Chapter 1

Introduction

and

Review of literature
Introduction
1. INTRODUCTION & REVIEW OF LITERATURE

1.1 INTRODUCTION

Malaria due to *Plasmodium falciparum* is the most serious infectious disease of humans, infecting 5-10% of the world's population, with 300-660 million clinical cases and more than 2 million deaths annually (Robert W. Snow *et al.*, 2005). A malaria vaccine is clearly needed and a better understanding of how the immune system responds to malaria parasites would facilitate this. Humoral immunity is maintained by long-lived plasma cells that constitutively secrete antibodies, and non-secreting resting memory B cells that are rapidly reactivated upon antigen encounter (Aribot *et al.*, 1996; Giha *et al.*, 1999). Numerous steps are involved in the differentiation of an antigen-experienced B cell to a memory B cell and long-lived plasma cell. Primary antibody response usually ceases within days or weeks after infection. However, following repeated infection, antigen-specific antibody titers consisting of IgG subclasses can persist for years and constitute an essential part of the protective immune memory (Slifka and Ahmed, 1996).

B cells and antibodies are necessary for eliminating the blood stages of malaria in most experimental models (Langhorne *et al.*, 1998, Brunet L.R, 2001; Metenou *et al.*, 2007). Although antibody response is critical in the blood stages of malaria, the level of these antibodies drops rapidly at the end of the malaria transmission seasons (Cavanagh *et al.*, 1998; Fonjourngo *et al.*, 1999). This drop is also seen in people after they have left the endemic areas. Even in mouse model, the B cell response to infected erythrocytes was
significantly reduced after one year of infection with *P. chabaudi* (Pearson *et al*., 1983). From these data it is evident that the generation of B cell memory and long-lived plasma cells is impaired in malaria infection (Wykes *et al*., 2005, Wykes *et al* 2006).

In the proposed study, two well defined *P. falciparum* recombinant antigens, 19kDa carboxyl terminal fragment of the Merozoite Surface Protein-1 (PfMSP1-19) and Merozoite Surface protein-3 (PfMSP-3F) have been used to quantitatively analyze de novo B cell differentiation into plasma cells, longevity of plasma cells, and the B-cell memory development.
Review of Literature
1.2 REVIEW OF LITERATURE

1.2.1 Life cycle of Plasmodium

Malaria parasites have a complex life cycle (Fig. 1.1). Infection in humans begins when an infected female Anopheline mosquito injects Plasmodium sporozoites into the bloodstream during a blood meal (Rosenberg et al., 1990; Ponnudurai et al., 1991). The injected sporozoites home to the liver and infect hepatocytes within an hour of infection where they multiply and differentiate into merozoites (Mota et al., 2001; Mota et al., 2002). The merozoites are released into the bloodstream and go on to invade red blood cells where they multiply by schizogony over a 48 hour period (in case of *P. falciparum* and *P. vivax*) to form the next generation of merozoites. *P. vivax* can form dormant hypnozoites in the liver, which may release merozoites and cause disease months or years later. *P. falciparum*, on the other hand, does not have the ability to form hypnozoites and differentiates completely from liver-stage schizonts to merozoites.

Within the red blood cell the parasite differentiates and multiplies asexually, periodically leading to rupture of the parasitized RBC (*Pf*-RBCs) and release of fresh merozoites in the blood circulation. It takes only a few seconds for merozoites to invade new blood cells, so merozoites spend most of its life inside RBCs and are relatively protected from the immune system. It is during this blood stage, that infected persons develop the periodic fevers, chills and other symptoms of malaria. During its life cycle within the RBCs several parasite proteins are introduced into the RBC plasma membrane and submembranous protein skeleton, thereby modifying a range of structural and functional properties of the parasitized RBCs (*Pf*-RBCs) (Cranston et al., 1984; Nash et al., 1989;
Then, this step of blood stage is followed by the maturation of *P. falciparum* merozoites to trophozoite (24–36 h) and schizont (36–48 h) stages in which *Pf-RBCs* display decreased membrane deformability (Cranston *et al.*, 1984; Paulitschke *et al.*, 1993; Suresh *et al.*, 2005), become spherical, and develop cytoadherence properties responsible for parasite sequestration in the postcapillary venules of different organs (Dondorp *et al.*, 2004; Miller *et al.*, 2002). Some of the blood-stage parasites differentiate into male and female gametocytes, which are picked up by the mosquito during a blood meal. In response to signals in the mosquito midgut, the gametocytes release male and female gametes by a process referred to as exflagellation. The gametes undergo fertilization in the mosquito midgut and form a zygote. The zygote transforms into an ookinete, which traverses the midgut epithelium of the mosquito to form an oocyst on the outer wall of the midgut. The oocyst matures to release thousands of sporozoites that invade salivary glands and are ready to be injected into the vertebrate host again thus completing the parasite’s life cycle.

All the clinical symptoms of malaria are attributed to the blood stage of the parasite’s life cycle. The periodic rupture of infected red cells and release of merozoites is accompanied with chills, headache, lassitude and a sharp increase in body temperature, which are classic symptoms of malaria (Glenister *et al.*, 2002). After the initial rise in parasitemia, the innate immune response of the host controls the exponential rise in parasitemia but can not completely eliminate the infection (Artavanis-Tsakonas *et al.*, 2002) and a naïve individual infected with *P. falciparum*, if left untreated, can quickly progress to one or
more severe malaria syndromes such as cerebral malaria, severe anemia, multiple organ failure, metabolic acidosis and respiratory distress, which can lead to death (Miller et al., 1994). Pro-inflammatory cytokines released as part of the innate immune response of the naïve host may in fact promote severe disease (De Souza et al., 1997; Artavanis-Tsakonas et al., 2002; Carnaud et al., 1999; Stevenson et al., 2004; Miller et al., 1994). In endemic areas, following repeated natural exposure to *P. falciparum*, individuals acquire immune responses that are able to limit parasite densities and provide protection against clinical malaria (Bruce-Chwatt et al., 1952; Wahlgren et al., 1986; Baird et al., 1991; Warsame et al., 1997; Sharma et al., 2004). Sterilizing immunity is never achieved even after repeated exposure. It is primarily non-immune children in endemic areas who are most affected by severe malaria and are at highest risk of death from malaria whereas adults acquire immunity and are protected against severe disease (Snow et al., 1994;
Review of Literature

Reyburn et al., 2005; reviewed by Padhmanand Sudhakar and Prasanth Subramani, 2007).

