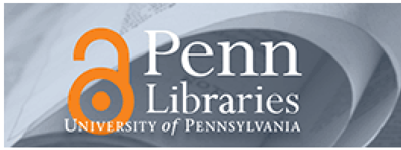

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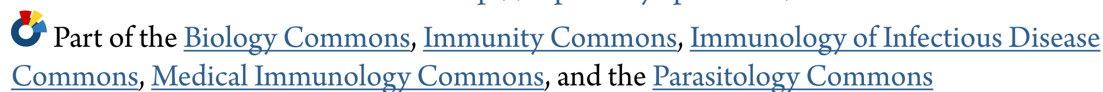
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The Acquisition of Human B Cell Memory in Response to Plasmodium Falciparum Malaria

Abstract
Immunity to Plasmodium falciparum (Pf), the most deadly agent of malaria, is only acquired after years of repeated infections and appears to wane rapidly without ongoing exposure. Antibodies (Abs) are central to malaria immunity, yet little is known about the B-cell biology that underlies Pf-specific humoral immunity. To address this gap in our knowledge we carried out a year-long prospective study of the acquisition and maintenance of long-lived plasma cells (LLPCs) and memory B cells (MBCs) in 225 individuals aged two to twenty-five years in Mali, in an area of intense seasonal transmission. Using protein microarrays containing approximately 25% of the Pf proteome we determined that Pf-specific Abs were acquired only gradually, in a stepwise fashion over years of Pf exposure. Pf-specific Ab levels were significantly boosted each year during the transmission season but the majority of these Abs were short lived and were lost over the subsequent six month period of no transmission. Thus, we observed only a small incremental increase in stable Ab levels each year, presumably reflecting the slow acquisition LLPCs. The acquisition Pf-specific MBCs mirrored the slow step-wise acquisition of LLPCs. This slow acquisition of Pf-specific LLPCs and MBCs was in sharp contrast to that of tetanus toxoid (TT)-specific LLPCs and MBCs that were vi vi rapidly acquired and stably maintained following a single vaccination in individuals in this cohort. In addition to the development of normal MBCs we observed an expansion of atypical MBCs that are phenotypically similar to hyporesponsive FCRL4+ cells described in HIV-infected individuals. Atypical MBC expansion correlated with cumulative exposure to Pf, and with persistent asymptomatic Pf-infection in children, suggesting that the parasite may play a role in driving the expansion of atypical MBCs. Collectively, these observations provide a rare glimpse into the process of the acquisition of human B cell memory in response to infection and provide evidence for a selective deficit in the generation of Pf-specific LLPCs and MBCs during malaria. Future studies will address the mechanisms underlying the slow acquisition of LLPCs and MBCs and the generation and function of atypical MBCs.

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THE ACQUISITION OF HUMAN B CELL MEMORY IN RESPONSE TO

PLASMODIUM FALCIPARUM MALARIA

Greta E. Weiss

A DISSERTATION

in

Immunology

Presented to the Faculties of the University of Pennsylvania

in Partial Fulfillment of the Requirements for the degree of Doctor of Philosophy

2010

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Alan Sher, Ph.D.

Dedication

This work is dedicated to the children who have suffered from and continue to suffer from *Plasmodium falciparum*-malaria.

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ABSTRACT

THE ACQUISITION OF HUMAN B CELL MEMORY IN RESPONSE TO
PLASMODIUM FALCIPARUM MALARIA
Greta E. Weiss

Susan K. Pierce

Immunity to *Plasmodium falciparum* (*Pf*), the most deadly agent of malaria, is only acquired after years of repeated infections and appears to wane rapidly without ongoing exposure. Antibodies (Abs) are central to malaria immunity, yet little is known about the B-cell biology that underlies *Pf*-specific humoral immunity. To address this gap in our knowledge we carried out a year-long prospective study of the acquisition and maintenance of long-lived plasma cells (LLPCs) and memory B cells (MBCs) in 225 individuals aged two to twenty-five years in Mali, in an area of intense seasonal transmission. Using protein microarrays containing approximately 25% of the *Pf* proteome we determined that *Pf*-specific Abs were acquired only gradually, in a step-wise fashion over years of *Pf* exposure. *Pf*-specific Ab levels were significantly boosted each year during the transmission season but the majority of these Abs were short lived and were lost over the subsequent six month period of no transmission. Thus, we observed only an incremental increase in stable Ab levels each year, presumably reflecting the slow acquisition of LLPCs. The acquisition *Pf*-specific MBCs mirrored the slow step-wise acquisition of LLPCs. This slow acquisition of *Pf*-specific LLPCs and MBCs was in sharp contrast to that of tetanus toxoid (TT)-specific LLPCs and MBCs that were

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Table of Contents

List of Tables……………………………………………………………………………………………………….xi
List of Figures …………………………………………………………………………………………………..xii
List of Abbreviations ……………………………………………………………………………………..xv

Chapter 1: Introduction

1.1 Overview…………………………………………………………………………………………..1

1.2 Malaria burden and epidemiology………………………………………………………1

1.3 *P. falciparum* life cycle………………………………………………………………………..3

1.4 Current control strategies and the need for a vaccine…………………………….5

1.5 Development of humoral memory in humans…………………………………………..7

1.6 The acquisition of immunity to malaria…………………………………………………12

1.7 The acquisition of antibodies and memory B cells in response to malaria..13

1.8 Hypotheses, goals and outline……………………………………………………………19

Chapter 2: Materials and Methods

2.1 Ethics Statements

2.1.1 U.S. Vaccine trials…………………………………………………………………**22**

2.1.2 U.S. blood bank samples………………………………………………………**22**

2.1.3 Kambila, Mali cohort study……………………………………………………**22**

2.1.4 Zungarococha, Peru cohort study…………………………………………**23**

2.2 Study sites and cohorts

2.2.1 U.S. Vaccine trials…………………………………………………………………**23**

2.2.2 U.S. blood bank samples………………………………………………………**24**

2.2.3 Kambila, Mali cohort study……………………………………………………**25**

2.2.4 Zungarococha, Peru cohort study…………………………………………**26**

2.3 Sample collection

2.3.1 U.S. Vaccine trials……………………………………………………………………27

2.3.2 U.S. blood bank samples………………………………………………………………28

2.3.3 Kambila, Mali cohort study…………………………………………………………28

2.3.4 Zungarococha, Peru cohort study………………………………………………29

2.4 Vaccine compositions and schedules……………………………………………………30

2.5 Assays and analysis

2.5.1 Research definition of malaria…………………………………………………30

2.5.2 Measurement of peripheral blood *Pf* parasitemia…………………………31

2.5.3 Identification of RBC polymorphisms…………………………………………31

2.5.4 Stool and urine exam for helminth infection………………………………32

2.5.5 Geographic information system data collection……………………………32

2.5.6 Antibody detection by ELISA…………………………………………………33

2.5.7 Memory B cell ELISPOT

2.5.7.1 MBC ELISPOT development……………………………………………33

2.5.7.2 Memory B cell ELISPOT assay…………………………………………35

2.5.7.3 Limiting dilution MBC ELISPOT ………………………………………37

2.5.8 Phenotypic analysis and sorting of B cell subsets…………………………39

2.5.8.1 Conjugation of detection reagents ……………………………………39

2.5.8.2 Basic B cell subset analysis……………………………………………39

2.5.8.3 Detailed B cell subset analysis…………………………………………40

2.5.8.4 Plasma cell analysis……………………………………………………41

2.5.8.5 B cell subset sorting……………………………………………………41

2.5.9 B cell fractionation…………………………………………………………………41

2.5.10 Antibody profiling by protein microarray

2.5.10.1 Chip fabrication…………………………………………………………42

2.5.10.2 Antibody profiling………………………………………………………44

2.5.10.3 Data normalization procedure…………………………………………44

2.5.10.4 Gene ontology and *Pf* stage-specific expression analysis of the immunogenic proteins……………………………………………45

2.5.11 Data management and analysis……………………………………………..……**46**

Chapter 3: Validation and optimization of the memory B cell ELISPOT assay

3.1 Introduction…………………………………………………………………………….………48

3.2 Results

3.2.1 Quantifying TT-specific MBC by flow cytometry**………………………….49**

3.2.2 Quantifying TT-specific MBC by limiting dilution ELISPOT**……………51**

3.2.3 Increasing the efficiency of the MBC ELISPOT assay**……………………51**

3.2.4 Establishing the selectivity of the modified assay for MBCs**……..…52**

3.2.5 Detecting Pf-specific MBCs in individuals living in malaria endemic areas. **………………………………………………………………………………53**

3.3 Discussion………………………………………………………………………………………55

Chapter 4: In malaria-naïve individuals *P. falciparum*-specific memory B cells and antibodies develop efficiently in response to subunit vaccination with CpG as an adjuvant

4.1 Introduction……………………………………………………………………………….……58

4.2 Results

4.2.1 The acquisition of MBCs in naïve individuals in response to vaccination**………………………………………………………………………..….60**

4.2.2 The acquisition of MBCs mirrors and predicts Ab responses in malaria-naïve vaccine recipients**………………………………………..……65**

4.2.3 Vaccination influences MBCs and PCs independently of antigen specificity**…………………………………………………………………….……67**

4.3 Discussion……………………………………………………………………………….…….69

Chapter 5: The design of the longitudinal study in Kambila, Mali and cohort description.

5.1 Introduction…………………………………………………………………………….……..75

5.2 Results

5.2.1 Description of the cohort and analysis of confounding factors ….**75**

5.2.2 Analysis of confounding factors…………………………………………….….77

5.3 Discussion……………………………………………………………………………..……84

Chapter 6: A prospective analysis of the antibody response to *P. falciparum* before and after the malaria season by protein microarray

6.1 Introduction……………………………………………………..…………………………87

6.2 Results

6.2.1 Validity and reproducibility of the protein microarray assay……….88

6.2.2 Analysis of *Pf*-specific antibody profiles before and after the malaria season…………………………………………………………………………..…..90

6.2.3 Identifying *Pf*-specific Ab profiles before the malaria season that correlated with subsequent protection from uncomplicated malaria…………………………………………………………………………..…..96

6.3 Discussion……………………………………………………………………………..…100

Chapter 7: *P. falciparum*-specific MBCs and Abs increase gradually over years with cumulative exposure in an area of intense seasonal *Pf* transmission

7.1 Introduction…………………….……………………………….………………………109

7.2 Results

7.2.1 Malaria immunity is acquired gradually despite intense exposure to the *Pf* parasite…………………….…………………………….…………….……..109

7.2.2 Analysis of *Pf*-specific and TT-specific MBCs and Abs in *Pf*-uninfected children and adults before the malaria season………..111

7.2.3 Longitudinal analysis of the *Pf*- and TT-specific MBC and Ab responses two weeks after acute malaria and after a prolonged period of decreased *Pf* exposure ……………………………………………..117

7.2.4 AMA1- and MSP1-specific MBC frequencies and Ab levels and malaria risk……………………….…………………………………………..…..120

7.3 Discussion…………………….………………………….……….………………….……121

Chapter 8: Atypical memory B cell expansion in individuals in the Kambila cohort

8.1 Introduction…………………….………………………………….……………………131

8.2 Results

8.2.1 Atypical MBCs are greatly expanded in individuals in malaria endemic areas……………………………………………………………………………**135**

8.2.2 Class switching in atypical MBCs in individuals in malaria endemic areas…………………………………………………………………………………**144**

8.2.3 Longitudinal profiling of B cell subsets in children before and after acute malaria……………………………………………………………………………**147**

8.3 Discussion……………………………………………………………………………………149

Chapter 9: Discussion…………………………………………………………………………154

Bibliography……………………………………………………………………………………184

List of Tables

Table 3.1 Efficiency of MBC ELISPOT with PSC stimulation……………………………………..51

Table 4.1 Sample size and mean vaccine-specific MBC percentage by vaccine type, CpG group, and study day. ……………………………………………………………………………….61

Table 5.1 Baseline clinical and demographic characteristics of entire cohort, according to age group. ………………………………………………………………………………………………76

Table 5.2 Frequency of red blood cell polymorphisms, according to age group………..78

Table 5.3 Malaria outcomes for the entire cohort, by age group…………………………..79

Table 5.4 Univariate analysis of baseline characteristics of children aged 2-10 years, stratified by hemoglobin (Hb) type………………………………………………………………82

Table 6.1 Gene ontology classifications overrepresented in the immunogenic *Pf* proteins relative to the entire *Pf* microarray……………………………………………………92

Table 6.2 Baseline characteristics of children aged 8-10 years classified as susceptible (≥1 malaria episodes) and protected (no malaria episodes) ……………………………97

Table 6.3 Proteomic features of *Pf* proteins associated with protection from uncomplicated malaria……………………………………………………………………………………99

Table 7.1 Baseline characteristics of MBC subset by age group……………………………110

Table 7.2 Malaria outcomes for the MBC subset, by age group……………………………112

Table S1. *Pf* proteins identified as immunogenic by protein microarray…………………171

List of Figures

Chapter 1

Figure 1.1 The *Plasmodium falciparum* life cycle……………………………………………………3

Figure 1.2 Clinical immunity to malaria is only acquired after repeated infections……………………………………………………………………………………………….12

Chapter 3

Figure 3.1 The frequency of TT-binding MBCs in vaccinated U.S. individuals……….50

Figure 3.2 The addition of IL-10 to PSC increased the efficiency of MBC differentiation to PC by 10 fold. ……………………………………………………………….52

Figure 3.3 Testing the selectivity of the PSC+IL-10 stimulation cocktail for MBCs..53

Figure 3.4 The frequency of antigen-specific MBCs by LD in PBMCs of *Pf*-exposed adults..……………………………………………………………………………………………….54

Figure 3.5 Numbers of total IgG^+ and antigen-specific ASC in bulk assay cultures of PBMC from malaria exposed adults………………………………………………………54

Figure 3.6 Correlations between the frequencies of antigen-specific MBCs determined by LD and antigen-specific ASC by bulk assay cultures…………55

Chapter 4

Figure 4.1 MBC are readily induced in malaria-naive individuals upon vaccination and CPG 7909 enhances the kinetics and magnitude of the AMA1-C1- and $MSP1_{42}$-C1-specific MBC response to vaccination.…………………………………62

