodriguez et al., 2008). Therefore, this complex must bind to another protein to tether to the merozoite surface. Reddy et al. suggested that the third protein partner of PfRh5 and PfRipr, CyRPA, acts as the tether to the merozoite surface through a GPI anchor (Reddy et al., 2015). However, a recent study identified another protein as a membrane tether of PfRh5. The protein is an abundant GPI-anchored merozoite surface protein called P113. This protein binds to PfRh5 in the N-terminal region, presents PfRh5 to the merozoite surface, and allows PfRh5 to interact with basigin. The binding of PfRipr and CyRPA to PfRh5 releases PfRh5 from P113 and results in PfRh5/PfRipr/CyRPA complex (Galaway et al., 2017). During the invasion process, the PfRh5/PfRipr/CyRPA complex appears to mediate the formation of a pore between the merozoite and the erythrocyte. This pore is essential for Ca\(^{2+}\) release from the merozoite into the erythrocyte. Moreover, this complex is also thought to be involved in tight junction formation (Volz et al., 2016; Weiss et al., 2015). A few immunology studies suggested that PfRh5, PfRipr, and CyRPA are targets of immunity and antibodies against them are correlated with malaria protection (Bustamante et al., 2013; Chen et al., 2011; Chiu et al., 2014; Douglas et al., 2015; Douglas et al., 2011; Douglas et al., 2014; Dreyer et al., 2012; Ntege et al., 2016; Reddy et al., 2015).

1.8.6. Duffy Binding Protein

Adams et al. proposed that the members of the EBL protein family and the Duffy binding protein belong to the same gene family because of their gene structure similarity and the amino acid homology in the 5' and 3' cysteine-rich regions (Adams et al., 1992). The P. vivax Duffy binding protein (PvDBP) gene consists of five exons. Exon 1 encodes a signal sequence followed by an Open Reading Frame (ORF) that is encoded by
Exon 2 (Adams et al., 1992). Exon 3 encodes a transmembrane domain of 18 amino acids, meanwhile Exon 4 and 5 encodes a cytoplasmic domain that is located after the transmembrane domain (Adams et al., 1992).

Exon 2 of the DBP gene contains six regions. Region I consists of the end part of Exon 1 and the beginning of Exon 2. Negatively charged amino acids dominate this region. According to Chitnis and Miller, the erythrocyte-binding domain is located in Exon 2. A conserved, 330 amino acids at the cysteine-rich region known as region II is served as an erythrocyte-binding domain and involved in the interaction between PvDBP and Duffy antigen receptor for chemokines (DARC) (Chitnis and Miller, 1994; Ranjan and Chitnis, 1999). The 5' and 3' cysteine-rich regions lie from region II to VI. The 5' cysteine-rich region has 12 conserved cysteine residues and highly conserved aromatic amino acids. Meanwhile, the 3' cysteine-rich region consists of 104 amino acids at the end of Exon 2. Further, the 5' and 3' cysteine-rich regions are separated by region III, IV, and V (Adams et al., 1992). Data from field studies suggest that having high-level of inhibitory antibodies targeting PvDBP Region II (PvDBPII) was associated with delayed time to \textit{P. vivax} reinfection and antibodies specific to PvDBPII reduced invasion efficiency (Grimberg et al., 2007; King et al., 2008). These data support the notion that a PvDBP-based vaccine might provide protection against \textit{P. vivax} infections.

1.9. Malaria Vaccine Development

An effective malaria vaccine is urgently needed, especially since insecticide resistance is emerging (Ranson et al., 2011) and artemisinin drug resistance is spreading in the Great Mekong Subregion (Ashley et al., 2014; WHO, 2016). The life cycle of \textit{Plasmodium} parasite is a complex process. One of the many challenges in malaria vaccine development is to provide information to decide which life cycle stage and which parasitic antigens should be targeted to induce a highly effective response. Several different approaches have been pursued to develop vaccines that target different stages of the complex life cycle of the malaria parasite. Other
factors including a high proportion of highly polymorphic antigens in *Plasmodium* species as well as an incomplete understanding of immune effector mechanisms required to confer protection are other important hurdles in the development of an efficient anti-malaria vaccine (Anders et al., 2010).

### 1.9.1. Pre-erythrocytic Vaccine

In order to prevent the development of clinical disease and malaria transmission, a pre-erythrocytic vaccine would be a desirable option. This vaccine should be able to completely block further parasite development in the liver before invading erythrocytes. An ideal pre-erythrocytic vaccine would need to induce high-titer, high-avidity, and long-lasting antibodies as well as induce MBCs in immunized individuals (Riley and Stewart, 2013). The identification of pre-erythrocytic antigens for vaccine production has been the topic of extensive research, but several trials to induce protective immunity to antigens have not produced the expected results (Lindner et al., 2012; Schwartz et al., 2012).

The first-ever malaria vaccine candidate entering large-scale phase 3 trials in Africa was RTS,S. This vaccine candidate is composed of C-terminal of the *P. falciparum* circumsporozoite (PfCS) that linked to the hepatitis B virus S antigen (HBs) and paired with AS01 adjuvant (Cohen et al., 2010; Gordon et al., 1995). Previous results of vaccine efficacy trial in African children showed moderate efficacy with 55.8% protection in children aged 5-17 months and 31.3% in infants aged 6-12 weeks over the first 12 months after dose 3 (Rts et al., 2012; Rts et al., 2011). However, vaccine efficacy of RTS,S/AS01 against clinical disease and severe malaria decreases over time. A recent report showed even lower vaccine efficacy in the absence of a booster dose (about 28% in children and 18% in young infants). Moreover, severe adverse events were also reported in 22 children (Rts, 2014; Rts, 2015).

Another potential pre-erythrocytic vaccine candidate that currently undergo phase 1 clinical trials is PfSPZ. The PfSPZ vaccine is a live
irradiated *P. falciparum* whole sporozoite vaccine, which is administered by direct venous inoculation into volunteers’ body. Data from clinical trials in Mali and Equatorial Guinea showed that PfSPZ vaccine was safe, protective (48% protective efficacy), and well tolerated in healthy adults. Nevertheless, study volunteers who received PfSPZ vaccine induced significantly lower antibody responses to PfSPZ compared to those in malaria-naïve adults (Olotu et al., 2018; Sissoko et al., 2017).

1.9.2. Blood-stage Vaccine

Blood-stage or erythrocytic-stage vaccines target merozoites and/or parasitized erythrocytes and are designed to prevent parasite replication in the erythrocyte and clinical symptoms. This type of vaccine is postulated to directly reduce parasite densities by blocking parasite invasion to erythrocytes thereby preventing clinical episodes (Moorthy et al., 2004). To date, several merozoite antigens have been involved in human trials, including AMA1, MSP1, MSP2, MSP3, EBA-175, GLURP, and serine repeat antigen 5 (SERA 5). Most of these have been assessed as single recombinant protein or in combination with other antigens. The vast majority of these blood-stage antigens have been clinical trials phase 1, some reached phase 2, and just a minor candidate-entering phase 1/2 (Beeson et al., 2016). In general, results of blood-stage vaccine trials so far have been disappointing with at least nine candidates being discontinued at or before phase 2 trials (Schwartz et al., 2012). However, encouraging results from a few numbers of phase 2 trials and follow-up data from phase 1b trials, support the rationale of development blood-stage antigens as vaccine candidates (Beeson et al., 2016; Schwartz et al., 2012).

Moreover, a new antigen with a strong potential as a vaccine candidate has been identified. This new vaccine candidate is PfRh5, an essential ligand for merozoite invasion discussed above. Studies in mice and rabbits have been shown that this antigen successfully induced parasite strain-transcending neutralizing antibodies and inhibitory
antibodies across common PfRh5 variants (Bustamante et al., 2013; Douglas et al., 2011). Pieces of evidence from the field have been confirmed the important role of PfRh5 in clinical malaria protection. The affinity-purified antibodies against PfRh5 that were collected from individuals living in Senegal and Mali are able to neutralize parasite in vitro (Patel et al., 2013; Tran et al., 2014). In addition, a study in PNG reported that anti-PfRh5 antibodies were correlated to malaria protection (Chiu et al., 2014). However, it remains obscure whether the promising results made in vitro will also show similar encouraging results in vivo. Recent results from a PfRh5 vaccine trial in Aotus monkeys (non-human parasite model) show for the first time that PfRh5-based vaccines induced antibodies and provided protection against a heterologous and highly virulent P. falciparum FVO (Vietnam oak-knoll) strain in vivo. The significant outcomes of this trial demonstrate that protection was correlated with anti-PfRh5 antibodies concentration and more than 60% GIA was observed at 2.5 mg/ml purified IgG against FVO strain (Douglas et al., 2015). Thus, PfRh5 promises a good prospect as a blood-stage vaccine candidate. Following-up all of the promising results, at the moment the open-label Phase1/2a clinical trial in malaria naïve adults is undergoing assessment for safety, immunogenicity, and efficacy of malaria vaccine candidate RH5.1/AS01 in Oxford and Tanzania (Coelho et al., 2017).