1.2.2 Overview of humoral immunity

B cells and their products, mainly secretion of antibodies, constitute the backbone of the humoral immune responses. Antibodies also known as immunoglobulins are produced in response to immunization or infection. Antibody levels gradually return to normal after the infection has resolved but upon rechallenge with the same pathogen/antigen, antibody response is faster and of much higher titer than the primary response. The rapid response to rechallenge is the defining characteristic of immunological memory (Burnet and Fenner 1949; reviewed in Gray D. 1997).

B cells exist in the periphery as naive cells and are able to specifically recognize antigen through unique receptor known as the B cell receptor (BCR). The BCR is a membrane-bound immunoglobulin (mIg) form of the antibody molecule (Batista et al., 2001; reviewed in Reth M. 1992). Upon binding of antigen to the BCR, the BCR-antigen complex is internalized, processed and presented as antigen-MHC class II complex to the CD4 T cells (Jordan et al., 2004; Bishop et al., 2001; reviewed in Colin Watts; 1997). The antigen specific T cells thereafter costimulate the B cells which will now proliferate and differentiate into effector, memory and antibody secreting plasma cells (PCs). An important subset of PCs reside in the bone marrow (BM) and are responsible for the long-term secretion of antibody that allows elimination or neutralization of a pathogen, upon rechallenge, before pathology develops (Slifka et al., 1995; Manz et al., 1997; Slifka et al., 1998). The conditions induced by the immune response such as cytokines,
chemokines, signal strength etc, and determines the fate of the B cell (Ho et al., 1986; Hargreaves et al., 2001; O’Connor et al., 2004).

1.2.3 The B cell response to antigen

B cells may first encounter antigen in any of several locations: blood, lymph nodes, the marginal zone of the spleen, or on follicular dendritic cells (FDC) and other antigen-transporting cells (Liu et al., 1991b; Cyster, 1999). Once a B cell has bound sufficient antigen it is redirected from the migration route of naive B cells through the spleen or lymph node and moves to the boundary of the B and T cell zones (MacLennan et al., 1990). B cells proliferate and can, depending on whether the antigen is a T cell-independent (TI) or T cell-dependent (TD) antigen, either undergo a proliferative burst and produce low-affinity IgM but no memory (Liu et al., 1991b; Lesinski and Westerink, 2001; Li et al., 2001) or induce a long-lasting immune response, forming memory B cells, and producing high affinity antibodies (Liu et al., 1991b).

1.2.4 T cell dependent immune responses

Naive B cells circulate through blood, lymph nodes and spleen until they encounter antigen. This often happens in the T cell area in the spleen or lymph nodes, to which antigen is brought by macrophages and dendritic cells. After encountering the specific antigen, the B cell stops migrating and remain in the T cell zone (reviewed in Kelsoe and Zheng 1993; Liu 1997). B cells specific for the antigen internalizes the antigen via the BCR complex. The antigen is then processed and presented on MHC class II molecules on the cell surface. In the T cell zone, naive antigen-specific T cells are activated,
probably by antigen presenting interdigitating dendritic cells. Upon antigen recognition activated CD4 T cells forms stable conjugates with the antigen-presenting B cells, and deliver both receptor mediated and soluble stimulatory signals to the B cells to proliferate extensively. Proliferation occurs initially in the T cell zone of the white pulp where B and CD4 T cells make contact (Liu et al., 1991b; Han et al., 1997). There are two possible pathways that a B cell can enter after contact with an antigen-specific CD4 T cell, one is to form a focus of antibody forming cells locally in the outer periarteriolar lymphocytic sheath (PALS) region of the white pulp (Claassen et al., 1986b) and this results in terminal differentiation of the B cells into plasma cells. These plasma cells produce the early wave of antibodies generated after antigen challenge (reviewed in MacLennan et al 2003). Some activated B cells do not undergo terminal differentiation, but instead migrate into the follicular region and initiate the germinal center (GC) formation (Han et al., 1997; Tarlinton and Smith, 2000). Although the complete requirement of the signals needed for differentiation either into plasma cells or GC B cells is unknown, the signaling via CD40 on the B cell induced CD40L on the T cell seems to be required for GC formation (Foy et al., 1994; Kawabe et al., 1994).

