General Conclusions
GENERAL CONCLUSIONS

I  Recombinant PfMSP-1_{19} generates defective memory B-cell response.

Vaccine development is widely recognized as one of the most cost-effective ways to improve public health and to protect humans against falciparum malaria. The approaches being followed for malaria vaccine development are entirely ‘subunit’ based, aiming by means of vectored, recombinant or synthetic fragments to induce antiparasite effects. In this doctoral research study a recombinant subunit vaccine, PfMSP-1_{19} was successfully expressed and purified to homogeneity (see methods) and then used to assess the development and maintenance of memory B cells in Balb/C mice. In this study, the time course of the immunizations and antibody titers showed that the responses to PfMSP-1_{19} were boosted after the second immunization but the specific antibody titers to PfMSP-1_{19} decreased two-fold by the end of the experiment.

The antibody responses induced by PfMSP-1_{19} were mainly IgG. The isotype distribution of IgG responses showed that antibodies of all isotypes were detected with the levels of IgG1 and IgG2a higher when compared to IgG2b and IgG3. No significant changes in IgG isotype patterns were observed during the time course of the antibody responses.

Development of spleen and bone marrow PfMSP-1_{19} -specific IgG-secreting plasma cells (ASC) in mice immunized with the PfMSP-1_{19} were investigated. PfMSP-1_{19} specific ASC in spleen showed rapid decrease after second boost, suggesting that ASC from spleen were short-lived. Serum PfMSP-1_{19} specific IgG specific decreased after the second boost which coincided with the fall of ASC in spleen. While anti- PfMSP-1_{19}
plasma cells in the spleen declined, a population of PfMSP-1\textsubscript{19}-specific plasma cells appeared in the bone marrow and constituted the major source of long-term antibody production. Bone marrow was the predominant anatomical site of specific ASC and it was noteworthy that after the third dose there was a 5-fold difference in the level of ASCs between spleen and bone marrow, indicating the generation of long-lived ASC by vaccination. The IgG subclasses of the ASC in the bone marrow and spleen matched the subclasses of PfMSP-1\textsubscript{19}-specific serum antibody, indicating that both spleen and bone marrow contributed to circulating antibody in the serum. However, when spleen and bone marrow contributions were compared, it was found that bone marrow contributed to total PfMSP-1\textsubscript{19}-specific ASC response after second boost indicating that bone marrow could be the major site of Ab production at later stages of immunization. It was also observed that when PfMSP-1\textsubscript{19} primed B cells from spleen or bone marrow were cultured with the PfMSP-1\textsubscript{19} there was decrease in the number of ASC production, therefore it could be argued that PfMSP-1\textsubscript{19} induced the propensity of ASCs towards cell death.

Antibody responses to selected Ags are produced by discrete B cell populations whose presence and functional relevance vary along the ontogeny (Andres \textit{et al.}, 2007). In this study, the expression of cell surface receptors such as IgD, IgM, and IgG on the spleen B-cell compartment during the early and late phases of PfMSP-1\textsubscript{19} immunization were used as a function of B cell activation in Balb/C mice. Surprisingly, there was no gradual diminution in the sIgM\textsuperscript{+} population coincident with an equivalent increase in the sIgG\textsuperscript{+} population observed in mice immunized with PfMSP-1\textsubscript{19}.
B cell memory is provided by (a) protective antibodies that are produced by long-lived plasma cells and (b) memory B cells that, upon antigenic challenge, rapidly generate plasma cells secreting high-affinity antibodies that play an important role in eliminating the erythrocytic stages of malaria in most experimental models. The response to the booster dose was consistent with development of memory B cells after primary immunization. One way of examining B cell memory generation is to assess the induction of germinal center reaction which is characterized by the presence of lectin PNA binding B cell in the spleen (Jacob et al., 1991; Atchman et al., 2003). Kinetics of germinal centre B cells in mice immunized with PfMSP-119 suggested that by day 7 after priming, PNA+ cells became detectable and peaked at day 15 before declining indicating that PfMSP-119 induced B cell activation and generated germinal centre compartment in the spleen.

