DYNAMICS OF IMMUNOLOGICAL MEMORY TO *PLASMODIUM FALCIPARUM* INFECTION AMONG GHANAIANS LIVING IN A MALARIA HYPO-ENDEMIC REGION

BY

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DECLARATION

I hereby declare that this submission is my own work towards the MPhil Biochemistry and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the university, except where due acknowledgement has been made in the text.

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This thesis is dedicated to Mrs. Amma Aboagyewaa Akorful. Thank you for your high involvement in my research career.

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ABSTRACT

Plasmodium falciparum (*Pf*) malaria remains an important cause of morbidity and mortality in sub-Saharan Africa particularly among children under 5 years of age and pregnant women. In Ghana, malaria alone accounts for about 33% of all deaths in children under 5 years of age. Malaria deaths may be averted if an efficacious vaccine that can offer long-term protection against the disease was available. However, such a vaccine is currently non-existent primarily due to lack of understanding of the dynamics of immunological memory in *P. falciparum* infection. Protection offered by the world's leading malaria vaccine candidate, RTS,S/AS01, showed an efficacy decline from 50% to 36% within a 4 year period, suggesting a waning immunological memory to drive an effective secondary immune response. B cells are the important custodians of immunological memory, capable of developing into antibody producing cells (plasma cells) to mount an immune response against invading pathogens. With a 1-year longitudinal design, sampling at 4 timepoints at quarterly intervals, the present study aimed to assess the immunological profiles of individuals living in the Greater Accra region of Ghana, a malaria endemic zone. The afro-immuno assay ELISA protocol was used to quantify antibody levels against candidate malaria vaccine antigens (AMA1, CSP, GLURP-R0 and R2, LSA1 and MSP3) and crude antigens of the schizonts stage parasite across the 4 sampling timepoints. A culture-based assay was used to determine memory B cell (MBC) responses against the whole parasite in the schizonts stage. A flow cytometry panel was also designed to profile the B cell phenotypes across the period of the study. The results showed transient changes in the antibody responses to the schizont extract antigen, the levels of memory B cell responses and mature B cell subsets over the study period. There was no significant difference in the antibody levels against all the 6 recombinant antigens across the timepoints. The present study speculates that the significant impact which was observed in the responses to the whole parasite antigen could be a result of a cumulative impact on the

antibody levels against the other numerous antigens expressed in the schizont stage of the parasite that were not considered in this study. Although the parasite detection data did not provide supporting evidence to dynamics in transmission seasons, the transient changes observed in the MBC responses over the timepoints may be attributed to possible exhaustion of the MBCs at the known characteristic high transmission seasons of the year, as have been reported by other studies. Moreover, the study also observed an inverse relationship between the MBC subsets and the atypical B cells, although the design of the flow cytometric panel was not limited to malaria-specific B cell phenotypes. The present study concludes that the wane of immunological memory to *P. falciparum* is due to possible exhaustion of memory B cells into the 'dysfunctional' B cell type, atypical B cells, as have been speculated by previous studies.

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LIST OF ABBREVIATIONS

Abbreviation	Full meaning
ADCI	Antibody dependent cell inhibition
atypMBCs	Atypical memory B cells
AMA1	Apical membrane antigen 1
BMI	Body mass index
CD	Cluster of differentiation
CSP	Circumsporozoite protein
GLURP R0	Glutamine rich protein region 0
GLURP R2	Glutamine rich protein region 2
IgG	Immunoglobulin G
LSA1	Liver stage antigen 1
MBC	Memory B cell
MSP3	Merozoite surface protein 3

CHAPTER ONE

1.0 INTRODUCTION

Malaria, a disease transmitted by the *Anopheles* mosquito is one of the world's major causes of deaths. The parasites that cause the disease are of the *Plasmodium* genus. There are five species of *Plasmodium* that infect humans; *Plasmodium falciparum, P. vivax, P. ovale, P. malariae* and *P. knowlesi. P. falciparum* is the cause of most malaria-related deaths across the globe (WHO, 2015).

In spite of the remarkable achievements in malaria control over the years, about half of the world's populace are said to be at risk of the disease (WHO, 2016). *P. falciparum* (*Pf*) malaria also remains an important cause of morbidity and mortality in sub-Saharan Africa particularly among children under 5 years of age and pregnant women (WHO, 2016).

Several methods have been employed in the control of the malaria parasite. The methods of control in use include vector control methods such as the application of insecticides and larvicides, and direct parasite control approach which mainly involves antimalarial drug usage (Control, 2011; Priest, 1992). However, over the years the parasite has shown resistance to drugs in use and the mosquito vector has shown resistance to insecticides which amongst many other factors is mainly due to genetic variations in the parasite and the vectors as well as the complexity of the life cycle of the parasite (Schofield, 1991).

A potent malaria vaccine is believed to be a major possible solution to malaria and the problems it poses globally. The development of malaria vaccines has targeted the parasites and the major stages of their life cycle; the pre-erythrocytic stage, the blood or asexual stage and the sexual stage (Dubovsky, 2001). Several antigens of the parasite are currently being employed in malaria vaccine development across the globe (WHO, 2016). The Apical membrane antigen (AMA1), Glutamine rich protein (GLURP), Merozoite surface protein 3 (MSP3), Circumsporozoite protein

(CSP) and the Liver stage antigen (LSA) are amongst the numerous malaria vaccine candidate antigens which are at different stages of vaccine development (Girard *et al.,* 2007). One major challenge in developing malaria vaccines is the decline in the efficacy of immune protection they offer. For example, protection offered by RTS, S/AS01 shows a decline from 36% to 50% efficacy within a 4-year period (Olotu *et al.,* 2013).

Clinical immunity also known as anti-disease immunity refers to a state where there is reduction in the disease symptoms even with the presence of parasites. Clinical immunity is attained during earlier in the lifetime of individuals in highly endemic areas but there is a delay in its acquisition in very low endemic areas (Doolan *et al*., 2009).

Through constant re-exposure individuals can become asymptomatic carriers of the blood stage and also the sexual gametocyte stage parasites. Antibodies are major players in conferring this clinical immunity. A previous study reported that blood stage parasitemia was controlled and disease alleviated in acutely infected children upon passive transfer of antibodies from clinically immune adults to infected children, demonstrating that antibodies are involved in conferring clinical immunity (Cohen *et al.,* 1961). However, clinical immunity to malaria appears to be shortlived and can be lost in the absence of regular exposure (Doolan *et al.*, 2009; Struik and Riley, 2004). The facts about actual causes of the slow acquisition of clinical immunity (Doolan *et al.,* 2009), as well as, its short-lived nature (Struik and Riley, 2004) remains to be established. It remains controversial whether these phenomena are indeed due to insufficient immune memory in malaria (Struik and Riley, 2004).

B cells are produced in the bone marrow and play major roles in acquired immunity to malaria (Scholzen and Sauerwein, 2013). Based on their various stages of development, B cells identified by delineating markers have been categorized into immature ($CD10^{+}$), naïve ($CD10^{-}CD21^{+}$ $CD27^{-}$), classical memory ($CD10^{-}CD21^{+}CD27^{+}$), atypical memory ($CD10^{-}CD21^{-}CD27^{-}$),

activated B cells ($CD10^{-}CD20^{+}CD21^{-}CD27^{+}$) and plasma cells ($CD10^{-}CD20^{-}CD21^{-}CD27^{+}$) (Bohnhorst *et al.*, 2001). Humoral immune memory has been demonstrated to be conferred by quiescent memory B cells (MBCs) and long-lived antibody producing plasma cells (PCs) (Tangye and Tarlington, 2009). Memory B cells in particular have the ability to rapidly proliferate and differentiate into PCs during subsequent infections for a more robust immune response to be made against the invading parasites (Tangye and Tarlington, 2009). One major speculation about the possible cause of the slow and incomplete induction of humoral and clinical immunity is the numerous antigens of the malaria parasite (Nogaro *et al.*, 2010) and the variation in antigens between different strains (Ferreira *et al.,* 2004). In addition, there is evidence that the parasite causes dysfunction of B cells similar to its modulative effects on T cell regulation and antigen-presenting cell function in *Plasmodium*-infected persons (Scholzen *et al.,* 2010; Wykes and Good, 2008). Others have proposed that the slow acquisition of protective immunity to malaria may be due to *P. falciparum* induced accumulation of atypical memory B cells (Weiss *et al.,* 2009) with similar phenotypic characteristics to the functionally "exhausted" B cells found in HIV-patients (Moir *et al.*, 2008). Therefore, an in depth understanding of the kinetics that *P. falciparum* infection impacts on immunological memory and the span of immunological memory to *P. falciparum* would be very important in the development of long-lasting vaccines.

1.1 PROBLEM STATEMENT

Naturally acquired immunity to malaria is not well understood. This includes a lack of understanding of immunological memory to the malaria parasite. It is also not clear whether the expansion of the atypical B cell subpopulation in malaria endemic populations is an immune combat strategy or immune evasion strategy of the parasites. The protection afforded by the

world's leading malaria vaccine candidates appear to be short-lived, suggesting a wane in immunological memory to the parasite.

1.2 JUSTIFICATION

An in depth understanding of naturally acquired immunity and immunological memory, and the kinetics involved would enhance malaria vaccine development strategies in the quest to inducing long lasting protection. The present study may provide further understanding on the acquisition and longevity of immunological memory to malaria.

1.3 Overall objective

To assess the acquisition and profiles of immunological memory in malaria exposed individuals living in a malaria endemic population.

1.4 Specific objectives

1. To measure and compare antibody responses against specific *P. falciparum* antigens (AMA1, CSP, GLURP R0 and R2, LSA1 and MSP3) and the whole parasite at the schizont stage in malaria exposed individuals across different parasite transmission seasons.

2. To assess the profile of B cell subpopulations in relation to *P. falciparum* infection across different parasite transmission seasons.

3. To measure and compare the levels of memory B cell responses against the whole parasite at the schizont stage across different parasite transmission seasons.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Malaria

Malaria is caused by protists from the genus *Plasmodium.* Several species of malaria parasites have been identified and have been found to infect many organisms such as reptiles, birds, murine and mammals. The parasites in most cases are host-specific (Butcher *et al.,* 1973). In spite of this, mammalian malaria parasites share many similarities including their life cycles (Aikawa and seed, 1980) and metabolic pathways (Trager and Jensen, 1976).

Until recently, four species had been known to cause malaria in humans; *Plasmodium falciparum, P. vivax, P. ovale, P. malariae.* But, *P. knowlesi,* which is known to infect macaques, has recently been found to infect humans, increasing the number of the infectious human parasite species to five (Kantele and Jokiranta, 2009). The classifications were much based on the

morphology of the malaria parasites and their developmental characteristics as well as characteristic symptoms of the disease they cause (Su, 2010). The introduction of molecular tools and techniques such as the Polymerase Chain reaction (PCR) and DNA sequencing has brought further advancement in the identification and classification of malaria parasites. Through the application of these techniques, the species, *P. ovale* has been classified into two distinct sub-species; *P. ovale wallikeri,* the variant type and *P. ovale curtisi,* the classic type (Sutherland *et al*., 2010). The species of the parasite that causes an infection determines how mild or severe the disease condition may be in infected individuals. The most severe and complicated form of the disease is however, caused by *P. falciparum* (WHO, 2016).

2.2 The life cycle of the malaria parasite

The malaria parasite has a complex life cycle (Figure 2.1). It is characterized by a liver (pre-erythrocytic) stage and a blood (erythrocytic) stage that occurs in the vertebrate host and a sexual stage which occurs in a female *Anopheles* mosquito (Pradel, 2007). Sporozoites are the infective form of the parasite. Upon biting of a human host by an infected mosquito, several of these sporozoites are injected into the skin. The sporozoites then migrate into the blood stream and invade the liver cells (hepatocytes) between a period of 30 to 60 minutes. Upon invasion of the hepatocytes, exoerythrocytic schizogony, an asexual form of reproduction begins (Sinnis and Coppi, 2007). Schizogony is a process of replication by which a progeny is produced through many divisions of the nucleus without division of the cytoplasm (Sinnis and Coppi, 2007). In some parasite species such as *P. ovale* and *P. vivax,* dormant forms known as hypnozoites may result from development of some sporozoites. These hypnozoites may later get activated and develop into schizonts (Mueller *et al.,* 2009). Each schizont that develops in the hepatocytes may contain about 30,000 merozoites and these are eventually released into the bloodstream after the rupture of the infected hepatocytes. This entire process may occur within about 8-14 days after inoculation of the sporozoites (Bray and Graham, 1982). In the blood stream, the merozoites invade the red blood cells within a few seconds and undergo a trophic or feeding phase. The parasite at its young feeding blood stage is referred to as a trophozoite and is characterized by a ringed morphology which disappears as the parasite matures. The trophozoite ingests the hemoglobin and cytoplasm of the host erythrocyte. The parasite then digests the hemoglobin to produce heme and amino acids using proteases (Francis *et al*., 1997). The trophozoites develop into schizonts and through erythrocytic schizogony they also further develop into merozoites. The infected erythrocytes rupture to release the merozoites into the bloodstream. The merozoites released invade new erythrocytes and this may be followed by another cycle of erythrocytic schizogony. The rupturing of parasite invaded erythrocytes is also accompanied by the release of parasite metabolites which

are associated with the periodic fever paroxysms observed in malaria infections (Bartoloni and Zammarchi, 2012). However, some trophozoites do not go through another erythrocytic schizogony cycle but undergo gametocytogenesis through which they develop into gametocytes that are taken up by a feeding mosquito. Within the first few minutes of uptake, the male gametocyte undergoes three consecutive rounds of replication of its nucleus in the midgut of the mosquito. This results into the formation of eight microgametes which locate and fuse with the female gametes to produce zygotes (Eksi *et al.,* 2006). This is followed by the development of the zygotes into motile ookinetes which traverse the epithelium of the midgut and develop into oocysts which are non-motile. Fully matured oocysts rupture, releasing numerous sporozoites which migrate into the salivary glands of the mosquito. The parasites are introduced into a new host from the salivary glands as the mosquito vector feeds, to repeat the cycle.

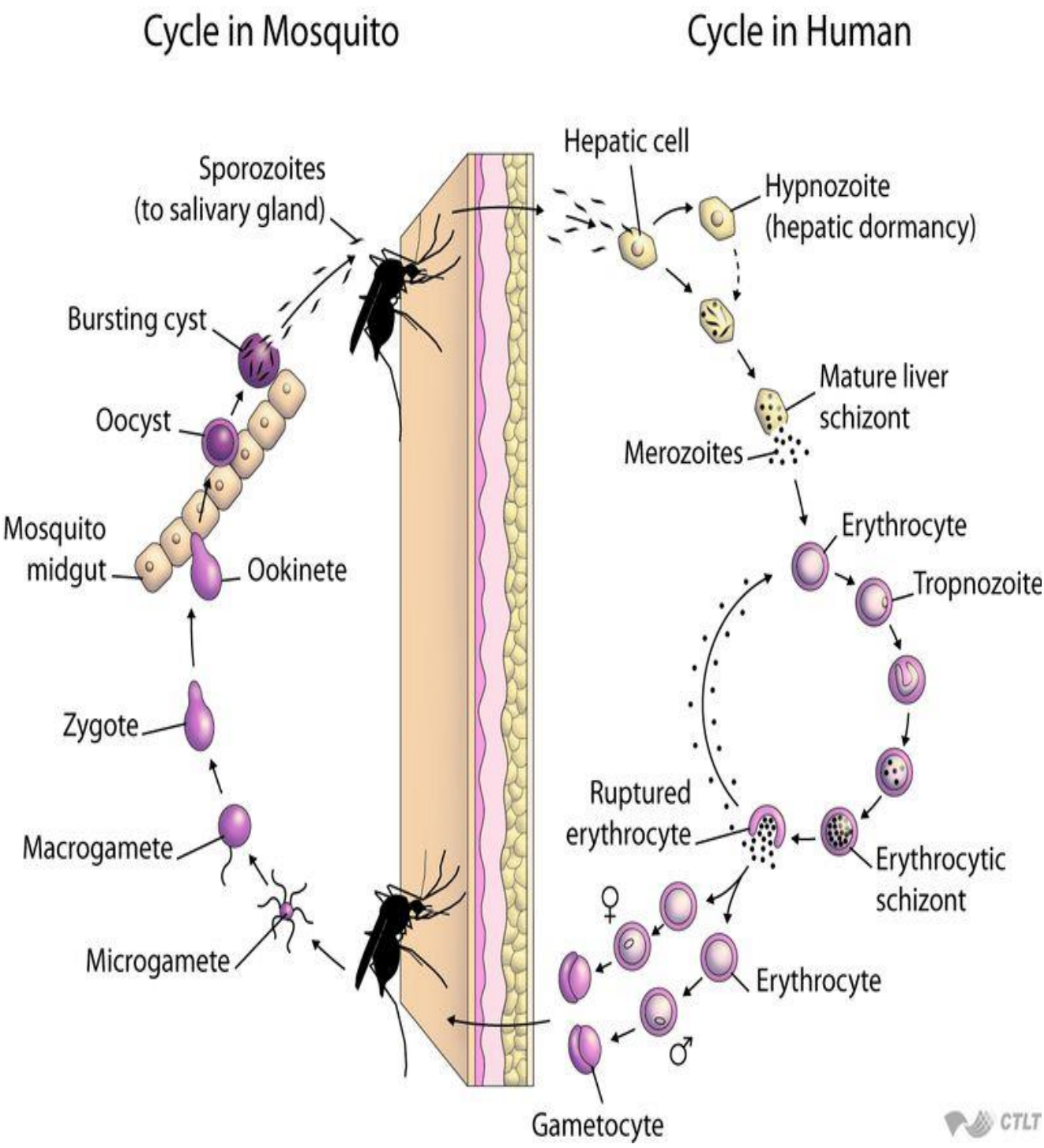

Figure 2.1: Life cycle of the human malaria parasite

Source: www.malariasite.com/lifecycle

Date accessed: 15th June, 2016.

2.3 Disease burden of malaria

Currently, 91 countries and territories have ongoing malaria transmission (Figure 2.2) (WHO, 2016).

Children under age 5 years are the most susceptible group to *Plasmodium* infection, illness and death in Ghana (Koram, 2016). By estimation, 303 000 malaria deaths occurred in children under age 5 years globally in 2015, with 292 000 out of these mortalities in children in the African Region (WHO, 2016).