1.2.5 Germinal centres

The GC is a site of intense B cell proliferation and supports somatic hypermutation and the subsequent selection of high affinity B cells (MacLennan and Gray, 1986; Berek et al., 1991; Jacob et al., 1991b; Kuppers et al., 1993) as well as production of memory and long-lived BM AFC (Weiss and Rajewsky, 1990; Han et al., 1997; Smith et al., 1997). GCs are formed in the B cell follicles of secondary lymphoid tissues during immune
responses (MacLennan, 1994). GCs first appear at days 4–5 post-immunisation, achieving maximum cell number by days 10–11 and declining after approximately 3-4 weeks (MacLennan et al., 1988). Although some GCs remains after initial immunisation, the peak response is seen by day 28 (Liu et al., 1991b). It takes as few as three B cell blasts to seed a GC (Kroese et al., 1987) that proliferate to become a population size of approximately 10,000 cells (Radmacher et al., 1998). Thus due to the large amount of expansion, a mass of death also occurs in GCs. GC B cells are therefore, highly susceptible to apoptosis (Liu et al., 1991a) and need continual survival signals from external sources. Two major signals that are known to prevent apoptosis of GC B cells are the crosslinking of the BCR and the CD40-CP154 interaction that act synergistically to extend the life of GC B cells (Liu et al., 1991a). FDCs or CD4 T cells may also provide continued survival signals in the GC (Christoph Huber et al., 2005).

1.2.6 Somatic hypermutation, affinity maturation and class switch

As the immune response matures there is a shift in antibody repertoire (Berek et al., 1991; Foote and Milstein 1991), the antigen-specific antibodies with higher affinity and other Ig classes are used. This phenomenon is called affinity maturation, and it mainly takes place in the environment of the GC. The range of B cell affinities is determined by somatic rearrangement of the Ig gene encoding the BCR and by repertoire development. Antigen-specific B cells that had entered the GC cycle undergo rapid clonal expansion coupled with BCR diversification and improved BCR affinity through somatic hypermutation (SHM) (Jacob et al., 1991; Berek et al., 1991). The consequence of the somatic mutation can either lead to better selection of mature B cells that shows better
binding to the antigen (affinity maturation) or apoptosis of B cells that expresses non-productive BCR or potential self-reactive variants (Longo and Lipsky 2001).

Generation of antibody variants and the subsequent selection of variants that bind antigen strongly drive affinity maturation (MacLennan and Gray, 1986; Takahashi et al., 1999). B cells of any affinity are recruited into the GC and the selective accumulation of high affinity B cells is driven by inter- and intraclonal competition for antigen retained on FDCs (Dal Porto et al., 1998; Takahashi et al., 1999). Successful affinity maturation is one of the mechanisms by which faster and a more sustained secondary humoral immune response compared to primary response is generated. Also, high affinity B cells are selected for differentiation into memory B cells or plasma cells.

Other somatic alterations to the BCR include isotype switching. In order to elicit an optimal antibody response, the B cells also have the ability to alter the antibody class used in the response by exchanging the C_{H} regions, which alters the effector function of the antibody molecule (Bruggemann et al., 1987). After antigen encounter and CD40-CD154 interaction, all naïve B cells exchange the IgM and IgD receptors for a different isotype, IgG, IgA or IgE. Cytokine induced during an immune response determines to what isotype the BCR receptor is switched. The correlations between cytokine production and selection of Ig isotypes in large part reflect direct effects of IL4 and IFN-γ (Snapper et al 1987). The predominant isotype induced by IL-4 is IgG1 (Maliszewski et al., 1993), and the induction of isotype switching requires the synergistic signaling of CD40 and IL-4. CD40 stimulation alone will induce proliferation with little or no isotype switching (Armitage et al., 1992) and IL-4 stimulus alone does not induce proliferation.
(Maliszewski et al., 1993). Other cytokines secreted by CD4 T cells can have enhancing effects on this switching, or induce differentiation (Maliszewski et al., 1993; Arpin et al., 1995; Choe and Choi, 1998; Zhang et al., 2001). On the other hand, Th1 cytokines such that IFN-γ promotes secretion of immunoglobulin G2a (IgG2a) antibody on B lymphocytes (Snapper et al., 1987). Evidences have shown that absence of IL-6 in vivo (Kopf et al., 1994, Kopf et al., 1998) results in the selective loss of IgG2a and IgG2b in thymus-dependent immune responses. B cell–specific ablation of TGFBRII has implicated TGFβ in the induction of IgA (Cazac et al., 2000). The change of isotype provides the secreted antibodies with a different effector function while their antigen specificity remains as before class switching.

1.2.7 Death and survival of mature B cells

Activation of B cells leading to proliferation and differentiation into long-lived mature B cells requires signaling through the BCR and co-stimulatory molecules. BCR signaling is crucial for B cell survival since mature B cells that loose their expression of the BCR is reported to die rapidly (Lam et al 1997). CD40L (reviewed in van Kooten and Banchereau 1997), BAFF (Moore et al 1999; Schneider et al 1999) and APRIL (a proliferating inducing ligand) (Stein et al 2002) mediate important signals for the B cell. Their receptors, CD40, TACI (transmembrane activator and CAML-interactor), BCMA (B cell maturation antigen) and BAFF-R signal through the transcription factors, the NF-κB family (Berberich et al 1994; Kayagaki et al 2002; reviewed in Laabi et al 2001; Marsters et al 2000; Yan et al 2000). Signaling induced by CD40, BAFF and APRIL, apart from upregulating genes important for B cell activation, also upregulates genes that
review of literature

promote survival, such as bcl-2, bcl-XL (Do et al. 2000) and A1 (Kuss et al. 1999). The negative selection in BM and spleen results in mature B cells either being deleted by apoptosis, rendered anergic or induced to undergo receptor editing (reviewed in Nemazee 2000).