1.9.3. Sexual Stage Vaccine

Transmission-blocking vaccines (TBV) target sexual stage antigens of Plasmodium parasite (gametocytes, zygotes, & ookinetes) to hamper the subsequent development of infectious sporozoite in the mosquito midgut. This vaccine contributes to the generation of herd immunity that reduces malaria transmission in the community but does not necessarily give protection from infection or disease. The development of TBV has targeted four P. falciparum ookinete surface proteins (Pfs25, Pfs28, Pfs48/45, Pfs230) (Carter et al., 1984). Among these antigens, Pfs25 is
the leading vaccine candidate. To enhance immunogenicity, Pfs25 has been chemically conjugated to *Pseudomonas aeruginosa* ExoProtein A (Jiang et al.) and formulated with Alhydrogel adjuvant. The Pfs25-EPA conjugate presents as a nanoparticle (Shimp et al., 2013). The Pfs25-EPA formulated on Alhydrogel elicited significantly higher antibody responses than Pfs25 formulated on Alhydrogel alone (Qian et al., 2007; Shimp et al., 2013). Antibodies to Pfs25-EPA reduced almost 100% of oocyst number in mosquito midgut (Qian et al., 2007). The Pfs25-EPA/Alhydrogel is currently undergoing phase 1 clinical trials in human volunteers. Perhaps, the potential obstacle of the TBV approach is that the antigens are not expressed naturally in humans. Therefore, antibodies induced by immunization may not be boosted by a natural infection, which might limit vaccine efficacy (Dinglasan et al., 2013).

1.9.4. *Plasmodium vivax* Vaccine Development

In contrast to *P. falciparum*, *P. vivax* has a short list of potential vaccine candidates (Mueller et al., 2015). The first candidate to reach phase 1 trial is Pvs25, the Pfs25 homologues. Phase 1 trial results showed low transmission blocking activity in membrane feeding assay (MFA) (Malkin et al., 2005). Another phase 1 trial examined Pvs25 formulated on Montanide ISA 51. The trials found that Pvs25 formulated on Montanide ISA 51 elicited transmission blocking activity, but the trial was terminated because of unexpected reactogenicity (Wu et al., 2008). A phase 1/2a clinical trial in healthy malaria-naïve adults has been conducted to evaluate Vivax Malaria Protein 1 (VMP001) vaccine that is paired with AS01 adjuvant. VMP001 consists of N- and C-terminal regions of the circumsporozoite protein (CSP) and repeat region from both the VK210 (type 1) and the VK247 (type 2) alleles. These vaccine-induced antibody responses, was well tolerated and resulted in a small delay in time to parasitemia (Bennett et al., 2016). Another preclinical study that examines virally vectored PvDBPII vaccine in mice and rabbit showed that antibodies elicited by the vaccine could block the binding of PvDBPII and
its receptor DARC (de Cassan et al., 2015). Further, phase 1a trial has been completed to assess virally vectored PvDBPII in the healthy UK (United Kingdom) adults. The results showed that the strain-transcending antibodies, B cell, and T cell responses can be induced specific to PvDBPII by immunization. Moreover, inhibitory antibodies were found to block the interaction between PvDBP and DARC by 50% (Payne et al., 2017).
AIMS

A better understanding of the immune response to malaria is required to develop a highly effective malaria vaccine. Thus the aim of this study:

• To investigate the association between antibody responses to *P. falciparum* & *P. vivax* invasion ligands with protection against symptomatic malaria

• To quantify the functional activity of parasite-specific growth inhibitory antibodies

• To measure the frequency of memory B cells specific for *P. falciparum* antigens
Figure 1.1. The Life Cycle of *Plasmodium* Parasites.

Malaria infection is started by the injection of sporozoites by a female *Anopheles* mosquito during a blood meal. The sporozoites then travel to the liver and invade hepatocytes where they multiply into thousands of exoerythrocytic merozoites. Exoerythrocytic merozoites are released into the bloodstream and then invade erythrocytes, but some stay in the form of hypnozoites (*P. vivax* and *P. ovale* infections). In the erythrocyte, they develop into ring, trophozoites, and schizonts that release merozoite daughters at egress. Free merozoites invade new erythrocytes to continue the erythrocytic stage. Some of the free merozoites develop into female and male gametocytes, the sexual form of the parasite. These areuptaken by a feeding mosquito and develop into gametes then fuse to form zygotes. Zygotes develop into motile ookinetes that cross the midgut and transform into an oocyst. The ruptured oocyst releases thousand sporozoites that then migrate to the salivary glands. The mosquito completes the parasites life cycle by injecting sporozoites in the next blood meal. Adapted from (Mueller et al., 2009).
Figure 1.2. Acquisition of Immunity to Malaria in an Endemic Population.

People who live in malaria-endemic areas are vulnerable to severe or life-threatening malaria through their childhood. However, as they get older and with more exposure, they develop immunity that prevents clinical disease. Typically, immunity to severe symptoms of malaria is acquired during childhood. After that, individuals progress to develop mild malaria and it is only after many years of constant exposure that individuals living in endemic area develop clinical immunity. This type of immunity is not sterilizing but essentially controls parasite burden and hamper the development of disease symptoms. Adapted from (Langhorne et al., 2008).
Figure 1.3. Antibody-mediated Immune Mechanisms to Malaria Blood-stage Parasite.

Antibodies to merozoites may mediate multiple protective immune mechanisms. This includes direct inhibition of invasion process to and growth of parasite within erythrocytes as well as inhibits the rupture of schizont. Antibodies directed to merozoites can interact with complement (red stars) to lyse merozoite, and agglutinate merozoites to prevent them from scattering after egress from schizont. Antibodies bind to the surface of merozoites for opsonization. The opsonized merozoites promote phagocytic clearance by monocytes and macrophages (via the Fc Receptor), interact with neutrophils that lead to the production of ROS, and stimulate monocytes to secrete soluble factors (represented by triangles) that inhibit parasite growth or kill parasites. Yellow and green antibodies represent different IgG subclasses. Adapted from (Teo et al., 2016).
Chapter 2. Materials and Methods

2.1. Study Site

A cross-sectional study of 126 individuals (5-40 years-old) was conducted in two villages (Pigapu and Hiripau) in Timika from July to December 2014. Almost 1000 people from 137 households were screened for \textit{P. falciparum} and \textit{P. vivax} malaria. Light microscopy positive individuals, as well as negative controls that provided written consent were enrolled in the study. A 10ml venous blood sample was taken at enrolment and PBMCs, pRBC pellets and plasma were frozen. Light microscopy positive participants received standard first-line anti-malarial treatment according to the Ministry of Health Republic of Indonesia guidelines. A short questionnaire covering baseline data, clinical examinations, usage of antimalarial drugs in the last one month and history of fever in the last 48 hours were administered to all participants at enrolment. The presence, species and parasite density in every blood sample was determined by microscopy and species-specific PCR. In addition, approximately 50 patients aged 5-40 years-old presenting with clinical malaria to \textit{P. falciparum} and \textit{P. vivax} at the Rumah Sakit Mitra Masyarakat (local hospital) were enrolled in the study. Sample collection was approved by Human Ethics Committees at the Eijkman Institute of Molecular Biology in Jakarta and The Walter & Eliza Hall Institute of Medical Research in Melbourne.

2.2. Enzyme-Linked Immunosorbent Assay (ELISA)

Immunoglobulin G responses were measured by ELISA. ELISA microtiter plates (Corning, USA) were coated with 50 µl of \textit{P. falciparum} or \textit{P. vivax} antigen in concentration 0.5-2.0 µg/ml in carbonate buffer pH 9.6 at 4°C overnight. Each antigen was individually optimized for coating, secondary antibody concentration (HRP-conjugated mouse anti-human IgG antibody), and development time. After washing with Phosphate-
buffered Saline (PBS), plates were blocked with 5% skim milk for 1 hour at 37°C. Serial two-fold dilution of plasma samples was added to plates after washing with 0.05% PBS-Tween20 (Sigma, USA). The plates then were incubated for 1 hour at 37°C. The plates were washed 3 times and incubated with an HRP-conjugated mouse anti-human IgG antibody (Southern Biotech, USA) at 1:1000 dilution for 1 hour at 37°C. After washing, plates were developed using tetramethylbenzidine (KPL, USA) and hydrogen peroxide. The reaction was stopped with phosphoric acid. Absorbance was read at 450 nm within 30 minutes after adding stop solution. After subtracting optical density (OD) of the background, antibody titres were calculated as the plasma dilution with an OD value higher than the mean of negative controls plus 2 standard deviations for each response. Negative controls for this ELISA were from naive Australian blood donors.