Figure 4.2 The AMA1-C1- and $MSP1_{42}$-C1-specific Ab response mirrors the corresponding MBC response and is enhanced by CPG 7909. ………………….65

Figure 4.3 The level of Ag-specific MBC at the time of booster vaccination predicts the Ab response 14 days later………………………………………………………………….66

Figure 4.4 At steady-state, levels of Ag-specific MBC and Ab are highly correlated……………………………………………………………………………………………67

Figure 4.5 Vaccination appears to have an Ag-independent effect on the total IgG^+ MBC pool…………………………………………………………………………………………..68

Chapter 5

Figure 5.1 Number of clinical malaria episodes per day from May 2006 to Jan 2007 for the whole cohort……………………………………………………………………………79

Figure 5.2 Kaplan-Meier estimates of the time from study enrollment to the first episode of malaria in children aged 2–10 years……………………………………81

Figure 5.3 Distribution of study participants and sickle cell trait (HbAS) carriers who are study participants in the entire Kambila village population……….83

Chapter 6

Figure 6.1 Validity and reproducibility of the protein microarray assay………………89

Figure 6.2 Gene ontology classification of the 491 immunogenic *Pf* proteins….....91

Figure 6.3 Impact of age and *Pf* transmission on *Pf*-specific Ab profiles………….…95

Figure 6.4 *Pf*-specific Ab profiles associated with protection from malaria……..…98

Chapter 7

Figure 7.1 Malaria immunity is acquired gradually despite intense exposure to the *Pf* parasite…………………………………………………………………………………… 111

Figure 7.2 The *Pf*-specific MBC and long-lived antibody compartments expand gradually with age……………………………………………………………………… 114

Figure 7.3 The size of the total IgG^{+} MBC compartment expands gradually with age………………………………………………………………………………………………116

Figure 7.4 Longitudinal analysis of the *Pf*- and TT-specific MBC and Ab response……………………………………………………………………………………….118

Chapter 8

Figure 8.1 Flow cytometry gating strategies for B cell phenotyping…………………..136

Figure 8.2 Atypical MBCs are significantly increased in Malian as compared with U.S. volunteers……………………………………………………………………………….137

Figure 8.3 Inhibitory and tissue-homing receptor expression is increased and lymph node-homing receptor expression is decreased on atypical MBCs relative to classical MBCs……………………………………………………………………………….139

Figure 8.4 Atypical MBCs increase with increased *Pf* transmission in individuals in two endemic transmission settings……………………………………………………….140

Figure 8.5 Comparison of atypical MBC percentages in Peruvian individuals separated by reported prior *Pf*-malaria……………………………………………….…141

Figure 8.6 The percent of atypical MBCs is larger in children with persistent asymptomatic *P. falciparum* parasitemia as compared with parasite-free children…………………………………………………………………………………….…143

Figure 8.7 The IgG expression of atypical and classical MBCs is similar……………..145

Figure 8.8 The percentage of IgG^{+} atypical MBCs increases with increased *Pf* transmission in individuals in two endemic transmission settings…………146

Figure 8.9 Acute malaria alters B cell subset composition in the periphery during convalescence……………………………………………………………………………….148

Figure 9.1 Model for the gradual acquisition of humoral immunity to blood stage *P. falciparum*……………………………………………………………………………………..162

List of Abbreviations

Ab	antibody
Ag	antigen
AMA1	apical membrane antigen 1 of *Plasmodium falciparum*
AMA1-C1	apical membrane antigen 1 of *Plasmodium falciparum* clinical grade
ASC	antibody secreting cell
CIDR1 α	cysteine-rich interdomain region 1α of the variant surface antigen of *P. falciparum*
CSP	circumsporozoite protein of *Plasmodium falciparum*
BCR	B cell receptor
FCRL4	Fc receptor-like-4
iRBCm	infected red blood cell membrane
EBA1	erythrocyte binding antigen 1 of *Plasmodium falciparum*
gam	gametocyte
HIV	human immunodeficiency virus type-1
Ig	immunoglobulin
IgD	immunoglobulin delta
IgG	immunoglobulin gamma
IgM	immunoglobulin mu
iRBC	*Pf*-infected red blood cell
KLH	keyhole limpet hemocyanin
LD	limiting dilution
LLPC	long-lived plasma cells
LSA1	liver stage antigen 1 of *Plasmodium falciparum*
MBC	memory B cell

MSP1	merozoite surface protein 1_{42} of *Plasmodium falciparum*
$MSP1_{42}$-C1	recombinant merozoite surface protein 1_{42} of *P. falciparum* clinical grade
MSP2	merozoite surface protein 2 of *Plasmodium falciparum*
Mtb	*Mycobacterium tuberculosis*
mero	merozoite
ODN	oligodeoxynucleotide
PAMP	pattern-associated molecular protein
PBMC	peripheral blood mononuclear cells
PC	plasma cell
PDC	plasmacytoid dendritic cell
PSC	pokeweed, SAC and CpG
PSC10	pokeweed, SAC, CpG and IL-10
Pf	*Plasmodium falciparum*
Pfs260	a sexual stage antigen of *Plasmodium falciparum*
PfSE	*Plasmodium falciparum* schizont extract
RBC	red blood cell
RAP1	rhoptry-associated protein of *Plasmodium falciparum*
SAC	*Staphylococcus aureus Cowen*
schiz	schizont
SIV	simian immunodeficiency virus
SLPC	short-lived plasma cells
SP	sulphadoxine–pyrimethanine
spor	sporozoite
TLR	toll-like receptor
TRAP	thrombospondin-related adhesive protein of *Plasmodium falciparum*

troph	trophozoite
TT	tetanus toxoid
TT-b/SA-APC	tetanus toxoid conjugated to biotin bound to streptavidin-conjugated allophycoerythrin
VV	vaccinia virus
VZV	varicella-zoster virus

Chapter 1: Introduction

1.1 Overview

Malaria is a major world health concern today, in spite of existing preventative measures. This is partially due to the failure to deliver existing anti-malarial measures to affected populations, and partially due to vector and parasite escape from these anti-malarial measures. A vaccine is widely regarded as a critical goal in malaria-control, but barriers to reaching this goal include identifying immune parameters defining protection from malaria and how these immune parameters are acquired over time in response to natural infection and determining how malaria infections might alter the immune system. Focusing on humoral immunity I present data addressing these issues, including: the identification of the specificity of protective antibody (Ab) responses (Chapter 6); a description of the development of Abs and memory B cells (MBCs) in response to subunit malaria vaccines in malaria-naïve individuals (Chapter 4) in contrast to the development of Abs and MBCs to natural malaria infection (Chapter 7) and the discovery of the presence of large numbers of atypical MBCs in individuals in malaria endemic areas (Chapter 8).

1.2 Malaria burden and epidemiology

Plasmodium falciparum (Pf) infections are the most deadly of the four species that cause malaria in humans, resulting in over 500 million cases of malaria and over one million deaths annually (157), with 1.38 billion people living in areas of stable *Pf*

transmission (98). In addition to the human toll, there is a significant economic toll in countries with a heavy malaria burden, as malaria can account for up to 40% of public health expenditures, 30% to 50% of inpatient hospital admissions, 60% of outpatient health clinic visits and can significantly impact the Gross Domestic Product of affected countries (157). Although approximately half of the world's population, about four billion people, is at risk of malaria, countries in Sub-Saharan Africa bear the brunt of the disease, with approximately 90% of deaths from malaria occurring in African children under the age of five(151). It is estimated that on average African children have 1.6-5.4 episodes of malaria each year, with 20% of all childhood deaths in Africa due to malaria. This means that every 30 seconds a child in Africa dies of malaria (157). Although malaria can be prevented by anti-malarial prophylaxis and uncomplicated malaria is curable with anti-malarial drugs, the infrastructure is not in place to deliver anti-malarials to affected populations. In addition, *Pf*-drug resistance inevitably emerges creating a constant demand for new drugs. Similarly, insecticides and insecticide-treated bed nets can be effective control measures but again the infrastructure is not in place to provide these consistently to all individuals at risk, and there is the predictable emergence of *Anopheles* insecticide resistance. Malaria disproportionately affects impoverished people in rural areas who either cannot afford treatment or have limited access, or a complete lack of access to health care. Thus, in addition to anti-malarial drugs and insecticides, a malaria vaccine is widely viewed as a priority in the control of malaria.

1.3 *P. falciparum* life cycle

The *Pf*-life cycle within the human host is complex and tightly regulated. *Pf* is transmitted by the infected female *Anopheles* mosquito, which transfers a small number *Pf* sporozoites from her salivary glands to the human (Fig 1.2, A) as she takes a blood meal. Upon contact with human skin the sporozoites travel to the draining lymph node where CD8+ T cells are primed (37) (B(a)). Sporozoites also burrow through tissue until reaching the bloodstream, which carries them to the liver (B(b)). Once inside the liver, sporozoites invade hepatocytes where they expand dramatically in a clinically silent stage of infection, with each sporozoite giving rise to tens of thousands of merozoites. At this point the parasites lyse the hepatocytes, leaving the liver clear of sporozoites, and enter the blood stage of infection as greatly expanded numbers of merozoites (C).

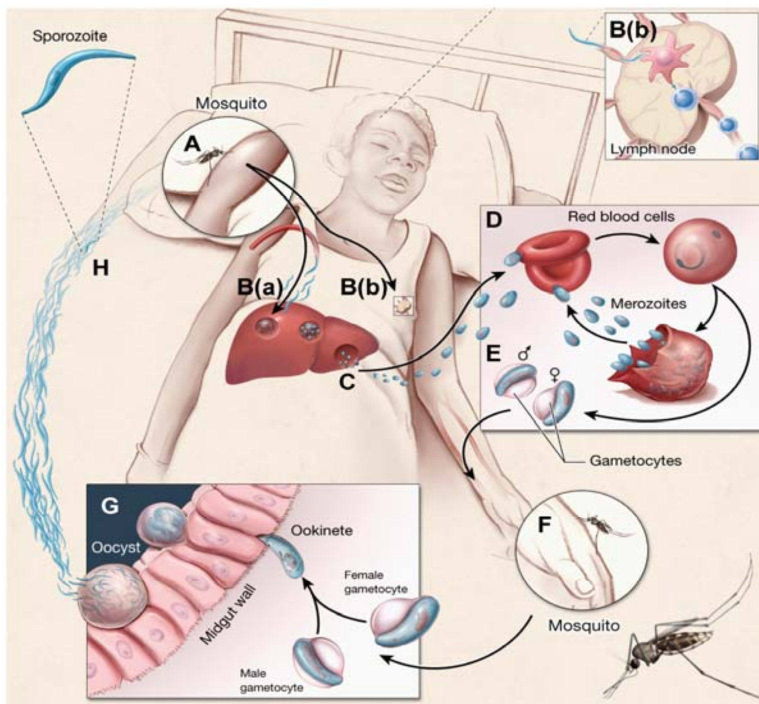

Figure 1.1 The *Plasmodium falciparum* life cycle. Sporozoites are transferred to the human host by a female Anopheles mosquito **(A)** where they prime CD8^{+}T cells in the draining lymph node **(B(b)** and invade hepatocytes **(B(a))** where they replicate for 10-14 days before entering the blood stage of infection as merozoites **(C)**, where they replicate exponentially inside erythrocytes in a 48 hour cycle **(D)**. Male and female gametocytes form **(E)**, are taken up by a mosquito during a blood meal **(F)**, and join in the mosquito midgut **(G)**. Haploid sporozoites result which travel to the mosquito's salivary glands and are transferred to another human host **(H)**.

During this stage of infection the parasites invade erythrocytes, where they digest hemoglobin for energy to replicate, then lyse their host erythrocytes and invade new erythrocytes, replicating exponentially in a 48 hour cycle (D). The blood stage is the symptomatic stage of infection typically causing headache, chills, sweating, and fever in uncomplicated malaria. During the blood stage of infection in a poorly understood process male and female gametocytes form (E), which are taken up by a mosquito during a blood meal (F). The gametocytes join in the mosquito midgut (G), differentiate into haploid sporozoites which travel to the mosquito's salivary glands and are transferred to another human during a blood meal (H) to complete the parasite's life-cycle (192).

A number of features of the parasite's life cycle within the human host appear to have evolved to evade the immune system. Firstly, only a small number of sporozoites are initially introduced into the host (as few as ten to fifteen sporozoites). The transport of merozoites from the liver to the bloodstream is accomplished inside hepatocyte-membrane derived vesicles, in which the parasite inhibits the exposure of phosphatidylserine (181) which is thought to have a role in mediating phagocytosis through protein kinase C activation (128). Thus, the parasite avoids antigen exposure, both by keeping contained within host membrane, and by controlling, to some extent, the composition of that membrane, decreasing the risk of phagocytosis. Merozoites invade and replicate inside erythrocytes, which lack MHC class I allowing parasitized red cells to evade CD8 T cell recognition. Merozoites also use multiple invasion pathways

mediated by multiple different receptors such that Ab responses to any one receptor do not block invasion (43, 189). *Pf*-infected red blood cells (iRBC) express the *Pf* protein PfEMP1 on their surface, allowing iRBC to sequester to blood vessel endothelium in the heart, brain, lung, kidney, subcutaneous tissue, and sometimes placenta, thus evading clearance by the spleen. PfEMP1s are encoded by the *var* gene family which has ~60 members, and although most parasites during an infection will express the same *var* gene product, the predominantly expressed PfEMP1 can change rapidly during infection, assisting iRBC in evading detection by the immune system (reviewed in (189)). The individual and cumulative effects of these mechanisms on the development of immunological memory to *Pf* are not fully known.

1.4 Current control strategies and the need for a vaccine

Current malaria control strategies depend on use of insecticides and drug therapies both as a prophylaxis and as treatment for infection, but the difficulty in expanding these measures to all malaria-endemic areas and maintaining this coverage as well as the growing resistance to these protective measures highlights the need for vaccine development. Both vector control and anti-parasite drug therapy are limited by the acquired resistance of mosquito to insecticides and parasites to antimalarials in addition to the inadequate infrastructure to implement these measures. Vector control includes indoor residual spraying (IRS) with insecticides including DDT and use of long-lasting insecticide-treated bed-nets (LLINs). Drug therapies involve mass drug administration (MDA) and intermittent preventive therapy (IPT)(75).