1.2.8 Memory B cells

B cell memory is characterized by the accelerated appearance of higher affinity and higher titers of antigen-specific antibody in response to rechallenge with antigen (Siskind and Eisen, 1965; Schittek and Rajewsky, 1990; Bachmann et al., 1996; Karrer et al., 2000). Generation of memory occurs in the GC since loss of the GC abrogates memory formation (Vieira and Rajewsky, 1990; Rajewsky, 1996; Han et al., 1997). Exit from the GC reaction signifies entry into the long-lived memory B cell compartment. Early studies have indicated that memory B cells are persistent, long-lived and antigen-specific (Gray and Skarvall 1988), and new models indicate that the survival of memory B cells is not dependent on signals through the BCR (Maruyama et al., 2000). A definite memory B cell population begins to appear approximately 21 days after primary immunisation (Vieira and Rajewsky, 1990; Ridderstad and Tarlinton, 1998). The memory B cells do not secrete antibody until expansion and differentiation into PC after rechallenge with antigen (Hayakawa et al., 1987; McHeyzer-Williams et al., 1991; Herzenberg et al., 1980). Most memory cells express switched Ig isotypes, but IgM memory cells can also be found (Klein et al. 1997). Furthermore, memory B cells can be divided into two populations based on the expression of B220 (Driver et al. 2001; McHeyzer-Williams et al. 2000). Memory B cells expressing B220 (B220+ ) have the highest potential of cellular
differentiation and proliferation capacity upon antigen re-challenge, while memory B cells lacking B220 (B220⁻) have the ability to self-replenish and are predisposed to form antibody secreting cells. A model of linear differentiation has been proposed, where B220⁺ memory cells give rise to B220⁻ cells from which antibody secreting cells are generated (McHeyzer-Williams et al 2000). The GC has since long been considered the main site for directing B cells to the memory pathway, however, it does not seem to be absolutely required as knock-out mice lacking GCs has been shown to have memory B cells (Toyama et al 2002). After leaving the GC, memory B cells can be divided into recirculating memory B cells and these cells are localized in the marginal zone in the spleen and lymph nodes (Liu et al 1988; Liu et al 1991; Liu et al 1996; Nieuwenhuis and Ford 1976; Oldfield et al 1988). Since marginal zone B cells are in regions where blood-borne antigens drain, they are capable to responding fast during secondary challenges. Rechallenge with an antigen leads to rapid and massive differentiation of memory cells into blasts and plasma cells (reviewed in MacLennan et al 1992; reviewed in van Rooijen 1990). Repeated antigen challenges can further increase the antibody affinity, which suggests that either the reactivated cells are capable of undergoing further affinity maturation or that the newly recruited naive B cells show higher affinity than the memory response to survive the GC process. The need for restimulation with antigen for maintaining memory B cells is under debate. Antigen can be stored for a long time as immune complexes trapped at the surface of FDC (reviewed in Szakal et al 1989; Tew and Mandel 1979). In agreement with the notion that maintenance of memory need antigen, it has been shown that transfer of memory B cells, in the absence of antigen, leads to the loss of the memory B cell population (Gray and Skarvall 1988). In contrast,
an experiment where the use of genetically modified mice enabled the specificity of the memory B cells to be changed indicated that long-lived memory B cells could survive without antigen (Maruyama et al. 2000). It has also been suggested that serological memory is maintained through polyclonal activation of memory B cells (Bernasconi et al., 2002).

Memory B cells generated by T cell-dependent antigens require cognate CD4 T cell regulation (MacLennan and Gray, 1986; MacLennan, 1994). Although the role of memory CD4 T cells remain unresolved, interaction between memory Th cells and memory B cells can substantially impact the developmental choices available to the memory responders (reviewed in McHeyzer-Williams and McHeyzer-Williams, 2005).

There are multiple subtypes of antigen-specific memory B cells (Lalor et al., 1992; Black et al., 1978; herzenberg et al., 1980; McHeyzer-Williams et al., 2000). As discussed previously, antibody isotype itself defines a major developmental and functional subdivision in the memory B cell compartment. The spectrum of isotype produced by memory B cells depends on the quality of cognate CD4 T cell regulation, which itself depends on the antigen, its dose, route of entry, and adjuvant context.

1.2.9 Plasma cell differentiation

An immune response will induce the formation of short-lived plasma cells that are able to create a fast increase in antibody levels. In a more long-term perspective, specific antibodies produced by long-lived plasma cells reside mainly in the BM. In contrast to
short lived plasma cells with life spans measured in days long-lived plasma cells live for several months (Manz et al 1997; Slifka et al 1998).

Plasma cells are the terminally differentiated B cells. They do not divide and during their lifetime, which can span between days up to months, they produce and secrete Ig. The phenotype of the cell changes to meet the new demands. The cytoplasmic compartment increases in size, and it contains large amounts of rough endoplasmic reticulum and secretory vacuoles. Various surface molecules are down-regulated when the B cell differentiates into a plasma cell, for example MHC class II, B220, CD19, CD21 and CD22. Also, the levels of different transcription factors change when B cell differentiates into a plasma cell. Some transcription factors such as B cell lineage-specific activator (BSAP), CIITA, early B cell factor (EBF) and Bcl-6 decreases, while the expression of others, like B lymphocyte-induced maturation protein I (Blimp-1), interferon-regulatory factor 4 (IRF4) and X box-binding protein I (XBP-I), are increased in plasma cells (reviewed in Calame et al 2003). XBP-1, expressed ubiquitously, is needed for plasma cell differentiation, but not during earlier steps of B cell development and strongly expressed in plasma cells (Reimold et al., 2001). It is negatively regulated by BSAP (Reimold et al., 1996), a transcription factor that decreases during differentiation to plasma cells (Barberis et al 1990; Rinkenberger et al 1996), which could in part explain the increase in XBP-1 during this stage. IRF4, expressed mainly in lymphoid cells, is also increased in plasma cells and a plasma cell-like subset of GC cells (Falini et al 2000). Blimp-1 has been shown in vivo to be expressed in all plasma cells, both in T cell independent and T cell dependent responses and in secondary responses (Angelin-Duclos et al 2000). Recently it was shown that Blimp-1 is required for plasma cell differentiation
Review of Literature