2.3. Parasite Culture

*Plasmodium falciparum* parasite strain 3D7 was cultured in washed human O+ erythrocytes. Washing buffer (14 ml of 7.5% NaHCO₃ and 500 ml of RPMI-HEPES) was added to the mixture of human O+ erythrocytes from two different Australian blood donors. The mixture was then centrifuged at 300 x g for 8 minutes and supernatant was aspirated. Tissue culture medium (14 ml of 7.5% NaHCO₃, 50 ml of heat-inactivated normal human serum, and 500 ml of RPMI-HEPES) was added to the culture flask (Corning, USA). Parasitemia was adjusted to 1% at 5% haematocrit with washed human erythrocytes. Cultures were maintained in the incubator at 37°C with a gas mixture of 95% nitrogen, 4% CO₂, and 1% O₂ environment for ~42 hours or 1 cycle of parasite replication.

2.4. Parasite Synchronisation

Parasite cultures were checked by light microscopy to determine parasitemia levels. When cultures reached 3-5% ring stage, parasites were harvested, transferred to 50ml tube and centrifuged at 300 x g for 8
minutes. Culture supernatants were aspirated and three to four volumes of pre-warmed filter-sterilised 5% sorbitol were added to the tube. The mixture of parasite and sorbitol was vortexed for 30 seconds and then incubated at 37°C for 12 minutes. Washing buffer was then added to a final volume of the 50ml and parasites were then centrifuged at 300 x g for 8 minutes. After a second wash, the percentage of ring stage parasitemia was determined by light microscopy. Culture medium was added to the synchronized parasites and then transferred to a new culture flask. Synchronized parasites were maintained under normal culture conditions.

2.5. Growth Inhibitory Assay (GIA)

Highly synchronous 3D7 late-pigmented-trophozoites (0.1-0.3% parasitemia) were added to erythrocytes at a 2% haematocrit in 96 wells sterile U-bottom plates (Corning, USA). Plasma samples (not heat inactivated at 56°C) were then added at 1:30 dilution to each well and the plates were incubated for ~90 hours at 37°C in a sealed, humidified and gassed box for a 2 replication cycle-assay. After incubation, each well was fixed with 0.25% glutaraldehyde diluted in PBS for 30 minutes at 22°C. The plates then were centrifuged at 290 x g for 2 minutes. Supernatants were discarded and parasites were stained with 5x SYBR Green (Invitrogen, USA) diluted in PBS. Parasitemia was assessed by flow cytometry by acquiring 50,000 events. Growth inhibition was calculated relative to parasites grown in non-immune serum. All samples were tested in triplicate.

2.6. B cells ELISPOT (Enzyme-Linked Immunosorbent Spot)

Peripheral blood mononuclear cells (PBMCs) were stimulated for 9 days in media (RPMI-HEPES with 10% HI-FCS, 2 mM L-glutamine, 50 µM 2-ME) with a cocktail of polyclonal activators: 2.5 µg/ml of CpG-ODN 2006 (Miltenyi Biotech, USA), protein A from Staphylococcus aureus Cowan at a 1:100,000 dilution (Sigma, USA), pokeweed mitogen at a 1:10,000
dilution (Sigma, USA), and IL-10 at 25 ng/ml (Miltenyi Biotech, USA). ELISPOT plates (Merck Milipore, IRL) were coated with either 10 µg/ml of anti-human IgG antibody (Mabtech, AUS); 1% bovine serum albumin (BSA); or 5 µg/ml of antigens of interest for 18 hours before use. After washing with 0.05% D-PBS-Tween20 (D-PBST) then with PBS, plates were blocked with 1% BSA in RPMI-HEPES for at least 2 hours at 37°C in 5% CO₂ incubator. Cells were then harvested, washed and added to pre-coated ELISPOT plates (5x10⁵ cells/well) and incubated for 18 hours at 37°C in 5% CO₂ incubator. Plates were washed four times with PBS and then D-PBST, and incubated overnight at 4°C in the dark with 1 µg/ml of anti-human IgG-biotin antibody (Mabtech, AUS). After washing with D-PBST, alkaline phosphate conjugate was added to the plates at 1:1000 dilution to detect antibody-secreting cells (ASCs) and plates were incubated for 1 hour at room temperature. The plates then were developed using 5-bromo-4-chloro-3-indolyl phosphate (BCIP)/nitro blue tetrazolium (NBT) for 1 hour at room temperature. After incubation, plates were washed with MilliQ water & dried overnight in the dark. ASCs then were quantified using an AID plate reader. Results were reported as frequencies of MBCs per 10⁶ PBMCs, where ‘MBCs’ refers to the number of ASCs derived from MBCs during the 9-day culture, and ‘10⁶ PBMCs’ refers to the number of PBMCs after culture.

2.7. Statistical Analysis

Statistical analysis was performed using STATA version 12.1 (StataCorp, USA) & GraphPad Prism version 7. Kruskal-Wallis tests were used to compare continuous variables between groups. The association between antibody levels and symptomatic malaria was studied using logistic regression model. A linear regression model was used to estimate the association between antibody responses and parasitemia levels. To measure the strength of the linear association, Pearson’s correlation was performed. Spearman’s Correlation Coefficient was performed to assess the relationships between growth-inhibition activities and antibody levels.
Antibody levels were analyzed as continuous and categorical variables. To analyze antibody levels as categorical variables, all samples were classified into two equal groups, reflecting high and low IgG responses for each antigen. Clinical and biological features were examined as potential confounders in regression analysis. The test showed that age was not a confounding factor whereas weight was identified as being significantly associated with antibodies to PfEBA-140 and asymptomatic individuals in comparison with individuals with symptomatic cases. Therefore, the confounding effect on antibody responses was controlled by adjusting for weight in regression analysis.
Chapter 3. Antibody Responses to 
*Plasmodium falciparum* and 
*Plasmodium vivax* Invasion Ligands

3.1. Introduction

The EBAs and PfRhs merozoite antigens are postulated to be targets of inhibitory antibodies that prevent parasite invasion and subsequent replication in the erythrocyte (Cowman and Crabb, 2006). In support of that proposition, rabbit antisera raised against PfEBA-175, PfEBA-140, PfRh2b, PfRh4, and PfRh5 have been shown to inhibit parasite replication *in vitro* (Baum et al., 2009; Duraisingh et al., 2003b; Jiang et al., 2011; Maier et al., 2003; Tham et al., 2009). Moreover, studies in human revealed that antibodies specific for PfEBA-175 and PfEBA-140 are significantly associated with protection from symptomatic malaria (Chiu et al., 2016; Irani et al., 2015; Richards et al., 2013; Richards et al., 2010).

From the very little available information on immune responses to PfRh antigens, PfRh2, PfRh4, PfRh5 are thought to be important targets of naturally acquired immunity. Despite promising results in experimental animals and *in vitro* assays, it is unclear which one of these invasion ligands are the most important targets of acquired immunity to malaria. This information is essential to identify the key antigens for new vaccine combinations and constitutes a critical validation step prior to further development.

Malaria disease caused by *P. vivax* is known for years as a neglected disease, but in fact this parasite causes majority (86%) of death cases outside sub-Saharan Africa (Gething et al., 2012; WHO, 2016). Severe cases and emerging *P. vivax* drug resistance drag attention back to this parasite. Therefore, it is also important to understand the
mechanisms that drive immunity to *P. vivax* malaria. Unlike immunity to *P. falciparum* that has been studying extensively, information regarding mechanisms of immunity to *P. vivax* is limited. *Plasmodium vivax* Duffy Binding Protein is one of a few *P. vivax* antigens that have been extensively characterized among others *P. vivax* antigens.

