Chapter 3

Memory B-cell response to recombinant C-terminal 19 kDa of Plasmodium falciparum merozoite surface protein 1 (PfMSP-1\textsubscript{19})
Introduction
3.1 Introduction

MSP-1 is a major surface protein present on the merozoite surface of all Plasmodium species. The *P. falciparum msp-1* gene consists of 17 distinct blocks, which code for conserved, dimorphic as well as polymorphic regions (Tanabe *et al.*, 1987; Miller *et al.*, 1993). All the blocks, except block 2 which is polymorphic, belong to either of the two families referred to as MAD20 or Wellcome/K1. Full length MSP-1 is a 190 kDa polypeptide which undergoes proteolytic cleavage before schizont rupture into four fragments of 83 kDa, 28kDa, 38kDa and 42 kDa, which are held together by noncovalent bonds (Holder *et al.*, 1992; McBride *et al.*, 1987). The GPI-anchored 42 kDa fragment undergoes secondary proteolytic cleavage into 33 kDa and 19 kDa fragments during red cell invasion (Blackman *et al.*, 1992). Only membrane bound C-terminal 19 kDa fragment (MSP-119), which contains two conserved epidermal growth factor (EGF)-like domains, is carried inside the erythrocyte during invasion while rest of the fragments are shed from the parasite surface (Blackman *et al.*, 1990; Blackman *et al.*, 1991). MSP-1 seems to play a vital role in red cell invasion since efforts to disrupt the *msp1* gene by homologous recombination have been unsuccessful. The C-terminal MSP-119 region appears to be functionally conserved across Plasmodium species (O’Donnell *et al.*, 2000). Several monoclonal antibodies directed against MSP-119 have been shown to inhibit erythrocyte invasion by the parasite *in vitro* suggesting that it plays an important functional role in invasion (Blackman *et al.*, 1990; Cooper *et al.*, 1992). The conformation of EGF-like modules of MSP-1 is important in generating invasion inhibitory antibodies. Antisera raised against correctly folded recombinant MSP-142 inhibit erythrocyte invasion *in vitro* while antibodies raised against incorrectly folded
recombinant MSP-142 did not inhibit parasite growth significantly (Chang et al., 1992). Some monoclonal antibodies that inhibit erythrocyte invasion were shown to block secondary cleavage of MSP1 to produce MSP-119 suggesting that secondary processing of MSP-1 is critical for invasion (Blackman et al., 1992). On the other hand antibodies against MSP-1 have also been identified which can block the function of invasion inhibitory antibodies in vitro (Guevara et al., 1997). The development of such antibodies may undermine efforts to elicit invasion inhibitory antibodies using a recombinant vaccine based on MSP-1.

The protective role of MSP-1 was first demonstrated in 1981 with rodent malaria parasite P. yoelli, where affinity purified MSP-1 induced protective immunity against P. yoelli blood stage challenge in mice (Holder et al., 1991). Later, immunization with recombinant MSP-119 was shown to provide protection in rodent and non-human primate models (Daly et al., 1993; Ling et al., 1994; Perera et al., 1998). Data from animal studies suggested that MSP-119 mediated protective immune responses were largely antibody concentration dependent with high antibody titers being essential for the protection (Daly et al., 1995; Hirunpetcharat et al., 1997). Antibodies that are generated against double EGF-like domain structure, and not against single EGF-like domain structure, mediated protection in rodent model (Ling et al., 1995). Importantly, reduction and alkylation of MSP-119 abolished the protection obtained with the protein suggesting the absolute requirement of the disulphide-bonded conformation for immunogenicity (Ling et al., 1994).
Studies performed with the sera of individuals living in malaria endemic regions showed the presence of naturally elicited high titer antibodies directed against MSP-1. In many cases the correlation between presence of antibodies against specific regions of MSP-1 and protection against malaria have been established (Egan et al., 1996; Branch et al., 1998; Egan et al., 1999; Perraut et al., 2005). Using correctly folded MSP-142, which maintained native conformation of the EGF-like domains, it was shown that concentrations of antibodies to MSP-142 were significantly higher in children who experienced asymptomatic infection than in children who developed clinical malaria (Riley et al., 1992). Protective role of the conserved C-terminal region of MSP-1 was further confirmed in a number of studies that used recombinant PfMSP-119. Study conducted with children and adults in Gambia demonstrated correlation between protection against clinical malaria and ability of sera to block binding of two monoclonal antibodies against different epitopes on MSP-119 by ELISA (Shai et al., 1995). Another study conducted with infants suggested that when infants were negative for IgG against PfMSP-119, they had 10 times greater risk of becoming parasitemic (Branch et al., 1998). A longitudinal study conducted in Liberia in West Africa with 100 infants suggested that presence of maternally derived antibodies against PfMSP-119 protects infants from clinical malaria in the first year of life (Hogh et al., 1995).

Analysis of IgG subtypes in human sera demonstrated that cytophilic antibodies of the IgG1 and IgG3 subclasses against blood stage parasite proteins provided protection whereas IgG2 and IgG4 subtypes did not (Bouharoun-Tayoun et al., 1992). The evaluation of natural immune responses against MSP-119 suggests that sera from endemic areas predominantly contain cytophilic subtypes IgG1 and IgG3. While defining the role
of these two IgG subtypes in attaining immunity against malaria, it was found that IgG1 antibodies against MSP-119 correlated with immunity to *P. falciparum* while IgG3 did not (Shi *et al.*, 1996; Cavanagh *et al.*, 2001; Braga *et al.*, 2002). Definition of specificity of IgG subtype against MSP-119 in malaria immune individuals may have implications in vaccine design based on MSP-119 and selection of appropriate adjuvants, which can elicit desired antibody subtype.