(Shapiro-Shelef et al. 2003). Blimp-1 acts as a transcriptional repressor of the c-myc (Lin et al., 1997), CIITA genes (Piskurich et al., 2000) and Pax5 (Lin et al., 2002). c-Myc is needed for proliferation and cell growth, and the repression of its transcription can explain the non-dividing state of terminally differentiated plasma cells. Likewise, the suppression of CIITA may explain the down-regulation of MHC class II expression in plasma cells. The repression of Pax5, coding for BSAP, is probably important for triggering the plasma cell development. BSAP is important during early stages of B cell development as well as for isotype switching in GCs (reviewed in Michaelson et al. 1996 and Morrison et al., 1998). The formation of plasma cells is dependent on down-regulation of BSAP (Lin et al. 2002; Usui et al. 1997), since BSAPs can repress XBP1 (Reimold et al. 1996), J chain (Rinkenberger et al. 1996) and Ig H chain (Singh and Birshstein 1993) gene transcription. Blimp-1 has also the ability to down-regulate Bcl-6, a protein necessary for GC formation (Dent et al. 1997; Ye et al. 1997). Bcl-6 can on the other hand repress the gene encoding Blimp-1, Prdm1, (Shaffer et al. 2000) creating a feedback loop controlling the B cell fate. While Bcl-6 is expressed in a GC B cell, Blimp-1 expression is repressed and the differentiation into plasma cell is blocked. However, when Prdm1 is induced, Blimp-1 inhibits the actions of Bcl-6 and the B cell terminally differentiates into a plasma cell.

Persistent of high-affinity antibody in the serum provides the first layer of protection against antigen/pathogen rechallenge. Although binding of high-affinity antibody to antigen is a clearance mechanism, it may also serve to increase the efficiency of antigen presentation to memory B cells through rapid immune complex formation and binding to
FcR or complement receptors on innate cells. In this manner, high-affinity antibody may amplify the sensitivity of the memory B cell response to antigen recall.

1.2.10 Bone marrow plasma cells

The major site of production of antibodies after infection is the bone marrow (BM) (Benner et al., 1974; Slifka et al., 1995). Since antibodies of the IgG isotype have a maximum half-life of three weeks (Talbot and Buchmeier, 1987; Vieira and Rajewsky, 1988) long term maintenance of antibody titers could be due to continued production by BM plasma cells (Ho et al., 1986). But these data had failed to explain the persistence of protective antibody titers (Slifka et al., 1995), leading to the theory that BM plasma cells are generated continually from memory B cell precursors (Schittek and Rajewsky, 1990; Sprent and Tough, 1994).

Long-lived antibody-secreting B cells can also be considered part of the memory B cell compartment. The long-lived PCs secrete isotype-switched antibody and display evidence of SHM with affinity-increasing mutation patterns, but they do not self-replenish through turnover (Smith et al., 1997; Takahashi et al., 1998 McHeyzer-Williams et al., 1999). This post-GC antigen-specific B cell compartment appears during the second week after initial antigen exposure (McHeyzer-William et al., 1993) and preferentially homes to the bone marrow for growth factor support of stromal cells (Minges et al., 2002). Bromodeoxyuridine (BrdU) labeling to measure plasma cell lifespan (Manz et al., 1997), or removal of persisting memory B cells by irradiation (Slifka et al., 1998) showed that BM plasma cells were long lived and continually generated antibody for up to a year post-immunisation.
1.2.11 Humoral immunity to malaria infection

Individuals living in Plasmodium falciparum malaria endemic regions face continuous exposure from a very early age that often leads to asymptomatic chronic infection over their entire lifespan. Immunity to malaria develops only after repeated infection and is lost without continued exposure to infection. It is widely perceived that immunity to malaria is, to an extent, defective and that one component of this defective immune response is the inability to induce or maintain long-term memory responses. This seems to be very different from the development and maintenance of immunological memory to other pathogens such as viruses, or immunisation with non-replicating antigens. A key distinguishing feature of immune memory maintenance between the malaria infection and most bacterial or viral diseases is long-term antigen persistence. Consequently, parasite immune memory is found to be in a continuous, dynamic flux between activation and deactivation producing functional parasite killing or functional memory cell death. Furthermore, due to the finite capacity of memory lymphocytes to proliferate, continuous parasite antigen stimulation may exceed a threshold level at some point in the chronically infected host. This may result in suboptimal effector immune memory leading to host susceptibility to reinfection, or immune dysregulation yielding disease reactivation or immune pathology (Atchman et al., 2005). Despite the enhanced efficacy of memory cells and the recall immune response, there are numerous experimental and empirical examples of malaria in which immunological protection or memory is short-lived (Dorfman et al., 2005; Cavanagh et al. 1998; Wykes et al., 2005). In the absence of active immune effector activities, the ability of memory cells to respond quickly enough to control this type of infection is limited.
A variety of experimental model and field studies support the view that B cells and antibodies are the crucial component of the protective immune responses and play a major role in eliminating the erythrocytic stages of Plasmodium malaria. Mice lacking B cells are unable to clear infections with P. yoelii and P. chabaudi chabaudi, although in the latter, the early acute phase is controlled to some extent in the absence of antibodies (Lenghorne et al., 1998; von der Weid et al., 1993; van der Hevde et al., 1994). Longitudinal studies had revealed the prevalence of antibodies against defined malaria antigens that have been associated with resistance against disease (Riley et al., 1992; Egan et al., 1996; Metzger et al., 2003). High antibody titers and in some cases restricted IgG isotypes to a particular malaria antigen or regions of an antigen correlate with protective immunity (Bouharoun et al., 1992; Braga et al., 2002; Shi et al., 1996; Cavanagh et al., 2001).