The socio-economic development of countries with the disease transmission is highly affected. Nevertheless, the high prevalence of the disease in less or undeveloped countries is not only due to the poverty situation but also favorable ecological factors which support the mosquito vectors (Gallup and Sachs, 2001). Heavy financial burden from the cost of health care, brain damage resulting from cerebral malaria, human resource losses, absence from work due to illness are amongst the economic impacts of malaria (Greenwood *et al.*, 2005). Malaria tends to be a major contributor to the difference between the gross domestic product (GDP) of unaffected countries and those affected (Okwa, 2012).

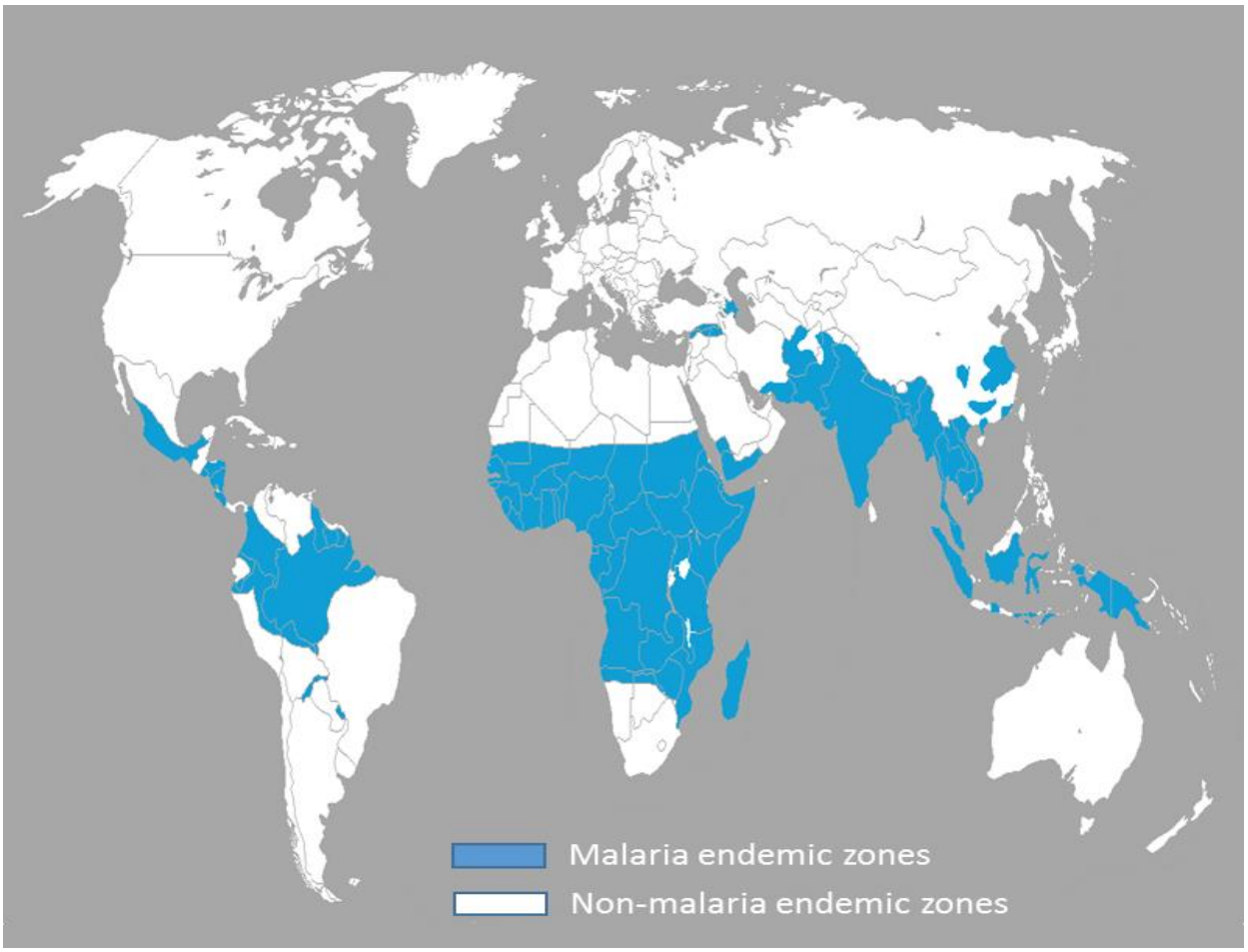

Figure 2.2: World Malaria Distribution

Source: www.bvgh.org

Date accessed: 20th June, 2015.

2.4 Signs and symptoms of malaria

The incubation period of malaria may last between 7 to 30 days. But, this duration highly depends on the species of the infecting parasite (WHO, 2010). However, it is also dependent on the immune status of the host prior to infection as well as last usage of malaria chemoprophylaxis. The incubation time observed in *P. falciparum* infections is about 14 days. Incubation periods are relatively shorter in all species of malaria when the mode of the transmission is transfusion. This is because the liver stage of the parasite life cycle is skipped (Bartoloni and Zammarchi, 2012).

There is also evidence that incubation periods may be prolonged in infected individuals when drugs that are ineffective in completely eliminating the parasite are administered (Gatton *et al.,* 2013). A similar trend of prolongation is also reported in immigrants who move into very low/non-endemic countries from endemic countries having very efficient immune systems. The incubation period may last as long as about 8 years in some cases (Szmitko, 2009).

The initial signs of malaria are nonspecific. They include: fever, headache, chills, joint pains, anorexia, general weakness and sometimes vomiting, diarrhea, thrombocytopenia, dry coughing, hepatosplenomegaly, and anemia (Grobusch and Kremsher, 2005). The typical fever symptom is such that the infected person feels very cold. Next, a rise in body temperature usually follows within an hour or two with the infected person desiring to get rid of the covering because the skin feels hot and dry. Finally, there is a gradual fall in body temperature accompanied with drenching sweat, weakness, tiredness and the individual likely to fall asleep (Tangpukdee *et al.*,2009). This cycle of paroxysm is irregular in the beginning but becomes more regular later with symptoms occurring every 72 hours for *P. malariae* ("quartan malaria"), every 48 hours in *P. ovale and P. vivax* infections ("tertian malaria"). Due to synchronized schizogony in *P. falciparum* infections, the symptoms occur every 36-48 hours and almost continuous (Crutcher and Hoffman, 1996).

Severe or complicated malaria may result from *P. falciparum* infections if diagnosis and treatment do not follow promptly (Trampuz *et al.,* 2003). This form of malaria may lead to neurological complication or cerebral malaria. It has clinical features of generalized convulsion and coma subsequently.

2.5 Malaria control

The impact of malaria on the socio-economic development of affected nations has led to the design and implementation of control measures and/or potential measures to eradicate the disease. The

geographical distribution of malaria caused by *P. falciparum* globally is such that about half the world's populations, at risk to malaria live in low-risks areas where elimination of the disease is achievable through the application of effective control measures (Guerra *et al.,* 2008). The World Health Assembly and Roll back malaria collaborated to reduce malaria cases and mortalities by 75% or more before the end of the year 2015. The strategy they launched aimed at reaching all individuals at risk of the disease with insecticide treated bed nets (ITNs) or indoor residual spraying (Newby *et al.,* 2016). Although the targeted reduction percentage was not met, these efforts and other inputs made global have resulted in a decrease in malaria mortality rate among children under ages of 5 by 53% between 1990 and 2015 (WHO, 2015).

The measures of control for malaria in use include vector control methods such as the use of insecticides and larvicides, direct parasite control approach by the use of drugs (chemotherapy) (Control, 2011). The use of universal and rapid diagnostic test kits has been very beneficial in providing effective control by supplying quick diagnosis of the disease, hastening the process of treatment (WHO, 2012).

2.6 Malaria immunity

Plasmodium parasite infection gives rise to an array of host responses which are controlled by both the innate and adaptive arms of the immune system (Marsh *et al.,* 1992). Adaptive immunity is known to be both stage- and species-specific. It is seldom sterile, but mostly associated with low levels of parasitemia and episodes of clinical disease often throughout life (Marsh *et al.,* 1992). Some studies have shown that children born in endemic areas to immune mothers have protection against the disease within the first half year of their lives through passively transferred maternal antibodies, although these were not observed in some other studies (Marsh *et al.,* 1992)**.** This period of passive immunity fades off into 1 or 2 years of increased susceptibility before active

immunity is acquired (Marsh *et al.,* 1992). Generally, the process of acquiring active immunity to malaria is slow and repeated exposure to the parasite is required for its sustenance. Parasite-induced immunosuppression, genetic variations in the human host as well as the parasite, and other factors account for this pattern (Greenwood *et al.,* 1972).

2.6.1 Innate immunity

Innate mechanisms that inhibit parasite growth by the human host contribute immensely for low parasitemia levels in acute *P. falciparum* infection (Mohan and Stevenson, 1998). Earlier studies in nonparasitic systems demonstrated that a family of germ line receptor proteins is essential for host innate defense system in both vertebrates and invertebrates. In mammals, macrophages which are activated through 'toll-like receptors' cause the induction of effector proteins that regulate and execute this innate defense against various invading microorganisms (Aderem and Ulevitch, 2000). Natural killer (NK) cells, mononuclear phagocytes and neutrophils play important roles in innate defense particularly observed in the early period of *Plasmodium* infections. NK cells have the ability to lyse *P. falciparum*-infected erythrocytes *in vitro* (Orago and Facer, 1991). However, NK cells also produce cytokines such as interferon-γ (IFN-γ) which leads to the activation of parasiticidal macrophages and this may be considered to be of higher importance for innate malaria immunity than their ability to lyse infected erythrocytes (Chua *et al.,* 2013).

Related cell types that are also involved in innate malaria immunity are the natural killer T (NKT) cells which in mice carry both the αβT-cell receptors (TCR) and the NK1.1 surface marker (Donda *et al.,* 2017) These cells are strong inhibitors of pre-erythrocytic parasite replication in mouse malaria systems mimicked *in vitro* (Pied *et al,* 2000). Moreover, NK1.1 CD4 murine T cells have also been reported to control antibody (IgG) responses to glycosylphosphatidyl inositol-anchored *P. falciparum* protein, which is an essential response for a rapid, specific but major

histocompatibility complex (MHC) unrestricted parasite control (Schofield *et al.,* 1999). Human NKT cells express TCR which are homologs to the murine NKT cells. Murine and human NKT cells can both be activated by invariant TCRs upon encountering a lipid antigen which is associated with the MHC class I like CD1 molecules (Porcelli and Modlin, 1999). Since this activation process does not require immunization it makes it important for the regulation of innate malaria immunity.

T cells that bear the γδTCR are also strongly increased in the early phases of *P. falciparum* infection and contribute to innate parasite control (Salerno *et al.,* 1998). It has been shown that, γδT cells but not αβT cells from malaria-naïve donors show inhibition to replication of parasites *in vitro* (Elloso *et al.,* 1994). The diversity in the recognition of the antigens by the two TCR types is thought to be the possible cause of the difference or, otherwise, to the existence on γδT cells of NK receptors (Hayday, 2000), the non-antigen-specific ligation of which leads to spontaneous secretions of proinflammatory cytokines.

2.6.2 Adaptive immunity

Adaptive immunity is the arm of the immune system that includes a complex array of genetically controlled, interactive and interdependent responses. The adaptive immune system is unique in its specificity to immunogens due to the presence of highly precise and selectively positioned antigen-recognition molecules on the surface of lymphocyte cells which are the major players in this arm of immunity. Another unique feature of this aspect of immunity is that it includes cells that maintain memory to invading parasites. The two major subtypes of adaptive immunity are cell-mediated immunity and humoral immunity.

2.6.3 Cell-mediated immunity

Cell-mediated immune responses to malaria infection may protect against both the liver and blood stages of the parasite. T cells are the major players in cell-mediated immunity (Sher and Coffman, 1992).

$CD4^+$ T cells are important for immune protection against the asexual stages of the parasite. $CD8^+$ T cells have regulatory and essential effector roles in pre-erythrocytic immunity which provides protection against severe malaria (Sher and Coffman, 1992).

$CD8^+$ T cells may also control immunosuppression in the situation of acute malaria and downregulate inflammatory responses (Schofield and Grau, 2005). Since human red blood cells do not express MHC antigens, lysis of infected RBCs by $CD8^+$ cytotoxic T lymphocytes do not play a major role in the protection against blood-stage parasites, although T cell responses against blood stage parasites have been reported (Lundie *et al.*, 2008). The existence of different functional types of $CD4^+$ T cells in naturally exposed donors has also been experimentally established. These cells show response to *P. falciparum* antigens by *in vitro* proliferation and/or cytokine secretions, e.g. IFN-γ or IL-4 (Constant and Bottomly, 1997). Generally, these *in vitro* responses have poor correlation with protection (Miller *et al.*, 2002). Nevertheless, $CD4^+$ T cells from malaria-exposed donors stimulated *in vitro* may result in IL-4 production combined with the serum concentrations of antibodies specific for the antigen used in stimulation of lymphocytes (Fell *et al.*, 1994). Furthermore, enhanced production and proliferation of IFNγ have been shown for T cells from donors recovering from a malaria (Constant and Bottomly, 1997).

Cells that express the γδTCR usually make up less than 5% of the total T cell population in the peripheral blood of healthy adults (Guenot, 2012). About 75% of the TCR of these cells is constituted of Vγ9 and Vδ2 chains with a minor fraction expressing Vδ1 with no preference to Vγ association (Guenot, 2012). The frequency of γδT cells in the blood of healthy West Africans is

about twice that of Caucasians, particularly because of an increase in the Vδ1 subset (Dieli *et al.*, 2001). γδT-cell activation results from *in vitro* stimulation with *P. falciparum* extracts of blood mononuclear cells with no previous exposure to malaria. The larger number of the responding cells express Vδ2/Vγ9 (Schwartz *et al.*, 1996) and a minority express Vδ1 (Glatzel *et al.*, 2002). In malaria-naïve donors, activated γδT cells but not αβT cells inhibit replication of parasites in erythrocytes *in vitro*, confirming their protective role, and particularly their function in innate defense against the malaria parasites (Grangeiro de Carvalho, 2011; McCall and Sauerwein, 2010). The activation of γδT-cell is in association with the IL-2 receptor (IL2R) signaling, which is triggered by cytokines such as IL-2, IL-4 and IL-15 (Hu and Xiong, 2013; Pennock *et al.*, 2013). γδT cells activated by *Plasmodium* antigens produce mainly but not exclusively pro-inflammatory cytokines (Robinson *et al.*, 2009). However, the activities of these cells may also have implications in malaria pathogenesis (Robinson *et al.*, 2009). Antigens from *P. falciparum* schizonts effectively stimulate γδT cells (Obiero *et al.*, 2015). Certain antigens are easily recognized by these cells through their conventional association with MHC class-I or II molecules (Braciale *et al.*, 1987; Shen *et al.*, 1997). Nevertheless, γδT cells also have the ability to recognize non-peptide antigens, in the absence of MHC presentation (Chien *et al.*, 1996). These activating ligands are relatively small, with molecular weights less than 500 kDa and typically containing phosphoesters (Kreslavsky and von Boehmer, 2010). These ligands bind specifically and directly to the γδTCR (Kreslavsky and von Boehmer, 2010).

2.6.4 The role of cytokines in immunity

Anti-malarial immune protection reflects cellular activities such as production of antibodies, cellular cytotoxicity, phagocytosis and parasite inhibition exerted by lymphocytes, mononuclear phagocytes and neutrophils. Yet, some of these cellular activities can also cause damage to tissues and the outcome of a malaria infection is highly dependent on a healthy balance between the

cytokines secreted by the various cells upon activation (Riley *et al.,* 2006). Proinflammatory cytokines like IFNγ, IL-1, IL-6 and others contribute to immune protection by inducing parasite killing by macrophages/monocytes and neutrophils (Kumaratilake and Ferrante, 1994). Mononuclear phagocytes and other cells produce IL-12 which was shown to contribute to immune protection against pre-erythrocytic and erythrocytic infection in mice and monkeys by initiating a Th1 anti-malaria response (Feghali and Wright, 1997; Stevenson *et al.,* 1995).

On the other hand, anti-inflammatory cytokines such as IL-10 counteract the possible cytopathic effects of the proinflammatory cytokines as well as their production (Ho *et al.,* 1995; Deloron *et al*., 1994). Studies of human *P. falciparum* malaria highlight the essence of a healthy balance between pro- and anti-inflammatory cytokines (Day *et al.,* 1999; Othoro *et al.,* 1999). Thus, increased IL-6 to IL-10 ratios in plasma as a result of relative IL-10 deficiencies may lead to a lethal outcome of severe malaria (Day *et al.,* 1999). Moreover, anemic children from some malaria holoendemic areas have lower IL-10 to TNF ratio compared to those with uncomplicated disease, suggesting that IL-10 can inhibit anemia induction via TNF production (Othoro *et al.,* 1999). Malaria-induced IL-10 is also known to contribute to resistance to *P. falciparum* infection, supporting the balancing function of anti-inflammatory cytokines (Kurtis *et al.,* 1999). The TNF cytokine also plays a vital role in both protection and malaria pathogenesis. TNF does not directly kill parasites but contributes to protection by activating the anti-parasitic features of several leukocytic effector cells (Kwiatkowski and Perlmann, 1999). In relation to pathogenesis, TNF-α levels have positive correlation to the severity of disease as well as with malaria fever (Grau *et al.,* 1989). The principal producers of this TNF-α are macrophages/monocytes activated by various parasite products (Riley *et al.,* 2006). However, immune complexes containing immunoglobin E (IgE) also contribute to local excess production of TNF in severe malaria (Perlmann *et al.,* 1997). Variation in the levels of TNF produced by these cells may be due to genetic reasons and is significant for the outcome of an infection. Thus, a single nucleotide polymorphism at the promoter

region–308 of TNF is associated with increased TNF production and an elevation in the risk of cerebral *P. falciparum* malaria (Wattavidanage *et al.,* 1999; Knight *et al.,*1999; Mcguire *et al.,* 1994). In contrast, children with low plasma concentrations of TNF as a result of a single nucleotide polymorphism at the TNF promoter allele –238A are prone to severe malarial anemia (Mcguire *et al.,* 1994). These mutations result from altered gene transcription due to variations in transcription factor binding to the corresponding promoter region of TNF (Mcguire *et al.,* 1994).