Mosquitoes and *Pf* parasites have both developed resistance to the respective treatments and resistance has spread rapidly and widely. DDT resistance developed less than two years after its introduction and now a number of mutations causing resistance through multiple mechanisms have been observed in the Middle East, India, Southeast and Central Asia, South-America and Africa (75), in more than 50 species of *Anophiline* mosquitoes(99). In addition, other health risks of DDT must be considered (reviewed in (195)). On the front of drug therapies, *Pf* has rapidly developed resistance to quinine, chloroquine, amodiaquine, sulphadoxine–pyrimethanine (SP), and may have acquired resistance to artemisinin (109). As an illustration of the rapidity of the acquisition of drug resistance in some cases, SP had a useful life of only five years in Thailand (178). *Pf* resistant to SP, originally reported in southeast Asia and South America, quickly spread to east and central Africa in the mid 1990's (126), chloroquine resistant *Pf* has spread to almost all malaria-endemic countries, and by 2001 choloroquine resistant *Pf* accounted for 25%-50% of all malaria cases in Africa (9), and multidrug resistant *Pf* has emerged in some areas, notably southeast Asia (204). A 12 year study in Papua New Guinea found that while using combinations of drugs increases clinical effectiveness, it does not decelerate growth of drug resistance (153). Thus, the difficulty in delivering insecticides or antimalarials to affected populations, the emergence of mosquitoes resistant to insecticides and increasing drug resistance in *Pf* highlight the importance of developing a vaccine.

1.5 Development of humoral memory in humans

The phenomenon of immunological memory is a fundamental property of the adaptive immune system and is the basis for all vaccine development. For most vaccines, neutralizing Abs play a critical role in protective immune responses (161), and thus understanding the mechanisms that underlie the generation and maintenance of humoral memory is of great importance. Long-term humoral immunity is encoded in MBCs and long-lived plasma cells (LLPCs) that are generated during the primary immune response in germinal center reactions (46, 47, 130). LLPCs are terminally differentiated cells that reside in the bone marrow constitutively secreting Ab and thus are responsible for the long-term maintenance of serum Ab levels which provide a critical first line of defense against reinfection (87). MBCs express somatically hyper-mutated and isotype switched B cell receptors (BCRs) and mediate recall responses to reinfection by proliferating and differentiating into plasma cells (PCs) resulting in rapid, high-titer, high affinity secondary Ab responses. Despite the central role of MBCs in protective immune responses, little is understood about how they are acquired in naïve individuals in response to antigen exposure and what factors influence this process. Efforts to develop new vaccines would benefit from a more detailed knowledge of the mechanisms underlying the acquisition of these cells.

Although the longevity of PCs and MBCs is a central feature of humoral memory, our understanding of the mechanisms that underlie the maintenance of these cell populations for the lifetime of an individual is only partial. The long-lived nature of LLPCs

in humans has been inferred from the stability of serum Ab levels induced by vaccination or infection. Virus-specific Ab levels were shown to be maintained for longer than 60 years after smallpox vaccination (48, 73, 95, 135), and the reported half-lives of Ab responses following infection ranges from 50 years for varicella-zoster virus (VZV) to over 200 years for measles and mumps viruses (12). These vaccines also induce stable, long-lasting Ab levels in the majority of individuals vaccinated, for example, more than 90% of those vaccinated 25-75 years prior to testing had substantial immunity to vaccinia (95). Typically, for the live-attenuated vaccines, vaccination causes a spike in Ab titers followed by a decrease in titers over one to three years, after which Ab titers remain relatively constant. For some vaccines Ab levels continue to decline but at a much slower pace, over decades with a half-life closer to ten to twenty years, as in the case of tetanus and diphtheria vaccination (46, 48, 73).

It remains an open question as to whether LLPCs are inherently long-lived or whether LLPCs are replenished by MBCs that proliferate and differentiate in response to persistent (216) or intermittent exposure to antigen, and/or through non-specific by-stander activation (e.g. cytokines or TLR ligands) (30). Based on data from humans, it seems likely that LLPCs are long-lived as Ab titers to tetanus, measles, mumps and rubella have been shown to persist at protective levels for years after rituximab treatment, which depletes $CD20^+$ cells, thus depleting naïve B cells and MBCs without depleting PCs (121), suggesting that MBC contributions to LLPC persistence are slight over time. As we might expect since MBCs differentiate into PCs, accounting for the

high titers of short-lived Abs during recall responses, recall responses after rituximab treatment are inhibited in both non-human primates (86) and in humans (196). The correlation observed between tetanus-, measles-, smallpox-, anthrax-, hepatitis B-, and rotavirus-specific MBCs and their respective Ab levels at steady state suggests that MBCs are ultimately responsible for replenishing PCs (30, 48, 64, 137, 165). Antigen-specific MBC levels and Ab titers were also shown to correlate after acute infection with measles, mumps and rubella, but not vaccinia (12). The story is not completely clear-cut however as MBC levels and Ab titers did not correlate in other studies for hepatitis B (199), varicella-zoster virus, EBV, tetanus and diphtheria (12).

Another key question is the role of antigen exposure in maintaining immunological memory in humans throughout the human life-span. MBCs specific for several pathogens after vaccination have been found to be remarkably stable in the absence of antigen exposure and the role of attenuated live or inactivated vaccine formulation is not entirely clear either with regard to the ability to induce long term immunity or MBCs. MBCs specific for pathogens following vaccination with live attenuated vaccines including vaccinia, measles, mumps and rubella, and inactivated vaccines including diphtheria, and tetanus as well as Epstein-Barr virus and varicella-zoster virus infection, were found to be remarkably stable in a recent cross-sectional analysis of adults (12). Inactivated tetanus and diphtheria vaccines induced shorter-lived antibody responses, however, the pathogen for which there was the most evidence for re-exposure to antigen following vaccination, varicella-zoster virus, had the

shortest-lived antibody response of the viruses studied. Interestingly, in the case of cholera the inactivated oral vaccine Dukoral® was shown to be effective at preventing severe diarrhea in large field trials in Bangladesh (40, 41), while the live attenuated oral cholera vaccine Orochol® did not show significant protection in a large field trial in Indonesia (167). There are several examples of the maintenance of Ab-mediated immunity in the absence of antigen exposure including immunity to measles on the Faroe Islands, yellow fever in the U.S., polio in remote Eskimo villages (reviewed in (46)), as well as the detection of antigen-specific MBCs more than 50 years after smallpox vaccination (48). Vaccinia-specific MBCs were detected over 50 years after smallpox vaccination and represented approximately 0.1% of total circulating MBCs, remaining unchanged at this percentage from 20 to 60 years post vaccination (48). The presence of these MBCs was correlated with a robust recall Ab response upon re-vaccination. Unlike PCs, which are terminally-differentiated, MBCs may be maintained through homeostatic proliferation (133), possibly through exposure to polyclonal stimuli (30).

Our current understanding of the acquisition of B cell immunity in humans is largely derived from studies of humans after vaccination due in large part to the difficulty in studying natural infections in humans when we cannot predict who within a population will be infected with a given pathogen at a given time. Although much can be learned from studies of the response to vaccination, the relative complexity of infection, where immune cells are typically stimulated by a panoply of PAMPs and multiple antigens and exposed to infected cells and the by-products of dead infected

cells, could significantly impact the development of immunity. Knowledge of the individual and cumulative effects of signaling through various receptors, antigen load and persistence, the cell types activated and the outcome of this activation on B cell differentiation in infection in response to various pathogens would give a much greater understanding of the requirements for or detriments to inducing long lived memory. While we still do not understand the factors that contribute to determining long- versus short-lived Ab responses, studying the response to vaccines allows us to gain some understanding of the development of long-lived MBCs and LLPCs. However, studying the differences between the responses to vaccines and those induced by infections could provide valuable information on the elements important for the induction of long-lived humoral immunity. Thus far, only three studies have analyzed the progression of a humoral response after infection and these were in individuals who presented with acute *Vibrio cholerae* infection, a pathogen that elicits long-term protection against subsequent disease in endemic areas, and found in the majority of patients, IgA and IgG MBCs specific for two antigens; cholera toxin B and toxin-co-regulated pilus major subunit A, increase from day two to thirty, and remain stable at this level for one year with no contraction (97, 112, 120). As will be shown in Chapter 7, this rapid acquisition of stable B cell immunity differs dramatically from that observed in malaria infections. Since at this point even some of the most basic questions surrounding the development of long-lived humoral immunity have not been addressed, I feel that studying the outcome in a highly complex disease such as malaria could ultimately offer important

insights into the factors that determine long-lived humoral memory. The study in Kambila presented here is the first prospective longitudinal study of MBC development in response to any natural infection.

1.6 The acquisition of immunity to malaria

Clinical immunity to *Pf* malaria develops gradually over years of repeated exposures. It is well known in medium to high transmission areas that malaria is a disease of children and pregnant women. Infants begin to get malaria around six months of age when maternal Abs start to wane, and during the first few years of life are at the greatest risk of severe malaria, manifested as severe anemia, acidosis or cerebral malaria. Children remain susceptible to uncomplicated malaria until about ten years of age, and adults remain susceptible to asymptomatic parasitemia throughout life (Fig 1.2). Although some partially-immune adults do have malaria episodes, these are

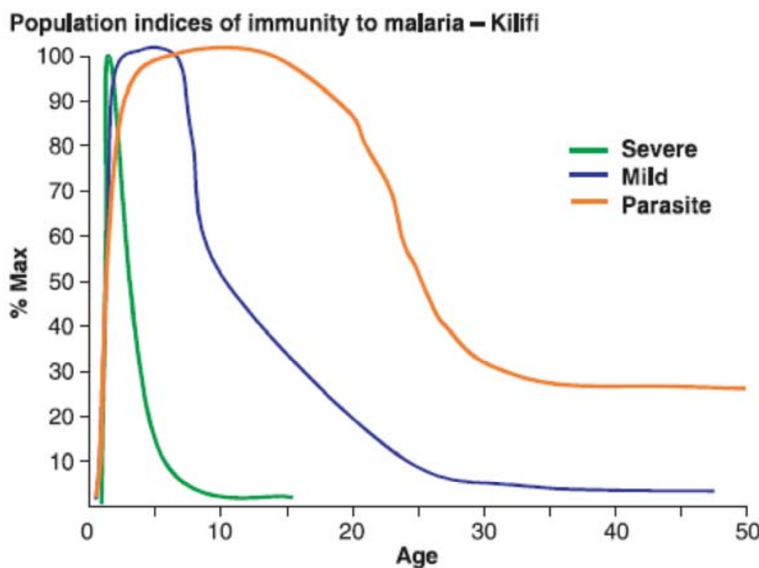

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Figure 1.2 Clinical immunity to malaria is only acquired after repeated infections. Representative data from a number of studies in Kilifi District on the coast of Kenya showing prevlence by age in relation to maximum prevalence recorded of severe or life-threatening malaria (green), mild or uncomplicated malaria (blue), and asymptomatic parasitemia (red) is shown. Children age five and under are at greatest risk for severe malaria, and remain susceptible to uncomplicated malaria until approximately ten years of age. Adults remain susceptible to asymptomatic parasitemia throughout life.

typically less severe and less frequent than episodes in children. In addition, adults who have achieved clinical immunity to *Pf* malaria who migrate to non-endemic areas have been empirically observed to become clinically susceptible after a one to two year time period. Sterile immunity to *Pf* has not been documented following natural exposure to the parasite. The pathologies and symptoms of severe malaria and uncomplicated malaria are different and resistance to these disease states is likely mediated via different immune mechanisms. My thesis will focus on the acquisition of B cell immunity to uncomplicated malaria.

1.7 The acquisition of Abs and MBCs in response to malaria

In 1961 Cohen et al. (42) conducted a study in humans, passively transferring purified IgG from malaria immune Gambian adults to children with severe malaria. As controls they gave children no treatment, IgG purified from malaria-naïve donors in the UK, or IgG-free serum from Gambian adults. No anti-malarial drugs were given and fever and parasitemia were monitored. Fever and parasitemia decreased dramatically in those given IgG from malaria-immune adults, while parasitemia decreased only slightly and insignificantly in all other groups of children, giving the first conclusive evidence that Abs play a key role in protection from malaria (42). Two human adoptive transfer studies followed in 1962 (67), and in 1963 where IgG from West African adults was given to East African children, and a fourth study 30 years after the initial manuscript, in 1991 (170) where West African IgG was given to Thai patients. In all cases, regardless of the geographical location or age of the patients, parasitemia and

malaria symptoms decreased, and in the most recent study it was shown that parasitemia decreased as rapidly in IgG treated patients as it did in drug-treated patients (170).

Several studies indicate that Abs specific for *Pf* proteins are generated inefficiently and inconsistently and lost rapidly in the absence of ongoing exposure to the parasite (reviewed in (129)). Many studies report Ab titers to *Pf* antigens in the days following malaria, but this Ab decreases in titer rapidly to the point of being undetectable in plasma weeks after malaria, suggesting that the entirety of, or the vast majority of, these Ab responses are due to SLPCs rather than LLPCs. A study in Kenya measured IgG to five *Pf* antigens, $MSP1_{19}$, MSP2 type 1, MSP2 type 2, EBA175, and AMA1, and showed that the half-lives of these responses were 9.8 days for IgG1 and 6.1 days for IgG3, regardless of antigen specificity, with Abs undetectable or nearly undetectable in individuals at six weeks (122). A four-year study in Sudan showed that *Pf*-RAP1 Ab was detectable only during and immediately after malaria infections and measureable Abs lasted only one to two months, although within an individual, responses increased in magnitude with repeated infections, suggesting a memory component (82). A study in The Gambia reported half-lives of 39.4 days for IgG1 and 32.6 days for IgG3 for *Pf*-AMA1 and *Pf*-MSP2, and further showed that Abs of these two specificities declined more slowly in children with persistent parasitemia, and in older versus younger children, with children four to six having mean half-lives of 52 and 47 days for IgG1 and IgG3 respectively, and children aged three and older having a mean

half-life of 16 days for both isotypes for both antigens (8). The rapid decline of *Pf*-specific Abs described in some studies may be explained by extrapolating the kinetics of Ab titers observed in vaccine trials where antigen-specific Ab levels rapidly increase and then rapidly decrease following immunization, as the subjects in these studies were assayed immediately following malaria (8, 122). However, the repeated reappearance and complete disappearance of Ab-reactivity in the plasma of individuals (36, 82, 188, 193) cannot yet be explained. These studies suggest that only short-lived Ab responses rather than long-lived Ab responses are generated following some infections. In one longitudinal study only 50% of adults had AMA1-specific Ab at any one of three timepoints tested and only 11% had Ab at all three timepoints tested (193). The percentage of adults in malaria endemic areas that have measureable Ab responses to those *Pf* antigens that have been tested after years of malaria exposure is also lower than would be expected based on vaccine studies, measured at 3-15% for MSP2 (207), 9-10% (207) and 58-66% for CSP (114), 20% for Pfs260 (188), 29-32% for RAP1 (82), 13-54% for PfSE (207), 19-41% for LSA1 (114), 61-64% for TRAP (114), 38% (188), 13-48% (207), 40% (68) and ~75% (152) for $MSP1_{19,}$ 70-75% for EBA1 (152), 11-50% (193), 23-37% (207) and ~90% for AMA1 (162). The low percentage of adults with positive Ab titers along with the observation that the Ab titer measured both increased with age, comparing adults ≤40 versus >40, and *Pf* transmission (152), indicate that even in individuals with decades of exposure to *Pf,* acquisition of stable long-lived Ab levels is remarkably slow.