Several studies have reported positive association between parasitemia and antibodies against ring-infected erythrocyte surface antigen (RESA), several merozoite surface antigens (MSP-1, MSP-2, MSP-3, MSP-6/7), rhoptry-associated protein 1 (RAP-1), apical membrane protein 1 (AMA-1), erythrocyte binding protein (EBA-75) and varient antigen of P. falciparum erythrocyte membrane protein 1 (PfEMP-1) (Mash et al., 1986; Ekala et al., 2002; Taylor et al., 1998; Ranford-Cartwright et al., 1996; Taylor et al., 1996; Wang et al., 2003; Kocken et al., 2002; Polley et al., 2004; Ockenhouse et al., 2001; Baum et al., 2003; Alifrangis et al., 1999; Migo-Nabias et al., 1999). Transfer of immune sera and antigen-specific antibodies into naive recipients (mouse, monkey and even humans) have shown to diminish or prevent infection in the recipients (Jara et al., 1986; Cohen et al., 1961). Individuals living in malaria endemic areas develop immunity
after repeated infections and it is lost without continued exposure to infection (Aribot et al., 1996; Foniungo et al., 1999; Fruh et al., 1991; Soares et al., 1999; Giha et al., 1999; Cavanagh et al., 1998). Studies have also shown that in Kenyan children there is variable kinetics of antibody production against blood stage antigens and these antibodies last for about six weeks (reviewed in Wykes et al., 2006). Yet studies of antibody response associated with protection revealed that they are short lived. Compared with other infection and immunization model, the antibody levels drop with unusual rapidity below the detectable threshold (Achtman et al., 2005).

1.2.12 Memory B cell response in malaria

There are limited data on the nature of immunological memory to malaria. A study from Sri Lanka (Ranawaka et al., 1988) had shown that there was inefficient ‘memory’ of transmission-blocking immunity for *P. vivax*. They found that if the interval of time from the previous infection exceeded 4 months, the boosting of antibody-mediated immunity was diminished. Similarly, Deloron and Chougnet (1992) in a longitudinal study in Madagascar demonstrated that after a sustained period of absence, parasite rates following a recent epidemic did not vary between people exposed for the first time and those who previously had immunity. However, in contrast to parasite immunity, clinical immunity was found to persist for decades in the absence of parasite exposure. In terms of antigen-specific responses, Egan and colleagues (1995) observed that up to 40% of subjects in a cross-sectional survey in malaria endemic region did not have antibodies to the major merozoite surface protein 1 (MSP1), a finding that could not be explained by HLA-linked immunological non-responsiveness.
Effect of vaccination with the malaria candidate vaccine *Plasmodium falciparum* apical membrane antigen-1 (AMA-1) formulated with Alhydrogel® on the differentiation of plasma cells and memory B cells was analyzed (cited from Wykes and Good, 2006). By flow cytometry, the CD19+ CD27+ memory B cells and effector plasma cells in the peripheral blood of five malaria naive volunteers enrolled in a Phase I vaccine trial were examined at several time points following primary, secondary and tertiary vaccination with AMA1 on Alhydrogel®. The percentages of CD19+ B cells were reported to have not change significantly over the course of vaccination and fluctuated within the normal range, between 10 and 20% of the total lymphocytes. They found that in most volunteers there was a significantly increase in the number of CD27+ memory B cells 7 days after the primary immunization and 3 days after the secondary and tertiary immunizations, suggesting that vaccination accelerated the expansion of the memory B cell population. In all cases they reported only a small increase in the number of plasma cell populations (CD27lowCD38high, CD27high CD38high, CD20−CD38high, and IgD−CD38high) after the first immunization. In contrast, the percentage of different plasma cell populations increased significantly, 3–14 days after the secondary and tertiary immunizations, showing an increases as large as 10-fold. In general, they found that volunteers with robust plasma cell response following immunization had higher levels of AMA-1-specific antibody.

These results indicate that immunization of a naive immune system resulted in an increase in both effector and memory B cells in the periphery, and that analyzing the memory and effector phenotypes of B cells in the periphery may be a valid indicator of the effect of vaccination on the humoral immune system (Wykes and Good, 2006).
The 19 kDa C-terminal fragment of the merozoite surface protein-1 (MSP1\textsubscript{19}) is an important vaccine candidate for malaria and high titers of anti-MSP1\textsubscript{19} antibody mediate complete protection in mice (Hirunpetcharat et al., 1997). Wykes and others (2005) showed that P. yoelii MSP-1\textsubscript{19} vaccine can generate functional memory cells as well as long-lived plasma cells. However, protection studies found that memory B cells specific for the vaccine per se, did not offer any protection and further investigation showed that P. yoelii parasite can induce deletion of vaccine specific memory B cells as well as long-lived plasma cells (Wykes et al., 2005). On the other hand, evidence from a study conducted with donors living in malaria endemic area showed that there is a defect in the establishment of the P. falciparum malaria blood stage circulating antigen-specific memory B cells (Dorfman et al., 2005). Achtman and others (2005) showed that following primary and secondary infection of P. chabaudi in mice there was a production of higher affinity antibodies but the establishment of stable B cell memory response was impeded. These findings indicate that the generation and / or maintenance of memory B cells and long-lived plasma cells are impaired in malaria infection.