Although MSP-119 is a highly conserved molecule, there are four allelic forms of MSP-119, E-KNG, E-TSR, Q-KNG and Q-TSR, reported in the field (Kang *et al.*, 1995). Human antibodies to all the four MSP-119 alleles have been found to cross-react with each other with minority of sera preferentially recognizing KNG compared to TSR (Apio *et al.*, 2000). In another study it was reported that IgG1 subtype antibodies, which play a role in protection against malaria, recognize all the variants tested, but IgG3 subtype antibodies react with MSP-119 variants differentially (Shi *et al.*, 1996). These results suggest that a recombinant vaccine based on a single allelic form of MSP-119 may be sufficient to induce protective antibody responses against diverse *P. falciparum* isolates.

While many studies show positive correlation of antibodies against MSP-119 with protection against clinical disease, some reports suggest no role of anti-MSP-119 antibodies in protective immunity. Two studies conducted in Tanzania and Ghana where malaria is endemic showed no association of antibody responses against MSP-119 with protection against clinical malaria in study infants and children respectively (Dodoo *et al.*, 1999; Kitua *et al.*, 1999).
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Analysis of naturally acquired immune responses to MSP-142, which is proteolytically processed into MSP-133 and MSP-119, using adult sera from individuals infected with *P. falciparum* demonstrated that MSP-133 was more immunogenic than MSP-119 (Egan *et al.*, 1997). Most of the donors made strong T-cell proliferative responses against MSP-133 but not against MSP-119. The poor T-cell response against MSP-119 may be due to the difficulty in processing the disulfide linked domain. Experiments conducted in animal models showed that although MSP-133 domain of MSP-142 elicited high antibody titers, there was no protection against blood stage challenge (Ahlborg *et al.*, 2002). Vaccine development based on MSP-1 has thus focused on trying to elicit high titer antibodies against MSP-119 by providing T-cell epitopes either from MSP-133 or from heterologous sources such as tetanus toxoid (Ahlborg *et al.*, 2002).

Individuals living in high endemic areas of *Plasmodium falciparum* malaria gradually develop substantial clinical protection against the disease over a period of several years and this reflects acquisition of protective immunity (Kitua *et al.*, 1999; Dodoo *et al.*, 1999). However, although a variety of immune responses directed against numerous parasite antigens have been identified (Bojang *et al.*, 2001; Benton *et al.*, 2002; Kawabata *et al.*, 2002), longitudinal studies conducted with individuals living in areas where malaria is endemic showed that antibody responses to blood-stage malarial antigens are often short lived (Cavanagh *et al.*, 1998; Giha *et al.*, 1999). However evidence showed that there are variable kinetics of antibody production against blood stage antigens in Kenyan children and these antibodies last for about six weeks (reviewed in Wykes *et al.*, 2006). On the other hand studies conducted with animal models showed that immunization with recombinant MSP-119 of *Plasmodium falciparum* or *P. yoelii* protects
monkeys or mice, respectively, against infection (Ling et al., 1994; Daly et al., 1995; Kumar et al., 1995; Hirunpetcharat et al., 1997). Though information regarding longevity of antibody responses / protection to Plasmodium malaria is limited, there is evidence which shows that immunization with MSP-119 either adjuvanted with CFA/IFA or CpG 19ODN/ISA gave rise to long term protection for up to at least 12 months after the last boost (Jeawwattanalert et al., 2007). Long term response of MSP-119-specific antibody reflect the generation and maintenance of long-lived plasma cells which survive for years (Manz et al., 1997; Slifka et al., 1998). However evidence showed that P. yoelii infection can cause apoptosis of MSP-119 specific memory B cells and long-lived plasma cells in mice (Wykes et al., 2005).

Recent study has shown that P. yoelii MSP-119-IgG2a antibody persists constantly longer than IgG1 antibody suggesting that memory of MSP119- specific IgG2a antibody last longer than IgG1 memory (Jeawwattanalert et al., 2007). The Th1-dependent IgG2a antibody response can persists stably over a period of 12 months after mice immunisation with PyMSP-119 while the Th2-dependent IgG1 antibody response declined over the period of time with vaccine formulations. (Rajewsky et al., 1989; reviewed in Finkelman et al., 1990; Jeawwattanalert et al., 2007). MSP-119-specific IgG2a antibody plays an important role in immunity against malaria infection (Smith et al., 2003). Evidences showed that transferring immune sera depleted of IgG2a did not confer protection in recipient mice following P. chabaudi infection but the immune serum depleted of IgG1 did (Su et al., 2003). IgG2a subclass prefers to bind FcγRI and then mediate antibody-dependent cell-mediated cellular cytotoxicity (ADCC), antibody-dependent cellular inhibition (ADCI) and phagocytosis but the MSP-119-specific IgG2a antibody has been
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reported not to use the Fc function for antibody-mediated protection (Rotman et al., 1998; Vukович et al., 2002). It may function by blocking parasite invasion or inhibiting MSP1 processing which is required for erythrocyte entry (Blackman et al. 1994; Waki et al., 1995).
3.2 Results and discussion

The 19kDa carboxyl terminus of the Plasmodium falciparum Merozoite Surface Protein-1, PfMSP-119, an essential component in the parasite's life cycle, is a primary candidate for a malaria vaccine. PfMSP-119 has been shown to induce protective immunity to challenge infection. Protection has been correlated, in the mouse model, with high level of PfMSP-119-specific antibodies that are predominantly of the IgG1 and IgG2b isotypes at the time of infection. Since serological memory after exposure to vaccination or malaria parasite is maintained by plasma cells and memory B cells, the present study was initiated to analyze the memory and effector phenotypes of B cells in the periphery that may be a valid indicator of the effect of vaccination on the humoral immune system.