Naturally acquired immunity to malaria parasites needs years of repeated exposures to develop and is not sterilizing. Experimental evidence suggests that antibodies to malaria parasites are short-lived and rapidly lost if individuals leave endemic areas, which indicates that constant exposure to malaria parasites is necessary for the establishment of functional immune memory cells, effector cells, as well as for their maintenance (Langhorne et al., 2008; Marsh and Kinyanjui, 2006). In spite of the fact that antibodies take a critical role in providing protection against malaria, only little is known about the fundamental cellular mechanisms of acquiring humoral immunity as the response to natural infections in malaria-exposed individuals. The current knowledge of the acquisition of immunity mostly came from animal malaria models and studies of vaccine-induced antibodies in humans. Those studies highlighted that the production and maintenance of MBCs and long-lived plasma cells (LLPCs) are required for long-lived antibody-based immunity (Gourley et al., 2004; Tarlinton, 2008). Further studies on the cellular basis of the ineffective acquisition of immunity to malaria are essential to gain a better understanding to inform in the design of an effective malaria vaccine. In this chapter, antibody responses to a range of merozoite invasion ligands as well as the frequencies of MBCs were quantified in PBMCs to determine if acquired immunity to malaria is associated with the development of MBCs specific to these antigens.
3.2. Results

3.2.1. Timika cohort study participants

After confirmation of diagnosis by microscopy and species-specific PCR, all samples from the cross-sectional study were divided into symptomatic malaria, asymptomatic malaria, and healthy controls. Individuals that were light microscopy positive (LM+) and PCR positive (PCR+) with malaria symptoms and/or high parasitemia were grouped as symptomatic malaria. While individuals that were LM+ and PCR+ without any symptoms were considered as asymptomatic cases. Healthy controls were individuals who had undetectable parasites by LM, with a small proportion (P. falciparum PCR+ = 6%; P. vivax PCR+ = 12%; mixed PCR+ = 3%) testing malaria positive only by PCR.

To confirm exposure to malaria among the PCR negative individuals in the healthy control group, total IgG to P. falciparum protein extract and PvDBP was determined by ELISA. From that group, 8 individuals were found to be seronegative. As exposure to malaria could not be confirmed in this group, these individuals were excluded from the analysis. The samples that were included in this analysis were as follows: 34 P. falciparum-asymptomatic, 30 P. vivax-asymptomatic, 33 P. falciparum-symptomatic, 31 P. vivax-symptomatic, and 31 malaria-exposed healthy controls.

The characteristics of the different groups are shown in Table 3.1. Briefly, all groups have a highly similar female and male ratio. No significant differences were found between hemoglobin levels of different groups, except for the P. vivax asymptomatic group, which was significantly lower than healthy control. The majority of (range from 87% to 100%) enrolled participants across groups were of Papuan origin, only a few of them came from free-malaria provinces. Papuans live in different geographic areas. Some of them live in Timika lowlands and some lives in Timika highlands. Highlands are considered as malaria-free areas whereas malaria exposure is common in lowlands. Migration of no pre-
existing immunity adults from highlands to work in lowlands reflecting that vulnerability to symptomatic malaria infection can appear in all age groups.

The average age of the participants in all groups ranges from 10.64-21.91 years old (Figure 3.1.A). The average age of healthy control and *P. vivax* symptomatic group were significantly higher than *P. vivax* asymptomatic group. No significant differences of age distribution were found between healthy control, *P. falciparum* asymptomatic, and *P. falciparum* symptomatic groups, indicating that age is not a confounding factor in the analysis of immune responses across these groups. Further, Figure 3.1.B demonstrates a comparison of parasitemia levels between asymptomatic and symptomatic group. Parasitemia levels of asymptomatic individuals were more than 60 times lower than symptomatic individuals for both *P. falciparum* and *P. vivax* infected individuals.

### 3.2.2. Antibody responses to *P. falciparum* & *P. vivax* blood-stage antigens

Total IgG levels of all samples were measured to seven *P. falciparum* (PfEBA-175 Region IV-V, PfEBA-140 Region III-V, PfRh2 a/b common region, PfRh4.9, PfRh5, PfRipr, & CyRPA) and one *P. vivax* (PvDBP Region III-V) merozoite-stage vaccine candidate antigens. In general, the vast majority of *P. falciparum*-positive samples recognized six antigens, except for CyRPA. Antibody levels to CyRPA appeared to be very low across all samples tested. Most of the participants (82%) of this study showed antibody levels below the assay cut-off, indicating that CyRPA is not a major target of naturally acquired immune responses in this population. In general, antibody responses to PfEBA-175 Region IV-V and PfEBA-140 Region III-V were higher compared to antibody responses to members of the PfRh family (Figure 3.2). Figure 3.3 demonstrates the relationship between age and antibody responses between four age groups (children, adolescent, young adult, adult). Except for responses to PfEBA-140 and PvDBP, in which antibody levels were significantly higher
in adults compared to children ($p<0.001$, $p<0.05$, respectively), there were no significant differences in the reactivity to the other merozoite antigens among children, adolescents, young adults, and adults.

To investigate associations between antibody responses and parasitemia levels among malaria positive individuals, a linear regression model was used. Individuals with *P. falciparum* parasitemia were correlated with antibody responses to *P. falciparum* antigens, while *P. vivax* parasitemia were correlated with antibody responses to PvDBP Region III-V. The increasing levels of anti-PfEBA-175 Region IV-V, PfRh4, PfRh5, and PfRipr antibodies were found to be significantly associated with the reduction of parasitemia in -0.301 parasite/µl ($p<0.05$), -0.519 parasite/µl ($p<0.05$), -0.555 parasite/µl ($p<0.05$), and -0.692 parasite/µl ($p<0.01$), respectively (Table 3.2). Consistent with these results, the Pearson’s correlation analysis that has been applied to determine the strength of the linear association shows that antibodies against those four antigens had relatively low negative correlations with parasitemia levels (Figure 3.4.A, D, E, & F).

Low negative correlations to parasitemia levels were also presented by anti-PfEBA-140 Region III-V ($r = -0.1130$, $p=0.363$) and PfRh2 ($r = -0.1085$, $p=0.382$) antibodies, but these observations failed to reach the level of statistical significance (Figure 3.4.B & C). On the other hand, Pearson’s correlation results show that antibody levels to PvDBP Region III-V ($r = 0.1115$) had low positive correlations with high-density parasitemia (Figure 3.4.G). Despite interesting trends in the relationship between parasite burden and antibody levels to PvDBP antigen, the association did not reach statistical significance ($p=0.392$).

The association between antibody responses and symptomatic malaria was studied using logistic regression model. For this particular analysis symptomatic malaria was individuals that had a history of fever (24-48 hours before enrolment day) or had a fever on the enrolment day, with *P. falciparum* parasitemia $>3,000$ parasite/µl. To avoid bias results, the confounding effects were controlled by adjusting for potential
confounders. For this regression analysis, age was not a confounder meanwhile weight was identified as being significantly associated with antibodies to PfEBA-140 and asymptomatic individuals. Therefore, Odd ratios were adjusted by weight to get the association between anti-PfEBA-140 and asymptomatic individuals in comparison with individuals with symptoms.

Analysing antibody titres as continuous variables on individuals experiencing symptomatic malaria compared to those of asymptomatic individuals revealed that increasing IgG levels specific for PfEBA-175 Region IV-V, PfRh4, PfRh5, and PfRipr were found to be significantly associated with a reduced risk of symptomatic malaria. Anti-PfEBA-175 antibodies were associated with an estimated 0.431-fold (56.9%) decrease in odds of getting a symptomatic case. Further, antibodies against PfRh4, PfRh5, and PfRipr were associated with a reduced risk of *P. falciparum* symptomatic case by 87.5%, 80.5%, and 89.4%, respectively. Moreover, antibodies against PfRh5 and PfRipr were also found to be associated with a predicted 0.306-fold (69.4%) and 0.222-fold (77.8%) decrease in odds of developing *P. falciparum* symptomatic infections in comparison with healthy controls (*p*<0.05) (Table 3.3).

A similar result was observed when analysing antibody titres as a categorical variable in *P. falciparum* symptomatic cases were compared against asymptomatic individuals. Individuals with high levels of anti-PfEBA-175 Region IV-V, PfRh4, PfRh5, and PfRipr antibodies were significantly associated with a decrease in chances of developing symptomatic malaria. When antibody levels of *P. falciparum* cases were compared to those of healthy individuals, a high level of anti-PfRipr antibody titres was found to be significantly associated with a decrease in odds of developing *P. falciparum* symptomatic malaria by 87.5%. Reduced odds of *P. falciparum* symptomatic case (0.278-fold) were also observed with increasing levels of anti-PfRh5 antibodies, although the *p*-value was only marginally significant (*p*=0.058) (Table 3.4). From both of logistic
regression analysis, anti-PfEBA-140 and PfRh2 were not significantly associated with malaria protection.