Pf-specific MBCs have been measured in only two studies to date, and both indicate defects in MBC development. One study (64) reported that the percentage of adults with detectable antigen-specific MBCs was 46% for AMA1, 36% for $MSP1_{19}$ and 64% for CIDR1α, compared to 71% for tetanus. Interestingly, children five months to nine years of age had comparable levels of MBCs to AMA1 and MSP1 and only differed for CIDR1α, where 21% of children had detectable MBCs. Children aged five months and adults had comparable numbers of *Pf*-specific MBCs and comparable but higher levels of tetanus-specific MBCs suggesting that there may be a limit to the size of the antigen-specific niche that cannot be overcome with repeated exposure. However these data are difficult to interpret as the sample numbers were quite low, with 15 adults and an average of six children per year of age studied. It is also difficult to compare these results with others in the literature as the MBC frequency was determined by a poisson distribution calculation based on the proportion of an average of six wells/sample which were positive, the translation of which to a direct frequency or percent is not entirely clear. In addition, the study was cross-sectional and parasitemic and aparasitemic individuals were analyzed as a group. If *Pf*-specific MBCs have some defect in maintenance, and are only observed transiently following infection as are *Pf*-specific Ab titers in some studies, analyzing parasitemic and aparasitemic individuals together could obfuscate age-related differences in MBC levels. *Pf*-specific MBCs and Ab titers were found to correlate, but for AMA1 and CIDR1α antigens, more individuals had Ab with no detectable MBCs than in the case of tetanus (64). In the

second study of *Pf*-specific MBCs, adults in a malaria endemic area were compared to those in the same city who had had a recent known episode of *Pf*-malaria, with the percentage positive for MBCs to each antigen given for these two groups respectively; for AMA1 14.2% and 48%, for $MSP1_{19}$ 14.2% and 33%, for MSP2 4.8% and 18%, and for CSP 0% and 1% (207). In this study no correlation was observed between MBCs and Ab titers. Again these data should be considered carefully, as the study was small, the time from the last *Pf* infection was not stringently known and groups were designated by a combination of volunteer-recalled malaria, seropositivity to schizont extract (PfSE), and prior recorded episodes of either *P. vivax* or *P. falciparum*. The failure to differentiate between prior *P. vivax* and *P. falciparum* infections, and, possibly even more critically, defining individuals as "recently exposed" based on seropositivity rather than documented recent exposure could bias the results. Collectively these data indicate an inefficient acquisition of Ab and MBC responses to *Pf*-malaria. Elucidating the cellular basis of the inefficient acquisition of malaria immunity and *Pf*-specific Ab responses may ultimately prove critical to the design of an effective malaria vaccine.

While Ab to several individual antigens confers protection in animal models, Ab reactivity to any single *Pf* antigen has not been conclusively correlated with protection in humans, and considering the complexity of the parasite's life cycle within the host, it is likely that Abs to multiple *Pf* antigens will be required to induce clinical immunity. One possibility is that immunity requires Abs to all of the PfEMP1 gene products of the ~60 members of the *var* gene family which *Pf* causes the expression of on infected red

blood cells (iRBC). Although most parasites during an infection will express the same *var* gene product, the predominantly expressed PfEMP1 can change rapidly during infection, thus evading elimination of iRBCs mediated by Abs to other PfEMP1's (reviewed in (189)). By this hypothesis, the slow development of immunity is due to the length of time required to expose individuals to all the various *var* gene products and recurrent infections are due to iRBC expressing PfEMP1's unfamiliar to that host. In addition to *var* genes, *Pf* has extensive genetic diversity, with nearly as many alleles of some genes reported as there are gene sequences, and a related hypothesis on the delay of acquisition of immunity is based on the length of time required to develop Abs to the wide natural genetic diversity (186). It has also been suggested that the difficulty in developing immunity to *Pf* could be a property of some *Pf* antigens themselves, either by inhibition of CD4 T cell activation (132), or by the presence of disulfide bonds that impede efficient antigen processing (100). Subunit *Pf* protein vaccines would allow the assessment of the ability of *Pf* antigens alone to induce MBCs independent of the effects of other *Pf* antigens and the complex effects of *Pf* infection. It would also be critical to test these subunit vaccines in malaria-naïve individuals who would not have had the opportunity to develop lasting immune modulation of the response to these antigens, as it is well known that preexisting immune reactions affect recall responses. Thus, by using subunit vaccines in malaria-naïve individuals we can explore this possibility.

In summary, as a result of the studies in *Pf*-specific Ab longevity, the delay in the development of immunity, and the renewed susceptibility to clinical infection of

previously immune individuals who return to endemic areas, there has been doubt in the malaria research community as to whether true immunologic memory to *Pf* develops or can be maintained. These are some of the central questions that we explore here.

1.8 Hypotheses, goals and outline

Our goals in these studies are to assess the ability of two blood stage *Pf* vaccine candidates to induce *Pf*-specific Abs and MBCs in malaria-naïve individuals in the context of vaccination, and in children in a malaria endemic area in the context of *Pf* infection. As a control antigen, to assess the effects of *Pf* infection as well as the development and maintenance of a non-*Pf*-related antigen in this population we will assess the Ab and MBC responses to tetanus vaccination. In addition we will analyze B cells for phenotypic changes associated with malaria by flow cytometry. In order to address the specificity of protective Ab responses, we will probe plasma samples by protein microarray to determine whether Ab reactivity to some antigens correlates with subsequent protection from clinical malaria, and to test this protein microarray platform as a way to identify protective Ab specificities.

Based on the observed delay in the development of immunity to *Pf*-malaria, the apparently short-lived nature of immunity to clinical *Pf*-malaria, the serology data indicating that Ab responses to *Pf* proteins are inconsistently generated, the rapid decrease in immunity in the absence of exposure, and the incredibly complex immune environment induced during *Pf*-malaria, we hypothesize that the MBC response to *Pf*

infection will be significantly impaired relative to that of tetanus vaccination within the same population, and/or relative to the response in malaria-naïve individuals vaccinated with *Pf*-antigens. We hypothesize that the MBC response in malaria exposed individuals will be either of decreased magnitude, inconsistently present within an individual, or inconsistently generated across individuals. In spite of the proposed idea that *Pf*-malaria can suppress non-related immune responses, we hypothesize that TT vaccination will likely have induced a relatively robust response in our Malian cohort, as it is unlikely that many individuals were infected with *Pf* at the time of vaccination, and there is little data to support a malaria-induced long-lasting general immune-suppression.

We do not hypothesize that we will see a correlation between AMA1- or MSP1-specific MBCs and malaria risk, as recent clinical trials showed that vaccination with either AMA1 or MSP1 did not confer protection (156, 171). Furthermore, we suspect that the frequency of MBCs *per se* may not reliably predict clinical immunity to malaria regardless of antigen specificity, but MBCs to protective antigens might predict future Ab titers that would be protective. This is based on the kinetics of *Pf* blood-stage infection, where symptoms can begin as early as three days after the blood stage infection begins (179), while the differentiation of MBCs into PCs peaks approximately six to eight days after re-exposure to antigen (30). Thus there may not be sufficient time for MBCs specific for *Pf* blood stage antigens to differentiate into the Ab-secreting cells such that existing MBCs could prevent the onset of malaria symptoms. Based on

the delay in acquisition of immunity and the data indicating defective Ab and MBC responses to *Pf* we hypothesize that there will be malaria-related phenotypic changes in B cells. We also hypothesize that a pattern of Ab reactivity to certain *Pf* antigens will correlate with subsequent protection from clinical malaria.

The data I present here address these questions and hypotheses. These data indicate an efficient development of Ab and MBCs to *Pf* antigens in response to subunit malaria vaccines in malaria-naïve individuals. This contrasts to the incremental, stepwise development of Abs and MBCs to the same *Pf* antigens in response to natural malaria infection. Further, all malaria-naive vaccinees had detectable *Pf*-specific MBCs following vaccination, while approximately half of adults with a lifetime of exposure to the antigens had detectable *Pf*-specific MBCs. The incremental development of *Pf*-specific MBCs also contrasted with the efficient and stable development of TT-specific Abs and MBCs in the malaria-exposed individuals. Potentially related to the inefficient development of *Pf*-specific Abs and MBCs, we identified an expansion of atypical MBCs phenotypically similar to the hyporesponsive $FCRL4^{+}$ MBCs which are similarly expanded in HIV patients. We also present a potential method of identifying the specificity of protective Ab responses, and identify 49 proteins to which higher antibody titer correlated with subsequent protection from malaria. While certain Ab specificities correlated with protection, MBCs to the two *Pf* antigens we tested did not correlate with protection, as predicted. Overall these observations give the first glimpse into the

development of humoral memory to *Pf* malaria and raise important questions for further research.

Chapter 2: Materials and Methods

2.1 Ethics Statements

2.1.1 U.S. Vaccine trials

Both the AMA1 and MSP1 vaccine trials were conducted under Investigational New Drug Applications reviewed by the U.S. Food and Drug Administration, and both were reviewed and approved by the National Institute of Allergy and Infectious Diseases Institutional Review Board and by the Institutional Review Boards at their respective sites and funding agencies. Written informed consent was obtained from all participants.

2.1.2 U.S. blood bank samples

Blood samples were obtained for research use by signed consent of the donors under approved human subjects protocol Institutional Review Board (IRB) no. 99-CC-0168.

2.1.3 Kambila, Mali cohort study

The ethics committee of the Faculty of Medicine, Pharmacy, and Odonto-Stomatology, and the institutional review board at the National Institute of Allergy and Infectious Diseases, National Institutes of Health approved this study (NIAID protocol number 06-I-

N147). Written, informed consent was obtained from adult participants and from the parents or guardians of participating children. The study was externally monitored by international monitors under contract with NIAID, in compliance with the International Conference on Harmonization Good Clinical Practices (ICH/GCP), 1) to verify the prompt reporting of all data points, including reporting severe adverse events, checking availability of signed informed consents; 2) to compare individual subject records and the source documents (supporting data, laboratory specimen records and medical records to include physician progress notes, nurse' notes, subjects' hospital charts); 3) to ensure protection of study subjects, compliance with the protocol, and accuracy and completeness of records. The monitors also will inspect the clinical site regulatory files to ensure that regulatory requirements are being followed.

2.1.4 Zungarococha, Peru cohort study

Ethical clearance for this study was received from New York University, the University of Alabama, and the Peruvian Ministry of Health National Institute of Health Internal Ethical Review Boards. All individuals enrolled in this study gave signed informed consent.

2.2 Study sites and cohorts

2.2.1 U.S. Vaccine trials

Malaria-naive adults (n=40) residing in the U.S. were enrolled in two separate phase 1 clinical trials (n=20 for each trial, half of which were vaccinated with CPG 7909-containing vaccines for each vaccine trial) of the blood stage malaria vaccine candidates, apical membrane antigen 1-combination 1 (AMA1-C1) and merozoite surface protein 1_{42}-combination 1 (MSP1_{42}-C1). Participants were healthy adults age 18–50. Exclusion criteria included prior malaria infection, recent or planned travel to a malaria endemic area, recent use of malaria prophylaxis, and pre-existing autoimmune disease. Subjects were required to be in good general health, without known significant medical conditions or significant medical history, and were required to have normal results for screening laboratories: complete blood count, alanine aminotransferase (ALT), and creatinine; no serologic evidence of hepatitis B, hepatitis C, or human immunodeficiency virus infection; and negative anti-double stranded DNA (dsDNA) as a marker for autoimmune disease. Urine pregnancy testing was performed at screening as well as prior to each vaccination for females.

2.2.2 U.S. blood bank samples

Venous blood samples from healthy U.S. adult blood bank donors (n=10) were analyzed as controls. Travel histories for these U.S. adults were not available, but prior exposure to *Pf* is unlikely.

2.2.3 Kambila, Mali cohort study

Individuals were invited to be screened for the study after being randomly selected from an age-stratified census of the entire village population and were enrolled (n=225) according to age categories; age 2-4 (n=75), age 5-7 (n=50), age 8-10 (n=50) and adults age 18-25 (n=50). Enrollment exclusion criteria were hemoglobin level <7 g/dL, fever ≥37.5°C, acute systemic illness, use of antimalarial or immunosuppressive medications in the past 30 days, and pregnancy. The study was carried out in Kambila, a small (~1 km^2), well-circumscribed, rural village with a population of 1500, situated 20 km north of Bamako, the capital of Mali. The transmission of *P. falciparum* is seasonal and intense, from July through December, peaking in September through November. The entomological inoculation rate measured in a nearby village was near zero during the dry season, and there were approximately 50–60 infective bites per person per month in October 2000 (59). Participants were encouraged to report symptoms of malaria at the village health center, which was staffed 24 hours a day by a study physician. For subjects with signs or symptoms of malaria, blood smears were prepared and examined for the presence of *P. falciparum*. Patients with positive smear results were treated with a standard three-day course of artesunate plus amodiaquine, following the guidelines of the Mali National Malaria Control Program. Children with severe malaria were referred to Kati District Hospital after an initial parenteral dose of quinine. At the end of the malaria season, participants (or their parents or guardians, in the case of children) were asked whether or not they had used a bed net nightly during the rainy season.