3.2.1 Expression and purification of *P. falciparum* MSP-119 recombinant protein.

Expression of the recombinant PfMSP-119 was done by growing the construct containing PfMSP-119 synthetic gene in pET28a(+) (kind gift from Mr. Suman Mazumdar, PhD student, ICGEB) a kanamycin based expression vector, transformed in *E.coli* BLR (DE3). The expression of PfMSP-119 was performed at 1L scale. Overnight culture of *E.coli* BLR(DE3) pET28a(+)PfMSP-119 grown in LB media containing kanamycin at 10 mg/mL was used to inoculate 100mL of LB media and grown to exponential phase. The exponential phase seed culture (200 mL) was used to inoculate a 2.5 L flask containing 1 L of LB media at 37 °C. Cell density of the culture was measured periodically at 600 nm (OD$_{600}$). Expression of PfMSP-119 was induced with 1mM IPTG when cell density of the culture reached an OD$_{600}$ of 0.6 to 0.8. *E. coli* cells were harvested by centrifugation 4 h post-induction. The harvested cell pellet was resuspended in lysis buffer (20mM Tris pH
8.0, 500mM NaCl, 5mM benzamidine hydrochloride, 10mM dithiothreitol (DTT), 100μg/mL lysozyme, 1.0% Tween 20) and cells were lysed by sonication. The sonicated sample was centrifuged to remove the insoluble fraction and supernatant was processed to purify PfMSP-119 by immobilized metal affinity chromatography (IMAC). The clarified sample was loaded on 10mL of Ni²⁺ charged streamline chelating resin packed in XK16 column and washed with 10mM imidazole. The PfMSP-119 was eluted from the column with 70mM and 500mM imidazole gradient at pH 7.4 and separated on SDS-PAGE gel to assess the purity (Fig 3.2.1). The PfMSP-119 obtained after IMAC was further purified by ion exchange chromatography.

![SDS-PAGE gel](image)

**Figure 3.2.1.** SDS-PAGE gel of total cellular protein showing expression of recombinant PfMSP-119 from synthetic genes in *E. coli* from shake flask culture. Protein samples were separated on 15% SDS-PAGE gel and detected by coomassie staining.
3.2.2 Ion exchange chromatography to purify recombinant PfMSP-1\textsubscript{19}

The IMAC purified PfMSP-1\textsubscript{19} was loaded on to XK50 column containing 100ml of Q sepharose fast flow resin. Column was washed with 25mM NaCl and monomeric PfMSP-1\textsubscript{19} was eluted from resin with 50mM NaCl. Purity of PfMSP-1\textsubscript{19} in ion exchange elutes was assessed by 15% SDS-PAGE (Fig. 3.2.2). Protein elutes were detected as single band on the coomassie treated gel indicating that the protein was monomeric and pure. The fractions containing monomeric PfMSP-1\textsubscript{19} were pooled and concentrated to 1mg/ml, filter sterilized and stored at -80°C in aliquots.

![Figure 3.2.2](image)

**Figure 3.2.2.** Purification profile of rPfMSP-1\textsubscript{19} by metal affinity and ion exchange chromatography. (A) rPfMSP-1\textsubscript{19} was purified from crude cell lysate using metal affinity chromatography and samples were analysed on 15% SDS-PAGE gel under reducing conditions.
3.2.3 Characterization of purified PfMSP-1\textsubscript{19}

Recombinant PfMSP-1\textsubscript{19} was characterized for its purity and homogeneity by HPLC and gel permeation chromatography. Conformation of expressed PfMSP-1\textsubscript{19} was assessed by using conformational specific monoclonal antibodies namely 5.2 and penta-His as well as sera collected from Pb-PfM19 infected mice. Presence of disulphide bond was assessed under reducing and non-reducing conditions. Purified PfMSP-1\textsubscript{19} was separated by SDS-PAGE and detected by coomassie staining. Recombinant PfMSP-1\textsubscript{19} migrated with expected mobility of $\sim$19kDa under reduced condition and $\sim$23kDa under non reduced condition. Only single band was observed after coomassie staining, suggesting that recombinant PfMSP-1\textsubscript{19} is highly pure. Differences in mobility of reduced and non-reduced PfMSP-1\textsubscript{19} on SDS-PAGE gel suggest the presence of disulphide bonds when the protein is in native condition as shown in Fig 3.2.3

Figure 3.2.3 Characterization of purified rPfMSP-1\textsubscript{19}. SDS-PAGE gel showing mobility of non-reduced and reduced recombinant PfMSP-1\textsubscript{19}. Mobility shift of purified rPfMSP-1\textsubscript{19} after reduction with BME suggest the presence of disulphide bonds. Lane 1, molecular weight marker and lanes labelled (1, 2 & 5) $\mu$g represent amount of PfMSP-1\textsubscript{19} loaded at each lane respectively. Arrows shows shift in mobility of reduced (19kDa) and non reduced (23kDa) form of rPfMSP-1.
3.2.4 Reverse-Phase HPLC

RP-HPLC profile of purified PfMSP-119 showed two peaks, a major peak (~85% peak area) at 16.7 min and a minor peak at 18.4 min (~15% peak area). Both the peaks have been shown to contain monomeric PfMSP-119 (Figure 3.2.4).

3.2.5 Gel Permeation Chromatography (GPC)

PfMSP-119 migrates as a single peak on an analytical gel filtration column with the expected retention time for a ~19 kDa monomeric protein suggesting that PfMSP-119 is present as monomeric form as shown in the Figure 3.2.5 below.

Figure 3.2.4. Reverse phase chromatography profile of purified recombinant PfMSP-119. Purified PfMSP-119 eluted as two peaks, a major peak (~85% peak area) at 16.7 min and a minor peak at 18.4 min (~15% peak area).
3.2.6 Western Blot

Recognition of recombinant PfMSP1\textsubscript{19} by p19 polyclonal antibody and penta-His antibody (Qiagen) was tested on western blot for identity testing (Figure 3.2.6). Both p19 polyclonal antibody and penta-His antibody recognized recombinant PfMSP-1\textsubscript{19} specifically.

Figure 3.2.5. Analytical gel permeation chromatography profile of rPfMSP-1\textsubscript{19} on superdex S-75 column. Recombinant PfMSP-1\textsubscript{19} is eluted as single symmetrical peak at its monomeric position of 19 kDa.