2.6.5 Humoral immunity

In individuals living in endemic areas, *Plasmodium* infection induces strong humoral immune responses, characterized by the production of predominately IgG and IgM but also of other immunoglobulin isotypes (Sabchareon *et al.,* 1991). A percentage of these immunoglobulins are non-malaria-specific, species- and stage-specific antibodies reacting with a wide variety of *P. falciparum* antigens and pointing out polyclonal B cell activation (Sabchareon *et al.,* 1991). A combination of Resiquimod (R848) and IL-2 has been shown to cause high polyclonal B cell activation and expansion in *in vitro* studies (Ampomah *et al.,* 2014; Walsh *et al.,* 2013). The mechanism is that R848 binds to the Toll-like receptors (TCR) 7 and 9 to induce the stimulation of these B cells to produce antibodies whilst suppressing T cell activation and activities. High proliferation and expansion in these cell types is also due to signaling by IL-2 to cause lymphocytic clonal expansion (Ampomah *et al.,* 2014; Walsh *et al.,* 2013). Earlier studies involving the passive transfer of IgG from immune donors showed that antibodies may be protective (Sabchareon *et al.,* 1991; Mcgregor *et al.,* 1964; Cohen *et al.,* 1961) by decreasing parasitemia and clinical disease. Through these early studies it was also established that some of the major antigens inducing such protective responses were common to *P. falciparum* parasites globally regardless of geographical origin (Sabchareon *et al.,* 1991).

Parasite antigens which are expressed on the surface of infected erythrocytes are of key importance in the development of humoral immunity to the malaria. Most of the antigens belong to highly variant families. This variability aids the parasites in evading immune response and, therefore, constitutes a vital virulence factor (Kraemer and Smith, 2003). Parasites cultured in the presence of anti-malarial antibodies have reduction in their susceptibility to antibody-mediated growth inhibition relatively to that of those cultured without antibodies previously (Dent *et al.*, 2008).

P. falciparum infection induces the production of both polyclonal and specific immunoglobulins. Different antibody isotypes may have protective functions, but, the most important in this respect is IgG. Cytophilic antibodies of IgG1 and IgG3 isotypes have often been found in protected individuals (Braga *et al.*, 2002; Roussilhon *et al.*, 2007). The functional relevance of these shown in studies found that the IgG1 to IgG3 antibody ratio appears to be highest in individuals whose antibodies are also most efficient in the neutralization of parasites *in vitro* (Perlmann and Troye-Blomberg, 2002). Some studies have also reported significant increments in levels of IgG3 antibodies in certain populations and established correlations of these levels to disease episodes (Bruce *et al.*, 2000). However, elevations in IgG2 antibody concentrations may also be associated with decreased risk of *P. falciparum* infection: This has been observed in certain persons whose monocytes bear a special allelic variant of a Fc receptor (RIIA), which enables binding to IgG2 (Aucan *et al.*, 2000). Higher levels of total IgE and IgE anti-malarial antibodies have also been observed in malaria infections of both humans and experimental animals (Duarte *et al.*, 2007). Induction of IgE also reflects a switch of regulatory T cell activities from Th1 to Th2 as a result of continuous exposure of the immune system to the parasites (Perlmann *et al.*, 1994). IgE elevation appears to be associated with malaria pathogenesis. The blood levels of this isotype are significantly higher in cerebral malaria patients or other forms of severe disease than in uncomplicated malaria patients (Perlmann *et al.*, 1994). A pathogenic effect of IgE is probably due to local overproduction in microvessels of TNF and nitric oxide (NO) caused by IgE-containing

immune complexes (Maeno et al., 2000). IgE is activated by such complexes through their induction and cross-link formation with CD23, the IgE low affinity receptor on monocytes and perhaps endothelial cells (Maeno *et al.,* 2000). However, these results do not imply that IgE antibodies may not be protective.

2.6.6 Antibody-dependent protection

Apart from inhibiting merozoite invasion of erythrocytes, antibodies may enhance clearance of infected erythrocytes (IEs) from the circulation by binding to their surface, preventing sequestration in small vessels and enhancing elimination by the spleen (Craig and Scherf, 2001). In particular, opsonization of IEs causes a substantial increase in their susceptibility to phagocytosis, cytotoxicity and parasite inhibition by various effector cells mediated by neutrophils and macrophages/monocytes (Mac-Daniel and Ménard, 2015; Urquhart, 1994). Interactions between opsonized erythrocytes and these effector cells induce the release of factors such as TNF which are toxic to the parasites, although it may also cause tissue lesions (Vassalli, 1992). Antigenic variation of the parasites also highly affects the protective efficiency of antibodies (Kraemer and Smith, 2003). For example, when the immune system is exposed to an infecting parasite, variant-specific anti-*Pf*EMP-1 antibodies inhibit cytoadherence and reduce the possibility of renewed infection by parasites expressing the same *Pf*EMP-1 antigens as the originally infecting one (Diatta *et al.,* 2004). But, the presence of such antibodies will also influence the immune selection of different variants to which these antibodies do not show protection (Perlmann and Troye-Blomberg, 2002).

2.6.7 B cells

B cell lymphopoiesis occurs in the bone marrow (McHeyzer-Williams *et al.,* 2012). Through this process, immature transitional B cells (TBC) are formed and released into the circulation which

serves as a means to migrate to the spleen where they undergo differentiation into mature naïve B cells. Upon primary encounter with antigens, this clone of mature naïve B cells expands. Further differentiation into memory B cells (MBCs) and plasma cells (PCs) takes place in germinal centers (GCs) of secondary lymphatic organs, aided by stromal follicular dendritic cells (FDCs) and follicular helper T cells (TFHs) (McHeyzer-Williams *et al.,* 2012). MBCs are unique in their ability to respond, proliferate, and differentiate more rapidly into plasma cells (PCs) in subsequent infections compared to naïve B cells which are antigen-inexperienced (Tangye and Tarlinton, 2009). FDCs and TFH direct the migration of B cells to GCs and secrete chemokines and survival factors for their survival, and orchestrate isotype switching (through class-switch recombination) as well as affinity maturation (through somatic hypermutation) by secreting cytokines and presentation of antigen–antibody complexes (McHeyzer-Williams *et al.,* 2012).

In the past decade a lot of progress has been made in identifying novel B cell subsets and delineating markers (Tangye and Tarlinton, 2009). Initially, delineation of B cells in human peripheral blood was based on the Bm1–5 gating strategy using surface IgD and CD38 expression, which was developed for classification of B cells in lymphoid organs (Bohnhorst *et al.,* 2001). This system discriminates $IgD^{+}CD38^{int/low}$ naïve B cells, $IgD^{-}CD38^{int/low}$ memory B cells (MBCs), $IgD^{+}CD38^{hi}$ GC-founder B cells or transitional B cells (TBCs) and $IgD^{-}CD38^{hi}$ GC B cells (in lymphoid organs) or circulating plasmablasts but is unable to differentiate naïve B cells as well as inactivated MBCs in the $IgD^{+}CD38^{int/low}$ population (Bohnhorst *et al.,* 2001). The identification of CD27 marker, which is an indicator of BCR somatic hypermutation in a GC reaction (Klein *et al.,* 1998), has brought transformation, allowing B cell subsets to also be subdivided on the basis of the surface expression of IgD and CD27 (Shi *et al*., 2003). Using this system, identification of naïve, inactivated IgD^{-} B cells lacking CD27 expression and three MBC populations: inactivated (marginal zone-like) MBCs [(IgM^{+}) $IgD^{+}CD27^{+}$], classical MBCs ($IgD^{-}CD27^{+}$) and activated double-negative (DN) MBCs ($IgD^{-}CD27^{-}$) is now achievable. Finally, $CD20^{+}$ B cells have

recently been delineated on the basis of the expression of CD27 in combination with the complement C3d receptor, CD21 (Moir *et al.,* 2008), distinguishing between naïve $CD27^{-}CD21^{+}$ B cells, resting MBCs ($CD27^{-}CD21^{+}$), activated MBCs ($CD27^{+}CD21^{-}$), and atypical MBCs ($CD27^{-}CD21^{-}$). TBCs are not considered under these two delineation schemes even as they overlap with the naïve B cell populations. But, by including the CD10 marker they can be identified (Sims *et al.,* 2005).

Atypical MBCs (atypMBCs), having the properties of low expression of the C3dg complement receptor CD21 and the hypermutation marker CD27, were first described in HIV-infected patients (Moir *et al.*, 2008; Moir *et al.*, 2001), and in malaria-exposed persons recently (Weiss *et al.*, 2011; Weiss *et al.,* 2009). They vary from classical MBCs in their markedly reduced ability to differentiate into antibody-secreting cells upon stimulation *in vitro* (Weiss *et al.,* 2009). $CD21^{-}$ $CD27^{-}$ atypMBCs are also constituents of the isotype-switched (IgD^{-}) $CD27^{-}$ MBC pool that expands upon malaria infection, systemic lupus erythematosus (SLE), respiratory syncytial virus infection and in elderly people (Asito *et al.*, 2011; Bulati, *et al.,* 2011).

There are currently three hypotheses about the origin of atypMBCs. First, they might be directly derived from a defective or alternative pathway of MBC differentiation (Weiss *et al.*, 2010; Wei *et al.,* 2007). A major indicator that atypMBCs result from an alternative pathway of MBC differentiation is the observation that fewer somatic hypermutations are shown by these cells compared to classical MBCs. Moreover, because they are not just present in SLE or HIV patients but also in healthy controls as well (Wei *et al.,* 2007), characterized with a consistent lack of the CD27 region (Klein *et al.,* 1998). This may be indicative of either extra-follicular MBC differentiation or an aborted GC reaction (Wei *et al.,* 2007). However, a previous study has shown that atypMBCs against the GLURP and MSP3 malaria antigens show relatively greater frequency of somatic hypermutations than their classical MBC counterparts, and no clonal relationship is

seen in both populations, supporting the hypothesis that atypMBCs originate from a distinct developmental pathway (Muellenbeck *et al.,* 2013).

Second, they might result from exhaustion caused by antigens through chronic activation of previously functional MBCs (Kardava *et al.,* 2011). This is possibly due to existing evidence of premature exhaustion following chronic activation in some other diseases. In chronic HIV-viremic patients, HIV antigen-specific B cells are enriched in the atypMBC compartment and have a less diverse Ig repertoire and a shorter replication history than classical MBCs (Moir *et al.,* 2008). The enhanced expression of CD86 and CD95 on atypMBCs is further proof of recent activation of these cells (Muellenbeck *et al.,* 2013; Isnardi *et al.,* 2010; Rakhmanov *et al.,* 2009).

Third, they depict the last stage of an abortive MBC life cycle when MBCs are removed from long-standing survival niches such as the bone marrow (Nogaro *et al.,* 2011). Finally, a more recent paper suggestion is that in spite of the distinct $CD27^{-}$ phenotype of atypMBCs, they might actually represent short-lived plasma blasts which are active contributors of IgG secretion (Muellenbeck *et al.,* 2013). This also may be a partial explanation to their reduced response to or survival during *in vitro* restimulation (Weiss *et al.,* 2009; Moir *et al.,* 2008).

2.6.8 Clinical immunity

Clinical immunity also known as anti-disease immunity refers to a state of immunity where there is reduction in disease symptoms at a certain parasite density and/or severe or complicated clinical disease (Doolan *et al.,* 2009). Studies have shown that clinical immunity is acquired during early childhood in highly malaria-endemic areas, but, may be delayed or even never attained in very low endemic areas. In most cases, clinical immunity and anti-parasite immunity is acquired simultaneously, but clinical immunity appears to be short-lived compared to anti-parasite immunity (Doolan *et al.,* 2009). There is currently no evidence that naturally acquired functional

sterile immunity against the malaria parasite is attainable on a large scale in malaria endemic areas. Instead, a state of clinical immunity towards severe disease may be acquired by adults, in which infection is controlled or tolerated (Doolan *et al.,* 2009). Thus, with constant reinfection they can become asymptomatic carriers of the asexual blood-stage, as well as infective, sexual gametocyte stages. Antibodies play a major function in conferring this clinical immunity through their control of blood-stage parasitemia as well as in alleviating disease (Cohen *et al.,* 1961). However, clinical immunity, begins to develop slowly after multiple exposures to the parasite (Doolan *et al.,* 2009). Moreover, clinically protective humoral responses to malaria are thought to be short-lived and can be lost in the absence of frequent exposure (Doolan *et al.,* 2009; Struik and Riley 2004). The controversy about these phenomena which result in insufficient immune memory in malaria is still pending (Struik and Riley 2004). Quiescent memory B cells (MBCs) and long-lived antibody-producing plasma cells (PCs) are known to confer humoral immune memory. Studies on the occurrence of antigen-specific MBC responses in individuals naturally exposed to malaria indeed suggest that these are acquired and maintained less efficiently as compared to vaccination-induced MBCs of other diseases during childhood (Struik *et al.,* 2004). The complex antigen diversity of the malaria parasite and the antigenic variation among different parasite strains are also thought to be possible major causes for the slow and incomplete induction of humoral immunity and clinical protection, and also the absence of sterile protection (Nogaro *et al.,* 2011; Ferreira *et al*., 2004). Furthermore, there is also increasing evidence that the malaria parasite interferes with the function of B cells similar to its direct modulatory effects in antigen-presenting cell function and T cell regulation during malaria (Scholzen *et al.,* 2010; Wykes and Good, 2008).

Just a few studies have addressed this issue of maintenance of MBC in malaria. In Thailand, a low-endemic area, no statistically significant decay was seen by using the longitudinal MBC data collected over 1 year and last reported clinical episode (Wipasa, *et al.,* 2010). By contrast, the magnitude and prevalence of malaria-specific MBCs were relatively lower in adults with no

clinical episodes over 6 years, compared to others who had one to three infections (Wipasa, *et al.,* 2010). Moreover, in Kenyan children, MBC prevalence was obviously less affected by prolonged non-exposure, for 7 years, than plasma antibody levels (Dorfman *et al.,* 2005). But, malaria antigen-specific but not tetanus-specific MBC prevalence were higher in children who were persistently exposed compared to others who were previously exposed (Ndungu *et al.,* 2012). Whether this is simply a result of the lack of boosting in the temporarily unexposed cohort or a specific defect in malaria-induced MBC maintenance remains to be determined (Struik *et al.,* 2004).

Studies in pregnant and non-pregnant females have also shown transient changes in MBC levels in naturally exposed pregnant women but with much stability in the levels over the period of pregnancy but with significant impact of parity. But, a wane in the antibody levels against *P. falciparum* antigens was observed in these studies (Ampomah *et al.,* 2014).

2.7 Vaccines as intervention for malaria

Vaccination is a term that refers to the process of administering an antigenic material (a vaccine) to an individual to stimulate the immune system to develop adaptive immunity against invading organisms (Perrie *et al.,* 2008). The advent of vaccines had a tremendous impact to public health. The overall objective of vaccination is to expose the immune system of an individual to optimal levels of non-pathogenic material so as to enable the stimulation of an enhanced immune response capable of preventing disease upon exposure to the pathogen. A good vaccine must be highly immunogenic, provide a long-lasting immunity, safely administered with other vaccines, heat stable and preferably not administered by injection (Spickler and Roth, 2003). The active component of a vaccine may consist of either the entire disease-causing organism in a weaken form or some of its parts (Saha *et al.,* 2011). Subunit vaccines are vaccines that are made up of

antigens of the pathogen that best stimulate the recipient's immune system and are preferred because there is assurance that the parasites are dead and impossible to revert into pathogenic forms as may occur for heat killed and live attenuated whole parasite vaccines respectively. A subunit vaccine may also be referred to as recombinant subunit vaccine if the antigen used in its preparation was produced by recombinant DNA technology (Liljeqvist and Ståhl, 1999). The search for a malaria vaccine was started as far back as 1961 when Cohen *et al (*1961) showed that administering gamma globulins from an individual from an endemic country to individuals who are naïve to the disease provided partial immunity to them. Nevertheless, only one vaccine has been approved for malaria because of the complexity of the parasite and the constant observable changes in its surface coat coupled with the different morphologies it shows at various stages of its life cycle (WHO, 2015).

Even though the complex nature of the life cycle of the parasite is a disadvantage, it also offers several opportunities for the parasite control. Each stage of the life cycle presents several antigens which are being explored for the development of potent vaccines (figure 2.3). In relation to the main stages of the life cycle, three types of vaccines could be made for malaria: the pre-erythrocytic stage vaccines, the erythrocytic stage vaccines and the sexual stage vaccines.