The decrease in the germinal center reaction corresponded with the gradual increase in the memory B cell compartment. Appearance of CD38 on B cells of PfMSP-119 immune mice as a marker of the memory phenotype, it was found that memory B cells become a discernible population after first boost by day 42. From this point on, the proportion of Ag-specific B cells displaying a memory phenotype continued to increase in the bone marrow but drastically decreased in the spleen. Therefore these findings indicate that B cell memory compartment developed in PfMSP-119 -immunized mice.

When PfMSP-119 specific memory B cells were transferred into naïve mice then challenged with Pb-PfM19 chimeric line of infection, Abs and ASC responses could be detected as early as day five post-transfer. This finding indicates that PfMSP-119-specific memory B cells differentiated into anti- PfMSP-119 ASCs within 5 days after challenge.
with the parasite. PfMSP-1,9-specific Abs and ASCs responses reached maximum by day 10 and then declined to basal level by day 14 indicating that following parasite challenge, PfMSP-1,9 -specific memory B cells failed to survive in the recipient mice. We further examined and confirmed that PfMSP-1,9 could generate functional short-live memory B cells but when transferred into naïve mice then challenged with Pb-PfM19 line of infection, transferred PfMSP-1,9 specific memory B cells failed to offer protection to the recipient mice.

In conclusion, this doctoral thesis provides evidences that PfMSP-1,9 could generate memory B cells and long lived plasma cells. Furthermore, in accordance with previous findings, the transfer of memory B cells into naïve recipient failed to offer any protection. These findings highlight the challenges that we face in the development of an effective malaria vaccine.

II Recombinant PfMSP-3F generates long-term memory B-cell response.

Merozoite surface protein 3 (MSP-3) is a malaria vaccine targeted by antibody-dependent cellular inhibition (ADCI), a protective mechanism against Plasmodium falciparum malaria. Antiparasitic activity of antibodies induced by Msp-3 has been demonstrated by both in vitro and in vivo methods. MSP-3 induces long-lasting antibodies responses and cytophilic antibodies mainly IgG3 in human directed to MSP-3 are associated with malaria protection in endemic areas. In this study a recombinant full-length fragment of Plasmodium falciparum merozoite surface protein 3 (PfMSP-3F) was successfully expressed and purified to homogeneity (see methods) and was used to assess the development and maintenance of B-cell memory response in Balb/C mice.
Immunization of Balb/C mice with the rPfMSP-3F formulated with CFA / IFA has revealed that the protein is able to elicit a significant total IgG antibody response after primary and secondary immunization. Following antibody decline at week 14, addition of a tertiary (third) booster immunization with soluble PfMSP-3F induced a remarkable recall of the immune memory established by earlier immunizations. This finding indicates that PfMSP-3F is able to activate and expand a small population of memory B cells established by previous immunization even after a long period of time as revealed by the established PfMSP-3F-specific serological memory shown in this study. We also observed that rPfMSP-3F is able to induce IgG isotype responses predominated with IgG1 and IgG2a. When we analyzed antiPfMSP-3F IgG isotype responses from immune mice, we observed that cytophilic antibodies mainly IgG2a last longer with higher titers up to 142 days after primary immunization.