Recognition with p19  
\begin{tabular}{c|c|c}
118 & M & 2 \\
79 & & 5 \mu g \\
47 & & \\
33 & & \\
25 & & \\
19.5 & & \\
\end{tabular}

Recognition with penta-His mAb  
\begin{tabular}{c|c|c}
118 & M & 2 \\
79 & & 5 \mu g \\
47 & & \\
33 & & \\
25 & & \\
19.5 & & \\
\end{tabular}

Fig 3.2 6. Western blot showing the recognition of recombinant PfMSP-1\textsubscript{19} by p19 polyclonal antibody and anti-penta histidine antibodies respectively.
3.2.7 Kinetic of Antibody response in mice immunized with recombinant PfMSP-19

Humoral immunity appears to be based on immunological memory provided by memory plasma cells, which secrete protective antibodies, and memory B cells, which react to antigen challenge by differentiating into plasma cells (Zinkernagel et al., 1996; MacLennan et al., 2000). The ability of memory B cells to generate a robust, prompt, and efficient Ab response to secondary challenge by an invading pathogen is a hallmark of immunological memory (Gray, 1993). B cells and antibodies are important component of protective immune response to the blood stages malaria infection. Yet, immunity to malaria infection is slow to develop and immunological memory appears to be short-lived (reviewed in Achtman et al., 2005). Also, the memory B cell pools to some malarial antigens (merozoite surface protein 1 (MSP1), apical membrane antigen 1 (AMA1), and the cysteine rich interdomain region 1a (CIDR1a) appear to be short-lived (Dorfman et al., 2005), indicating that the long-lived humoral response to malarial antigens may not be functioning optimally in natural infection. To gain insight into why humoral responses to malarial antigens have a propensity to be short lived, we studied the generation and maintenance of antigen-specific memory B cells in a mice model. We tracked the antibody and memory B cell responses to the C-terminal portion of P. falciparum merozoite surface protein 1 (MSP-19). PfMSP-19 is considered to be one of the leading candidates for inclusion in a vaccine against blood stages of Plasmodium falciparum. These data would give some insight into the differentiation of naive B cells into effector cells and stability of circulating antigen-specific memory B cells in antigen primed mice.
In this study, mice were immunized with recombinant PfMSP-1_{19} formulated in Complete Freund’s Adjuvant (CFA) as per the schedule (Table I) to investigate the effect of PfMSP-1_{19} on the kinetics of antigen specific antibody responses and composition of B cell subsets in spleen and bone marrow blood.

We observed that there was no increase in antibody titer after primary immunization. Mice elicited heightened serum anti-PfMSP-1_{19} antibody responses following the first boost that peaked 2-3 weeks after the first boost and declined thereafter (Fig. 3.2.7 A). After first boost, there was 30-fold increase in the antibody titer compared to the primary immunization (48000.0 ± 18475.21 versus 1600.0 ± 577.3503) (Fig. 3.2.7 B). Next, we assessed the ability of a booster dose of PfMSP-1_{19} to effect the antibody responses previously established by the primary/secondary immunization regimen.

90 days after the first boost, mice were boosted with an identical booster dose. Following the second booster dose there was only a marginal increase in the antibody titer (32000 ± 0.0) and this increase was only 1.3-fold compared to the response elicited by first boost (Fig.3.2.7 B). Unlike first boost when peak response was seen after three weeks of injection, the peak antibody titer was evident within one week after the second boost and remained at plateau levels for ~1 month (Fig. 3.2.7 B). The antibody response in PfMSP-1_{19} immune mice gradually decreased over the 4 months after the last immunization. Compared to the antibody levels after first boost, the antibody levels at 4 months post immunization were noticeably lower (mean ± SD, 44800 ± 18475.21 versus 2800 ± 1154.701 at days 67 and 196, respectively). Our results are consistent with the findings observed by Jeamwattanalert et al., 2007, whereby the antibody levels induced by
immunization of MSP-119 with Freund's adjuvant in mice gradually declined to significantly lower levels 12 months after last immunization.

Figure 3.2.7. Duration of PfMSP-119-specific antibody responses after immunization. BALB/c mice were immunized with PBS or PfMSP-119 plus CFA/IFA. Sera were collected at indicated time point and assayed for antibody titer by ELISA. A. Kinetics of PfMSP-119 specific total IgG antibody titers in individual mice. B. Kinetics of PfMSP-119 specific total IgG antibody titers in pooled sera from immunized mice. Values are means plus SDs for five mice.
3.2.8 PfMSP-1<sub>19</sub> induces both antigen-specific IgG1 and IgG2a subtype responses in mice

IgG subclass antibody titer responses to MSP-1<sub>19</sub> have been demonstrated to play an important role in malaria immunity and protection (Smith et al. 2003; Su et al. 2003). Since the isotype of an antibody may be a significant determinant of the protective nature of the immune response (Ling et al., 1997), the serum antibody isotype profile induced by vaccination with PfMSP-1<sub>19</sub> was determined. The dominant serum IgG subtype elicited in PfMSP-1<sub>19</sub>-immunized mice was IgG1 followed by subclasses IgG2a, IgG2b, and IgG3 (Fig. 3.2.8). 3 weeks after first boost the levels of both IgG1 and IgG2a increased substantially and persisted for a month then gradually declined thereafter. However after the second boost there was no increase in the anamnestic IgG1 and IgG2a responses (Fig 3.2.8). Antibodies of the IgG2b and IgG3 subclass were detected at low levels and there was no notable change in the levels of these subtypes after boosting. Longevity of these responses have been reported by Jeamwattanalert et al., 2007. Contrarily our investigation on the longevity of PfMSP-1<sub>19</sub> specific IgG subclasses revealed that both IgG1 and IgG2a declined progressively after the first boost and yet second boost did not alter the profile significantly. The increased response of the the total IgG after the second boost indicates that PfMSP-1<sub>19</sub> elicited short-term antibody responses that persisted for a while then declined progressively (Fig. 3.2.7).
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Figure 3.2.8. Kinetics of PfMSP-19 -specific IgG isotypes after immunization. BALB/c mice were immunized with PBS or PfMSP-19 plus CFA/IFA. Sera were collected at indicated time points and assayed for antibody titer by ELISA to study kinetics of IgG Isotypes of PfMSP-19-specific antibodies. Values are means plus SDs for five mice.