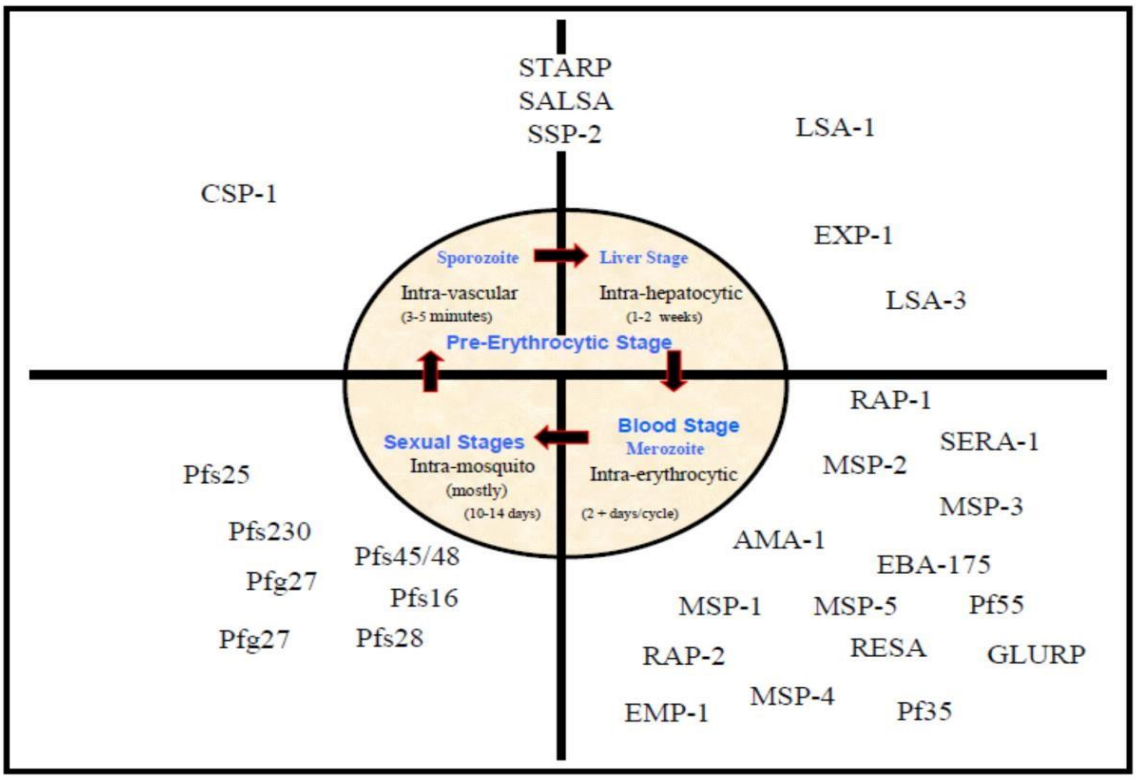

Figure 2.3: Target antigens for vaccine development at various stages of life cycle of *P. falciparum.*

CSP-Circumsporozoite protein 1, STARP- Sporozoite Threonine and Asparagine-Rich Protein, SALSA- Sporozoite and liver stage antigen, SSP-2-Sporozoite surface protein 2, LSA -1 and -3- Liver stage antigen-1 and -3, AMA-1-Apical membrane antigen 1, MSP-1, -2, -3, -4 and -5- Merozoite surface protein -1,-2,-3,-4 and -5, RAP-1 and -2-Rhoptry associated protein-1 and -2, RESA- Ring-infected erythrocyte surface antigen, EBA-175-Erythrocyte binding antigen-175, EMP-1-Erythrocyte membrane protein-1, *Pf*55- and -35 *Plasmodium falciparum* antigen-55 and -35, Pfs -25, -28- 48/45, -230- *Plasmodium falciparum* sexual stage antigen -25, -28, -48/45, -230 and Pfg-27- *Plasmodium falciparum* gametocyte antigen-27

Source: (Dubovsky, 2001)

2.7.1 Pre-erythrocytic stage vaccines

Pre-erythrocytic stage vaccines have the ultimate aim of providing sterile immunity by blocking sporozoites from invading the liver cells and/or destruction of infected hepatocytes (Schwartz *et al.*, 2012). The hope of such a vaccine was drawn from a murine study where the mice acquired sterile immunity after receiving X-radiation attenuated sporozoites (Nussenzweig *et al.*, 1967) and a parallel study in humans resulted in 24 individuals protected out of a total of 26 challenges (Hoffman *et al.*, 2002). There have been limitations in large scale production through this method of vaccine production due to inherent safety issues related to this method, particularly the possibility of reversion into pathogenic forms (Duclos, 2004).

2.7.2 Erythrocytic stage vaccines

Erythrocytic or Blood stage vaccines are thought to be curative. This is because they aim at inducing immune responses against the parasite when it gets into the bloodstream, where the signs and symptoms of the disease are induced. Candidate blood stage vaccine antigens that have been under vaccine trials include: Apical Membrane Protein 1 (AMA1), Merozoite Surface Protein 1, 2 and 3 (MSP1, MSP2 and MSP3 respectively), Glutamate Rich Protein (GLURP), Erythrocyte Binding Antigen (EBA-175), Serine Repeat Antigen (SERA 5), *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) and Ring-infected Erythrocyte Surface Antigen (RESA), GLURP-MSP 3 fusion protein (GMZ-2) (Dhanawat *et al.*, 2010; Dubovsky, 2001). The general progress of the blood stage vaccines has been quite slow due to the numerous antigens serving as alternative pathways for red blood cell invasion by the parasites (Hill, 2011).

2.7.3 Sexual stage vaccines (Transmission-Blocking Vaccines)

The malaria parasite has an obligatory sexual stage in the female *Anopheles* mosquito. Transmission of the parasite from a human host to another requires a successful completion of the sexual stage within the mosquito. Inside the mosquito, the male and female gametes fuse to form the zygote which subsequently develops into sporozoites which can be passed on to another human. A sexual stage vaccine targets sporogenic development within the mosquito, preventing passage of plasmodium from an individual to another, thus, the name Transmission-Blocking Vaccine (TBV) (Carter, 2001). The rationale behind such a vaccine is that, antibodies to the vaccine, when picked up by a mosquito would prevent sporogenic development by preventing fertilisation or ookinete from traversing the mosquito midgut. These vaccines are altruistic in importance because they would prevent an individual from passing malaria to another but not from becoming infected with the disease. TBVs would block the transmission of drug resistant parasites or any that escape immune responses elicited by pre-erythrocytic and blood-stage vaccine and serve as essential components in combination vaccines for malaria (Dinglasan and Jacobs-Lorena, 2008). TBV vaccine development has been either vector based or parasite-based approach.

The less popular vector-based approach involves development of antibodies against midgut ligands that are utilised by the ookinetes within the midgut. Work done in this field suggests that the midgut ligands alanyl amino peptidase and carboxypeptidase are important in parasite invasion (Dinglasan *et al.*, 2007; Lavazec *et al.*, 2007)**.** Antibodies against these midgut enzymes significantly decrease ookinete traversing of midgut epithelium with subsequent oocyst formation (Dinglasan *et al.*, 2007; Lavazec *et al.*, 2007).

Parasite based TBV approaches are based on antigens on surfaces of gametocytes, zygote and ookinete. These antigens include the pre-fertilisation antigens *Pf*s48/45 and *Pf*s230 which are expressed on the surface of gametocytes and gametes, and whose corresponding homologues have

been found in murine and avian species of *Plasmodium* (Carter, 2001; Kaushal *et al.*, 1983). Post-fertilisation antigens whose antibodies have been shown to completely block malaria transmission in membrane feeding assays are *Pf*s28 and *Pf*s25 (Duffy and Kaslow, 1997). TBVs based on *Pf*s48/45 and/or *Pf*s230 are thought to be likely more advantageous because these antigens are expressed by the parasite while it is still in the human host, making them amenable to natural boosting (Carter, 2001; Kaushal *et al.*, 1983).

2.8 Malaria vaccine candidate antigens

2.8.1 Apical membrane antigen (AMA1)

The Apical membrane antigen 1 (AMA1) is a micronemal protein of the malaria parasite that has been found to be essential during the invasion of RBCs (Remarque *et al.*, 2008). AMA1 has a conserved type I integral membrane protein structure which varies between 556 to 563 amino acids (aa) in most species of *Plasmodium.* It has a 622aa structure in *Plasmodium falciparum* and it is closely related to the chimpanzee malaria parasite, *Plasmodium reichenowi* (Chesne-Seck *et al.*, 2005). The cytosolic region constitutes about 50 aa. The bulk of AMA1 forms the ectodomain, which is comprised of 16 conserved cysteine amino acids which contribute to disulfide bonding, the pattern of which suggested that the mature ectodomain folds as an N-terminal pro-sequence and 3 domains (DI, DII and DIII) (Hodder *et al.*, 1996).

During late schizogony of asexual erythrocytic development of *Plasmodium*, there is maximum expression of AMA1 and it is targeted to merozoite micronemes (Healer *et al.*, 2002). AMA1 is processed proteolytically just like other most microenemal proteins, and this is such that around the time of merozoite release and red blood cell invasion, the prosequence is cleaved away. In *Pf*AMA1, the 83kDa *Pf*AMA183, the precursor protein is converted to *Pf*AMA166 (Narum and

Thomas, 1994), an event required to precede circum-merozoite relocalisation of AMA1 (Narum and Thomas, 1994).

By *in vitro* and animal model studies, immune responses to *Plasmodium* AMA1 have shown profound parasite-inhibitory effects, suggesting AMA1 as a malaria vaccine candidate. The polymorphic nature of AMA1 is thought to be a result of immune selection towards the antigen as an important target of naturally occurring immunity (Remarque *et al.*, 2008).

2.8.2 Circumsporozoite protein (CSP)

The circumsporozoite protein (CSP) is a protein that is found on the surface of sporozoites and infected hepatocytes. CSP is made up of two major B cell epitopes consisting of tandem repeats (Asn-Ala-Asn-Pro and Asn-Val-Asp-Pro). CSP has been the main antigen of interest in the quest for a non-whole parasite pre-erythrocytic vaccine. RTS,S which is the most advanced and well-documented pre-erythrocytic vaccine candidate is derived from CSP. The RTS,S candidate vaccine is being developed by Glaxo Smith Kline (GSK) in collaboration with the Walter Reed Army Institute of Research (WRAIR) and comprises the C-terminus (amino acids 207–395) of CSP fused to the hepatitis B surface antigen and expressed in the form of virus-like particles (VLPs) in *Saccharomyces cerevisiae* (Bojang *et al.*, 2001; Kester *et al.*, 2001; Stoute *et al.*, 1997).

The resultant hybrid protein that makes up the vaccine consist of a central repeat ‘R’ fused with the ‘T’ lymphocytes epitope bearing C-terminus of CSP and Hepatitis B virus surface coat protein ‘S’. The last letter ‘S’ in the name of the vaccine represents the unfused Hepatitis B virus surface coat protein that is required to be co-expressed with recombinant RTS to generate a high yield of the vaccine (Hill, 2011). But the efficacy of the RTS,S vaccine showed a wane from 50% to 36% over 3 years (Agnandji, 2011).

2.8.3 Glutamate rich protein (GLURP)

The glutamate rich protein (GLURP) is a 220-KDa exo-antigen expressed in both the liver and blood stages of the *Pf* parasite, which includes the surface of newly released merozoites in human hosts (Borre *et al.,* 1991). It has high immunogenecity and is a target for antibodies involved in Antibody dependent cyototoxicity inhibition (ADCI) activities in the presence of monocytes (Oeuvray *et al.,* 2000). Monocytes in synchrony with cytophilic antibodies (mainly IgG3 and IgG1) in ADCI prevent the multiplication of *Plasmodium* (Tebo *et al.,* 2001). Immuno-epidemiological studies conducted in different endemic areas have reported the presence of high levels of GLURP antibodies in malaria-exposed individuals, which were also seen to provide protection against high parasitaemia and clinical disease (Nebie *et al.,* 2008; Lusingu *et al.,* 2005). The gene that encodes GLURP in *P. falciparum* is made up of three regions; N-terminal region (R0) which is nonrepetitive, central region (R1) which is repetitive and an immunodominant C-terminal region (R2) which is repetitive (Borre *et al.,* 1991). The recombinant *Pf*GLURP R0 and R2 regions expressed in *Escherichia coli* were found to cause the induction of humoral response *in vivo* (Theisen *et al.,* 1995). A study on initially naive Dutch volunteers who were later exposed to malaria found much higher antibody level against the R2 repeat region of GLURP in plasma than that of the R1 and R0 regions (Turner *et al.,* 2011). This study and others further suggested that the R2 repeat region plays a key role in the induction of immune protection to *P. falciparum* malaria (Turner *et al.,* 2011). The R2 repetitive region has also been reported as a good genetic marker for genotyping of *P. falciparum* and also for distinguishing new infections from recurring infections (Barrera et al., 2010).

2.8.4 Liver stage antigen (LSA)

The liver stage antigen is a malaria vaccine candidate antigen found on both the sporozoite and liver stages of the parasites. The liver stage antigen has two known variants that are malaria vaccine candidates; LSA1 and LSA3 (Takala and Plowe, 2009).

LSA1 is a 230-kda protein. It has a central repeat region made up of about 80 repeats of 17 aa. This large central repeat region is flanked by 2 known highly conserve C- and N-terminals which also contain epitopes to CD4+ and CD8+ T cells as well as B cells (Fidock *et al.,* 1994). This highly conserved regions of the antigen have been of interest in vaccine development studies of the antigen because of its potential to trigger cross-reactive immune responses with other P. falciparum strains. Also, the abundance of LSA1 throughout schizogony is thought to create an opportunity by providing possibly sufficient time for memory-recalled and circulating effector cells to infiltrate the liver and effect their immune functions (Kurtis *et al.,* 2001).

*Pf*LSA3 is a protein made up of 1786 aa in the K1 strain of the parasite. This antigen has been of much interest particularly of its abundance on both the liver stage and the sporozoite stage of the parasite. Another reason is its consistent recognition by serum samples from protected individuals immunized with irradiated sporozoites and absence of recognition to it by sera from unprotected immunized individuals (Aidoo *et al.,* 1999). LSA3 contains a conserved central repeat region that is known to define a B cell recognition marker that antibodies from malaria exposed individuals are able to recognize (Hollingdale and Krzych, 2002).

2.8.5 Merozoite surface protein 3 (MSP3)

Merozoite surface proteins (MSPs) are considered important malaria vaccine candidate antigens. This is mainly due to their relatively greater exposure to the host immune system during the asexual stage of the parasite development at which they are usually expressed (Chauhan *et al.,* 2010). The

surface location of these proteins also suggests their possible role in the invasion of erythrocytes (Schwartz *et al.,* 2012). Merozoite surface protein 3 (MSP3), a 40 kDa protein encoded by a single locus on chromosome 10 of the *P. falciparum* parasite is a nonintegral protein that triggers antibody responses during experimental immunization and natural malaria infection (Druilhe *et al.,* 2005; Carvalho *et al*., 2004).

MSP3 is a major target of antibody response to *P. falciparum* infection and antibodies to MSP3 were found to mediate ADCI of the parasite (Oeuvray *et al*., 1994). Monkeys immunized with recombinant forms of MSP3 showed protection to malaria infection (Hisaeda *et al.,* 2002). A vaccine based on the N-terminal fragment of MSP3 is already undergoing human trials (Audran *et al*., 2005; Sirima *et al.,* 2007). Apart from the vaccine development with MSP3, two other characteristics of MSP3 are of important consideration; firstly, because MSP3 while a soluble protein, forms oligomers, and secondly because it can bind to heme. But, the actual significance of these characteristics is not clear (Imam *et al.*, 2013).

Although MSP3 heme binding activity has been described, the mode and extent to which it binds heme is still not well understood (Spycher *et al*., 2008). MSP3 positioned on the surface of merozoites does not have any 'unique' structural features, possibly high histidine or cysteine content etc. that would explain its binding ability to heme (Oeuvray *et al*., 1994). On the other hand, MSP3 contains a C-terminus leucine zipper like motif, three domains of alanine heptad repeats and a glutamic acid rich region (McColl *et al.,* 1994). The formation of dimers and tetramers of MSP3 has been attributed to the presence of a specific forty residues sequence in the leucine zipper region (Campanale *et al.,* 2003).

2.8.6 *Plasmodium falciparum* erythrocyte membrane protein 1 (*Pf*EMP1)

The first identification of *Pf*EMP1 was done by immunoprecipitation with immune sera from parasite infected *Aotus* monkeys. The *Pf*EMP1 protein is a high molecular weight protein of about 200–350 kDa. The protein is encoded by a highly polymorphic *var* multigene family (~60 var genes) (Baruch *et al.,* 1995; Smith *et al.,* 1995). *Pf*EMP1 is the main parasite ligand responsible for cytoadhesion and is also known to bind to a range of endothelial and erythrocyte molecules which includes: ICAM-1 (Smith *et al.,* 2000), CD36 (Baruch *et al.,* 1997), complement receptor 1 (CR1) (Rowe *et al.,*1997), chondroitin sulphate A (CSA) (Reeder *et al.,* 1999; Buffet *et al.,* 1999), heparan sulfate (HS) (Chen *et al.,* 1998) and others.

As a result of mutually exclusive transcription of *var* genes, just a single *Pf*EMP1 variant is generally expressed on infected erythrocyte (IE) surface at a given time (Chen *et al.,* 1998; Scherf *et al.,* 1998). However, more recent studies have reported the potential of more than one *Pf*EMP1 variant being expressed on the IE surface by live confocal microscopy demonstration, *in vitro* adhesion assays and cell sorting by flow cytometry (Joergensen *et al.,* 2010). The biochemical properties of *Pf*EMP1 (Triton X-100-insoluble and SDS-soluble) demonstrates its anchorage to the IE membrane (Kriek *et al.,* 2003) and its high sensitive cleavage to mild trypsin treatment of intact IEs (10 μg/ml) (Leech *et al.,* 1984).

HB3VAR06 and IT4VAR60 are *var* genes of *Pf*EMP1 that are known to be associated with erythrocyte resetting (Ghumra *et al*., 2012). Rosetting is an event that may occur during the asexual stages of *P. falciparum* infection where the parasite utilizes molecules on the surface of uninfected red blood cells (RBC) for rosette formation: binding of infected RBCs (iRBC) to uninfected RBCs. Rosetting has been found to be associated with severe malaria which includes cerebral malaria and anemia (Barragan *et al.,* 2000).

2.8.7 GMZ-2 (GLURP-MSP3 fusion protein)

GMZ-2 is a protein which has been expressed in *Lactococcus lactis* as a recombinant construct from the fusion of *Pf*GLURP and *Pf*MSP3 (Dhanawat *et al.*, 2010). The reason for the design of this recombinant protein was to elicit high levels of cytophilic antibodies. This is because studies suggested that hybrid antigen protein vaccines may enhance immune antigen presentation (Dhanawat *et al.*, 2010). The GMZ2 vaccine was tested in a multicenter phase 2b randomized, controlled trial in African children (N=1849) aged 12-60 months old in Burkina Faso, Gabon, Ghana and Uganda and the efficacy was only 14%. Although GMZ2 was well tolerated and reduced malaria incidence in the study population, the authors concede its efficacy would need to be substantially improved, possibly with better adjuvants for the vaccine to have a good public health benefit (Sirima *et al.*, 2016).

A comprehensive review of GMZ2 has been recently published describing the details of the whole rational behind the vaccine's design through preclinical testing to safety and efficacy trials (Theisen *et al.*, 2017).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study site and study participants

The study was conducted at the Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, Legon which is situated in the city of Accra in the Greater Accra region of Ghana. The Greater Accra region falls within the coastal savannah and the transmission of *P. falciparum* parasites remains high in Ghana, with two peak transmission seasons (high and low) in each year which coincide with the rainfall seasons (WHO, 2016; Afari *et al.*, 1995).

Forty (40) staff members of the Institute (27 males and 13 females) who consented to participate in the study after having been properly informed about the study design and purpose were recruited. Prior to this, ethical approval for the study was obtained from the institutional review board (IRB) of the NMIMR. All the individuals recruited were adults between the ages of 18 to 49 years who live in Accra. Pregnant females and persons who had had a malaria episode and/or been on antimalarials 3 weeks before the study began were excluded. Basic demographic information was also collected from participants; sex, age, malaria history, use of insecticide-treated bed nets. At enrolment and at quarterly intervals, about 20 ml of venous blood, filter paper blood blots and microscope blood smears were obtained from all participants. Samples were collected over a 1-year period, covering two malaria transmission seasons. The baseline, follow-up 1, follow-up 2 and follow-up 3 sampling time fell within high, low, low and high transmission periods respectively. Laboratory tests and studies were performed at the Immunology Department of the NMIMR, University of Ghana, Legon.