2.2.4 Zungarococha, Peru cohort study

In the small collaborative study reported here Peruvian adults (n=18) were analyzed, both presenting with *Pf* parasitemia with no prior reported history of malaria (n=6), and presenting with *Pf* parasitemia and a prior reported history of one or more episodes of *Pf* malaria (n=12). This study, done in collaboration with the Malaria Immunology and Genetics in the Amazon (MIGIA) study (32), is ongoing in a region called Zungarococha, south of Iquitos, Peru. The transmission of *Pf* is seasonal and low, from January through July and the incidence of infection is 0.13 *Pf* infections/person/malaria season. The study began in 2003 and from 2003-2007 active case detection (ACD) surveillance (by using community surveys and longitudinal prospective sampling of sentinel houses), and passive case detection (PCD; presentation of symptomatic community members at the clinic) were done. At each visit (ACD or PCD) the individual is given an epidemiologic questionnaire, asking how many malaria infections they have had in their life time, who diagnosed them, and if and where they received treatment, in order to define if individuals had 0, 1, 2 or >2 prior clinical *Pf* malaria infections. Comparing self reports with the health center records, there was complete agreement in the 0, 1, 2 or >2 classification in 56.3% of individuals, and in classification within one difference (e.g., a "0" in patient report versus a "1" in the health post records) there was complete agreement in 83.1% of individuals. At least once a year a demographic survey, pedigree, GPS/GIS, nutritional questionnaire, weight, height, helminthes examination, and self report of lifetime malaria (matched with health records) are taken for each individual.

All malaria treatments are administered by the Peruvian Ministry of Health (MOH) at no charge. There is no known black market for malaria drugs in this region and since 1995 all fever cases presenting in health centers and any diagnosis of malaria have been recorded, specifying *P. falciparum* and/or *P. vivax*.

2.3 Sample collection

2.3.1 U.S. Vaccine trials

Peripheral venous blood samples were drawn into heparanized tubes (BD) on day 0,3,7,28,31,35,56,59,63,84,140 and 236 after vaccination for AMA1 and on day 0,7,28,31,56,59, and 140 for MSP1. For blood draw days that were on vaccination days, i.e. day 0, 28, and 56, blood draws were done prior to vaccination. Plasma was isolated and stored at -80C. PBMC were isolated from whole blood by Ficoll-Hypaque density gradient centrifugation (Amersham Biosciences) and frozen at ten million cells/ml in 90% heat-inactivated FBS (Gibco) and 10% DMSO (Sigma-Aldrich) at -80 °C for 24 h before storage in liquid nitrogen. For each individual, frozen PBMC from all available time points were thawed and assayed simultaneously. PBMC were rapidly thawed in a 37°C water bath and then added to complete media [RPMI-1640 plus L-glutamine (Gibco) supplemented with 10% heat-inactivated FBS, penicillin (10,000 IU/ml) streptomycin (10,000μg/ml) (Gibco), and β-ME (50μM) (Gibco)] warmed to 37°C. Cells were washed, resuspended in complete media, and counted using trypan blue (BioWhittaker) dye exclusion to detect viable cells.

2.3.2 U.S. blood bank samples

PBMC obtained from healthy donors at the NIH blood bank were isolated from whole blood or elutriated mononuclear cell buffy coats (obtained by lymphapheresis) by Ficoll-Hypaque density gradient centrifugation. Peripheral blood mononuclear cells were washed twice with sterile phosphate buffered saline (PBS; KD Medical), platelets were removed by low-speed centrifugation at 350g for 10 minutes through 5-10 ml underlayed FBS, and used immediately or resuspended in 90% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), kept at -80°C for 24 h, and then stored at -196°C in liquid nitrogen.

2.3.3 Kambila, Mali cohort study

Stool and urine samples were examined at enrollment for the presence of helminth and *Schistosoma haematobium* infection, respectively. Venous blood samples and blood smears were collected before the malaria season (May 2006), at cross-sectional time points every two months during the malaria season (July, October, and December 2006), prior to the second malaria season (May 2007), and 14 days after the first episode of malaria. Blood samples (8 ml for children and 16 ml for adults) were drawn by venipuncture into sodium citrate-containing cell preparation tubes (BD, Vacutainer® CPT™ Tubes) and transported to the laboratory (20 km) for processing within two h. Following centrifugation according to the manufacturer's instructions (1800 relative centrifugal force; 20 min), plasma was collected and stored at -80°C. Peripheral blood

mononuclear cells were collected, washed twice with sterile phosphate buffered saline (PBS; KD Medical), resuspended in 90% heat-inactivated fetal bovine serum (FBS; Gibco, Grand Island, NY) and 7.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO), kept at -80°C for 24 h, and then stored at -196°C in liquid nitrogen. For each individual, PBMC and plasma samples from all time points were thawed and assayed simultaneously. Two hundred microliters of whole blood was collected from the sterile tubing and used to identify RBC polymorphisms HbS (sickle cell trait), HbC, α-thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, as well as blood group. Participants were instructed to report symptoms of malaria at the only village health center, staffed 24 hours per day by a study physician. For individuals with signs or symptoms of malaria, blood smears were examined for the presence of *Pf*. Patients with positive smear results (i.e. any level of parasitemia) were treated with a standard three-day course of artesunate plus amodiaquine, following the guidelines of the Mali National Malaria Control Program. Individuals with *Plasmodium* malarias other than *P. falciparum* and co-infections were excluded from analysis. Anti-malarial drugs were provided exclusively by the study investigators. Children with severe malaria were referred to Kati District Hospital after an initial parenteral dose of quinine.

2.3.4 Zungarococha, Peru cohort study

At each visit (ACD or PCD) the individual is examined by a physician, and has a finger-prick blood sample (500µl) taken for a blood smear, haematocrit, serum sample, and red blood cell sample. If diagnosed with malaria, a 4-8mL Vacutainer tube of blood is taken.

PBMC were isolated using ficoll density-gradient centrifugation and frozen in FBS and DMSO. Frozen vials were obtained from our collaborators and all assays and analyses were performed at NIAID.

2.4 Vaccine compositions and schedules

Apical membrane antigen 1-combination 1 (AMA1-C1) and merozoite surface protein 1_{42}-combination 1 (MSP1_{42}-C1), both formulated on Alhydrogel and mixed with 564 µg of CPG 7909 (149) (www.clinicaltrials.gov no. NCT00320658). Both vaccines contained an equal mixture of antigen from two different clones of *P. falciparum* (FVO and 3D7) produced separately as recombinant proteins. Individuals received i.m. vaccinations on days 0, 28, and 56. For the AMA1-C1 trial, individuals received 80 µg of AMA1-C1 protein with the exception of four volunteers in the CPG 7909 group who received 20 µg. Since the dose of AMA1-C1 was not associated with a difference in the magnitude of the AMA1-C1-specific MBC response at any time point ($p > 0.100$ for all time points), the high- and low-dose groups were analyzed as a single group. For MSP1_{42}-C1, all individuals received 80 µg of protein.

2.5 Assays and analysis

2.5.1 Research definition of malaria

The research definition of malaria was an axillary temperature ≥37.5°C, *P. falciparum* asexual parasitemia ≥5000 parasites/µL, and a nonfocal physical exam by the study physician. Severe malaria, as defined by the WHO (1), was included in this definition.

2.5.2 Measurement of peripheral blood *Pf* parasitemia

Thick blood smears were stained with Giemsa and counted against 300 leukocytes. *P. falciparum* densities were recorded as the number of asexual parasites per microliter of whole blood, based on a mean leukocyte count of 7500 cells/µL. Each smear was evaluated separately by two expert microscopists blinded to the clinical status of study participants. Any discrepancies were resolved by a third expert microscopist.

2.5.3 Identification of RBC polymorphisms

Hemoglobin was typed by high-performance liquid chromatography (D-10 instrument; Bio-Rad). The mutation responsible for G6PD deficiency in Mali (*G6PD**A-) was identified by restriction-fragment length polymorphism analysis of PCR-amplified DNA samples, as described elsewhere (91). ABO blood groups were determined by use of a monoclonal Ab– based kit (Linear Chemicals). The 3.7-kb deletional determinant of α-thalassemia ($\alpha^{-3.7}$) was identified by a nested PCR protocol. Approximately 5 ng of extracted genomic DNA (Qiagen) was amplified in a 25-µL reaction volume consisting of 20 mmol/L Tris-HCl, pH 8.5, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 1 mol/L betaine (Sigma), 0.3 µmoles/L of each primer, 0.2 mmol/L each dNTP and 1.25 units Platinum *Taq* polymerase (Invitrogen). In the first round (multiplexed), forward 5'-CCCCTCGCCA AGTCCACC C-3' [40] and reverse 5'- AAAGCACTCTAGGGTCCAG CG-3' (39) primers were used to generate a product that would only amplify if $\alpha^{-3.7}$ was deleted. A different reverse primer 5'- AGACCAGGAAGGGCCGGTG-3' (39) was used in the same reaction mixture to generate a product that would only amplify if $\alpha^{-3.7}$ was present. Denaturation

at 95°C for five min was followed by 35 cycles of denaturation at 97°C for 45 s, annealing at 60°C for 75 s, and extension at 72°C for 2.5 min with a final extension at 72°C for five min. Separate nested amplifications of the first round products (1 µL of 1:20 dilution) were performed in the same reaction buffer with forward primer 5'-CTTTCCCTACCCAGA GCCAGGTT -3' (25) and reverse primer 5'-AGGAGGGCCCGTTGGGAGGC-3' (to generate a 1.8-kb product that amplifies in the absence of $\alpha^{-3.7}$) or forward primer 5'-CTTTCCCTAC CCAGAGCCAGGTT-3' (25) and reverse primer 5'-CCACTTTCCCTCCTCCATCCC-3' (to generate a 2.0-kb product that amplifies in the presence of $\alpha^{-3.7}$). Thermal cycling steps were the same as for the first round. Amplified products were separated and visualized on 1.2% agarose gels (Lonza). The sole presence of the 1.8-kb band indicated no deletion, the sole presence of the 2-kb band indicated $\alpha^{-3.7}$ homozygosity (-α/-α), and the presence of both the 1.8-kb and 2-kb bands indicated $\alpha^{-3.7}$ heterozygosity (-α/αα).

2.5.4 Stool and urine exam for helminth infection

At enrollment, duplicate stool samples were examined for *S. mansoni* eggs and other intestinal helminthes by use of the semi quantitative Kato-Katz method. To detect *S. haematobium* eggs, 10 mL of urine were poured over Whatman filter paper. One or two drops of ninhydrin were placed on the filter and left to air dry. After drying, the filter was dampened with tap water, and helminth eggs detected by microscopy.

2.5.5 Geographic information system data collection

Latitude and longitude coordinates and the altitude of study subjects' households were measured by a handheld global positioning system receiver (GeoXM; Trimble).

2.5.6 Antibody detection by ELISA

ELISAs were performed by a standardized method as described previously (141). For both AMA1 and MSP1, a 1:1 mixture of FVO and 3D7 AMA1 and MSP1 isotypes was used to coat the ELISA plates for the Kambila cohort, and AMA1-C1-FVO and AMA1-C1-3D7, and $MSP1_{42}$-C1-FVO and $MSP1_{42}$-C1 -3D7 for U.S. vaccine recipients. It is noteworthy that there was a strong correlation between AMA1-C1-FVO and AMA1-C1-3D7 titers (149), as well as $MSP1_{42}$-C1-FVO and $MSP1_{42}$-C1-3D7 titers (Martin et al, unpublished, www.clinicaltrials.gov #NCT00320658). The limit of detection for the AMA1 and MSP1 ELISA is derived by a calculation developed by Kazutoyo Miura based on the range of values that gives reproducible results at the Malaria Vaccine and Development Branch at NIAID where the assay is routinely performed. The limit of detection is the ELISA unit value at the lowest point on the standard curve, multiplied by the dilution factor at which samples are tested. The minimal detection levels for the MSP1 and AMA1 ELISA assays were 11 and 33 ELISA units, respectively. For analysis, all data below the minimum detection level were assigned a value of one half the limit of detection (i.e. 6 units for MSP1, 17 units for AMA1). The limit of detection for the TT ELISA was not determined because we did not have access to TT-naïve serum.

2.5.7 Memory B cell ELISPOT

2.5.7.1 MBC ELISPOT development

Whole PBMCs or sorted populations of MBCs, naïve B cells or PCs were cultured at $1x10^6$cells/ml in complete media in 24 well plates alone or with 2.5 µg/ml CpG oligodeoxynucleotide-2006 (Operon Technologies); 1/10,000 dilution SAC, 1/100,000 dilution pokeweed mitogen (Sigma-Aldrich). When indicated the wells of 24 well culture plates were incubated overnight at 4°C with 0.5 µg/ml CD40-specific Ab and washed. In addition, when indicated 10 ng/ml IL-2, 15 ng/ml IL-4, 150 ng/ml IL-5, 150 ng/ml IL-6, 10-150 ng/ml IL-10, or 100 ng/ml IL-21 (Cell Sciences) or 50 ng/ml BAFF (R&D Systems) were added to cultures. Cells were incubated for five to seven days, washed in complete media warmed to 37°C, counted and plated on prepared ELISPOT plates. Filterbottom 96-well ELISPOT plates (Millipore Multiscreen-HA) were prepared by incubating plates overnight at 4°C with either: 10 µg/ml polyclonal goat Abs specific for human IgG (Caltag) to detect all IgG-secreting cells; 1% bovine serum albumin (BSA) as a non-specific protein control or 5 µg/ml of tetanus toxoid (TT), MSP1 or AMA1 in PBS. Plates were blocked by incubation with a solution of 1% BSA in RPMI for 2 h at 37°C. Cells from five to six day cultures were serially diluted in duplicate or triplicate at concentrations of $4x10^4$-$3x10^2$ PBMC/well to detect total IgG^+ ASCs and $5x10^5$-$4x10^3$ PBMC/well to detect antigen-specific ASCs in the culture media described above at 37°C for five to six days. ELISPOT plates were kept at 37°C in a 5% CO_2 incubator for five hours, then washed four times with PBS and four times with PBS-0.05% Tween 20. Goat Abs specific for human IgG Fc conjugated to alkaline phosphatase (Jackson ImmunoResearch Laboratories) diluted 1:1000 in PBS-0.05% Tween 20 with 1% FBS was

added to wells and incubated overnight at 4°C. Plates were washed four times with PBS-0.05% Tween 20, three times with PBS, and three times with distilled water before adding BCIP/NBT 100 µL/well (Calbiochem). Plates were dried in the dark and spots were quantified with the Immuno Spot series 4 analyzer (Cellular Technologies LTD) and results analyzed using Cellspot software.