3.2.9 Antibody Secreting Cell (ASC) response induced by PfMSP-19 in vivo

The isolation of mononuclear cells from spleen and bone marrow at various intervals from mice immunized with PfMSP-19 provided further corroboration of the induction of Ag-specific antibody responses at the cellular level. PfMSP-19-immunized mice were monitored at various time intervals to enumerate antibody-secreting cells or plasma cells by flow cytometry. Splenocytes from PfMSP-19-immune mice were stained with B220 (CD45R), a marker for B cells and CD138 was used as a marker for plasma cells in flow cytometric analyses. 2 weeks after primary immunization, there were very few CD138 (syndecan-1)+ splenic cells in the PfMSP-19-immune mice that were comparable with PBS treated mice. 10 days after first boost, a small proportion of the B220+ cells expressed CD138, and the increase in the numbers of CD138+ cells peaked at day 56.
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There were two subsets of plasma cell cells detected: B220+CD138+ and B220-CD138+. While B220+CD138+ subset dominated the early phase of the splenic response before declining by the third week of first boost, B220-CD138+ reached peak levels during the late phase of the splenic response but declined after second boost (Figs. 3.2.9 and 3.2.10). After the second boost was a marked decrease in B220+CD138+ in the spleen of PfMSP-119 immunized mice. In bone marrow, there was a rapid increase in plasma cells expressing B220+CD138+ after second boost that reached maximal levels by day 90 (Fig 3.2.9). The number of CD138+ plasma cells in the bone marrow was slightly higher than that observed in the spleen in PfMSP-119 immune mice. Therefore, the plasma cell response dominated by the B220-CD138+ subset persisted as a long-term reservoir of quiescent plasma cells in the bone marrow.

3.2.10 Frequency of PfMSP-119-specific antibody secreting cell (ASC).

It has been suggested that the serological memory or the sustained antibody production may be maintained by long-lived plasma cells that survive in appropriate bone marrow niches with half-lives of 3 months (Manz et al., 1997; Slifka et al., 1998; Manz and Radbruch, 2002). The frequency of PfMSP-119-specific antibody secreting cell (ASC) in spleen and bone marrow was investigated by the B cell ELISPOT assay. Splenocytes and bone marrow cells were harvested from PfMSP-119 immunized mice as well as PBS treated mice at various time after immunization and stimulated in vitro with recall antigen for 5 days. After in vitro stimulation, ASC responses were measured in the splenocytes and bone marrow cells. In PBS treated mice no PfMSP-119-specific ASC could be detected in either spleen or bone marrow. The PfMSP-119-specific ASC response in the
spleen slightly preceded the responses in the bone marrow. Although, PfMSP-1\textsubscript{19}-specific IgG-secreting ASCs were evident (11 ASC per $10^6$ cells) in the spleen within 1 week after the first boost, peak numbers of PfMSP-1\textsubscript{19}-specific IgG-secreting ASC (26 ASC per $10^6$) were found in the spleen 21 days after first boost. The numbers of PfMSP-1\textsubscript{19}-specific

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{spleen_bone_marrow.png}
\caption{Presence of plasma cells in the spleen and bone marrow cells of mice immunized with PfMSP-1\textsubscript{19}. Spleens and bone marrow cells were harvested at indicated time following primary immunization and Ag challenge and analyzed for the presence of CD138\textsuperscript{+} cell in B220\textsuperscript{-} cells by flow cytometry. A, Dot blot shows shows the presence of CD138\textsuperscript{+} populations in the spleen. B, shows the presence of CD138\textsuperscript{+} cells in the bone marrow.}
\end{figure}
IgG ASC in the spleen progressively decreased from third week onward in PfMSP-19 immunized mice. Even after the second boost, number of PfMSP-19-specific IgG ASC in spleen continued to decrease (Fig. 3.2.10 a).

The kinetics of the PfMSP-19-specific ASC response in the bone marrow was quite distinct from that in the spleen. In bone marrow, there was a steady increase in the PfMSP-19-specific ASC response after the first antigen boost, the peak ASC response was observed approximately 2 week after the second boost. After the peak response there was a gradual decline in PfMSP-19-specific ASC responses in bone marrow (Fig 3.2.9). PfMSP-19-specific ASC in the bone marrow constituted 20 to 30% of the total ASC and averaged 1.8 x 10³ anti-PfMSP-19 specific plasma cells per total bone marrow. After the first boost, most of the PfMSP-19-specific ASCs were present in the spleen and only few in the BM. But after the second boost, the number of PfMSP-19-specific ASCs in the spleen but not in the bone marrow were about 16-fold reduced in immunized mice.

The kinetic analysis of the ASC response indicates that the elevated PfMSP-19-specific serum Ab levels were probably maintained by the ASC in the bone marrow. When the percentage of PfMSP-19-specific IgG ASC in the spleen and bone marrow compartments were compared (Fig. 3.2.10 b), it was found that 60-70% of the PfMSP-19-specific ASC was located in the spleen after first boost. After the second boost, about 50 to 55% of PfMSP-19-specific plasma cells were located in the bone marrow indicating that the anti-PfMSP-19 antibodies at later time points were mainly constituted by the bone marrow ASCs. Thus, the presence of ASCs in the BM after second boost is probably responsible
for the presence of circulating PfMSP-19-specific IgG titers in the blood. By day 187, there was a 5-fold difference of ASCs between spleen and bone marrow (3 ± 1/10^6 in spleen vs 15 ± 4/10^6 bone marrow cells) (Fig. 3.2.9). Thus it is possible that some of the plasma cells generated following antigen boost become long-lived, possibly because they

![Graph showing cell populations](image)