3.2 Materials

3.2.1 Reagents and consumables

Blood collection sets (HUMAN Gassellschaft fur, Wiesbaden, Germany), Syringes (BD, New Jersey, USA), EDTA Vacutainer tubes (BD, Plymouth, UK), Microscope slides (Thermoscientific, Braunschweig, Germany), cuvettes (HemoCue, Angelholm, Sweden), 15 ml and 50 ml centrifuge tubes (FALCON, BD, New Jersey, USA), RPMI-1640 with $NaHCO_3$ and L-glutamine (Sigma-Aldrich, Steinheim, Germany), Foetal Bovine Serum (Sigma-Aldrich, Steinheim, Germany), Penicillin-Streptomycin (Sigma-Aldrich, Steinheim, Germany), Ficoll-Paque (GE Healthcare, Uppala, Sweden), 10 ml and 25 ml serological pipettes (Sarstedt, Marnay, France), Dimethyl sulphoxide (Sigma-Aldrich, Steinheim, Germany), Guava Viacount solution (Merck Millipore, Darmstadt, Germany), Ethanol (Sigma-Aldrich, Steinheim, Germany), 10 µL, 200 µL and 100 µL pipette tips (Sarstedt, Marnay, France), 0.2 Filtropur (Sarstedt, Marnay, France), cryotubes (NUNC, Thermoscientific, Braunschweig, Germany), CO_2 gas (Air liquid, Tema, Ghana), FACS cell wash (BD, New Jersey, USA), FACS Clean (BD, New Jersey, USA), FACS Rinse (BD, New Jersey, USA), FACS tubes (FALCON, BD, New Jersey, USA), Resiquimod (R848) (Mabtech, Nacka Strand, Sweden), Recombinant human IL-2 (Mabtech, Nacka Strand, Sweden), 24-well sterile tissue culture plates (NUNC, Thermoscientific, Braunschweig, Germany), 96-well flat-bottom Nunc plates (Thermoscientific, Kamstrupvej, Denmark), Phosphate Buffered Saline (Gibco, Paisley, UK), Tween-20 (Sigma-Aldrich, Steinheim, Germany), Skimmed milk (Premier foods, Hertfordshire, UK), Sodium azide (Sigma-Aldrich, Steinheim, Germany), $3,3^1,5^1,5^1$-tetramethylbenzidine (KEM-EN-TEC, Taastrup, Denmark), H_2SO_4 (Sigma-Aldrich, Steinheim, Germany) and Liquid nitrogen (NMIMR, Accra, Ghana).

3.2.2 Antibodies

PE Mouse IgG1κ isotype control (Biolegend, California, USA), APC Mouse IgG1 κ isotype control (Biolegend, California, USA), FITC Mouse IgG1 κ (Biolegend, California, USA) isotype control (Biolegend, California, USA), PerCP/Cy5.5 Mouse IgG1 κ isotype control (Biolegend, California, USA), CD19-PE (Biolegend, California, USA), CD10-APC (Biolegend, California, USA), CD21-FITC (Biolegend, California, USA) and CD27-PerCP/Cy5.5 (Biolegend, California, USA) and Goat anti-human antibody conjugated to Horse-radish peroxidase (KPL, Milford MA, USA).

3.2.3 Equipment

Water bath (Clifton, Germinston, South Africa), Microscope (OLYMPUS, Tokyo, Japan), Incubator (Thermo Electrocorporation, Massachusetts, USA), FACS Calibur (BD, New Jersey, USA), Guava EasyCyte HT flow cytometer (Merck Millipore, Darmstadt, Germany), Biosafety cabinet (Walker, Nottinghamshire, UK), Centrifuge (Thermoscientific, Massachusetts, USA), Pipette aids (Thermoscientific, Massachusetts, USA), Pipette Boy (Bibbyjet pro, Staffordshire, UK), Weighing balance (AND, Tokyo, Japan), Vortex (Scientific Industries, New York, USA), Spectrophotometer (BioTek, Winooski, USA).

3.2.4 Antigens

The recombinant antigens used in this study include are AMA1, CSP, GLURP R0, GLURP R2, LSA1 and MSP3. Recombinant AMA1 is the N terminal domain of the antigen from the FVO strain of *P. falciparum.* It comprises amino acids 25 - 545 and produced by expression in the methylotrophic yeast, *Pichia pastoris* (donated by A. Thomas of Biomedical Primate Research Center, Netherlands). The CSP recombinant antigen is composed of three regions; an N terminus,

a 4 amino acid repeat region (NANP) and a C terminus containing a thrombospondin-like type I repeat (TSR) domain (obtained as a kind gift from Dr. Kwadwo Asamoah Kusi, NMIMR, Legon). GLURP R0 contains the conserved non-repeat N-terminal region of GLURP, (amino acids 25–514) whiles GLURP R2 (amino acids 705–1178) contains the carboxy-terminal repeat region which were all expressed in *Escherichia coli* (obtained as a kind gift from Professor Michael Theisen of Staten Serum Institute, Copenhagen). Recombinant LSA1 is a 230-kda protein made up of about 80 repeats of 17 amino acids (obtained as a kind gift from Dr. Kwadwo Asamoah Kusi, NMIMR, Legon). The MSP3 antigen is of the 3D7 strain of *P. falciparum* and was produced by expression in in *E. coli* (supplied by Dr. Richard Shimp from NIAID, NIH, USA).

3.5 Parasite detection and infection status

At each time point of sample collection, Giemsa-stained blood smears were examined by microscopy to determine the parasite infection status of the participants. The Giemsa stain was prepared by a 1:10 dilution of the stock Giemsa in distilled water. Slides were stained for 10 min and followed with rinsing off the stain with running tap water. Both the stained thick and thin blood films were examined for parasitaemia. A participant was considered negative if no parasite was seen within 200 oil fields of a thick film. For slides that were positive, parasites were counted per 200 white blood cells (WBCs) and parasite densities calculated by assuming 8,000 WBCs/µL blood.

3.6 Blood sample collection and preparation

Plasma and buffy coat were separated by centrifugation at 800 x g for 10 min at 4°C from about 20 ml of venous blood samples collected from each participant in EDTA tubes. Plasma was stored at -40°C until use. For the peripheral blood mononuclear cells (PBMC) to be isolated, the buffy

coat obtained after plasma collection was diluted 2-fold with R0 (a mixture of RPMI-1640, L-glutamine, $NaHCO_3$ and 1% Penstrep of the total mix). The buffy coat-R0 mixture was then overlaid gently on 11 ml of Ficoll-Paque each in two 50 ml centrifuge tubes. The tubes were centrifuged again at 800 x g for 10 min at 4°C. After centrifugation, the PBMC layer was gently taken into fresh 50 ml accuspin tubes. This was washed twice using about 35 ml of R5 (a mixture of RPMI-1640, L-glutamine, $NaHCO_3$, 1% Penstrep and 5% Heat-inactivated Foetal Bovine Serum (FBS)) and by centrifugation at 800 x g for 10 min at 4°C. The final pellet obtained was then resuspended by adding 5 ml of R10 (a mixture of RPMI-1640, L-glutamine, $NaHCO_3$, 1% Penstrep and 10% Heat-inactivated Fetal Bovine Serum (FBS). Counting was then done by a 1:20 dilution in Guava Viacount solution and using the Guava easyCyte HT flow cytometer (Millipore Sigma, USA). The obtained PBMCs were stored at 20 x 10^6 cells/ vial in cryopreservation solution (10% Dimethyl Sulfoxide (DMSO) with 90% FBS) in liquid nitrogen filled tanks.

3.7 Retrieval of cryopreserved PBMCs

The stored PBMCs were placed on ice in transportation to the laboratory and rapidly thawed in the water bath at 37°C. The cells were gradually released into 10 ml of warm R10 media. The cells were washed by centrifugation at 800 x g for 10 min at 37°C and resuspended in 5 ml of R10 media. The cells were re-counted to ascertain the viability of each sample upon recovery using the Guava easyCyte HT flow cytometer.

3.8 Profiling of B cell phenotypes by flow cytometry

Upon retrieval of PBMCs and determination of cell numbers and viability by Viacount, 1×10^6 PBMCs were aliquoted into two tubes (labelled Tube 1 and Tube 2) for each sample. The cells were centrifuged at 800 x g for 10 min at 4°C and resuspended in 100 µL of R10 media. Tube 1

was stained with the isotype controls: PE Mouse IgG1κ isotype control, APC Mouse IgG1 κ isotype control, FITC Mouse IgG1 κ isotype control, PerCP/Cy5.5 Mouse IgG1 κ isotype control and Tube 2 was stained with the B cell monoclonal antibodies: CD19-PE, CD10-APC, CD21-FITC and CD27-PerCP/Cy5.5. The stained cells were incubated for 20 min at room temperature in the dark. After the incubation, the cells were washed with 2 ml of FACS cell wash with centrifugation at 800 x g for 7 min at 4°C. The cells were resuspended in 300 µL of FACs cell wash. The acquisition was then performed on the BD FACS Calibur flow cytometer.

3.9 Stimulation of B cells in culture

About 1×10^6 cells were plated for each sample in sterile 24-well tissue culture plates. The B cell polyclonal activator, Resiquimod (R848) was added at 0.5 µg/ml and recombinant human IL-2 was also added at 5 ng/ml to induce stimulation and proliferation of B cells respectively. Appropriate volumes of R10 media were added to attain a 2 ml culture in each well. The cultures were incubated in a 37°C incubator with conditions of 5% CO_2 for 6 days. A volume of 400 µL of the culture supernatant was sampled on Days 3 and 6 of the culture to be used in measuring the levels of antibodies produced by the B cells over the period. The sampled volume of the culture was replenished with media as well as the mitogens after collection on Day 3 to ensure no bias in culture conditions.

3.10 Culturing of *P. falciparum* parasite to produce schizont extract antigen

P. falciparum parasites stored in liquid nitrogen tanks were retrieved and maintained in parasite medium (O+ human Red blood cells (RBCs) at 3% hematocrit in RPMI-1640 medium at a pH of 7.4 and supplemented with HEPES (25 mg/ml), Hypoxanthine (50 µg/ml), 10% pooled human serum, Sodium bicarbonate (2 mg/ml) and Gentamycin).

Cultures were incubated in a 37°C incubator at 1% O_2, 4% CO_2 and 95% N_2. Parasites were monitored by microscope examination of methanol fixed, Giemsa-stained blood smear slides. The cultures were maintained at a parasitaemia of below 5% infected RBC. This was performed by dividing the cultures and adding uninfected RBCs until the parasites were acclimatized to *in vitro* conditions. After acclimatization was achieved, 20 IU/ml of Percoll was added to the cultures at the ring-stage to synchronize growth. When most of the parasites were at the schizont stage, the culture was centrifuged at 300 x g for 5 min to pellet the cells. The media containing Percoll was removed and the cells were resuspended in parasite medium to allow schizont rupture and merozoite invasion. After about 4 hours, Percoll (20 IU/ml) was re-added to the cultures. The parasitaemia and maturation stage of the parasite was assessed by daily examinations of stained smears.

Thirty-six hours after the last addition of Percoll, the cultures were centrifuged at 300 x g for 5 mins and the pellet was resuspended in parasite medium at 25% hematocrit. A large magnetic column attached to a magnet was equilibrated with column parasite medium. The *P. falciparum* culture was added to the column and allowed to pass through. Then, 1 X PBS was used to wash the column until the flow-through was clear. The parasites were then eluted into 30ml of parasite medium. A smear was prepared to count estimate the parasitaemia. Eight vials of the schizont extract antigen were obtained at a concentration of 11 x 10^6/vial, averagely 180 ug/ml protein concentration by readings on the Nanodrop spectrophotometer.

3.11 Determination of antibody levels by Enzyme-linked Immunosorbent Assay (ELISA)

The 96-well microtilter plates were coated individually with 100 µL/well of the various antigens, AMA1, CSP, GLURP R0, GLURP R2, LSA1 and MSP3 at 0.5 µg/ml, except for the Schizont extract antigen which was coated at 20 ng/ml. The plates were incubated overnight at 4°C. The

blocking buffer (1 X PBS with 0.1% Tween-20 and 5% skimmed milk of the total mix) was then added at 200 µL/well and incubated for 1 h at room temperature. The standard and positive control plasma samples (obtained as pools of samples that showed relatively high responses to a broad range of malaria antigens in a previous study) were diluted to their appropriate pre-determined concentrations in the dilution buffer (1 X PBS with 0.1% Tween-20 and 2.5% skimmed milk of the total mix) and added to each plate (see appendix I). The standard sample was 3-fold serially diluted across 12 wells of the plates in duplicates. A volume of 100 µL of the plasma/culture supernatant test samples were also added in duplicates at their pre-determined appropriate concentrations (see appendix I). The plates were then incubated for 2 h at room temperature. A volume of 100 µL of the secondary antibody, goat anti-human IgG conjugated to horse-radish peroxidase at a dilution of 1:3000 was added to each at 100µL/well. For measurements of IgG1, IgG2, IgG3 and IgG4 levels, the secondary antibodies were diluted at 1:1000. The plates were then incubated for 1 h at room temperature. In cases where culture supernatants were the test samples, the plates were incubated for 2 h at room temperature. A volume of 100 µL of the substrate, 3,3′,5,5′-Tetramethylbenzidine (TMB) was added to each well. The plates were then incubated in the dark for 10 min in the case of Plasma Sample Total IgG measurements. In the case of measurements of plasma IgG subclasses, the plates were incubated for 40 min. For culture supernatant sample isotype IgG measurements, the plates were incubated for 20 min. The enzyme-substrate reaction was stopped by the addition of 100 µL of 0.2 M H_2SO_4 stop solution to each well. The plates were washed 4 times with 100 µL of wash buffer (1 X PBS with 0.1% Tween-20 of the total mix) in between steps. The plates were read at a wavelength of 450nm using the Biotek EL808 spectrophotometer to obtain the optical densities.

3.12 Management of ELISA data

The ADAMSEL FPL b040 software (developed by Edmond Remarque of Biomedical Primate Research Center, BPRC, Netherlands) was used to convert optical densities to antibody units by fitting the standard curve. The ADAMSEL Merge b009 software was also used to merge the converted data files into single combined data files.

3.13 Statistical analysis

Data analysis was conducted with STATA (special edition 14; Statacorp, 2015), GraphPad prism (version 5; GraphPad Software Incorporated, 2017) and R statistical software (version 3.3.0; The R foundation for Statistical Computing Platform, 2016). Generalised estimating equations with identity link function following either Gaussian or Gamma distribution with robust standard error were used to compare the memory B cell responses between days of culture sample harvest. Kruskal-Wallis test was used to measure differences in the antigen-specific antibody levels, memory B cell responses and the frequency of B cell subsets across the sampling time points. A Bonferroni pair-wise test was performed when the Kruskal-Wallis showed statistical significance. The longitudinal active surveillance measurements were explored graphically with tools from time-series analysis coupled with box and whisker plots. Statistical significance was set at $\alpha=0.05$.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Plasma IgG responses to *P. falciparum* antigens

Levels of antigen-specific IgG against different *P. falciparum* recombinant antigens; AMA1, CSP, GLURP R0, GLURP R2, LSA1 and MSP3 as well as the whole parasite schizont extract were measured in plasma samples from study participants. The IgG responses of the participants to the various *P. falciparum* antigens were compared over time. Out of the 40 participants recruited (Table 4.1), 10 were lost to follow-up over the one-year study period.

Table 4.1: Characteristics of study participants at November 2015 (baseline)

	Males	**Females**
Donors, n (%)	27 (67.5)	13 (32.5)
Median age (range) (years)	28.5 (22-46)	27.5 (23-38)
Median BMI (range) (Kg/m^2)	31.1 (19.4-41.5)	26.8 (20.9-50.4)
Median Hb (range) (g/dl)	15.35 (11.8-16.8)	12.25 (12-14)
Parasitaemia (proportion)	0.00	0.00

BMI, Body mass index; Hb, Haemoglobulin

There was no statistically significant difference in the levels of AMA1-specific total IgG, IgG 1 and IgG 2 antibodies between the timepoints of sampling (i.e. Nov. 2015, March 2016, July 2016 and Nov. 2016) ($P>0.05$ in all cases, Kruskal-Wallis test) (Fig 4.1 A and B). However, there were significantly higher levels of AMA1-specific IgG3 (Fig. 4.1 B) at July 2016 compared to

Nov. 2015 (P=0.02, Bonferroni post-hoc test) and March 2016 (P=0.02, Bonferroni post-hoc test). A similar trend of higher levels at July 2016 compared to Nov. 2015 (P=0.004, Bonferroni post-hoc test) and March 2016 (P=0.01, Bonferroni post-hoc test) was observed for IgG4 (Fig. 4.1 B). All other paired comparisons of AMA1-specific IgG3 and IgG4 levels did not show significant differences at the various timepoints (P>0.05 in all cases, Kruskal-Wallis test). But at nearly all timepoints, there were higher levels of IgG2, followed by IgG1, IgG3 and IgG4 (Fig. 4.1 B).