3.2.3. Frequency of memory B cells

The frequency of ASCs was quantified in PBMCs to determine if clinical immunity to malaria is associated with the development of MBCs specific for merozoite invasion ligands. The assay was performed on *P. falciparum*-infected and healthy control samples from children and adults who are Lowlanders, Highlanders, and non-Papuans. The PBMC samples have travelled a long distance from Timika to Melbourne and were collected from people with conditions. Therefore, optimization experiments are required to obtain optimum conditions to perform B cell ELISPOT assays. The 9 days of polyclonal stimulation was applied to these assays because based on the preliminary experiments a longer stimulation day provides more time for MBCs to better differentiate into detectable ASCs.

Figure 3.5 shows that immune healthy controls, asymptomatic individuals and symptomatic individuals had similar frequencies of PfEBA-175 RIII-V-specific MBCs. In contrast, whereas anti-PfRh5 MBCs could be readily detected in healthy controls and asymptomatic individuals, these cells could not be found among individuals with symptomatic *P. falciparum* infection. Although the small sample size in this cohort did not allow performing a powerful statistical analysis, these trends suggest that PfRh5-specific MBCs could be a new predictor of protection from symptomatic malaria.

3.3. Discussion

The design of a widely effective anti-malaria vaccine requires a better understanding of which are the main antigenic targets of naturally acquired immunity across different geographic locations. Only a few immunological studies were performed to determine the association between antibody levels and protection against malaria parasites outside African regions. In this study, those associations were assessed from field
samples that were collected in Timika, Indonesia. Plasma samples from asymptomatic individuals, symptomatic individuals, and healthy immune controls were tested against a panel of merozoite invasion ligands.

Study participants were either long-term residents of Timika, an area where malaria transmission is perennial, or migrants from malaria low or free areas. As a result, there were a high proportion of malaria-naïve individuals that was susceptible to symptomatic infection in their adult life because of their limited malaria exposure. It is possible that similar antibody responses across age groups to majority antigens tested in this study, except for PfEBA-140 and PvDBP, reflect a large number of participants having their first malaria infection during adulthood. Consequently, the level of antibodies was not different compared to the children.

The vaccine candidate PfRh5 has been described to form a complex with PfRipr and CyRPA on the surface of the merozoite during the invasion. Despite the close proximity of these proteins on the parasite’s surface, antibody responses to these antigens may not be correlated. In particular, antibody levels to CyRPA were very low with most of the samples in the cohorts, including Timika cohort, being seronegative to this antigen. Interestingly, serum samples from individuals living in Ghana showed no CyRPA-specific IgG (Dreyer et al., 2012). This lack of reactivity appeared to be associated with polymorphism within the CyPRA molecule, as only one nonsynonymous SNP was found among a range of P. falciparum isolates. It might suggest that the high degree of CyRPA sequence conservation is associated with low-level of natural immune pressure, due to the limitation of exposure time to antibodies during the invasion process.

Compared to antibody responses to EBAs, the reactivity level to PfRh5 and PfRipr was in general lower. Despite inducing low antibody levels, these proteins were found to be important targets of naturally acquired immunity to malaria, as individuals that had high levels of antibodies to PfRh5 and PfRipr were found to be protected from
symptomatic malaria and high parasitemia levels. This finding is consistent with previous studies from a longitudinal cohort conducted in PNG, where antibodies specific for PfRh5 were associated with protection from high-density malaria and clinical symptoms in children (Chiu et al., 2014). Moderate to high levels of antibodies specific to both these antigens were strongly associated with protective immunity (Richards et al., 2013). In addition, studies on immune responses in two different malaria-endemic areas, Senegal and PNG, revealed that the predominant IgG subclass responses to PfRh5 and PfRipr appear to be IgG1 and IgG3 (Patel et al., 2013; Weaver et al., 2016).

Previous results in other cohort studies indicated that antibody responses to PfEBA-175 and PfRh4 predicted protection from clinical malaria (Chiu et al., 2016; Reiling et al., 2012; Richards et al., 2013). In the present study, similar results were reported. Increasing levels of antibody responses to conserved regions of PfEBA-175, Region IV-V, and PfRh4 in asymptomatic individuals but not healthy controls (presumably due to the small sample size) were associated with a reduced risk of having high parasite densities and with protection to *P. falciparum* symptomatic malaria. This data suggests that low levels of circulating parasites might be required to maintain protective levels of antibodies against these antigens in asymptomatic individuals. On the other hand, acquired antibodies to PfEBA-140 Region III-V and PfRh2 were not associated with an altered risk of asymptomatic infections compared with symptomatic malaria. In contrast to antibody responses to PfEBA175, PfRh4, PfRh5, and PfRipr that were associated with malaria protection and low parasitemia, anti-PvDBP Region III-V antibodies showed a positive correlation to high parasitemia. These findings suggest that PvDBP Region III-V could be potentially used as serological markers for recent exposure.

Previous studies identified the presence of MBCs specific for highly abundant merozoite surface antigens such as MSP1 and AMA1 (Ndungu et al., 2013; Nogaro et al., 2011; Weiss et al., 2010; Wipasa et al., 2010).
A study in Mali revealed that parasite-specific MBCs are acquired gradually in areas of high seasonal malaria transmission (Weiss et al., 2010). To date, the development of MBCs specific for *P. falciparum* invasion ligands has not been investigated. Here we reported for the first time, that individuals in a malaria endemic area of Indonesia acquire MBCs specific for PfEBA-175 and PfRh5.

The frequency of PfEBA-175-specific MBCs was not significantly different between symptomatic, asymptomatic individuals or malaria-exposed healthy controls. In contrast, whereas PfRh5-specific MBCs were found in malaria-exposed healthy controls and asymptomatic individuals, no MBCs-specific for this antigen could be detected among *P. falciparum* symptomatic individuals. This finding is consistent with the serology data revealing a strong association between antibody responses to PfRh5 and protection of symptomatic malaria. Thus, together these results suggest that PfRh5 is an essential target of natural immunity to malaria and a strong vaccine candidate.
Table 3.1. Epidemiological characteristics of the Timika study

<table>
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Values for hemoglobin level were indicated as median and other characteristics were indicated as the percentage of individual values. * Significantly different to healty control, Kruskal-Wallis test (p<0.05).

Figure 3.1. Age distribution and parasitemia level of study participants.

(A) Average age of study participants. (B) Parasitemia level. Data are shown as median with 95% confidence interval (CI).

* p<0.05  ** p<0.01  **** p<0.0001
Figure 3.2. Antibody levels to seven *P. falciparum* merozoite-stage antigens.

Antibody titres to PfEBA and PfRh proteins were measured by ELISA. Analysis was done with Kruskal-Wallis test and data are shown as median with 95% CI.

* $p<0.05$  ** $p<0.01$  *** $p<0.001$  **** $p<0.0001$
Figure 3.3. Antibody levels to a panel of *P. falciparum* and *P. vivax* merozoite-stage antigens by age.

Antibody responses were compared between four age groups (children, adolescent, young adults, and adults) to examine the relationship between age and antibody responses. Analysis was done with Kruskal-Wallis test. Data are shown as median with 95% CI. * *p < 0.05  *** *p < 0.001
Figure 3.4. Correlation between antibody responses and parasitemia level.