In this study, investigation of the development of spleen and bone marrow PfMSP-3F -specific secreting plasma cells (ASC) in immune mice reveals that primary immunization of PfMSP-3F is able to establish a population of antigen-specific ASC in the spleen and bone marrow as early as day 14 and 21 respectively. The induced antigen-specific ASC responses showed rapid increase after the first and second booster immunizations suggesting that ASC from both the spleen and bone marrow were long-lived. We have observed that though the early peak ASC response was located in the spleen, but at later phase of humoral response, about 60% of PfMSP-3F -specific ASC were localized in the bone marrow. Since IgG isotypes play an important role in P. falciparum malaria protection, in this study we have investigated the distribution pattern of antiPfMSP-3F ASC- IgG isotypes and found that IgG1 and IgG2a isotypes contributed 41.1% and
35.3% respectively at the peak of first boost humoral response in the spleen. Similarly IgG isotype profile in the bone marrow showed that IgG1 (36.2%) and IgG2a (33.3%) were the predominant total IgG subclasses at the peak of responses. These results show that both the bone marrow and the spleen contribute to serum antibody production, but total PfMSP-3F-specific ASC in the bone marrow outnumber those in the spleen by approximately 2 to 1. We also observed that the profile of IgG isotype distribution of splenic and bone marrow ASC and anti-PfMSP-3F-specific antibodies in the serum were similar indicating that both spleen and bone marrow contributed to circulating antibody in the serum. In vitro culture investigation of PfMSP-3F specific memory B cells with the recall antigen revealed that there was 2 to about 3-fold increase in the numbers of IgG PfMSP-3F-specific ASCs compared with splenocytes cultured with medium, suggesting that the increase in ASC seen in PfMSP-3F cultures reflects an increased propensity of antibody secreting cells towards proliferation.

The ability of PfMSP-3F specific memory B cells from immune mice to transfer memory response in to naïve mice was then investigated. We observed that anti-PfMSP-3F antibodies could be detected in blood plasma of naïve mice received antigen-specific memory B cells within three days after the antigenic challenge indicating re-stimulation of PfMSP-3F-specific memory B cells. Similarly the number PfMSP-3F-specific ASC were detected as early as day 3 which increased gradually till day 15. However, by day 40 there was a significant reduction in the numbers of PfMSP-3F-specific ASC in the spleen of mice challenge with rPfMSP-3F. These experiments showed that following antigenic challenge, PfMSP-3F-specific memory B cells survived over a period of time in the recipient mice. We also observed that the antibody response of mice boosted with the
soluble rPfMSP-3F matched the profile of memory response in the naïve mice that had received memory B cells from PfMSP-3F immune mice. This finding indicates that PfMSP-3F-specific serological memory can portray PfMSP-3F-specific memory B-cell response in the lymphoid organs (spleen and bone marrow). This information is of practical value for future clinical trials for the rational design of subunit vaccine constructs derived from MSP3.

III Comparison between antigen-specific memory B-cell responses induced by PfMSP-119 and PfMSP-3F

- In contrast to PfMSP-119 where by it targets antibodies that inhibit red blood cell invasion by the malaria parasite, PfMSP-3F is targeted by cytophilic antibodies that inhibit intra-erythrocytic parasite growth in a monocyte-dependent manner and this mechanism of defense is eventually termed as antibody dependent cellular inhibition (ADCI).

- While PfMSP-119 immunizations showed that antibody responses could be detected after the secondary immunization and responses were boosted after the tertiary booster immunization, PfMSP-3F immunization could elicit antibody response as early as 14 days after primary immunization and the responses increased as mice received further booster immunizations. The maximum antibody titer induced by PfMSP-119 during the course of immunizations was 64,000 while that of PfMSP-3F was around 300,000. Both in PfMSP-119 and PfMSP-3F immunizations were predominated by IgG1 and IgG2a isotype
responses but antibody titers of PfMSP-3F was far higher as compared to that of PfMSP-119 isotype responses

- Both the PfMSP-119 and PfMSP-3F immunizations could establish a profile of antigen-specific antibody secreting cell response similar to their respective serological antibody responses. While PfMSP-119 was found to induce death of antigen-specific memory B cells in vitro, PfMSP-3F induce about 3-fold proliferation of antigen-specific memory B in vitro.

- The ability of PfMSP-119 specific memory B cells from immune mice adoptive transfer into naïve mice, a rapid antibody response was observed on day 5 after parasite challenge as revealed by the antigen specific antibody response as well as ASC response that waned rapidly by day 15. On the other hand, adoptive transfer of PfMSP-3F-specific memory B cells from immune mice into naïve mice were found to induce antigen-specific antibody and ASC responses as early as day 3 and the responses persisted till day 40.

- In conclusion, findings from this doctoral study reveal that while recombinant PfMSP-119 generates defective short-term memory B-cell response, recombinant PfMSP-3F could generates a potent long-term memory B-cell response.