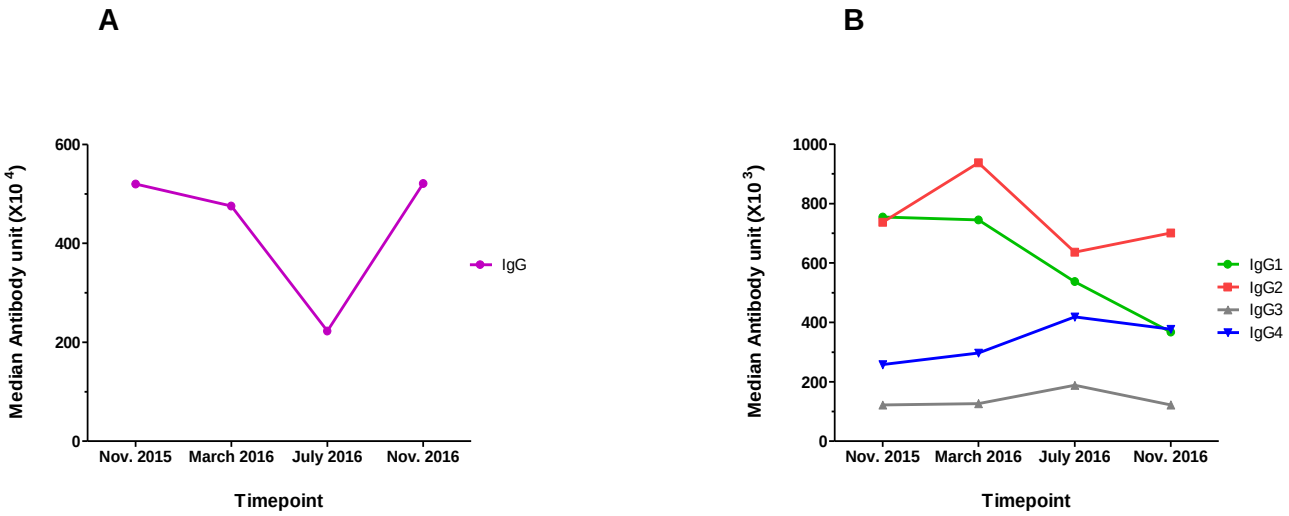

Figure 4.1: AMA1 IgG levels across the 4 sampling timepoints. Median levels of AMA1-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4 green line, red line, gray line and blue line respectively. Abbreviations: AMA1, Apical membrane antigen 1.

In the case of CSP-specific antibodies (Fig. 4.2 A and B), there was no significant difference in the levels of total IgG, IgG2 and IgG3 across the timepoints of sampling (P>0.05 in all cases, Kruskal-Wallis test). However, there were significantly lower levels of IgG1 at Nov. 2016 compared to Nov. 2015 (P=0.006, Bonferroni post-hoc test), March 2016 (P=0.008, Bonferroni post-hoc test) and July 2016 (P=0.006, Bonferroni post-hoc test). Pair-wise comparisons between all other timepoints were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). A similar trend of lower levels of IgG4 at Nov. 2016 compared to Nov. 2015, March 2016 and July 2016 was observed (P<0.0001 in all cases, Bonferroni post-hoc test). All other paired comparisons

were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). For the antibody subclasses, IgG 2 levels were the highest at nearly all the timepoints, followed by IgG4, IgG3 and IgG1 (Fig. 4.2 B).

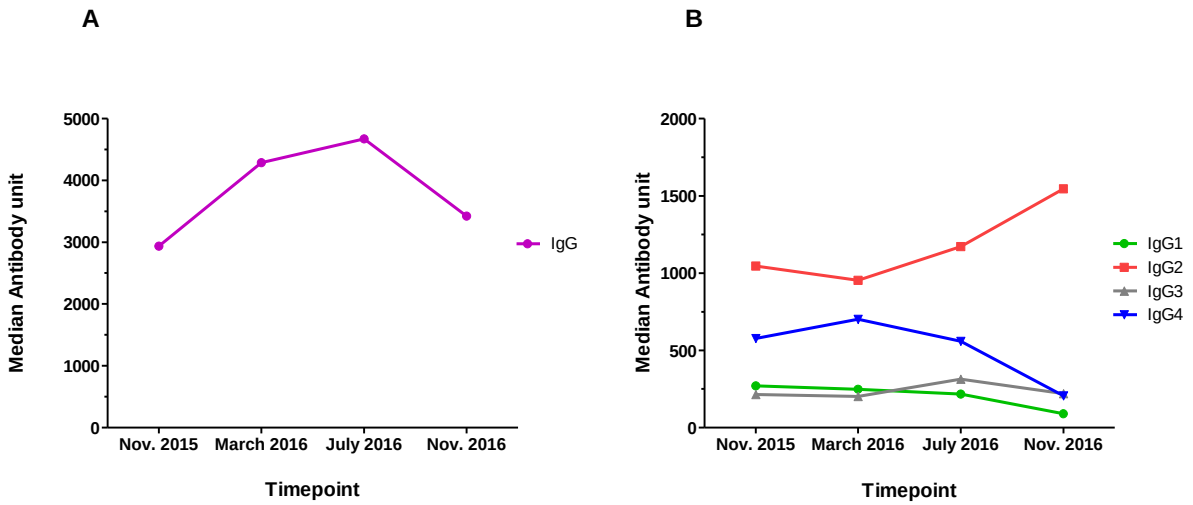

Figure 4.2: CSP IgG levels across the 4 sampling timepoints. Median levels of CSP-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4 green line, red line, gray line and blue line respectively. Abbreviations: CSP, Circumsporozoite protein.

The statistical results also showed that there was no significant difference in the levels of R0-specific total IgG, IgG1 and IgG3 across the sampling timepoints (P>0.05 in all cases, Kruskal-Wallis test) (Fig. 4.3 A and B). There were significantly lower levels of R0-specific IgG2 at Nov. 2016 compared to Nov. 2015 (P=0.005, Bonferroni post-hoc test), March 2016 (P=0.003, Bonferroni post-hoc test) and July 2016 (P<0.001, Bonferroni post-hoc test) (Fig. 4.3 B). All other pair-wise comparisons were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). There were also significantly higher levels of IgG4 at July 2016 compared to Nov. 2015 (P=0.002, Bonferroni post-hoc test) and March 2016 (P=0.009, Bonferroni post-hoc test) (Fig. 4.3 B). Pair-wise comparisons between all other timepoints were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). At almost every timepoint, IgG2 was the highest followed by IgG1, IgG3 and IgG4 (Fig. 4.3 B).

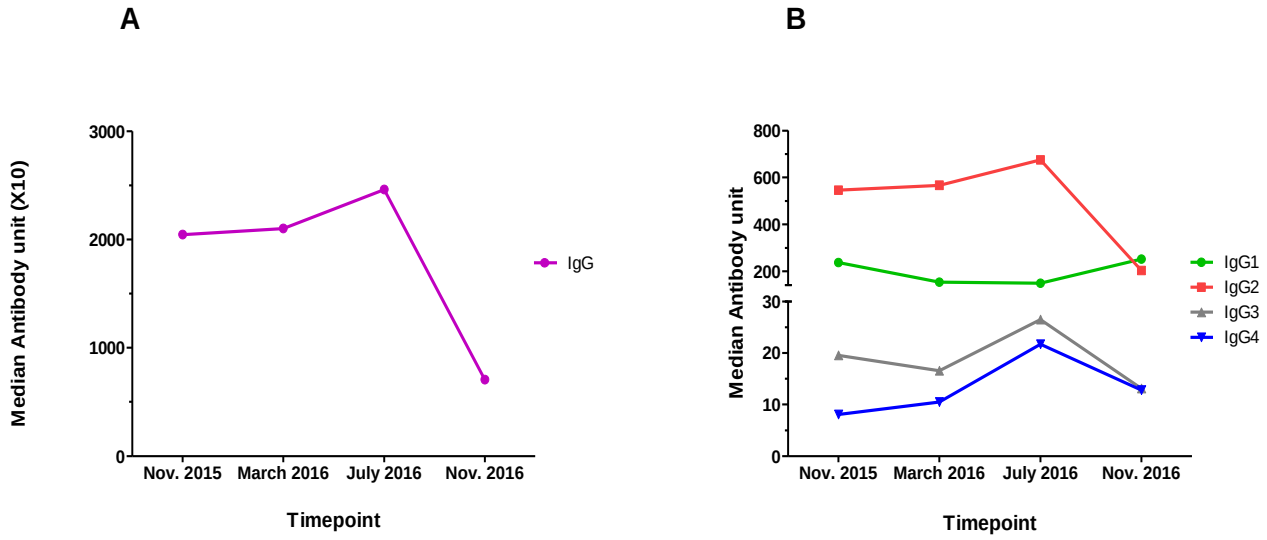

Figure 4.3: R0 IgG levels across the 4 sampling timepoints. Median levels of R0-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4, green line, red line, gray line and blue line respectively. Abbreviations: R0, Region 0 of the Glutamine-rich protein.

There was no significant difference in the levels of R2-specific total IgG, IgG1 and IgG3 across the 4-timepoints ($P>0.05$ in all cases, Kruskal-Wallis test) (Fig. 4.4 A and B). But, there were significantly lower levels of IgG2 at Nov. 2016 compared to July 2016 ($P=0.01$, Bonferroni post-hoc test) (Fig. 4.4 B). There was no significant difference in all other pair-wise comparisons ($P>0.05$ in all cases, Kruskal-Wallis test). There were also significantly higher levels of IgG4 at March 2016 ($P=0.006$, Bonferroni post-hoc test), July 2016 ($P<0.001$, Bonferroni post-hoc test) and Nov. 2016 ($P<0.0001$, Bonferroni post-hoc test) compared to Nov. 2015 (Fig. 4.4 B). There were also higher levels of IgG4 at Nov. 2016 compared to March 2016 ($P=0.02$, Bonferroni post-hoc test) (Fig. 4.4 B). All other pair-wise comparisons were not significantly different ($P>0.05$ in all cases, Kruskal-Wallis test). At nearly every timepoint, IgG2 antibody levels were the highest followed by IgG1, IgG3 and IgG4 (Fig. 4.4 B).

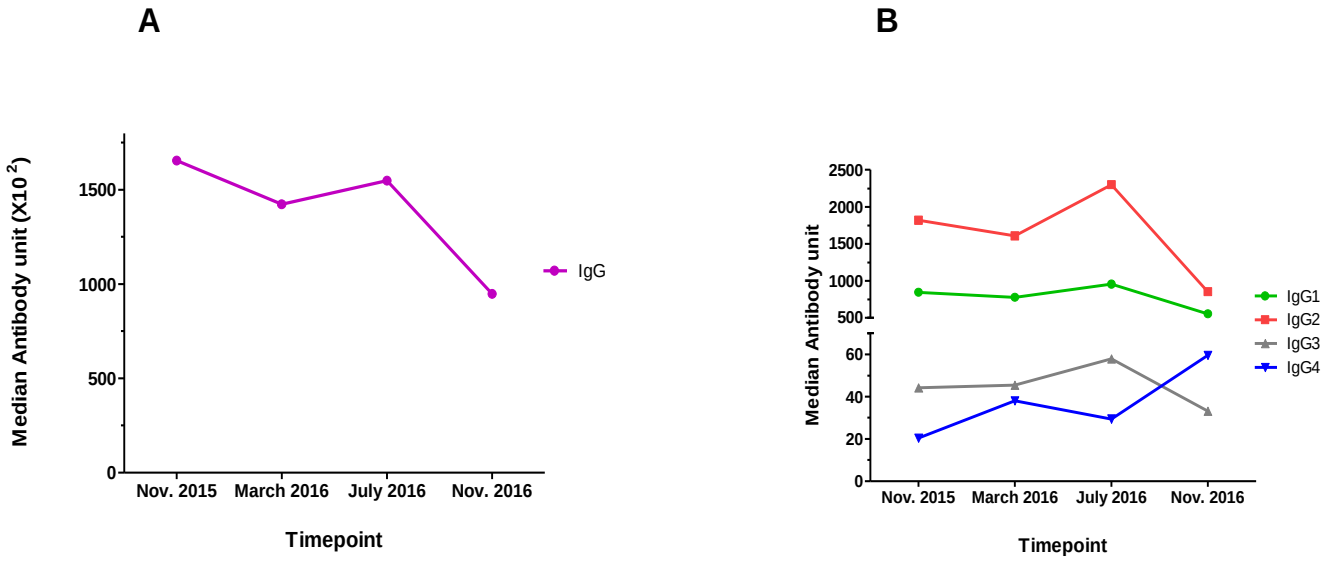

Figure 4.4: R2 IgG levels across the 4 sampling timepoints. Median levels of R2-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4, green line, red line, gray line and blue line respectively. Abbreviations: R2, Region 2 of the Glutamine-rich protein.

The statistical results also showed that the levels of LSA1-specific total IgG, IgG1, IgG2 and IgG3 were not significantly different across the timepoints of sampling (Fig. 4.5 A and B). However, there were significantly lower levels of LSA1-specific IgG4 at July 2016 compared to Nov. 2015 (P=0.04, Bonferroni post-hoc test) (Fig. 4.5 B). All other pair-wise comparisons were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). At almost all timepoints, the levels of LSA1-specific IgG1 antibodies were the highest followed by IgG2, IgG4 and IgG3 (Fig. 4.5 B).

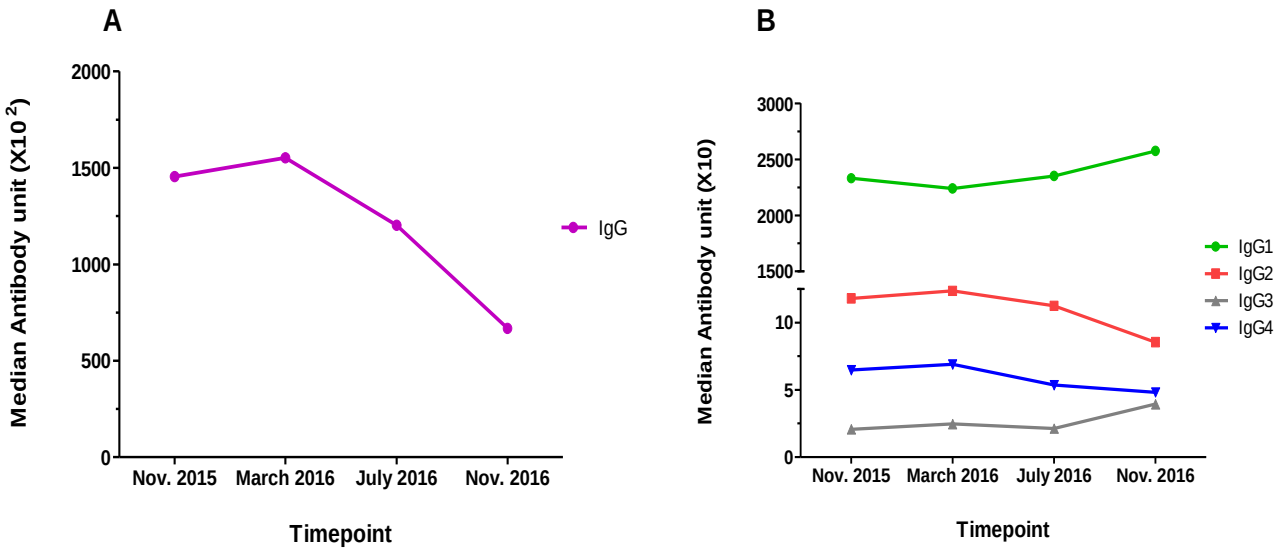

Figure 4.5: LSA1 IgG levels across the 4 sampling timepoints. Median levels of LSA1-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4, green line, red line, gray line and blue line respectively. Abbreviations: LSA1, Liver stage antigen 1.

There was also no significant difference in the levels of MSP3-specific total IgG, IgG1, IgG2 and IgG3 across the sampling timepoints (P>0.05 in all cases, Kruskal-Wallis test) (Fig. 4.6 A and B). However, significantly higher levels of MSP3-specific IgG4 antibodies were recorded at Nov. 2016 compared to July 2016 (P=0.02, Bonferroni post-hoc test) (Fig. 4.6 B). But, all other pair-wise comparisons showed no significant difference (P>0.05 in all cases, Kruskal-Wallis test). At nearly all timepoints of sampling, IgG2 levels was the highest followed by IgG1, IgG3 and IgG4 (Fig. 4.6 B).

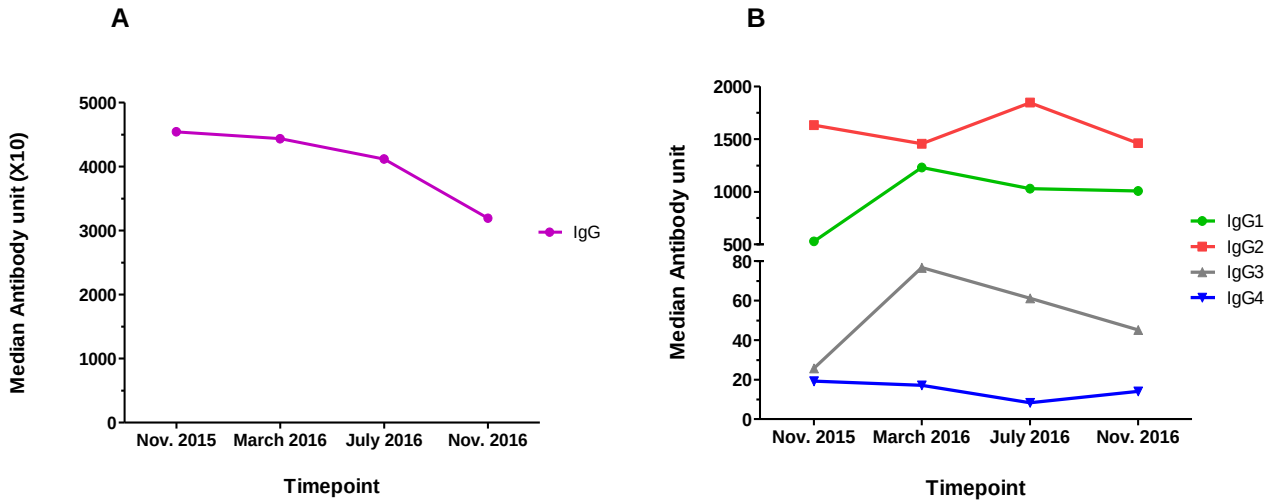

Figure 4.6: MSP3 IgG levels across the 4 sampling timepoints. Median levels of MSP 3-specific (A) total IgG, purple line (B) IgG 1, 2, 3 and 4, green line, red line, gray line and blue line respectively. Abbreviations: MSP3, Merozoite surface protein 3.

There were significantly lower levels of anti-schizont extract IgG at July 2016 compared to Nov. 2015 (P=0.002, Bonferroni post-hoc test) and March 2016 (P=0.01, Bonferroni post-hoc test) (Fig. 4.7). All other pair-wise comparisons showed no statistical significant difference (P>0.05 in all cases, Kruskal-Wallis test).