2.5.7.2 Memory B cell ELISPOT assay

PBMCs were thawed and cultured in 24 well plates at 37°C in a 5% CO_2 atmosphere for six days in media alone (RPMI 1640 with L-Glutamine, Penicillin/ Streptomycin 100 IU/ml, 10% heat-inactivated FBS, 50 μM β-Mercaptoethanol) or media plus a cocktail of polyclonal activators: 2.5 μg/ml of CpG oligonucleotide ODN-2006 (Eurofins MWG/Operon, Huntsville, AL), Protein A from Staphylococcus aureus Cowan (SAC) at a 1/10,000 dilution (Sigma-Aldrich, St. Louis, MO), pokeweed mitogen at a 1/100,000 dilution (Sigma-Aldrich), and IL-10 at 25 ng/ml (BD Biosciences). Cells were washed and distributed on 96-well ELISPOT plates (Millipore Multiscreen HTS IP Sterile plate 0.45um, hydrophobic, high-protein binding) to detect Ab-secreting cells (ASCs). ELISPOT plates were prepared by coating with either: a 10 µg/ml solution of polyclonal goat Abs specific for human IgG (Caltag) to detect all IgG-secreting cells; a 1% solution of bovine serum albumin (BSA) or keyhole limpet hemocyanin (KLH; Pierce) at 2.5 µg/ml in PBS as a non-specific protein control; or 5 µg/ml solutions of either tetanus toxoid (TT), AMA1, or MSP1 to detect antigen-specific ASCs. For AMA1 and MSP1, a 1:1 mixture of FVO and

3D7 isotypes (2.5 µg/ml each for a final concentration of 5 µg/ml) was used to coat the ELISPOT plates. For the vaccine trial these recombinant proteins were clinical grade and lot matched to the vaccines administered to the study participants. Plates were blocked by incubation with a solution of 1% BSA (Sigma-Aldrich) in RPMI-1640 for 2 h at 37°C. For the detection of antigen-specific ASCs, cells were plated in duplicate in eight serial dilutions beginning with $5x10^5$ cells/well. At this stage it is critical to keep cultured cells at 37°C to maintain optimal readout. For detection of total IgG ASCs cells were plated at six serial dilutions beginning at $4x10^4$ cells/well. As controls, stimulated PBMC were distributed onto BSA- or KLH-coated wells at 2-4 x 10^4 cells/well and unstimulated PBMC at 2-20 x 10^4 cells/well on anti-human IgG-coated wells. After a five hour incubation of the cells in the ELISPOT plates, plates were washed four times each in PBS and PBS-Tween 20 0.05% (PBST), and incubated overnight with a 1:1000 dilution of alkaline phosphatase-conjugated goat Abs specific for human IgG (Zymed) in PBST/1% FCS. Plates were washed four times each in PBST, PBS, and ddH_2O; developed using 100 µL/well BCIP/NBT (Calbiochem) for 10 minutes; washed thoroughly with ddH_2O and dried in the dark. ELISPOTS were quantified using Cellular Technologies LTD plate-reader and results analyzed using Cellspot software. Laboratory investigators were blinded to the CPG 7909 status for the vaccine trials, or clinical parameters and timepoint for the Kambila cohort, of study participants. Results are reported either as frequencies of MBCs per 10^6 PBMCs after the six-day culture or percentage:

$$\left(\frac{\text{the frequency of antigen-specific MBCs per million PBMC (after 5 day culture)}}{\text{the frequency of IgG secreting MBCs per million PBMC (after 5 day culture)}} \right) \times 100$$

The limit of detection of the MBC ELISPOT assay for the Kambila cohort analysis was five ASCs per 10^6 PBMC based on the average number of ASCs on the BSA control. This was determined for this set of assay plates and the background and subsequent limit of detection could vary depending on the immune experience of the cohort. Assay failure was defined as fewer than 1000 IgG^+ ASCs per 10^6 PBMCs after the six-day culture which resulted in the exclusion of 15% of individuals at month 0, 13.2% 14 days after the first malaria episode, and 7.3% at month 12. For individuals with a limited number of PBMCs, priority was given to performing the ELISPOT assay for MSP1, then TT, and then AMA1.

2.5.7.3 Limiting dilution MBC ELISPOT

The limiting dilution (LD) assay was modified from Pinna et al, 2009. After isolation, PBMCs were resuspended in complete medium with 10% FCS and cultured at concentrations of $5x10^3$-$5x10^2$ PBMC/well in 96 well plates to detect total IgG^+ MBCs and $2x10^5$-$2.5x10^4$ PBMC/well to detect TT-specific MBCs in section 3.2.2. For these experiments cells were directly transferred from 96 well culture plates to 96 well ELISPOT plates. For AMA1, MSP1 and TT LDA's in section 3.2.5 cells were cultured in 96 well plates at five-graded concentrations: (1.0 X 10^5, 0.75 X 10^5, 0.5 X 10^5, O.25 X 10^5,

0.1×10^5). Eighteen replicate wells for each PBMC concentration were cultured in 200 µL of complete medium containing 10% FCS with, or without with the polyclonal activators; pokeweed mitogen, SAC, IL-10 and CpG. Cells were incubated at 37 °C in 5% CO2 for 10 days. At the end of the culture period, supernatants were cleared of debris by centrifugation, harvested and frozen. For the detection of antigen specific IgG in section 3.6 culture supernatants, 96-well flat bottomed ELISA plates were coated with the respective recombinant (including the negative control protein, KLH) antigens at pre-determined concentrations of 10 µg/ml. After overnight incubation at 4°C, plates were blocked with 200 µl/well of PBS/10% FCS (Sigma-Aldrich) for 1 h at room temperature. To assess the secretion of antigen specific Ab, 30 µl of cell culture supernatant was added to each ELISA well following exactly the same plate-layout as the corresponding culture plate. After overnight incubation at 4 °C, plates were washed and bound IgG Abs were revealed by adding 100 µl/well of an alkaline phosphatase-conjugated goat anti-human IgG diluted 1/2000 in PBS with 1% FCS and 0.05% Tween (Sigma-Aldrich), followed by 100 µl/well p-nitrophenyl phosphate (pNPP) in alkaline phosphatase buffer (Sigma-Aldrich). The reaction was stopped after 30 min with 2 M NaOH (50 µl/well) and the optical density (OD) of each plate was read at 405 nm. Based on the OD obtained from ELISA results, the number of wells that were positive and negative for the presence of antigen-specific Ab was determined for each PBMCs dilution. An OD equal to the average + 3 SD OD observed in 6 replicate wells with supernatants from unstimulated cells was taken as a cut-off value to score a well as

positive. Wells cross-reactive with KLH were not included in the analysis.

2.5.8 Phenotypic analysis and sorting of B cell subsets

For assays described in 2.5.8.1-5 all phenotypic analyses were performed using mouse mAbs specific for human B cell markers conjugated to fluorophores or biotin with a secondary streptavidin-conjugated fluorophore. Stains were done on 0.5-1x10^6 PBMC at 4°C for 20-30 min. FACS data analyses were performed using FlowJo software, (Tree Star; Ashland, OR). Other details are described per section below.

2.5.8.1 Conjugation of detection reagents

Reagents used for antigen-specific B cell detection by FACS were prepared in our lab. Tetanus toxin (TT) was provided by Biologic Laboratories, University of Massachusetts Medical School, Jamaica Plains, MA. TT and human serum albumin (HSA) were biotinylated using the EZ-Link™Sulfo-NHS-LCBiotin kit (Pierce), according to the manufacturer's protocol. In brief, 450 µg of TT protein or 300 µg of HSA protein were incubated with biotin at a molar ratio of 1 mole of protein per 10 moles of biotin and dialyzed to remove excess biotin. TT was conjugated to Alexa Fluor® 488 (Molecular Probes) according to the manufacturer's direction.

2.5.8.2 Basic B cell subset analysis

For basic B cell subset analysis during the MBC ELISPOT assay development the following markers were used: CD19-PE-Cy5.5, CD27-PE, CD20-APC Alexa Fluor 750

(Caltag Laboratories) and CD38-APC and IgD-FITC (BD Pharmingen). Stained cells were resuspended in PBS, fixed with 1% paraformaldehyde, and analyzed on a FACSCalibur flow cytometer (BD Biosciences).

2.5.8.3 Detailed B cell subset analysis

The source of mAbs specific for the following markers conjugated to fluorophores is as follows: PECy7-CD19, PE-CD20, APC-CD10, APC-CD27 and PE-IgG, BD Biosciences (San Jose, CA); FITC-CD21, Beckman Coulter (Fullerton, CA); PE-CD85j, PE-CXCR3, PE-CCR6, PE-CCR7, PE-CXCR4, and PE-CXCR5, R&D Systems (Minneapolis, MN) and PE-CD11c, Invitrogen (Carlsbad, CA). The FCRL4-specific mAb was kindly provided by M.D. Cooper (Emory University School of Medicine, Atlanta, GA)(69). PE-conjugated rabbit Abs specific for mouse IgG2a were purchased (Invitrogen) and used to detect the mouse FCRL4-specific mAb. A four color two-stain strategy was used (stain 1: FITC-CD21, PE-CD20, PECy7-CD19, APC-CD20; stain 2: FITC-CD21, PE-IgG, PECy7-CD19, APC-CD27) in which within the $CD19^+$ gate the number of plasma cells ($CD21^-$ $CD20^-$) are subtracted from the number of $CD21^-$ $CD27^+$ cells to obtain the number of activated MBCs. The number of immature B cells ($CD10^+$) is subtracted from the number of $CD21^+$ $CD27^-$ cells and the number of GC B cells ($CD10^+$ $CD27^+$) is added to obtain an accurate number of naïve B cells. The number of $CD21^-$ immature B cells is subtracted from $CD21^-$ $CD27^-$ cells to obtain the number of atypical MBCs. FACS analyses were performed on a FACSCalibur flow cytometer.

2.5.8.4 Plasma cell analysis

The kinetics of the PC response to vaccination was examined in a subset of individuals from the AMA1-C1/Alhydrogel study. The lymphocyte fraction of peripheral venous blood samples was separated by Ficoll-Hypaque density gradient centrifugation, and 1 x 10^6 fresh PBMC were placed in each well of a 96-well plate and stained at 4°C for 30 min with fluorescently-labeled Ab to CD19 (PE-Cy5.5, Invitrogen), CD3 (Alexa 405, Invitrogen), CD27 (PE, Invitrogen), CD38 (PE-Cy7, Invitrogen), and IgD (FITC, Invitrogen), and then washed with PBS. Stained cells were resuspended in PBS, fixed with 1% paraformaldehyde and analyzed on a Becton-Dickinson LSR-II (BD).

2.5.8.5 B cell subset sorting

For sorting, PBMC were washed in PBS and platelets were removed by low-speed centrifugation through FBS. For MBC ELISPOT development assays cells were stained with fluorescently-labeled Ab to CD19 (PE-Cy5.5, Caltag), CD27 (PE, Caltag), and CD38 (APC, BD) and sorted on a FACSAria cell-sorting system (BD Biosciences). B cell subsets were defined as naïve B cells ($CD19^+$ $CD27^-$), MBCs ($CD19^+CD27^+$ $CD38^-$), plasma cells (PCs) ($CD19^+CD27^{++}$ $CD38^{+++}$), and non-B cells ($CD19^-$).

2.5.9 B cell fractionation

Mature ($CD10^-$) B cells were isolated from PBMCs by negative magnetic bead-based selection using a B cell enrichment cocktail supplemented with tetrameric CD10-specific mAb, Stem Cell Technologies (Vancouver, B.C., Canada). Mature B cells were separated

into $CD19^+$, $CD27^+/CD21^{hi}$ and $CD27^-/CD21^{lo}$ fractions using a two step magnetic bead selection process as detailed elsewhere (143). The subpopulations were cultured as previously described (44) in complete media alone or complete media plus a cocktail of polyclonal activators which included 2.5 μg/ml of CpG oligonucleotide ODN-2006 from Eurofins MWG/Operon (Huntsville, AL), Protein A from *Staphylococcus aureus* Cowan at 1/10,000 dilution from Sigma-Aldrich (St. Louis, MO), pokeweed mitogen at 1/100,000 dilution from Sigma-Aldrich, and IL-10 at 25 ng/ml from BD Biosciences. Cells were kept at 37°C in a 5% CO_2 incubator for five days, washed twice with complete media warmed to 37°C, counted and distributed onto 96 well ELISPOT plates coated with human IgG-specific goat Abs to detect all IgG-secreting cells as described (47).

2.5.10 Antibody profiling by protein microarray

2.5.10.1 Chip fabrication

Protein microarrays were constructed in four steps: (1) PCR amplification of each complete or partial *Pf* open reading frame, (2) *in vivo* recombination cloning, (3) *in vitro* transcription/translation, and (4) microarray chip printing. Open reading frames were derived from the *Pf* genomic sequence database (www.plasmodb.org) and selected based on stage-specific transcription or protein expression (80), subcellular localization in the parasite infected red blood cell, secondary protein structure, and documented immunogenicity in humans or animal models. The 1,204 unique proteins on the array are represented by 2,320 whole or partial proteins because open reading frames >3,000

base pairs were cloned as overlapping segments. Custom PCR primers comprising 20 base pairs of gene-specific sequence with 33 base pairs of "adapter" sequences are used in PCRs with *Pf* clone 3D7 genomic DNA as template. We optimized the PCR conditions to increase the efficiency of target sequence amplification using low temperature annealing and elongation protocols (182). The adapter sequences, which become incorporated into the termini flanking the amplified gene, are homologous to the cloning site of the linearized T7 expression vector pXT7 (52) and allow the PCR products to be cloned by *in vivo* homologous recombination in competent DH5α cells. We have recently introduced a more efficient method which allows the recombination to occur *in vitro* with much lower amounts of recombination product to be transformed into *E. coli* (26). The resulting fusion protein also incorporates a 5' polyhistidine epitope, an ATG translation start codon, and a 3' hemagglutinin epitope and T7 terminator. Sequence-confirmed plasmids are expressed in five hour *in vitro* transcription/translation reactions (rapid translation system (RTS) 100 Escherichia coli HY kits; Roche) according to the manufacturer's instructions. Protein expression is monitored either by dot blot or microarray using monoclonal anti-polyhistidine (clone His-1, Sigma) and anti-hemagglutinin (clone 3F10, Roche). Microarrays are printed onto nitrocellulose coated glass FAST slides (Whatman) using an Omni Grid 100 microarray printer (Genomic Solutions). Each microarray chip contains the following controls: (1) a 'no DNA' negative control in which an empty plasmid vector is placed in the RTS reaction, (2) serially diluted human IgG—a positive control and standard curve to normalize data from arrays

probed at different times, and (3) serially diluted Epstein-Barr nuclear antigen-1—a positive control given the high prevalence of latent Epstein–Barr virus infection in Africa.