**Figure 3.2.10 a.** Kinetics of B220^+CD138^+ and B220^−CD138^+ populations in the spleen and bone marrow cells of mice immunized with PfMSP-19. Bar graphs depict frequency of CD138^+ cells in the spleens and bone marrow cells of PfMSP-19 immunized mice analyzed by flow cytometry.
are rescued in survival niches in the bone marrow as long-lived plasma cells. By day 200 after immunization, PfMSP-19-specific antibody titers decline below detectable amounts. These antibodies are thus likely thus secreted by short-lived plasma cells that reside in the bone marrow. The experimental data obtained in the present study suggest that plasma cells have a limited life span, the antibody they produce accumulate initially and then decline with the half-life of the plasma cells themselves. In addition, it was found that upon culturing splenocytes from immunized mice with PfMSP-19, there was a 2-fold decrease in the numbers of IgG PfMSP-19-specific ASCs per spleen compared with splenocytes cultured with medium (Fig. 3.2.10 b). In these experiments, the average anti-PfMSP-19 ASC frequency in the PfMSP-119 culture was 26 ASC per $10^6$ spleen cells whereas in the medium cultures it was 31 ASC per $10^6$ spleen cells (Fig. 3.2.10 b). Similarly in the bone marrow, PfMSP-19-specific ACSs was 17 per $10^6$ BM cells when compared with medium, 24 ASC per $10^6$ B cells. Thus, it could be argued that the decrease in ASC seen in PfMSP-19 cultures reflects an increased propensity of antibody secreting cells towards cell death.

To further analyse the IgG subclass profile of PfMSP-19-specific plasma cells after boosts with recombinant PfMSP-19, the four subclasses, IgG1, IgG2a, IgG2b, and IgG3, were quantitated by subclass-specific ELISPOT (Fig 3.2.10 c). IgG subclass profile showed IgG1 as the predominant subclass (64%), followed by IgG2a (35%) at the ASC peak response in the spleen and also in the bone marrow. The IgG subclass distribution of splenic ASC matched the IgG subclass distribution of PfMSP-19-specific antibody in the serum at all the time points tested (days 47, 57, 77, 124 and 187). These results show that both the bone marrow and the spleen contribute to serum antibody production, but total
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PfMSP-1\textsubscript{19} specific ASC in the bone marrow outnumber those in the spleen by approximately 2 to 1.

![Graph showing the initial humoral response to recombinant PfMSP-1\textsubscript{19}](image)

**Figure 3.2.10 a.** The initial humoral response to recombinant PfMSP-1\textsubscript{19} occurs in the spleen, but later antibody synthesis occurs in the bone marrow. BALB/c were immunized with 20\mu g of recombinant PfMSP-1\textsubscript{19}. The data shown are averages of two mice assayed at various time points after primary immunization. The ELISPOT assay was used to quantitate individual PfMSP-1\textsubscript{19}-specific ASC isolated from the spleen and bone marrow.

![Spleen and Bone marrow ASC response graphs](image)

**Figure 3.2.10 c.** Kinetics of PfMSP-1\textsubscript{19}-specific ASC response in mice immunized with PfMSP-1\textsubscript{19}. ELISPOT assay was used to measure the number of cells producing PfMSP-1\textsubscript{19}-specific IgG1, IgG2a, IgG2b, or IgG3 at different times after immunization. Results are expressed as the number of ASC/1 x 10\textsuperscript{6} cells. The mean ± SD is shown for two to three individual mice.
3.2.11 Phenotype of B cells from PfMSP-119 immune mice

Next, phenotype of splenic B cells from mice immunized with PfMSP-119 was examined by two-color flow cytometry. Splenic cells from PfMSP-119-immune or PBS treated mice were stained 1 wk postimmunization for the B cell marker B220 vs IgD, IgM, IgG PNA reactivity as markers of B cell activation. As Fig. 3.2.11 shows, there is a significant reduction in the proportion of IgD-bearing B cells in PfMSP-119-immune mice in comparison to the naive mice. After secondary immunization, some of the B cells expressed surface IgM, but majority of B cells had switched to IgG in PfMSP-119 immune mice. Since surface IgD is normally down-regulated on naive B cells upon T-dependent germinal center activation (Kelsoe, G., 1996), this suggested that most naive B cells in the spleens of PfMSP-119-immune mice had received activation signals. Our findings are consistent with other reported observations on T cell-dependent B cell activation (Jacob et al., 1991; Lalor et al., 1992; McHeyzer-Williams et al., 1993, Smith et al., 1996). Surprisingly, there was no gradual (or sudden) diminution in the sIgM\(^+\) population coincident with an equivalent increase in the sIgG\(^+\) population observed in mice immunized with PfMSP-119 (Fig. 3.2.11). The kinetics and distribution of Ig isotypes observed during the anti-PfMSP-119 response are consistent with earlier studies demonstrating the rapidity of isotype switching in vivo (Jacob et al., 1991; Lalor et al., 1992; McHeyzer-Williams et al., 1993, Smith et al., 1996).
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3.2.11 Flow cytometric analysis of B cell populations in spleen of mice immunized with PfMSP-1 \textsubscript{19}. Spleens were harvested 7 days after primary immunization and Ag challenges and analyzed for the presence of surface IgD, IgM and IgG cell in B220\textsuperscript{+} cells. Numbers in the upper right hand quadrant of dot plots represent the percentage of sIgD, sIgM and sIgG cells in the B220\textsuperscript{+} cell population.