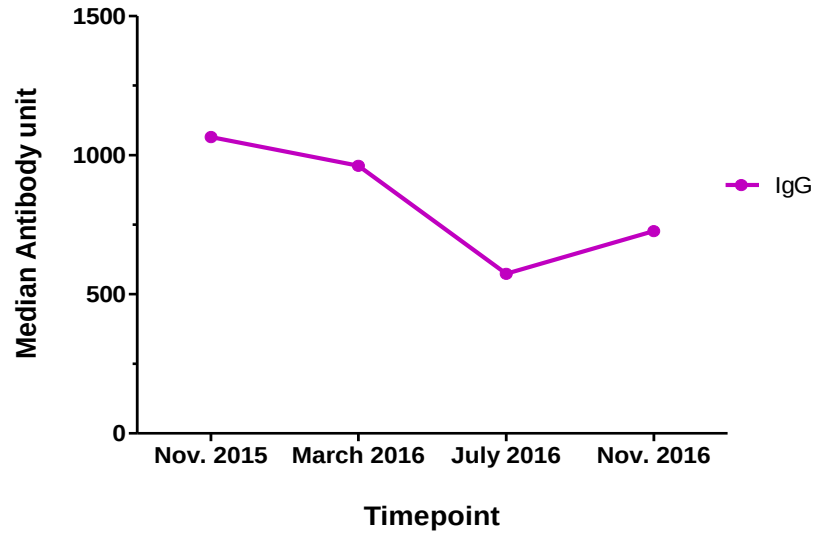

Figure 4.7: Schizont extract antigen specific-IgG levels across the 4 sampling timepoints. Median levels of Schizont extract antigen-specific total IgG, purple line.

4.0.2 *P. falciparum*-specific memory B cell responses to stimulation and activation

The memory B cell (MBC) responses were determined by measuring the total IgG levels against the schizont extract in culture supernatant samples that were taken on days 3 and 6 of the cell culture. The median memory B cell response on day 6 was significantly higher than the responses on the day 3 (Fig. 4.8). The results showed that there was a significant difference between the responses measured on the two days (P<0.0001, Generalised estimating equation).

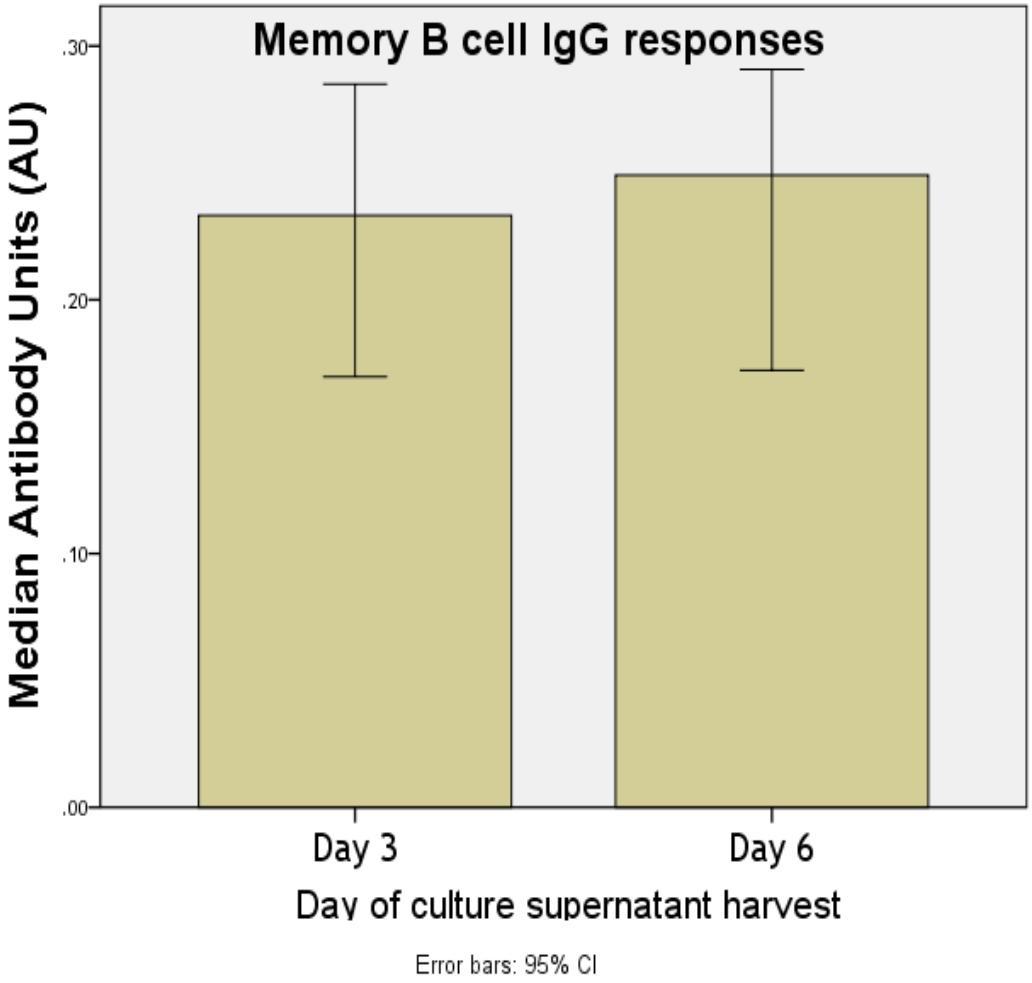

Figure 4.8: Memory B cell responses at day 3 against day 6. Abbreviations: IgG, Immunoglobulin G.

For the purpose of analyzing the MBC responses across the four-time points, the MBC responses at Day 3 were used.

There were significantly higher memory B cell responses at March 2016 (P=0.003, Bonferroni post-hoc test) and July 2016 (P<0.0001, Bonferroni post-hoc test) compared to Nov. 2015 (Fig. 4.9). There were significantly lower levels of MBC responses at Nov. 2016 compared to July 2016 (P<0.001) (Fig. 4.9). All other pair-wise comparisons were not significantly different (P>0.05 in all cases, Kruskal-Wallis test).

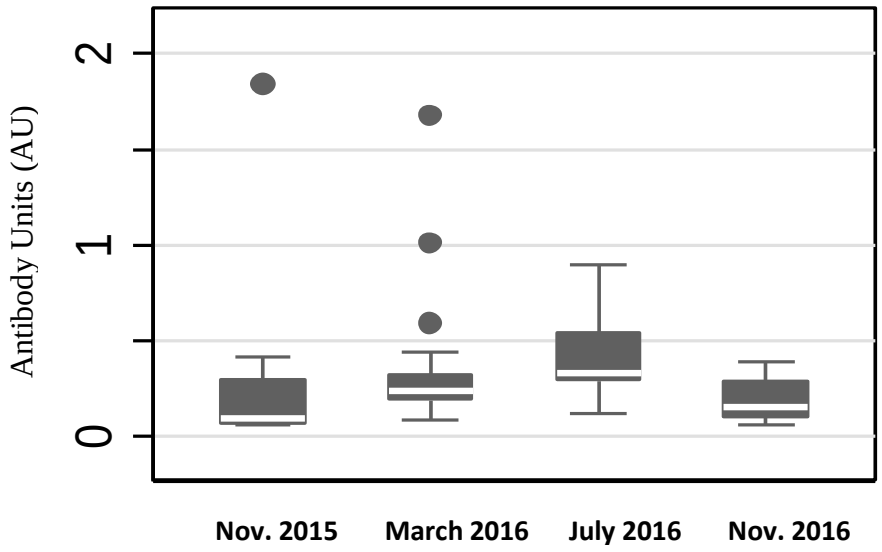

Figure 4.9: Levels of MBC responses at Day 3 of culture across the 4 sampling timepoints. The middle line, median value of the dataset; the upper limit of the box, the mean of the values above the median value; lower limit of the box, the mean of the values below the median value; the upper limit of the whisker, the highest value in the dataset; lower limit of the whisker, lowest value in the dataset.

4.0.3 B cell subpopulation profiling

The proportion of various subsets of B cells (CD 19^{+} cells) of the entire population of lymphocytes as determined across the four-time points of sampling are presented in Fig 4.10.

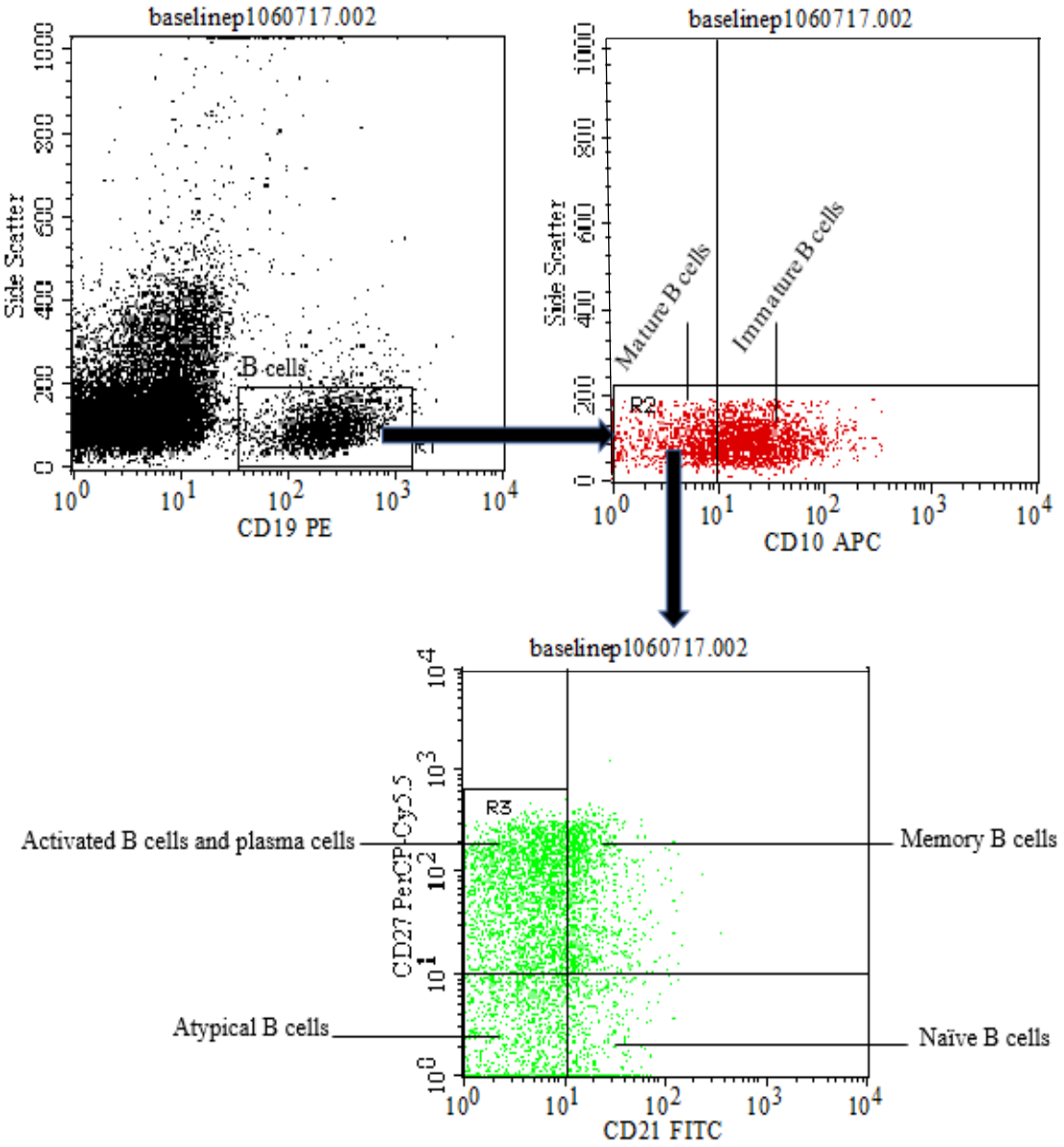

Figure 4.10: B cell immunophenotyping gating strategy. Abbreviations: CD, Cluster of differentiation; PE, Phycoerythrin; APC, Allophycocyanin; PerCP-Cy 5.5, Peridinin chlorophyll protein-Cyanine 5.5; FITC, Fluorescein isothiocyanate.

The results showed that the frequency of mature B cell subsets ($CD19^{+}CD10^{-}$) was lower at Nov. 2016 compared to Nov. 2015 and March 2016 (P=0.04 in both cases, Bonferroni post-hoc test). There was no significant difference in all other pair-wise comparisons made (P>0.05 in all cases, Kruskal-Wallis test). Contrary to the mature B cell frequency, the frequency of the immature B cell subsets ($CD19^{+}CD10^{+}$) was significantly higher at Nov. 2016 compared to Nov. 2015 and

March 2016 (P=0.04 in both cases, Bonferroni post-hoc test). There was no significant difference in other timepoint pair-wise comparisons made (P>0.05 in all cases, Kruskal-Wallis test).The gating strategy (Fig. 4.10) enabled further distribution of the mature B cell subsets into various subpopulations. The frequency of naïve B cells ($CD19^{+}CD10^{-}CD27^{-}CD21^{+}$) was higher at March 2016 (P<0.001, Bonferroni post-hoc test), July 2016 (P=0.02, Bonferroni post-hoc test) and Nov. 2016 (P=0.001, Bonferroni post-hoc test) when all were compared pairwise with Nov. 2015 (Fig. 4.11 C). There were no significant differences in all other timepoint pair-wise comparisons (P>0.05 in all cases, Kruskal-Wallis test). The memory B cell ($CD19^{+}CD10^{-}C27^{+}CD21^{+}$) frequency was significantly higher at Nov. 2016 compared to Nov. 2015 (P<0.0001, Bonferroni post-hoc test) and March 2016 (P=0.02, Bonferroni post-hoc test) (Fig. 4.11 D). All other timepoint pair-wise comparisons were not significantly different (P>0.05 in all cases, Kruskal-Wallis test). The frequency of activated B cells and plasma cells ($CD19^{+}CD10^{-}C27^{+}CD21^{+}$) were significantly higher at Nov. 2016 compared to July 2016 (P=0.003, Bonferroni post-hoc test) (Fig. 4.11 E). There was no significant difference in all other timepoint pair-wise comparisons (P>0.05 in all cases, Kruskal-Wallis test). The frequency of atypical B cells ($CD19^{+}CD10^{-}C27^{-}CD21^{-}$) was significantly lower at Nov. 2016 compared to Nov. 2015 (P<0.0001, Bonferroni post-hoc test), March 2016 (P=0.003, Bonferroni post-hoc test) and July 2016 (P=0.006, Bonferroni post-hoc test) (Fig 4.11 F). All other timepoint pair-wise comparisons showed no significant differences (P>0.05 in all cases, Kruskal-Wallis test)

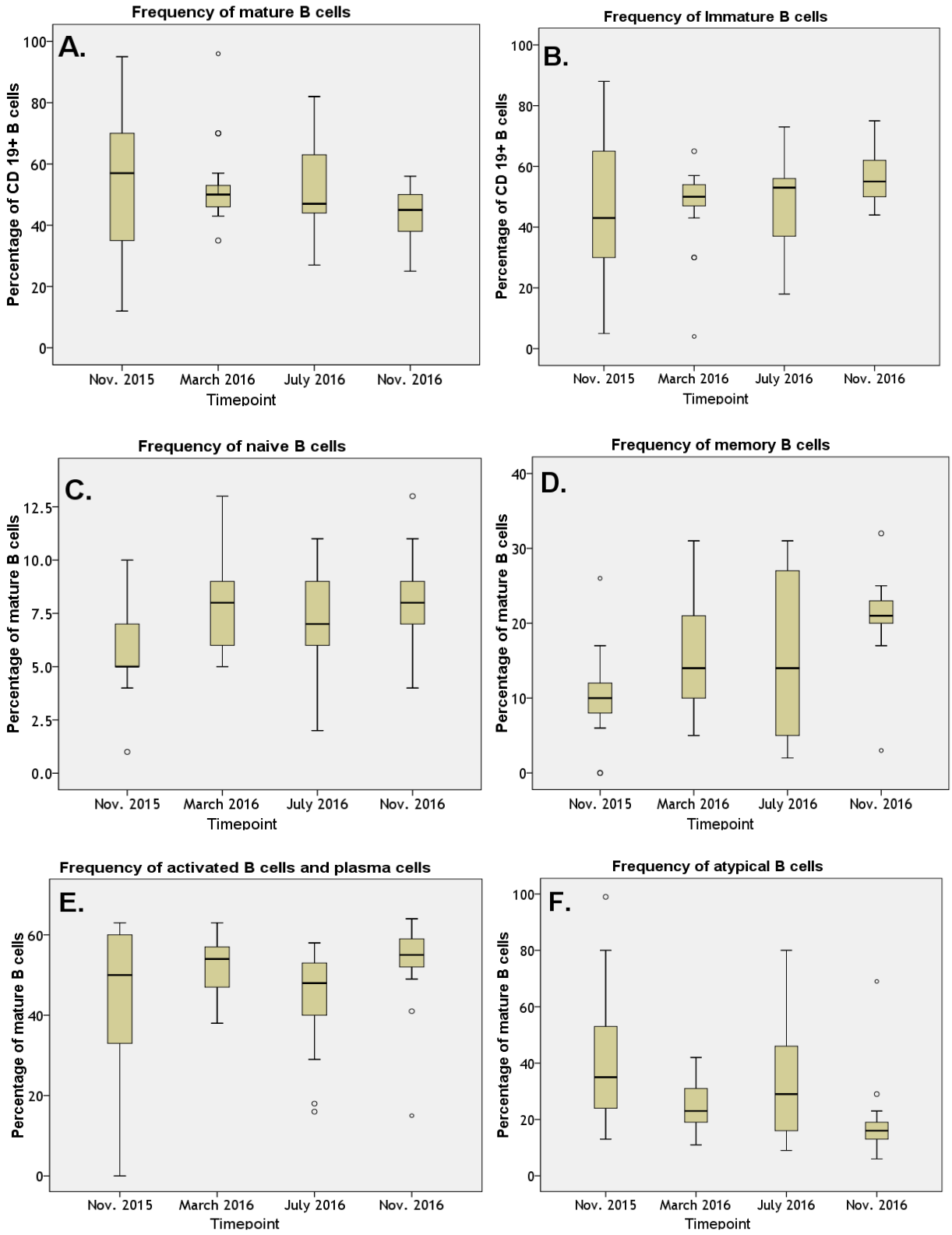

Figure 4.11: B cell subpopulation profiles across the 4 sampling timepoints. Frequency of (A) mature, (B) immature (C) naïve, (D) memory (E) activated and plasma cells and (F) atypical B cells. The middle line, median value of the dataset; the upper limit of the box, the mean of the values above the median value; lower limit of the box, the mean of the values below the median value; the upper limit of the whisker, the highest value in the dataset; lower limit of the whisker, lowest value in the dataset; circles, outliers.