2.5.10.2 Antibody profiling

Prior to incubating with the microarray chip, the plasma samples are diluted to 1/200 in Protein Array Blocking Buffer (Whatman) containing *E. coli* lysate at a final concentration of 30% and incubated at room temperature for 30 minutes on a rotating platform. The arrays are rehydrated in blocking buffer for 30 minutes and probed with the pretreated plasma overnight at 4°C with constant agitation. The slides are then washed five times in tris (hydroxymethyl)-aminomethane (Tris) buffer, pH 7.6 containing 0.05% (v/v) Tween 20, and incubated in biotin-conjugated goat anti-human immunoglobulin (anti-IgGFcγ, Jackson Immuno Research) diluted 1/200 in array blocking buffer. After washing the slides three times in Tris buffer containing 0.05% (v/v) Tween 20, bound Abs are detected by incubation with streptavidin-conjugated SureLight® P-3 (Columbia Biosciences). The slides are then washed three times in Tris buffer containing 0.05% (v/v) Tween 20 and three times in Tris buffer without Tween 20 followed by a final water wash. The slides are air dried after brief centrifugation at 1000g for four minutes and analyzed using a Perkin Elmer ScanArray Express HT microarray scanner. Intensities are quantified using QuantArray software.

2.5.10.3 Data normalization procedure

It has been noted in the literature that data derived from microarray platforms is heteroskedastic (21, 66, 108). This mean-variance dependence has been observed in

the arrays presented in this dissertation (183, 184). In order to stabilize the variance, the vsn method (105) implemented as part of the Bioconductor suite (www.bioconductor.org) in the R statistical environment (www.r-project.org) is applied to the quantified array intensities. In addition to removing heteroskedacity, this procedure corrects for non-specific noise effects by finding maximum likelihood shifting and scaling parameters for each array such that the variances of each negative 'No-DNA' and positive human IgG probes are minimized. This calibration has been shown to be effective on a number of platforms (23, 123, 172).

2.5.10.4 Gene ontology and *Pf* stage-specific expression analysis of the immunogenic proteins

We electronically annotated the entire *Pf* genome by using the default settings of Blast2GO (http://blast2go.bioinfo.cipf.es/home). From a total of 5,679 genes, we assigned gene ontologies to 3,416. To determine which gene ontology terms were overrepresented among the immunogenic *Pf* proteins relative to the 2,320 proteins on the *Pf* microarray, we used a one-tailed Fisher's Exact Test with a false discovery rate filter set at $P < 0.05$. For each chosen subset, we plotted multilevel pie charts filtered by the number of sequences per gene ontology term. To determine the proportion of immunogenic proteins expressed during the sporozoite and blood stages of the *Pf* life cycle, we retrieved DNA microarray expression data from PlasmoDB (www.plasmodb.org). Because many proteins are expressed during more than one stage, we assigned each protein to the stage at which it is maximally expressed.

2.5.11 Data management and analysis

Data was analyzed using Stata software (version 10.0; StataCorp), GraphPad Prism (version 5.01 for Windows; GraphPad Software), and R 2.0 (www.r-project.org). Blast2Go (www.blast2go.org) was used for gene ontology annotation and enrichment analysis.. Clinical data collected from the Kambila site were double entered and verified in a database (Microsoft Access 2003; Microsoft). Three clinical endpoints were used to evaluate the relationship between *Pf*-specific immune responses and malaria risk: 1) whether or not malaria was experienced, 2) the incidence of malaria, and 3) the time to the first malaria episode. The Kruskal-Wallis test was used to compare continuous variables between groups, and the Fisher's exact test was used to compare categorical variables and binary outcomes. The probability of a subject remaining malaria-free over an eight-month period was estimated by the Kaplan-Meier method, and the time-to-event curves of different groups were compared by the log rank test. The Cox proportional hazards model was used to assess the effect of the following factors on risk of malaria: age, sex, weight, ethnicity, distance of residence from clinic, bed net use, baseline *Pf* parasitemia, helminth infection, HbAS phenotype, HbAC phenotype, *G6PD**A- genotype (hemizygosity, heterozygosity, and homozygosity), -α/αα genotype, and ABO blood group. The same list of variables was included in a Poisson regression model to determine their impact on malaria incidence. The covariates included in the final Cox and Poisson regression models were determined by applying the stepwise model selection procedure, in which *P* values <.1 and >.2 were set as criteria for

covariate inclusion and removal, respectively. For all tests, 2-tailed *P* values were considered significant if they were ≤.05. The nonparametric Wilcoxon rank-sum and Wilcoxon matched pairs tests were used to compare continuous variables between unpaired and paired groups, respectively. The correlation between different continuous measures was determined by using the Spearman correlation coefficient. To account for the correlation among multiple measurements for the same subject, the generalized estimating equations (GEE) method (Liang, 1986) was employed to study the association between continuous outcomes and covariates of interest. An "exchangeable" correlation structure was used as the working assumption for all GEE analyses. The strength of association between two continuous variables was determined by calculating the Pearson correlation coefficient (r). The microarray data was normalized and calibrated as described above, then Bayes-regularized t-tests were used to identify significant differential Ab reactivity (22). The Benjamini-Hochberg method was used to correct for the false discovery rate. With the exception of gene ontology analysis, for all tests, 2-tailed *P* values were considered significant if they were < 0.05.

Chapter 3: Validation and optimization of the memory B cell ELISPOT assay

3.1. Introduction

Despite the importance of immunological memory, we still have an incomplete understanding of the cellular and molecular mechanisms that underlie the generation, maintenance and re-activation of immunological memory. An inherent limitation to the analysis of MBCs in humans has been the restricted access to tissues other than peripheral blood and the unknown relationship between the relative frequency of MBCs in the peripheral blood and lymphoid tissue. However, a recent study of individuals decades after smallpox vaccination showed that although the majority of vaccinia-specific MBCs were in the spleen, their frequency in the spleen reflected the frequency in peripheral blood (134). Furthermore, the frequency in the spleen and peripheral blood remained correlated throughout revaccination in these individuals. Based on this observation, the frequencies of MBCs in peripheral blood are a good reflection of the total number of MBCs in an individual.

An assay frequently used to detect antigen-specific human MBCs in human peripheral blood, described by Crotty *et al.* 2004 (47), relies on the selective ability of MBCs to proliferate and differentiate into Ab secreting cells (ASC) *in vitro* in response to a combination of pokeweed mitogen (PWM), fixed *S. aureus*, Cowan strain (SAC) and the TLR9 agonist CpG oligonucleotide (ODN-2006) over a five to six day culture period. Antigen-specific and total ASCs are quantified in ELISPOT assays using plates coated with either antigen or human Ig-specific Abs to capture total IgG. Crotty *et al.* showed that

peripheral blood MBCs (defined as $CD19^+CD20^+CD27^+$) from individuals immunized with anthrax vaccine differentiated into anthrax protective antigen (PA)-specific ASCs, while naïve B cells (defined as $CD19^+CD20^+CD27^-$) did not. PA-specific MBCs represented up to 2% of circulating IgG^+ B cells in immune individuals and were essentially undetectable in non-immune individuals. Thus, this assay provided a means of identifying antigen-specific MBCs in human peripheral blood. Here we assess the efficiency of this assay, and optimize it, and determine that this modified assay can be successfully used to quantify *Pf*-specific MBCs in semi-immune adults in Kenya. The increased efficiency of this modified assay allows for the measurement of MBCs from smaller blood volumes than was possible with the original assay, facilitating field studies.

3.2 Results

3.2.1 Quantifying TT-specific MBCs by flow cytometry

To quantify the number of TT-specific MBCs in the peripheral blood of vaccinated U.S. individuals we used a sensitive flow cytometry-based assay for detecting MBCs described by Amanna and Slifka (11). This method allows the detection of low frequency antigen-binding MBCs by using antigens labeled with two different fluorophores at sub-saturating levels. To detect TT-specific MBCs we incubated peripheral blood PBMCs with TT conjugated to Alexa Fluor 488 (TT-Alexa 488) and biotinylated TT (b-TT) followed by streptavidin-conjugated APC (SA-APC). As a control antigen we used biotinylated human serum albumin (b-HSA) and SA-APC. Gating on

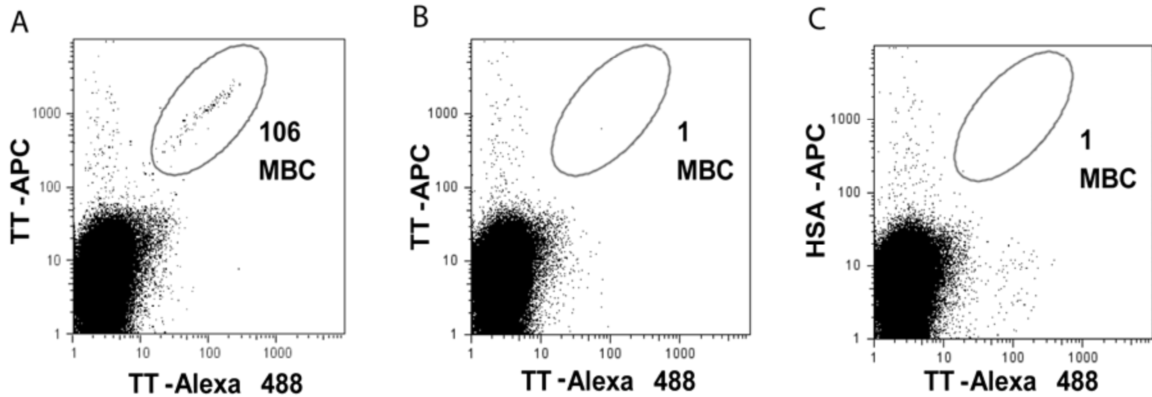


Figure 3.1 The frequency of TT-binding MBCs in vaccinated U.S. individuals. (A) TT-specific MBC are identified by gating on MBCs that stain positive for TT- and TT-b/SA-APC Alexa 488. **(B)** Cold competition control, where cells are incubated with unlabeled TT before staining with TT-b/SA-APC and TT-Alexa 488. **(C)** Mis-matched antigen control, where cells are stained with TT-b/SA-APC and HAS-Alexa 488.

$CD20^+$, $CD27^+$, IgD^- MBCs, we detected approximately 30 cells/10^6 PBMCs that bound to both b-TT/SA-APC and to TT-Alexa 488 (Fig. 3.1 A). Similar results were obtained defining MBCs as $CD19^+$ and $CD27^+$. The specificity of the staining was confirmed using a cold competition control where PBMCs were incubated with unlabelled TT prior to staining with TT-Alexa 488 and b-TT/SA-APC (Fig. 3.1 B). A mis-matched antigen control, was also used in which case PBMCs were stained with TT-Alexa Fluor 488 b-HSA/SA-APC (Fig. 3.1 C). Essentially no double labeled cells were detected in either case. The frequency of approximately 30 TT-specific MBCs per 10^6 PBMCs detected in our PBMC samples is on the order of that reported by Amanna and Slifka (11) who observed 107 TT-specific MBCs per 10^6 $CD20^+$ B cells, assuming that $CD20^+$ B cells were 15%-20% of total PBMC in their assay.

3.2.2 Quantifying TT-specific MBCs by limiting dilution ELISPOT

To determine the frequency of TT-specific MBCs in the *in* vitro culture assay described by Crotty *et al.* we modified the assay, culturing PBMC in complete media containing pokeweed mitogen, Staphylococcus aureus Cowen and CpG (PSC) at limiting dilution (LD) in 96 well plates rather than in bulk culture 24 well plates. We determined that LD, when ≥ 66% of the wells were negative, was reached when cells were cultured at 100,000-50,000 PBMC per well in 96 well plates for TT-specific MBCs, and 5,000-500 PBMC per well for total IgG^+ MBCs. Wells were scored positive when there were three or more ASCs per well, and a minimum of 48 wells were used per dilution per condition. We detected approximately three TT-specific MBCs per 10^6 PBMCs (Table 3.1). Thus, only approximately one in ten TT-specific MBCs detected by flow cytometry responded by differentiating into ASCs in the LD assay *in vitro*.

3.2.3 Increasing the efficiency of the MBC ELISPOT assay

To optimize the efficiency of the ELISPOT assay, several cytokines and stimulatory factors known to have a role in the differentiation of MBCs and ASCs, including anti-CD40, IL-2, IL-21, IL-6, IL-4, IL-10 and BAFF, were added to in various

Table 3.1 Efficiency of MBC ELISPOT with PSC stimulation

Number of cells per well	Percent of wells positive	# responding tetanus-specific MBCs /10^6 PBMC	Assay efficiency
100 x 10^5	27%	2.7	11.1%
50 x 10^5	12.5%	2.5	10.3%

combinations with PSC to cultures in 24 well plates. Combinations included: PSC with IL-4, IL-5, IL-6, IL-10, BAFF or anti-CD40; PSC+ IL-4+ BAFF; PC+ IL-10; PSC+ IL-4+ BAFF; IL-4+ BAFF, anti-CD40+ IL-4; anti-CD40+ IL-10, and BAFF+ IL-21. The number of total ASCs detected on anti-Ig coated ELISPOT plates from the 24 well plate cultures was only consistently significantly increased by a combination of PSC and 25ng/ml IL-10. Adding concentrations greater than 25ng/ml IL-10 did not further increase the number of ASCs. LD analyses showed that as compared to PBMCs cultured in PSC alone, the addition of IL-10 to PSC increased the number of responding MBCs by approximately ten fold (Fig. 3.2 A). We also determined the burst size or the number of ASCs in each positive well. Within individuals we found no difference in the average ASC burst size in PSC- versus PSC+IL-10-containing cultures (Fig. 3.2 B).