In a longitudinal study, long-term antibody responses to malaria in Kenyan children were found to be very brief (Kinyanjui et al., 2007). The brevity of the response has been traditionally attributed to the skew in the response towards IgG3. Human IgG3 antibody has a half-life of 8 days, whereas the half-lives of IgG1, 2, and 4 are up to 23 days. This study had compared the antibody responses in children, at admission to the hospital with uncomplicated malaria and subsequently during the convalescent period; 1, 2, 3, 6, 9, and 12 weeks after infection. The study reported that children showed variable kinetics of IgG1 and IgG3 responses specific for MSP-1\textsubscript{19}, MSP-2, EBA-175 and AMA-1 and that
IgG1 responses increased with age. However, they found that although an antibody response was generated to the infection that peaked after 7 days, the antibody response only lasted for approximately 6 weeks. This loss of specific antibody production occurred regardless of isotype of antibody measured and raised an important question as to whether antibody decay was faster in children or if there was a failure to produce long-lived plasma cells to secrete antibody for extended periods. Since IgM responses were minimal in these children, the antibody titers seen in the infected children were of secondary responses. However, re-infection did not boost the antibody responses. Several explanations were put forth for this intriguing observation. Although it had been suggested that memory B cells were deleted, alternatively it could be that high levels of serum antibody gave negative signals to B cells or that regulatory T cells blocked re-activation of memory responses. Finally, the splenic architecture was disrupted following infection, and this could prevent activation of memory B cells.

Dorfman and others (2005) had compared the number of memory B cells per million peripheral blood mononuclear cells (PBMC) in endemic areas, specific for tetanus toxoid (TT), apical membrane antigen 1 (AMA1), MSP1 and cystein rich inter-domain region 1 α (CIDRI α ), which is part of the PfEMP1 protein. In the blood there were measurable numbers of memory B cells and the mean frequency of memory B cells was the same for all antigens. The specific titers of antibody in the serum correlated with the numbers of memory B cells. Whereas 71% of individuals tested had memory B cells specific for TT, only 46% and 31% of individuals had memory B cells specific for AMA1 and MSP-119, respectively. However, responses for CIDRI α were unusual, in that 21% of children and 64% of adults had memory B cells for this antigen. Furthermore, they found that while
significant proportion of individuals (81 and 57%) showed AMA-1- and CIDR1α-specific antibodies, respectively, approximately 50% had no memory B cells for these antigens. In contrast, whereas 70% of individuals had memory B cells for TT, only 36% of individuals had memory B cells for MSP-1₁₉. This suggested that there was a problem with the maintenance of memory B cells specific for the parasite and that the main difference was that infants were immunized at birth with TT, but MSP-1₁₉ specific response was dependent on naturally acquired immunity.

Data from Kenya showed that antibody prevalence by proxy could be a useful measure of malaria transmission intensity as acquisition and maintenance of seropositivity requires exposure to the parasite (Drakeley et al., 2005). In this study prevalence of IgG specific for three Plasmodium falciparum asexual stage antigens was assessed in individuals of all ages, living at varying altitudes, encompassing a range of transmission intensities. The prevalences of antibodies to MSP-1₁₉ and MSP-2 correlated with altitude. Furthermore, seroprevalence was also found to highly correlate with P. falciparum point prevalence in children under the age of 2 years, but not in older individuals. The interpretation drawn from these data was that seropositivity reflected cumulative exposure to malaria over a number of years and that variation in seroprevalence with age could be used to infer variations in transmission intensity over the recent past. Perhaps most interestingly, from the point of view of functional B cell memory, simple mathematical modeling of age-stratified seroprevalence data revealed that the half-life of remaining seropositive after seroconversion was approximately 50 years (Drakeley et al., 2005).
Achtman et al., 2003 had investigated the longitude of antibody response to *P. chabaudi* after a primary infection of mice and whether treatment with chloroquine to eliminate parasites affected longevity and affinity of primary and secondary antibody responses. Following infection with *P. chabaudi*, there was both a long-lasting germinal centre B cell responses (suggesting B cell memory), as well as a pronounced splenic extrafollicular plasma cell response, characteristic of short-lived antibody responses. However, although a primary infection with *P. chabaudi* led to a chronic parasitaemia that lasted up to 3 months, malaria-specific antibody responses dropped rapidly after the acute infection. Treatment of mice with chloroquine to eliminate chronic parasitaemia did not affect this, but resulted in a higher proportion of higher affinity antibodies. Immunity to a second infection required B cells, which were rapidly activated after challenge. Chloroquine treatment between the primary and secondary infection resulted in higher but shorter secondary parasitaemia and a higher antibody response than chronically infected and challenged mice. Thus, both long-lived and short-lived antibody responses might be generated during a *P. chabaudi* infection and chloroquine treatment did not affect this. However, it is possible that chronic infection may aid the development of higher affinity antibodies but impede memory B cell response.

In another mouse model study, mice infected with *P. berghei ANKA*-parasitized erythrocytes, an early (day 3) strong activation of T cells in secondary lymphoid organs was observed and, on days 6-8 of infection, there was overwhelming activation of B cells, with loss of conventional germinal center architecture, intense centroblast activation, proliferation and apoptosis but little differentiation to centrocytes. In the spleen, the marginal zone disappeared and the limits between the disorganized germinal
center and the red pulp were blurred. Intense plasmacytogenesis was observed in the T
cell zone. The observed alterations, especially the germinal center architecture
disturbance with poor centrocyte differentiation suggested that B cell responses during \textit{P. berghei} ANKA infection in mice were defective that could have with potential impact on
B cell memory responses.