4.2 DISCUSSION

In the quest to solve the problem that malaria poses globally, there is the need to adequately understand naturally acquired immunity to malaria. However, little is known about how immunological memory is maintained against malaria. Several malaria vaccines are at various stages of development and the limited efficacy of malaria vaccine candidates is thought to be an outcome of the wane in vaccine-specific immunological memory. Nevertheless, it is yet to be shown whether immunological memory is maintained in a similar manner against various *P. falciparum* antigens. Adequate understanding of the antibody kinetics in immunological memory with *P. falciparum* infection as well as the memory B cell profiles of individuals living in endemic populations may enhance malaria vaccine development.

In this study, antigen-specific antibody, memory B cell responses and B cell immuno-phenotyping data was used to elucidate the immunological memory profiles to *P. falciparum* infection in individuals living in a hypo-endemic area, the Greater Accra region of Ghana. With a 1-year longitudinal design, sampling was done at 4 timepoints; November 2015, March 2016, July 2016 and November 2016. The first (Nov. 2015) and last (Nov. 2016) timepoints are characteristic of high parasite transmission whiles March and July are characteristic of low transmission (Afari *et al.,* 1995).

P. falciparum infection induces the production of both polyclonal and specific immunoglobulins (Roussilhon *et al.,* 2007; Braga *et al.,* 2002). Different antibody isotypes may have protective functions, but IgG antibodies have been demonstrated to play a key role in immune combat against *P. falciparum* infection (Sabchareon *et al.,* 1991; McGregor *et al.,* 1964). Clinical immunity to malaria is known to be associated with levels of exposure and age (Doolan *et al.,* 2009). In a study conducted by Nebie *et al.* (2008) in Burkina Faso, they found an impact of the malaria transmission intensity on antibody levels to the four malaria recombinant antigens they considered (GLURP R0 and R2, circumsporozoite repetitive sequence $(NANP)_5$ and MSP3). Contrary to their results, the

present study found no statistical difference in the level of total IgG antibodies to all the recombinant antigens studied. In spite that the design of their study was cross-sectional, the differences in the analytical procedures, sample size as well the reagents used in both studies may have contributed to the contrasting results. However, the relative stability of the total IgG antibody levels against all the 6 recombinant antigens in the current study may be due to the age range of the study participants (18-49 years) and possibly an outcome of many infections of the malaria parasite within their lifetime that has led to the build-up of a robust antibody reservoir. Meanwhile, previous studies have shown that clinical immunity to malaria may be attained by most individuals within this age range in malaria endemic areas (Dodoo *et al.,* 2008; Baird, 1998; Baird, 1995). However, except for CSP and AMA1, the results of the present study showed that there was a trend of decrease in the plasma total IgG levels against all recombinant antigens (R0, R2, LSA1 and MSP3) as well as the whole parasite extract between the first and last time points of sampling. Data from a previous study approximates the overall catabolic half-life of IgG as 26 days (Mankarious *et al.,* 1988). This may in part explain the overall lower antibody levels observed after the 1-year period for all these antigens, with the relatively low parasite infection recorded over the study period. However, the present study was unable to ascertain the possibility of this trend of decrease in the antibody levels over the one-year period leading to a gradual depletion of the antibody reservoir to *P. falciparum* in this population if it remains the same (or similar) over a long period. Nevertheless, a larger sample size study may be required to strengthen and confirm the impact of this observed trend in this endemic zone. The trends observed in the antibody responses against the schizont extract antigen seems to have followed the malaria transmission patterns reported by Afari and others (1995), suggesting an impact of the transmission season at the sampling time, even though this was not confirmed by the microscopic detection (probably due to its relatively less sensitivity to submicroscopic parasites). There was also a possibility of losing information on infections that might have occurred within the quarterly intervals that existed

between the sampling timepoints. The trend is seen in the decline in the antibody levels from Nov. 2015, which was within the high transmission season to July 2016 which was within the low transmission season. As might have been expected, there was an increase in the levels from July 2016 to Nov. 2016, because Nov. 2016 was the same month of sampling as the earliest timepoint, but in the subsequent year. Only AMA1-specific total IgG followed this same trend that was observed in the schizont extract-specific total IgG responses, although this was observed at the antibody subclasss level for MSP3 IgG4 and R0 IgG1. Possibly, this similarity in the trends between AMA1 and the whole parasite antibody responses may be due to the relative abundance of the protein on the surface of the parasite during invasion of RBCs by the parasite (Cowman and Crabb, 2006; Triglia *et al.*, 2000).

As expected, the levels of IgG 1, 2, 3 and 4 subclasses varied for all the antigens as has been reported in other studies (Nebie *et al.*, 2008; Dodoo *et al.*, 2008; Roussilhon *et al.*, 2007). IgG1 and IgG3 have also shown to be the key IgG subclasses in response to malaria antigens (Nebie *et al.*, 2008; Dodoo *et al.*, 2008). These two cytophilic antibodies are important in conferring protection to the malaria parasite, particularly due to their high affinity binding properties to Fc receptors (Adu *et al.*, 2013; Braga *et al.*, 2002). In this study, IgG2 showed the highest responses amongst all the subclass antibody types for AMA1, CSP, R0, R2 and MSP3. Moreover, for these antigens, there was a trend of IgG2 being the highest at nearly all timepoints, followed by IgG1, IgG3 and IgG4. However, this has not been observed by others (Nebie *et al.*, 2008; Dodoo *et al.*, 2008; Theisen *et al.*, 1998). The ability of an antigen to elicit antibody responses that can lead to parasite growth inhibition via antibody-dependent cellular inhibition (ADCI) may be an advantage in contributing to its success as a malaria vaccine candidate (Malkin *et al.*, 2005; Theisen *et al.*, 1998). Cytophilic antibodies are more efficient at recruiting phagocytic cells such as macrophages and monocytes through their Fc receptors (Ravetch and Bolland, 2001). Meanwhile, some other studies have reported that non-cytophilic antibodies such as IgG2 and IgG4 are inhibitors of the

bridging of merozoites to human monocytes by cytophilic antibodies against the same antigenic targets. This tends to lead to a reduction in the ability of cytophilic antibodies to control parasite multiplication through the ADCI mechanism (Bouharoun-Tayoun and Druilhe 1992). This constitutes why antigens that induce strong cytophilic IgG responses are often thought to be better vaccine candidates. The patterns being observed in this study are also similar to reports from studies in Eastern Sudan, a malaria hypoendemic zone, where high levels of IgG2 were also reported in Fulani adults (Nasr *et al.,* 2009) and in regions of low malaria transmission in Burkina-Faso (Aucan *et al.,* 2000). In these other populations, high levels of IgG2 were associated with protection and there was a high prevalence of the 131H allele of FcγRIIA which has been shown to efficiently bind to IgG2 opsonized parasites. The present study also recorded very low malaria parasite transmission which makes it similar to hypo-to-mesoendemic transmission patterns. These findings suggest that in areas where malaria transmission is low, there is a high prevalence of IgG2 antibodies to most malaria antigens and could confer protection against clinical malaria. However, this may be due to differences in the half-life of these IgG subclass antibodies (Cavanagh *et al.,* 2001). In this regard, IgG3 for example is thought to require a more stable exposure to the malaria parasite for its maintenance due to its relatively shorter half-life (Cavanagh *et al.,* 2001). Notwithstanding, genetic variations have been associated with differences in the induction of various immune responses (Modiano *et al.,* 1999a; 1999b).

Several studies in different malaria-endemic regions have sought to establish the impact of malaria infection on the B cell profile of individuals living in these areas (Portugal *et al.,* 2015; Ampomah *et al.,* 2014; Muellenbeck *et al.,* 2013; Weiss *et al.,* 2009; Ehrhardt *et al.,* 2005). Most authors have reported findings that have suggested that chronic exposure to the malaria parasite causes an exhaustion of memory B cells into atypical B cell phenotypes that have been shown by other studies to be dysfunctional (Weiss *et al.,* 2009). This notwithstanding, Muellenbeck and others (2013) also provided functional-assay based evidence that atypical B cells produced polyreactive

neutralising antibodies. However, most of these reports, including Muellenbeck *et al.* (2013), also showed that these B cell phenotypes showed hyporesponsiveness to *in vitro* stimulation by various mitogens including *Staphylococcus aureus* cowan (SAC), CPG-ODN, CD40 ligand (Portugal *et al.,* 2015; Weiss *et al.,* 2008; Ehrhardt *et al.,* 2005). In the current study, the malaria-specific MBC antibody responses in culture showed a trend that also follows a pattern that suggests an impact of malaria transmission at the time of sampling. The comparably lower level of memory B cell responses measured in the two high transmission sampling timepoints may be associated with the persistent stimulation and activation of the MBCs at these periods in the year. There was also an evidence of increase in the MBC responses at the two low transmission timepoints. This data agrees with findings of previous cross-sectional studies that showed reduced levels of MBCs in malaria endemic populations compared with malaria naïve individuals (Illingworth *et al.,* 2008; Weiss *et al.,* 2009). Considering also that the mitogens used in this study have shown efficiency in stimulating and activating memory B cells into antibody producing cells over time (Walsh *et al.*, 2013), the memory B cell response at day 6 was expected to be about twice that at day 3, but was many folds lesser than expected. However, this may be due to the inability of the cells to thrive under *in vitro* conditions over long periods. Despite this possibility, the overall lower levels of memory B cell responses at the high transmission timepoints and the reduced level of antibody production is in accordance with previous studies that showed that continual stimulation of malaria MBCs led to dysfunctionalities and exhaustion of the cells to produce antibodies (Portugal *et al.,* 2015; Weiss *et al.,* 2009). This study was not able to establish direct associations between the levels of memory B cell responses in culture and the levels of antibodies against the various recombinant antigens studied. This is in part because the assessments of the memory B cell responses were only in relation to the schizont extract antigen but not against various recombinant antigens. However, the antibody levels against the schizont extract antigen showed trends that suggests direct associations with the memory B cell responses and agrees with findings from other

similar studies (Ampomah *et al.,* 2014; Weiss *et al.,* 2008). It is evident in the increased antibody levels during high transmission season accompanied with decreased memory B cell levels and a decrease in the low transmission season accompanied with increased memory B cell levels. It might be tempting to speculate that there was chronic stimulation of the existing pool of malaria specific-MBCs to produce high levels of antibodies at the high transmission seasons which contributed to the lower responsiveness in culture as was observed. Meanwhile, evidence of somatic hypermutations of memory B cells into atypical B cells due to chronic stimulation has also been shown by a previous study (Erhardt *et al.,* 2005). Atypical B cells have been described as phenotypically similar to plasmablasts because of their short life span. In addition, similar to plasmablasts, atypical B cells have the CD69, CD80 and CD86 markers of activation, but lack the unique CD138 marker of long-lived plasma cells. These findings may explain the transient changes in the levels of memory B cell responses observed in this population and may also confirm results from a previous study which established a positive correlation between atypical B cells and transmission intensity in Mali and Peru (Weiss *et al.,* 2011).

Mature B cells are known to constitute various B cell subsets that are $CD19^{+}CD10^{-}$. The present study observed a steady decline in the mature B cell subpopulation and a corresponding increase in the immature B cell subpopulation from the first to the last timepoints of sampling. Meanwhile, a previous study in advanced HIV patients reported that there was a continuous increase in the immature B cell subsets in the peripheral blood of the patients (Malaspina *et al.,* 2006). This study speculated that the increased frequency of immature B cell subsets against the decrease in the mature B cell subsets may be due to possible exhaustion of the mature B cell subsets as a result of the HIV infection. This trend also agrees with the trend of this study. Possibly, there may exist a similarity in the impact of HIV and the malaria parasite on immune profiles of exposed individuals as have been speculated by several authors (Weiss *et al.,* 2009; Moir *et al.,* 2008). However, even though the study participants of this present study are from a malaria endemic zone, the design of

the flow cytometric panel for this study was not limited to malaria-specific B cell phenotypes. This implies that these observations could be due to the impact of several other infections and confounders that these individuals are naturally exposed to; placing a limitation on possible conclusions on the observations made. Yet, this observation may also be due to an overall higher frequency of mature B cells that are short-lived in the blood. Even if this may be the underlying cause, this trend may not be the same in other lymphoid tissues. A study in HIV infected individuals in Mali reported an observation of higher frequency of activated B cells and plasma cells compared with uninfected individuals (Moir *et al.*, 2008). Another study in Mali involving malaria exposed adults and children by Weiss and others (2009) reported higher frequencies of activated B cells and plasma cells compared to malaria naïve US adults. Both studies showed associations of the heightened frequencies of these B cell phenotypes to the pathogens that affected their cohort. Similarly, the present study found the frequency of the activated B cells and plasma cells to be the highest among the mature B cell phenotypes across all the timepoints of sampling. This finding may support the observations made in the Malian studies and the thought that relatively higher frequencies of activated B cells and plasma cells may be indicative of ongoing parasite transmission within a malaria endemic zone (Weiss *et al.*, 2009). Nevertheless, the overall relatively low frequencies of the antigen-inexperienced, naïve B cells and the increase in the frequency of memory B cells from the earliest timepoint to the last timepoint in this present study may be providing support for this claim as well. The low frequencies of naïve B cells coupled with the increase in the memory B cell frequency may also suggest a build-up of immunological memory. An inverse relationship was also observed between the memory B cell subsets and the atypical B cell subsets, where an increase in one of the subsets was associated with a decrease in the other and vice-versa. These findings may also be providing supporting evidence for the possibility of exhaustion of the memory B cell phenotypes to the atypical B cell phenotypes from deductions on the memory B cell culture data as well the findings of other studies. Yet, the non-

malaria specific design of our flow cytometric panel and the low parasite prevalence detected by microscopy across the period of this study places limitations on the evidence used to explain these kinetics.

CHAPTER FIVE

5.0 CONCLUSION AND RECOMMENDATION

5.1 Conclusion

With a 1-year longitudinal design, the present study has been able to expound several dynamics to *P. falciparum* infection in the immunological profile of individuals living in a malaria hypo-endemic area;

- This study found transient changes in antibody responses to the schizont extract antigen, the level of memory B cell responses as well as various mature B cell subsets.
- The study also observed an inverse relationship between the MBC subsets and the atypical B cells.

The present study concludes that the wane of immunological memory to *P. falciparum* is due to possible exhaustion of memory B cells into the 'dysfunctional' B cell type, atypical B cells, as have been speculated by previous studies.

5.2 Recommendation

- It is recommended that similar studies would be replicated in other territories and endemic populations with larger number of study participants and shorter sampling intervals to provide further understanding to naturally acquired immunity to malaria.
- Studies to assess the levels of MBC responses to various malaria antigens over time may also be of much importance to understanding the kinetics in immunological memory to malaria.
- Also, there is the need to perform more functional assays to establish the functionalities and abilities of atypical B cells.

- There is also the need to establish the impact of genetic variations in the malaria parasite on the B cell phenotypes and the maintenance of immunological memory to the parasite.

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Appendices

Appendix I

ELISA Plate layout

3-fold dilution 

	1	2	3	4	5	6	7	8	9	10	11	12
A	STANDARD											
B	STANDARD											
C	Positive control	Positive control	Sample 01	Sample 01	Sample 02	Sample 02	Sample 03	Sample 03	Sample 04	Sample 04	Sample 05	Sample 05
D	Sample 06	Sample 06	Sample 07	Sample 07	Sample 08	Sample 08	Sample 09	Sample 09	Sample 10	Sample 10	Sample 11	Sample 11
E	Sample 12	Sample 12	Sample 13	Sample 13	Sample 14	Sample 14	Sample 15	Sample 15	Sample 16	Sample 16	Sample 17	Sample 17
F	Sample 18	Sample 18	Sample 19	Sample 19	Sample 20	Sample 20	Sample 21	Sample 21	Sample 22	Sample 22	Sample 23	Sample 23
G	Sample 24	Sample 24	Sample 25	Sample 25	Sample 26	Sample 26	Sample 27	Sample 27	Sample 28	Sample 28	BLANK	BLANK
H	Sample 29	Sample 29	Sample 30	Sample 30	Sample 31	Sample 31	Sample 32	Sample 32	Sample 33	Sample 33	BLANK	BLANK

Dilutions for Standard pool, Positive control and Plasma samples for ELISA assay

Antigen	Standard Control	Positive control	Sample
AMA1			
Total IgG	1:10000	1:15000	1:5000
IgG 1	1:4000	1:5000	1:1000
IgG 2	1:2000	1:3000	1:1000
IgG 3	1:4000	1:5000	1:1000
IgG 4	1:2000	1:3000	1:1000
CSP			
Total IgG	1:100	1:150	1:500
IgG 1	1:20	1:30	1:100
IgG 2	1:20	1:30	1:30
IgG 3	1:20	1:30	1:30
IgG 4	1:20	1:30	1:30
GLURP R0			
Total IgG	1:500	1:1000	1:500
IgG 1	1:150	1:150	1:100
IgG 2	1:50	1:50	1:100
IgG 3	1:50	1:50	1:100
IgG 4	1:50	1:50	1:100
GLURP R2			
Total IgG	1:1000	1:1000	1:500
IgG 1	1:150	1:150	1:100
IgG 2	1:50	1:50	1:100
IgG 3	1:50	1:50	1:100
IgG 4	1:50	1:50	1:100
LSA1			
Total IgG	1:3000	1:4000	1:2000
IgG 1	1:1000	1:1000	1:100
IgG 2	1:20	1:30	1:100
IgG 3	1:20	1:30	1:100
IgG 4	1:20	1:30	1:100
MSP3			
Total IgG	1:500	1:1000	1:500
IgG 1	1:150	1:150	1:100
IgG 2	1:50	1:50	1:100
IgG 3	1:50	1:50	1:100
IgG 4	1:50	1:50	1:100

Appendix II

Statistical commands used in R statistical package and their functions

Using the commands for AMA1 antibody responses analysis over the 4 sampling timepoints of the study as an example:

Command	**Function**
setwd("c:/users/user.user-pc/desktop/r data")	To set a working directory to the folder containing data to be analysed in R.
ama<-read.csv("ama1.csv")	To assign a specific name, 'ama' to the CSV file containing AMA1 IgG data.
library(agricolae)	To access the agricolae analysis package tool and call it to function.
kruskal.test(amaIgG,amafollowup)	To test for significant differences in the AMA1 IgG levels across the 4 timepoints
igg.data<-kruskal(amaIgG,amafollowup,p.adj="bonferroni",group=F)	To perform Bonferroni pair-wise comparisons of AMA1 IgG levels at the 4 timepoints.