3.2.12 Kinetics of GC B cell induction.

An increase in the ability to bind to the lectin PNA normally accompanies T-dependent B cell activation and germinal center formation, and PNA binding is in fact treated as a hallmark of germinal center B cells (Kelsoe, G., 1996). As seen in Fig. 3.2.12 a B220\textsuperscript{+}PNA\textsuperscript{high} cells were evident in the spleen of PfMSP-1\textsubscript{19}-immune mice 7 days after primary immunization and increased in number until day 15 and then decreased to one-half this maximum frequency by day 21 postimmunization while only a minor proportion of B cells in PBS treated mice were PNA. After first and second boosting there was further reduction in the percentage of B220+PNA\textsuperscript{high} cells (>2 wk p.i.). Thus, soon after
primary immunization, before the peak of hypergammaglobulinemia is reached, the majority of splenic B cells in PfMSP-1_{19}-immune mice were in an activated state. To confirm that B220^+PNA^{high} cells were indeed GC B cell population, we further characterized the phenotype of this population. B220^+PNA^+ B cells were purified by using B220-and PNA-specific magnetic bead separation and tested for expression of GL7, CD95 and CD38. On B220^+PNA^+ B cells, expression of GL7 and CD95 (Fas) were upregulated whereas expression of CD38 was downregulated (Figure 3.2.12 b). Thus the kinetics of germinal center appearance after PfMSP-1_{19} immunization are similar to those observed after immunization with classical hapten Ags (e.g., (4-hydroxy-3-nitrophenyl acetyl nitropropene), where PNA^+ germinal centers are visible within 8 days of immunization and also by others (Jacob, Kassir, and Kelsoe. 1991; Liu et al., 1991; Ridderstad and Tarlinton., 1998; Atchman et al., 2003; Carvalho et al., 2007). The lifespan of germinal centers depends on the duration of Ag exposure, and hapten-specific germinal centers were found to be present in the spleen for 3 wk after immunization (Liu et al., 1991).

3.2.13 PfMSP-1_{19}-specific memory B cells in the spleen and bone marrow.

To directly analyze development of the memory B cells following boosting with the PfMSP-1_{19} by flow cytometry, expression of CD38 (in combination with B220) was used as a marker of memory B cells. In mice, CD38 expression is down-regulated on Ag-specific GC B cells and subsequently reexpressed on a progressively increasing fraction of the memory B cell population (Ridderstad, A., D. M. Tarlinton. 1998). Decline in the
Results & Discussion

FIGURE 3.2.12 a. Kinetics of the B220\(^{+}\)PNA\(^{\text{high}}\) population elicited by PfMSP-1\(_{19}\) immunization. On the indicated days postimmunization, splenocytes from mice immunized with PfMSP-1\(_{19}\) were stained with PE-anti-B220 and FITC-PNA. Numbers within the upper right hand quadrant of the dot plots are the percentages of cells that are B220\(^{+}\)PNA\(^{\text{high}}\) phenotype observed at a given time point.
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Figure 3.2.12 b. B220<sup>+</sup>PNA<sup>high</sup> cells exhibit the phenotypic attributes of GC B cells. At day 15 postimmunization, B220<sup>+</sup>PNA<sup>+</sup> cells were purified from splenocytes and stained with FITC-labeled Abs specific for the indicated surface determinants. The histograms depict the expression of GL-7, CD95, and CD38 on B220<sup>+</sup>PNA<sup>+</sup> gated population.

Number of B220<sup>+</sup>PNA<sup>+</sup> cells in the spleen was evident by the end of the third week of primary immunization and was clearly diminished by day 37 after first boost (Fig 3.2.12 a). After first boosting there was a gradual increase in the number of B220<sup>+</sup>CD38<sup>+</sup> cells that became visible around day 42 and at successively later time points, there was further increase in B220<sup>+</sup>CD38<sup>high</sup> (Figure 3.2.13 a). However, after day 90, the percentage of B220<sup>+</sup>CD38<sup>high</sup> cells increased in the bone marrow before plateauing whereas in the spleen there was significant reduction in the percentage of B220<sup>+</sup>CD38<sup>high</sup> cells (Figures 3.2.13 b and 3.2.13 c). Together, with the data presented in the earlier section, suggests that after both active GC reaction and the humoral recall response have resolved, a B cell memory compartment develops in PfMSP-1<sub>19</sub>-immune mice.
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Spleen

Figure 3.2.13 a. Kinetics of the B220⁺CD38⁺ population elicited by PfMSP-1₁₉ immunization. On the indicated days postimmunization, splenocytes from mice immunized with PfMSP-1₁₉ were stained with PE-anti-B220 and FITC-CD38. Numbers within the upper right hand quadrant of the dot plots are the percentages of cells that are B220⁺CD38⁺ phenotype observed at a given time point.

Bone marrow

Figure 3.2.13 b. Kinetics of the B220⁺CD38⁺ population elicited by PfMSP-1₁₉ immunization. On the indicated days postimmunization, bone marrow cells from mice immunized with PfMSP-1₁₉ were stained with PE-anti-B220 and FITC-CD38. Numbers within the upper right hand quadrant of the dot plots are the percentages of cells that are B220⁺CD38⁺ phenotype observed at a given time point.

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Figure 3.2.13 c. Kinetics of the B220<sup>+</sup>CD38<sup>+</sup> population elicited by PfMSP-1<sub>19</sub> immunization. On the indicated days postimmunization, memory B cells were enumerated by staining splenocytes (grey bars) and bone marrow cells (red bars) from mice immunized with PfMSP-1<sub>19</sub> with PE-anti-B220 and FITC-CD38.