Pearson’s Correlation was performed to measure the strength of linear association between antibody response and parasitemia level. r values represent Pearson’s correlation coefficients. Antibody responses to six *P. falciparum* merozoite-stage antigens were negatively correlated with parasitemia level, but only anti-(A) PfEBA-175 Region IV-V, (D) PfRh4, (E) PfRh5, and (F) PfRipr showed significant correlations. On the other hand, positive correlations were found in (G) PvDBP Region III-V (not significant).
Table 3.2. Correlation between antibody responses and parasitemia levels

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Coef</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>PfEBA-175 RIV-V</td>
<td>-0.301</td>
<td>0.013</td>
<td>-0.535 -0.067</td>
</tr>
<tr>
<td>PfEBA-140 RIII-V</td>
<td>-0.129</td>
<td>0.363</td>
<td>-0.409 0.152</td>
</tr>
<tr>
<td>PfRh2</td>
<td>-0.169</td>
<td>0.382</td>
<td>-0.551 0.214</td>
</tr>
<tr>
<td>PfRh4</td>
<td>-0.519</td>
<td>0.026</td>
<td>-0.974 -0.065</td>
</tr>
<tr>
<td>PfRh5</td>
<td>-0.555</td>
<td>0.011</td>
<td>-0.981 -0.130</td>
</tr>
<tr>
<td>PfRipr</td>
<td>-0.692</td>
<td>0.001</td>
<td>-1.100 -0.284</td>
</tr>
<tr>
<td>PvDBP RIII-V</td>
<td>0.179</td>
<td>0.392</td>
<td>-0.237 0.596</td>
</tr>
</tbody>
</table>

Antibody responses to *P. falciparum* and *P. vivax* invasion ligands were analyzed as continuous/numerical variable using linear regression model. Values represent ratios unadjusted with 95% CI.
Table 3.3. Association between antibody responses and protection from *P. falciparum* symptomatic malaria (Numerical variable)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Symptomatic vs Asymptomatic</th>
<th>Symptomatic vs Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>p-value</td>
</tr>
<tr>
<td>PIEBA175 RIV-V</td>
<td>0.431</td>
<td>0.045</td>
</tr>
<tr>
<td>PIEBA140 RIII-V</td>
<td>0.549</td>
<td>0.192</td>
</tr>
<tr>
<td>PfRh2</td>
<td>0.614</td>
<td>0.296</td>
</tr>
<tr>
<td>PfRh4</td>
<td>0.125</td>
<td>0.009</td>
</tr>
<tr>
<td>PfRh5</td>
<td>0.195</td>
<td>0.015</td>
</tr>
<tr>
<td>PfRipr</td>
<td>0.106</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Antibody responses to six *P. falciparum* invasion ligands were analyzed as continuous/numerical variable using logistic regression model. Association of antibody responses and malaria protection are expressed as odd ratio (OR). Odd ratio values less than 1 means a reduced risk/odd of outcome of interest. Odd ratio values represent with 95% CI.
Table 3.4. Association between antibody responses and protection from *P. falciparum* symptomatic malaria (Categorical variable)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Symptomatic vs Asymptomatic</th>
<th>Symptomatic vs Healthy control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>OR</td>
<td>p-value</td>
</tr>
<tr>
<td>PfEBA175 RIV-V</td>
<td>0.143</td>
<td>0.008</td>
</tr>
<tr>
<td>PfEBA140 RIII-V</td>
<td>0.425</td>
<td>0.206</td>
</tr>
<tr>
<td>PfRh2</td>
<td>0.691</td>
<td>0.545</td>
</tr>
<tr>
<td>PfRh4</td>
<td>0.182</td>
<td>0.012</td>
</tr>
<tr>
<td>PfRh5</td>
<td>0.263</td>
<td>0.047</td>
</tr>
<tr>
<td>PfRipr</td>
<td>0.088</td>
<td>0.004</td>
</tr>
</tbody>
</table>

Antibody responses to six *P. falciparum* invasion ligands were analyzed as categorical variable using logistic regression model. Antibody levels were divided into low and high responder groups. Odd ratio values represent with 95% CI.
Figure 3.5. The total IgG and antigen-specific frequency of MBCs.

The frequency of IgG\(^+\) MBCs per million PBMCs measured by ELISPOT to PfRh5 and PfEBA-175 RIII-V proteins. Analysis was obtained by Kruskal-Wallis test. Data are shown as mean with SEM.
Chapter 4. Functional Activity of Antibodies Against *Plasmodium falciparum* *in vitro*

4.1. Introduction

Understanding the role of antibodies to *Plasmodium* parasites is essential to gain further insights on key mechanisms by which such antibodies protect against malaria disease. Several effector mechanisms have been described including i) direct inhibition against merozoite proteins during the invasion process; ii) inhibition of intraerythrocytic parasite development; iii) antibody-mediated activation of the classical complement pathway; iv) opsonization of merozoite followed by phagocytosis; v) activation of monocytes and production of pro-inflammatory cytokines to intensify parasite killing (Boyle et al., 2015; Dent et al., 2008; Hill et al., 2013; Hill et al., 2017; Jensen et al., 1982; Osier et al., 2014).

The assessment of antibody responses to *P. falciparum* has relied mainly on serologic measurements. When used alone, this approach has limitations, as it does not portray the functional activity of the antibodies. Therefore, functional assays are also often needed to provide comprehensive information on protective immunity to malaria in addition to serologic approaches.

Growth inhibition assays are one of the functional approaches commonly used in immunogenicity studies and development of blood-stage vaccines. This functional assay measures the capacity of antibodies to prevent parasite invasion into the erythrocyte by measuring parasite growth *in vitro* in the presence of malaria-exposed plasma samples compared to malaria-unexposed plasma samples. Several different vaccine studies have used this assay in animal models and human donors (Dutta et al., 2009; Otsyula et al., 2013; Remarque et al., 2012; Singh et
al., 2006; Spring et al., 2009). To date, parasite growth inhibitory studies in human populations have been revealed inconsistent findings on whether inhibitory activity is a good predictor of reduced risk of malaria infection or not (Crompton et al., 2010; Dent et al., 2008; John et al., 2004; Marsh et al., 1989; McCallum et al., 2008; Perraut et al., 2005; Rono et al., 2012). This chapter explores the relationship between \textit{P. falciparum} growth-inhibitory function of antibodies and symptomatic malaria to advance the knowledge in the acquisition of human antibodies in a malaria endemic area of Indonesia.

4.2. Results

4.2.1. Profile of Inhibitory Activity Measured in the Timika cohort study

The functional activity of plasma samples to prevent \textit{in vitro} growth of 3D7 \textit{P. falciparum} laboratory strain was determined using a previously described (Healer et al., 2013; Persson et al., 2006) two-growth cycle assay with a few modifications. Briefly, highly synchronous late-pigmented-trophozoites to schizont stage parasites were adjusted to 0.1-0.3%-infected erythrocytes with a final 2% haematocrit and an optimum 1:30 plasma dilution from \textit{P. falciparum}-infected individuals were added to 96-well sterile U-bottom plates. Preliminary experiments have been done to determine the optimum plasma dilution. The 1:30 dilution was decided to be the best dilution with minimum background or non-specific inhibition compared to 1:10 dilution. Moreover, Inhibition variations were observed at 1:30 dilution in different samples. The plates then were incubated at 37°C for 2 cycles of parasite replication. Parasitemia was determined using flow cytometry and inhibitory activity was calculated relative to parasites grown in non-immune serum.

The distribution of growth-inhibitory activity for all \textit{P. falciparum}-positive plasma samples and healthy immune controls shown in Figure 4.1. Inhibitory activity ranged from -3.44% to 71% with a median of
14.73%. To investigate the relationship between inhibitory antibodies and age, percent inhibition was compared between children, young adults, and adults. Figure 4.2.A illustrates that growth-inhibitory activity did not increase with age. Similar median inhibitory activities were observed among four age groups (15.31%, 15.45%, 12.83, & 16.63%, respectively). Further, plasma from LM P. falciparum positive parasitemia (both symptomatic and asymptomatic) exhibited significantly higher growth-inhibitory activity compared to the healthy controls while individuals with malaria symptoms showed no significantly different in growth inhibition with asymptomatic individuals. The median of growth-inhibitory activity was 10.59% for healthy control group (range: -3.17%-39.84), 17.44% for asymptomatic group (range: -3.44%-55.57%), and 18.91% for symptomatic group (range: 4.07%-71.00%) (Figure 4.2.B).

4.2.2. The relationship between Growth-Inhibitory Activity and antibody levels to P. falciparum invasion ligands

It is reasonable to postulate that antibodies against merozoite surface antigens could exert parasite growth inhibitory activity. However, the association between protective immunity and inhibitory activities in humans remains to be explored. The relationship between growth inhibitory activity and antibody levels against different P. falciparum invasion ligands was explored using Spearman’s correlation test. Antibody levels to all invasion ligands were significantly correlated with parasite growth inhibitory activity albeit different magnitudes (Figure 4.3). Whereas antibody responses to PfEBA-140 Region III-V ($r_s = 0.4552, p<0.0001$) and PfRh4 ($r_s = 0.4226, p<0.0001$) were strongly correlated with GIA, anti-PfRh5 and PfRipr had lowest correlations to inhibitory activities ($r_s = 0.2643, p<0.01; r_s = 0.2620, p<0.01$, respectively).