In conclusion, it appears that memory responses to \textit{Plasmodium} parasites are affected in
humans as well as in rodents. There are, however, no consensuses as to whether infection
prevents the development of memory responses, the maintenance of the memory B cells,
or if memory B cell survives infection. Given these characteristics of the immune
responses during blood stage malaria, understanding of the mechanisms that results in
poor immunity and immunopathology is crucial for the rational development of
prophylactic and therapeutic interventions, such as vaccines.
Rationale, Aim & Objectives
1.3 RATIONALE, AIM AND OBJECTIVES

1.3.1 RATIONALE

Immune memory is the foundation of the practice of vaccination. Serological memory after exposure to antigen or vaccine is maintained by plasma cells and memory B cells (Manz et al., 1997; Slifka et al., 1998; Bernasconi et al., 2002). Protein subunit vaccination is an effective public health initiative with demonstrable utility in the prevention of infectious diseases. Although attenuated microorganisms have been used historically, protein subunit vaccines provide a safer, more targeted preventative therapy against a variety of infectious agents.

MSP-1 is considered to be a major anti-malarial vaccine candidate present on the merozoite surface of all Plasmodium species. During red cell invasion MSP-1 undergo two proteolytic cleavages to form 19 kilodalton product of the MSP1 C-terminal region known as MSP-1\textsubscript{19}. The protein (MSP-1\textsubscript{19}) contains two conserved epidermal growth factor (EGF)-like domains and is carried inside the RBC during invasion [Blackman et al., 1990; Blackman et al., 1991; Blackman et al., 1992]. The 19 kilodalton of the MSP1 C-terminal region is functionally conserved across Plasmodium species [O’Donnell et al., 2000]. It has been reported that MSP-1\textsubscript{19} is an important vaccine candidate for malaria and its high antibody titers mediate complete protection in mice (Hirunpetchar et al., 1997). Evidences have shown that in spite of MSP-1\textsubscript{19} vaccine being able of generating functional memory B cells and long lived plasma cells, the vaccine has a tendency of deleting vaccine specific memory B cells as well plasma cells leading to the notion that memory B-cell response to MSP-1\textsubscript{19} is defective (Wykes et al., 2005). Evidences showed
that there is an occurrence of cell depletion in the marginal zone, lack of GC formation and a decrease in the number of B cells in the spleens of patients died of severe falciparum malaria (Urban et al., 1994). Furthermore, it has been reported that there is a defect in the establishment of falciparum malaria blood stage circulating antigen-specific memory B cells in individuals living in malaria endemic area (Dorfman et al., 2005).

MSP-3 is expressed on the surface of a merozoite during schizogony stage of a malaria parasite and released as soluble product when schizonts rupture. MSP-3 is loosely associated with the merozoite surface since it lacks transmembrane domain or a glycosyolphosphatidylinositol anchor. MSP3 has been selected as a vaccine candidate on the basis of a mechanism found to correlate with protection of malaria in humans through the induction of cytophilic antibodies that are the key agents of antibody-dependent, monocyte-mediated inhibition of growth of the parasite (McColl et al., 1994; Oeuvey et al. 1994a; Singh et al. 2004; Druilhe et al., 2005). The immunogenicity and protective efficacy of various antigen-adjuvant formulations derived from P. falciparum MSP-3 have been evaluated in Saimiri sciureus monkeys and were shown to be able to control parasitaemia upon experimental P. falciparum blood-stage infection (Carvalho et al. 2004; Soe et al. 2004; Theisen et al. 2004). A long synthetic peptide of PfMSP-3F (PfMSP-3F-LSP) has been reported to induce strong T-cell and B-cell responses that could persist over a year long (Audran et al., 2005; Druilhe et al., 2005). Anti-PfMSP-3F cytophilic antibodies mainly IgG3 has been associated to malaria protection in individuals living in endemic areas and these antibodies could be observed over six years (Roussilhon et al., 2007). Evidences showed that the C-terminal part of MSP-3 is highly conserved among the various isolates of the parasite (McColl et al., 1997; Huber et al.,
Rationale, Aim & Objectives

1997). These findings suggest that MSP-3 as a potent vaccine candidate can induce long-term memory responses.

The notion of impaired memory B-cell responses is further supported by Achtman et al, (2005) and Carvalho et al, (2005) where by they used Plasmodium chabaud chabaud AS and Plasmodium berghei ANKA infections (respectively) in murine models to study germinal centres which is considered to be a tightly controlled microenvironment for B cell expansion, differentiation and/or death [Eijk et al., 2001; Guzman-Rojas et al., 2002; Calame et al., 2003]. They found that a major defect in the germinal centre formation is in the establishment of B cell differentiation (centrocyte transformation), and not activation and proliferation (centroblast reaction), which, instead, are overwhelming. These findings indicate that the generation and / or maintenance of memory B cells and long-lived plasma cells are impaired in malaria infection.

Short-lived nature of many immune responses to P. falciparum blood-stage antigens in malaria endemic areas warrant to a question whether memory B cells are established at all in such individuals or they are being established with some defects. Here, in this doctoral study we have systematically examined the establishment of PfMSP-119 and PfMSP-3F specific memory B cell population in spleen and bone marrow of murine model and the correlation of these cells with anti- PfMSP-119 and anti-PfMSP-3F specific antibody responses in the peripheral blood of mice.
Rationale, Aim & Objectives

1.3.2 AIM

Given the fact that generation and maintenance of memory B cells and long-lived plasma cells is impaired in the malaria infection, we need to understand the mechanism behind this lack of long-term immunological memory. Immune memory is the foundation of the practice of vaccination. In the present study, the protective efficacy of *P. falciparum* vaccine candidates, PfMSP-119, and PfMSP-3F in identifying the mechanisms by which formation of germinal center, the survival of memory B cells and plasma cells will be examined.

1.3.3 OBJECTIVES:

1. To determine kinetics of antibody and antibody secreting cells (ASCs) in response to PfMSP-119 and PfMSP-3F

2. To determine the B cell response to PfMSP-119 and PfMSP-3F

3. To determine the B-cell memory response in mice immunized with PfMSP-119 and PfMSP-3F