3.2.14 Recombinant PfMSP-1<sub>19</sub> generates short-lived memory B cells

Next, to determine whether PfMSP-1<sub>19</sub>-induced memory B cells were capable of transferring memory responses to naive mice, splenocytes and bone-marrow cells were harvested from immunized mice after they were rested for 8 weeks. Splenocytes and bone marrow from immunized mice were depleted of plasma cells (CD138<sup>+</sup> cells) with CD138 microbeads. This depletion resulted in a cell population that did not contain any plasma cells but contained PfMSP-1<sub>19</sub>-induced memory B and memory T cells. CD138<sup>-</sup> fractions from immunized mice and also from PBS treated mice were transferred into naive mice. 24 hours after cell transfer, the recipient mice were challenged i.p. with 5x10<sup>3</sup> chimeric parasite line, Pb-PfM19. This chimeric parasite line is a rodent malaria parasite.
Plasmodium berghei that expresses Plasmodium falciparum form of PfMSP-1\textsubscript{19} in place of its own domain. 5, 10 and 14 days following parasite challenge, recipient mice were analyzed for anti-MSP-1\textsubscript{19} titers in the serum by ELISA and for the presence of IgG-secreting Ag-specific AFCs by ELISPOT analysis in the spleen (Figure 3.2.14). Five days after challenge, anti- PfMSP-1\textsubscript{19} antibodies could be detected in blood plasma, indicating the restimulation of PfMSP-1\textsubscript{19}-specific memory B cells. Antibody titers reached a maximum at 10 days after treatment and then the titer fell by day 14 (Figure 3.2.14A). Recipient mice that were not infected with PbPfM19 parasites after cell transfer or mice that received cells from PBS treated group and were challenged with PbPfM19 parasites did not develop anti- PfMSP-1\textsubscript{19} antibodies. This result indicates that PfMSP-1\textsubscript{19}-specific memory B cells differentiate into anti-PfMSP-1\textsubscript{19} ASCs within 5 days after challenge with PbPfM19. However, by day 14 there was a significant reduction in the numbers of PfMSP-1\textsubscript{19}-specific ASC in the spleen of mice challenge with PbPfM19. Similarly, there were even fewer numbers of PfMSP-1\textsubscript{19}-specific ASC in the bone marrow of recipient mice. These experiments showed that following parasite challenge, PfMSP-1\textsubscript{19}-specific memory B cells failed to survive over a period of time in the recipient mice. The absence of any PfMSP-1\textsubscript{19}-specific ASC in spleens of recipient mice that received cells from PBS-treated mice but treated with PfMSP-1\textsubscript{19} showed that new ASC were not generated within 5 days of antigenic challenge. Anti-PfMSP-1\textsubscript{19} IgG response in recipient mice was contributed by the peak titers of IgG1 (16,000) followed by IgG2a (8000) and IgG2b (2000) whereas IgG3 responses were less detectable (1000) (Fig. 3.2.14 B).
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Figure 3.2.14. PfMSP-1\textsubscript{19} specific total IgG titer and ASC responses from mice cohort injected with the Ag-specific memory B cells then 24 hrs later infected with PbPf infection. Mice injected with naive B cells did not show any of the responses. (A) PfMSP-1\textsubscript{19} specific total IgG titer responses, sera were collected on day 5, 10 and 14. (B) PfMSP-1\textsubscript{19} specific IgG isotypes titer responses showing IgG1 is the dominant response followed by IgG2a, IgG2b and IgG3 is less detectable. (C) PfMSP-1\textsubscript{19}-specific ASCs total IgG with the peak responses at day 10. (D) PfMSP-1\textsubscript{19}-specific ASCs responses to IgG subclasses dominated by IgG1 followed by IgG2a subclass response.

To determine whether the adoptively transferred memory B cells could protect recipient mice from blood stage infection, cohorts of mice that had received memory cells from PfMSP-1\textsubscript{19} immunized or PBS treated mice were infected with a transgenic parasite line,
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PbPfM19. 24 hours after adoptive transfer of CD138- cells from spleen and bone marrow from PfMSP-119 immunized mice or PBS-treated, the recipient mice were injected i.p. with 5000 PbPfM-19 iRBCs. The progression of infection (parasitemia) was monitored by counting infected RBCs in blood smears prepared from the recipient mice. Recipient mice that received CD138- cells either from PfMSP-119 immunized and rested mice or from PBS treated group developed parasitaemia within 6 days and the parasitaemia increased gradually up to 80% by day 21 (Fig. 3.2.15 A). There was no significant difference on the survival between these two groups, both the groups of recipient mice died within 22-23 days (Fig. 3.2.15 B). These studies clearly showed that vaccination with PfMSP-119 did not confer any protection against PfPbM-19 infection.

The results from the present study show that the recombinant PfMSP-119 could generate a "short-term serological memory", which is mediated by the antigen-driven production of short-lived and long-lived plasma cells and memory B cells. The decline in the PfMSP-119-specific IgG antibody levels after second boost indicates that a large fraction of the plasma cells generated during secondary immunization was short-lived. The subsequent persistence of antibodies at low but stable levels additionally indicates the existence of either long-lived PfMSP-119-specific plasma cells or of plasma cells generated by repeated activation of memory B cells (Slifka et al., 1998; Bernasconi et al., 2002; Manz and Radbruch, 2002).

The observed faster kinetics and higher avidity of the antibody response after infection in mice with adoptively transferred CD138- cells from PfMSP-119 immune mice demonstrate that PfMSP-119-specific memory B cells also develop. Rapid anamnestic
antibody responses after infection appear to be primarily driven by the size of the reactive memory B cell compartment and the quality of its antibodies. Studies on protection found that while memory B cells were functional, they fail to offer any protection to mice infected with the transgenic parasite line, PbPfM19. The two compartments of memory B cells and long-lived plasma cells provide separate lines of humoral immunity. Long-lived plasma cells secrete protective antibodies of high specificity and adapted function. Memory B cells provide the potential to react fast and with even more adapted antibodies against reinfection. Parasite evasive strategies reflect the essential role of protective long-

![Figure 3.2.15](image)

**Figure 3.2.15.** Lack of protection by PfMSP-139-specific memory cells. Cohorts of six mice were immunized with PfMSP139 or PBS in adjuvant and were then rested for 10 wk and then challenged with PbPfM19. (A) Parasitemias in mice given memory cells from mice immunized with either PfMSP-139 or PBS and then challenged with PbPfM19 (B) Percentage survival of mice given memory cells from mice immunized with either PfMSP-139 or PBS and then challenged with PbPfM19.
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lived plasma cells and reactive memory B cells in antiparasite defense. Understanding the rules governing the differential generation of memory B cells versus long-lived plasma cells from the same precursors, and the mechanisms maintaining them, will provide exciting new options to develop novel, efficient antimalaria vaccines.