To determine whether the growth-inhibitory activity was associated with malaria risk, the relationship between GIA and the risk of symptomatic malaria was also analyzed using logistic regression models. For this test, growth-inhibitory antibodies were analyzed as a numerical variable. Most
of the participant (>98%) in this study did not use antimalarial in the last one month, suggesting that the use of antimalarial might not be a confounding factor in the analysis of growth-inhibitory activity. Table 4.1 shows that increasing levels of parasite growth inhibitory antibodies were not associated with a reduced risk of symptomatic infection when compared with either asymptomatic infected individuals or healthy exposed donors.

4.3. Discussion

The identification of targets of naturally acquired immunity to malaria as well as the understanding of effector mechanisms underlying protective immunity is an important key to design an effective malaria vaccine. In order to gain a better understanding of basic mechanisms that drive protective immunity, growth inhibition assays were performed in a cross-sectional study conducted in Timika. The results in this chapter suggest that parasite growth inhibitory remain relatively stable as age increases. Children and adults living in a malaria endemic area like Timika exert the same level of inhibitory antibodies. These findings are similar to those from McCallum et al who showed that although malaria risk decreases with age, inhibitory activity negatively correlates with age and remains stable throughout the years (McCallum et al., 2008). Dent et al and Marsh et al also found negative correlations between age and inhibitory activity. Younger participant in both studies showed significantly higher growth-inhibitory activity compared to the older participants. Further, a study in Kenya by Dent et al reported that inhibitory activity decreased with age for all parasite lines tested (Dent et al., 2008; Marsh et al., 1989). Thus, these findings together with the data provided in this study suggest that acquisition of growth-inhibitory antibodies could be achieved at an early age after a few infections.

Not many studies investigated associations between protective immunity and antibody-mediated parasite growth-inhibitory activity. Data from field studies in Gambia, Kenya, and Senegal found no correlation
between growth inhibition and protection against clinical malaria (Marsh et al., 1989; McCallum et al., 2008; Perraut et al., 2005). In contrast, John et al. stated that having a high level of inhibitory antibodies specific to MSP1-19 were associated with 66% reduction of experiencing malaria (John et al., 2004). Furthermore, a study in Mali showed that inhibitory activity was associated with a decrease in odds of malaria (Crompton et al., 2010). Most recently, Rono et al. performed growth inhibition assay on human sera from Tanzania. They found that growth-inhibitory activity to 3D7 parasite line was associated with a reduced risk of malaria (Rono et al., 2012). To date, the reason for these inconsistencies is unclear. Several factors including, serum/plasma preparation protocols, genetic diversity of study participants and *P. falciparum* rates could influence assay outcomes (Crompton et al., 2010). In the Timika cohort, individuals with active infections had more inhibitory antibodies compared to healthy donors. This suggests that recent exposure to malaria parasites induces growth-inhibitory activity, which does not necessarily translate in protection from clinical malaria, as symptomatic individuals displayed a similar level of growth-inhibitory antibodies than individuals who did not show clinical symptoms.

Logistic regression analysis confirmed that increasing levels of inhibitory activity were not associated with a reduced risk of symptomatic malaria. Interestingly, the analysis of the correlation between antibody levels and growth-inhibitory activity revealed that antibody responses to PfEBA-140 Region III-V and PfRh4 had the strongest correlation with inhibitory activity, followed by PfEBA-175 Region IV-V and PfRh2. Antibodies against PfEBA-140 Region III-V appear to be highly abundant within plasma samples of the Timika cohort. Furthermore, high levels of anti-PfEBA140 Region III-V antibodies were not correlated with protection to symptomatic malaria. It is possible that the growth inhibitory activity detected in the assay with total plasma primarily reflects the reactivity of highly abundant antibodies such as those against PfEBA-140. Interestingly, antibodies against PfEBA-175 Region IV-V that were found
to be correlated with growth inhibitory activity were also abundant and associated with a decreased probability of symptomatic malaria. Thus, these assays together do not allow to conclusive determine the precise process by which anti-PfEBA-175 Region IV-V prevent symptomatic infection.

Acquired antibodies to PfRh5 and PfRipr that were associated with protection from symptomatic malaria were also found to correlate with parasite growth inhibitory activity. It is possible that the low level of these antibodies in plasma samples does not allow identifying associations between parasite growth inhibitory activity and protection from symptomatic malaria. In support to that view, previous studies using purified specific for PfRh5 and PfRipr reported invasion inhibition and robust *P. falciparum* growth inhibitory activity that was associated with reduced risk of clinical malaria (Bustamante et al., 2013; Chiu et al., 2014; Ntege et al., 2016; Tran et al., 2014). Further studies using antigen-specific antibodies will be required to determine whether anti-PfRh5 and anti-PfRipr parasite growth inhibitory antibodies are predictors of reduced risk of clinical malaria in the Timika cohort study.
Figure 4.1. Distribution of growth-inhibitory activity.

Inhibitory activities for *P. falciparum* symptomatic and asymptomatic individuals as well as healthy immune controls were measured relative to parasites grown in non-immune serum. Percent inhibitions ranged from -3.44% to 71% with most of the samples showed nearly 20% inhibition.

Figure 4.2. Growth Inhibition Profile of Study Population.

(A) Level of growth inhibition by age group. (B) Level of growth inhibition across malaria outcome group. Analysis was obtained by Kruskal-Wallis test. Data are shown as median with 95% confidence interval (CI).

**p<0.01 *** p<0.001
Figure 4.3. Correlation between in vitro growth inhibition and antibodies to *P. falciparum* antigens.

(A) PIEBA-175 Region IV-V. (B) PIEBA-140 Region III-V. (C) PfRh2. (D) PfRh4. (E) PfRh5. (F) PfRipr. $r_s$ value represents Spearman’s correlation coefficients.
Table 4.1. Association between growth-inhibitory antibodies and risk of symptomatic malaria

<table>
<thead>
<tr>
<th></th>
<th>OR</th>
<th>p-value</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptomatic vs Asymptomatic</td>
<td>2.181</td>
<td>0.340</td>
<td>0.439</td>
</tr>
<tr>
<td>Symptomatic vs Healthy control</td>
<td>18.493</td>
<td><strong>0.003</strong></td>
<td>2.615</td>
</tr>
<tr>
<td>Asymptomatic vs Healthy control</td>
<td>11.710</td>
<td><strong>0.014</strong></td>
<td>1.661</td>
</tr>
</tbody>
</table>

Growth-inhibitory antibodies were analyzed as a continuous/numerical variable using logistic regression model. Associations of growth inhibition and protection from malaria outcomes are expressed as odd ratio (OR). Ratios were presented with 95% (CI).
Chapter 5. Discussion

The WHO Global Technical Strategy (GTS) for malaria has established a target to eliminate malaria from at least 35 endemic countries by the year 2030. Despite a great reduction of clinical cases and mortality rates, malaria still takes the lives of estimated 639,000 children and adults every year (WHO, 2016). This makes malaria a major killer of children and a significant public health issue. Therefore, to achieve this ambitious elimination target new and advanced tools such as an effective malaria vaccine should be considered.

There are still many challenges to overcome in order to develop a highly effective malaria vaccine, including high genetic diversity of the *Plasmodium* parasite antigens and incomplete understanding of immune effector mechanisms required to provide immunity. The role of antibodies against blood-stage malaria parasites is known to be important to confer protection from clinical disease and to control parasitemia (Cohen et al., 1961), but the primary antigenic targets of naturally acquired immunity are not completely understood. Several immunology studies revealed that antibodies specific for invasion ligands block successfully parasite growth and are associated with reducing malaria risk (Chiu et al., 2014; Chiu et al., 2015; Egan et al., 1999; Reiling et al., 2012; Richards et al., 2013; Tran et al., 2014). These findings highlight the importance of identifying antigenic targets of immunity by investigating associations between acquired antibodies and protection against malaria, especially from field studies in order to inform in the design a highly effective malaria vaccine.

To address that challenge and also increase our understanding of the immune response to malaria, this thesis assessed associations between antibody responses and protection against symptomatic malaria from individuals living in Timika, Indonesia to identify targets of naturally acquired immunity to malaria in this region. The key findings of this thesis revealed important associations between protection from experiencing
symptomatic malaria and antibodies specific to PfEBA-175 Region IV-V, PfRh4, PfRh5, and PfRipr. Moreover, the result from this thesis describes for the first time the presence of MBCs specific for PfRh5 and their association with protection from *P. falciparum* symptomatic malaria.

Natural exposure to *Plasmodium* parasites did not induce high antibody responses to the recently characterized member of the PfRh5 complex, CyRPA. This protein is highly conserved, with only single SNP found across several *P. falciparum* laboratory strains and field isolates studied to date (Dreyer et al., 2012). These findings suggest that CyRPA might play an important role in parasite fitness and/or that it is not under immune pressure. Further work is required to understand more the reasons for the low immunogenicity of CyRPA during natural infection.

The level of antibody responses to PfRh5 and PfRipr were in general lower compared to response specific for EBL antigens. These findings are in agreement with those of Douglas et al., who found that PfRh5 have low natural immunogenicity (Douglas et al., 2011). The reasons why these antigens induce poor responses to natural infection are not fully understood, but it is possible that it reflects the short exposure time of this complex during the invasion. In the invasion process, the PfRh5/PfRipr/CyRPA complex is involved in the pore formation on erythrocyte surface, which allows Ca^{2+} flux from merozoite to erythrocyte (Volz et al., 2016; Weiss et al., 2015). As the complex is secreted to the surface of the merozoite only seconds before the invasion, its exposure to human immune system is limited (Weiss et al., 2016). Despite this limitation, low levels of naturally acquired antibodies against the complex appear to be highly protective, as they might block an essential step in the merozoite invasion process.

The recognition of PfRh5 as a malaria vaccine candidate has been increasing in recent years and the outcomes from this study provide support to that notion. Protective immunity to symptomatic malaria was found to be associated with anti-PfRh5 as well as anti-PfRipr antibodies. Analysis of plasma sample from malaria-exposed individuals in Timika
revealed that with increasing level of anti-PfRh5 and PfRipr antibodies significantly promoting the development of asymptomatic malaria rather than symptomatic malaria. In addition, the present study also reported that having a high level of antibodies specific to both ligands were substantially associated with a reduced risk of getting symptomatic malaria and advanced to remain healthy. This result highlights the importance of PfRh5 and PfRipr as the antigenic targets of naturally acquired malaria immunity considering that increasing anti-PfRh5 and PfRipr may favour clearing malaria infections.

These findings are consistent with results from Richards et al. that found a strong association between high antibody levels to PfRh5 and PfRipr with protective immunity (Richards et al., 2013). Interestingly, a longitudinal study of children in PNG showed that anti-PfRh5 but not anti-PfRipr antibodies were associated with protective immunity to a clinical episode and high-density parasitemia (Chiu et al., 2014). Inconsistent results of a protective role for antibodies to PfRipr may be partly due to different methods applied for the analysis. To date, the association between malaria protection and antibody responses to PfRipr has not been widely studied. Therefore, further investigations in other settings will be important in determining the importance of PfRipr as a target of naturally acquired immunity to malaria.

Besides anti-PfRh5 and PfRipr antibodies that were associated with symptomatic malaria protection, antibodies to PfEBA-175 Region IV-V and PfRh4 were associated with acquired immunity that prevented malaria infection leading to symptomatic malaria but might not against malaria infection per se. Consistent with those findings, previous studies reporting the role of antibodies to PfEBA-175 and PfRh4 in protection from clinical episodes (Chiu et al., 2016; Reiling et al., 2012; Richards et al., 2013; Richards et al., 2010). Recent studies revealed the PfEBA175, Region IV-V domain is highly conserved across different parasite strains (Healer et al., 2013). Similar to previous finding (Chiu et al., 2016), our results here revealed that antibodies specific these highly conserved regions are
associated with protection from symptomatic malaria. The fact that this domain is highly conserved suggests that it may be essential for PfEBA-175 function, even though the precise role of this domain has not been fully explained. Indeed, there is an indication that PfEBA-175 Region IV-V are involved in dimerization of this ligand that is essential in binding to its receptor glycoporphin A (Tolia et al., 2005). Interaction of PfEBA-175 with its associate receptor is required for the release of rhoptry proteins onto the parasite surface (Singh et al., 2010). Thus it is possible that antibodies against conserved regions of PfEBA-175 Region IV-V prevent the release of apical organelle proteins, an important event required for merozoite invasion.

Previous immunology studies reported that high levels of antibody to PfEBA-140 Region III-V and PfRh2 were strongly predictive of protection from high parasitemia and prevent disease symptoms (Reiling et al., 2010; Richards et al., 2013; Richards et al., 2010). However, this study failed to reproduce those findings. The increasing anti-PfEBA-140 Region III-V and PfRh2 antibodies were not associated with an increased probability of acquiring asymptomatic parasitemia in comparison with getting symptomatic malaria. Antibody to PfEBA-140 is not always reported to have an association with reduced parasitemia. A study in Nigeria found the presence of significantly higher antibody levels against PfEBA-140 Region III-V in individuals with high-frequency parasitemia compared to individuals with low parasitemia level (Tijani et al. 2017).

Due to its key function to bind to DARC receptor for parasite invasion (Ranjan and Chitnis, 1999), PvDBP Region II has become the major focus on immunology studies in P. vivax biology. Antibodies to PvDBPII have been shown to be associated with reduced risk of P. vivax infection (Grimberg et al., 2007; King et al., 2008). However, Region II is likely to be under natural selection by the human immune system and has been shown to be polymorphic (Ampudia et al., 1996; Gosi et al., 2008; Kho et al., 2001; Sousa et al., 2006; Tsuboi et al., 1994; Xainli et al., 2000). Thus, the high polymorphism of PvDBPII somehow discourages the
idea of this protein being used as an effective anti-malaria vaccine. On the other hand, antibody responses to the more conserved and hydrophobic regions of PvDBP, Region III-V have not been investigated. In this study, antibodies against PvDBP Region III-V were not found to be protective of *P. vivax* symptomatic malaria. Rather, antibody levels to this antigen were correlated with high parasitemia suggesting that these responses could be a good marker or recent *P. vivax* exposure. Indeed, the development of biomarkers of recent exposure to malaria is an area of growing interest in order to generate robust surveillance tools to monitor transmission after the implementation of various control strategies.

To date, very little information is available about cellular mechanisms of acquired humoral immunity to natural infection. Previous studies in several different malaria areas found that malaria-exposed individuals acquire MBCs specific for AMA1, MSP1, MSP2, MSP3, and circumsporozoite protein (CSP) (Ayieko et al., 2013; Ndungu et al., 2013; Nogaro et al., 2011; Weiss et al., 2010; Wipasa et al., 2010). This thesis for the first time demonstrates that residents of malaria endemic region, like Timika, produce MBCs specific for the leading vaccine candidate PfRh5 as well as the best-characterized ligand among the EBL family members, PfEBA-175 Region III-V.

Notably, anti-PfRh5 MBCs were not detected among individuals with symptomatic *P. falciparum* infection, but were only found in asymptomatic and malaria-exposed healthy individuals. Although further investigations with larger sample sizes will be required to confirm these interesting trends, the results support the notion that the acquisition of clinical immunity to *P. falciparum* malaria might be associated with the induction of anti-PfRh5 MBCs. Anti-PfRh5 MBCs might be able to become rapidly activated upon re-infection and secrete antibodies required to protect the host from high-density parasitemia and illness. Thus together the serology and cellular results presented here have provided further information supporting the idea that PfRh5 is an essential target of natural
immunity to malaria and a strong candidate, which should be considered for the next generation of anti-malaria vaccines.
Conclusion and Future Perspectives

A better understanding of the immune response that mediates naturally acquired immunity to malaria parasites is required to develop a highly effective malaria vaccine. The findings presented in this thesis support the notion that antibody responses to PfEBA-175, PfRh4, PfRh5, and PfRipr are important predictors of protective immunity to symptomatic malaria and are strong malaria vaccine candidates. Furthermore, having a high level of IgG specific to PfRipr significantly promoting to remain healthy rather than to develop symptomatic malaria. On the other hand, PvDBP could be suitable as a serological marker for recent \textit{P. vivax} exposure. Lastly, the functional assay demonstrates significant \textit{in vitro} growth inhibition of \textit{P. falciparum} by total IgG to six \textit{P. falciparum} merozoite-stage vaccine candidates in this study, but not necessarily associated with protection from malaria. The findings highlight the importance of performing growth inhibition assay using antigen-specific antibodies to PfEBA-175, PfRh4, PfRh5, and PfRipr and/or evaluating other effector mechanisms to further our understanding on the mechanisms that drive the acquisition of immunity to malaria to support vaccine development and malaria eradication.


strategy for enhancing immunogenicity of malaria vaccine candidates. Vaccine 25, 3923-3933.


predict protection from malaria. The Journal Of Infectious Diseases 209, 789-798.


Author/s:
Utami, Retno Ayu Setya

Title:
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2018

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File Description:
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