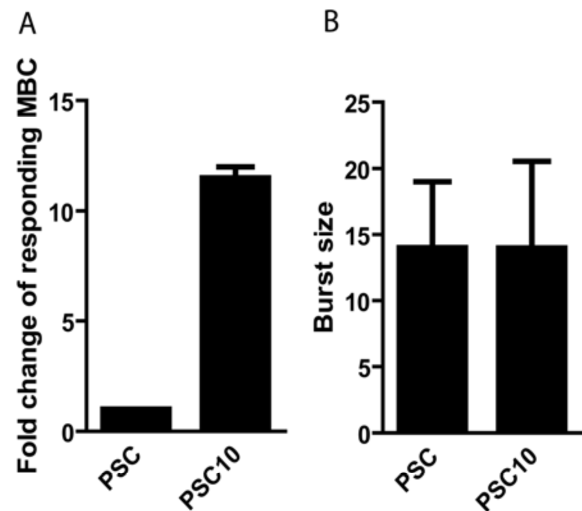


Figure 3.2 The addition of IL-10 to PSC increased the efficiency of MBC differentiation to PC by 10 fold. Comparison of two stimulation cocktails at LD, PSC, or PSC+IL-10. **(A)** Average fold change in the number of IgG^+ MBC differentiating into ASC in PSC or PSC+IL-10, with PSC set to 1 in order to compare multiple experiments. **(B)** Average number of daughter ASC arising from each responding IgG^+ MBC in PSC or PSC+IL-10.

3.2.4 Establishing the selectivity of the modified assay for MBCs

In order to confirm that only MBCs were responsive to stimulation with PSC+IL-10, B cells were sorted into MBCs ($CD19^+$, $CD27^+$ gating out $CD27^{++}$, $CD38^{+++}$ plasma cells), naïve B cells ($CD19^+$, $CD27^-$) and PCs ($CD19^+$, $CD27^{++}$, $CD38^{+++}$). These populations

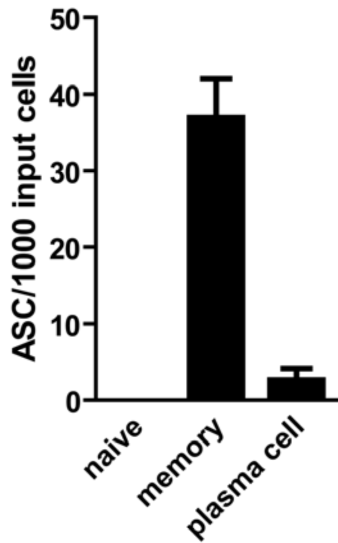


Figure 3.3 Testing the selectivity of the PSC+IL-10 stimulation cocktail for MBCs. Naïve B cells ($CD19^+CD27^-$), MBCs ($CD19^+$ $CD27^+$), and PCs ($CD19^+CD27^{++}$ $CD38^{+++}$) were sorted and cultured with $CD19^+$ B cell-depleted PBMCs in PSC+IL-10. Shown are the number of IgG+ ASC per 1000 sorted input B-cells.

had a purity of ≥ 98.2% for MBCs, 99.9% for naïve B cells, and 94.1% for plasma cells, of B cells as estimated by flow cytometry. For these experiments non-B-cells ($CD19^-$) were added back to sorted populations of B cells to simulate the environment present in whole PBMC cultures. The combination of PSC + IL-10 (PSC10) stimulated MBCs to differentiate into ASCs and had no measurable effect on the differentiation of naïve B cells (Fig. 3.3). Approximately four ASCs were detected per 1000 input cells in the PC population that could be due to a low frequency of PCs surviving in the five-day cultures. If so, in whole PBMCs in which PCs represent approximately 0.5-2.0% of B cells, these PCs would contribute an additional 0.012% of the IgG producing ASCs.

3.2.5 Detecting *Pf*-specific MBCs in individuals living in malaria endemic areas.

In collaboration with Francis M. Ndungu and Kevin Marsh of the Kenya Medical Research Institute we used the PSC10 LD cultures to determine the frequency of MBCs specific for TT and two *Pf* antigens in the PBMCs of adults living in a malaria endemic area of Kenya. The results showed an average frequency of 19 TT-specific MBCs per 10^6 PBMC in 12 adults (Fig. 3.4), similar to the frequency of $27/10^6$ PBMCs reported here for U.S. volunteers. The average frequencies of MBCs specific for two *Pf* proteins, namely

AMA1 and MSP1, were 11 and 18 per 10^6 PBMCs measured in 13 and 11 individuals respectively (Fig. 3.4). LD analyses are more expensive, time consuming and laborious than the bulk cultures for reading out total ASCs. We therefore determined if there was a correlation between the frequency of MBCs determined in LD and the number of ASCs measured from bulk cultures (Fig. 3.5).

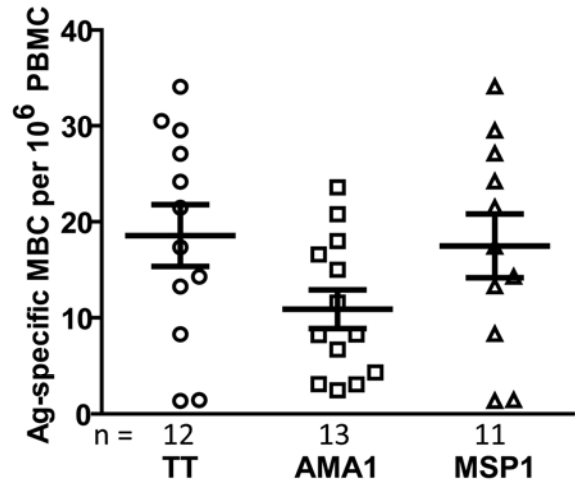


Figure 3.4 The frequency of antigen-specific MBCs by LD in PBMCs of *Pf*-exposed adults. Total PBMC were plated in 5-graded dilutions (from 10^4 to 10^5) in 18 replicate wells, each, and stimulated with PSC10 over a 10 day culture period. Supernatants were analyzed by ELISA for TT-, AMA1-, and MSP1-specific IgG. Shown are the mean ± SEM of antigen specific MBC/10^6 PBMC. Data obtained by Francis M. Ndungu

Shown are the numbers of total IgG-ASCs (Fig. 3.5 A), TT-, MSP1- and AMA1-specific ASCs per 10^6 PBMCs (Fig. 3.5 B). We observed a correlation between the number of MBCs detected in LD and the number of ASCs in five day bulk culture for TT and AMA1

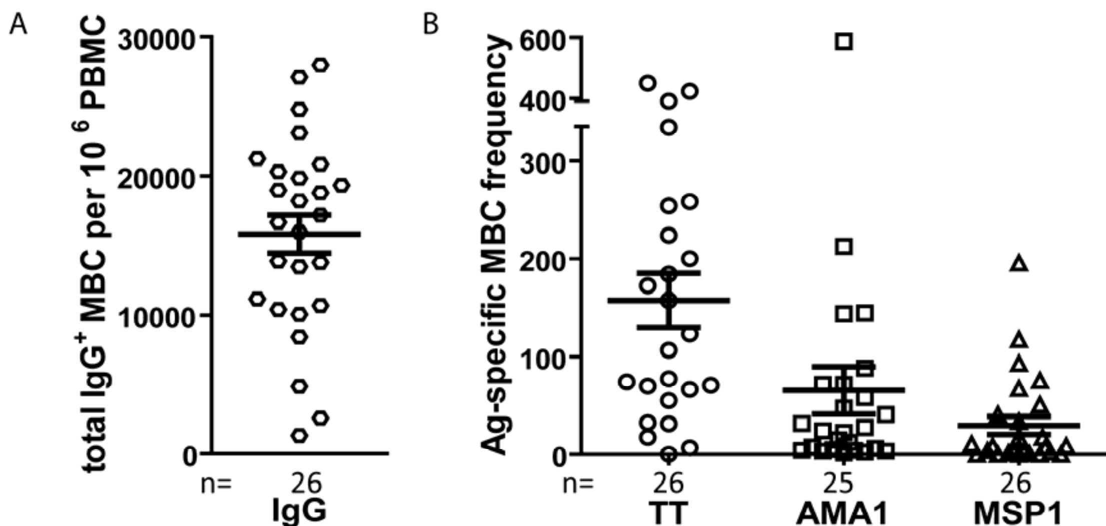


Figure 3.5 Numbers of total IgG⁺ and antigen-specific ASCs in bulk assay cultures of PBMC from malaria exposed adults. PBMCs were cultured for 5 days with PSC10. Shown are the mean ± SEM of IgG^+ **(A)** or antigen-specific **(B)** ASC/10^6 cultured PBMC. Data obtained by Francis M. Ndungu.

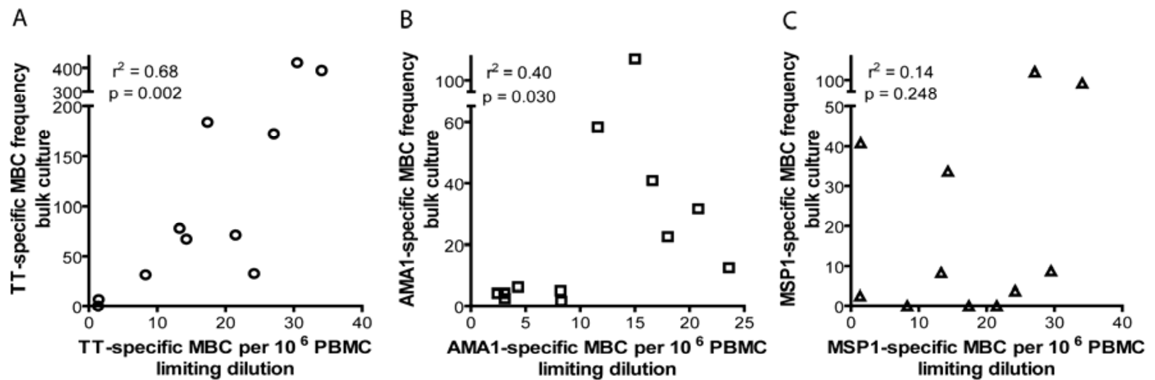


Figure 3.6 Correlations between the frequencies of antigen-specific MBCs determined by LD and antigen-specific ASC by bulk assay cultures. Antigen-specific correlations shown for adults exposed to malaria for **(A)** TT, **(B)** AMA-1 and **(C)** MSP-1. Spearman rank correlations are shown. Data obtained by Francis M. Ndungu.

(Fig. 3.6). Thus, the 24 well cultures appear to be a good tool to measure the relative frequencies of MBCs. It is of interest that the ratio of MBCs from LD and ASCs from bulk cultures is approximately 1:7 for TT-specific cells and approximately 1:2 for *Pf*-specific MBCs, suggesting that the ability of *Pf*-specific MBCs to differentiate into ASCs *in vitro* may be reduced. It will be of interest to determine if this is a phenomenon associated with the differentiation of MBCs during *Pf* infections.

3.3 Discussion

MBCs are important in immunity and it is of interest to develop highly efficient assays that provide the tools for assessing MBCs. We have optimized and characterized the MBC ELISPOT assay described by Crotty et al., and specifically tested its applicability for detecting *Pf*-specific MBCs in the PBMCs of individuals in a malaria endemic area. Here we report that only approximately ten percent of antigen-specific MBCs enumerated by flow cytometry respond in a limiting dilution version of the *in vitro* assay described by Crotty *et al.* and that the efficiency of this assay can be significantly

improved to nearly one hundred percent by the addition of IL-10 to the five day cultures. IL-10 is known to induce MBCs to differentiate into PCs (17, 211) and the addition of IL-10 has been shown to result in a 17 fold increase in ASCs derived from MBCs as compared to cultures without IL-10 (38). It has also been shown that IL-10 preferentially induces MBCs to differentiate into ASCs, while having little effect on the differentiation of naïve B-cells (16). Thus, IL-10 was one of the better candidates in increasing the sensitivity of the assay without disrupting the specificity for detecting MBCs. As IL-10 causes an increase of MBCs that respond to stimulation without affecting burst size, IL-10 could contribute to controlling the stringency of MBC differentiation into ASCs *in vitro*. As IL-10 is well known to decrease a cellular immune response and influence the development of CD4+ helper T cells, this cytokine could have other functions in furthering a humoral immune response, however the relevance of this observation *in vivo* has yet to be determined. We provide evidence that this modified assay can be successfully used to quantify *Pf*-specific MBCs in semi-immune adults in Kenya.

Collectively, the results presented here demonstrate that the efficiency of the *in vitro* assay described by Crotty *et al.* to detect antigen-specific MBCs can be increased significantly by the addition of IL-10 to the cultures. The addition of IL-10 increased the frequency of the MBCs activated in culture but did not influence the ASC burst size nor did it alter the selectivity of the assay for MBCs. In addition the assay proved efficacious in detecting MBCs in field samples from adults living in a malaria endemic area in Africa.

The increased efficiency of this modified assay allows for the measurement of MBCs frequencies from smaller blood volumes than was possible with the original assay, which is important in field studies in children where blood volumes are limiting. The further application of this assay to the study of the acquisition and maintenance of *Pf*-specific B cell memory through natural infection may ultimately aid in the design and development of a badly needed malaria vaccine.

Chapter 4: In malaria-naïve individuals *P. falciparum*-specific memory B cells and antibodies develop efficiently in response to subunit vaccination with CpG as an adjuvant

4.1 Introduction

Because of the delay in development of clinical immunity to malaria, reports of very short half-lives of some *Pf* antigens, and the observation that some individuals with life-long exposure to *Pf* fail to have measureable Ab titers to commonly expressed proteins, it has been proposed that properties of the antigens in question prevent the efficient development of MBCs or LLPCs (100, 132). These observations as well as the renewed susceptibility to clinical infection of previously immune individuals who return to endemic areas, has caused some doubt as to whether immunologic memory to *Pf* can develop, and if memory is induced, whether it can be maintained. By using subunit vaccines in malaria-naïve individuals we can explore the ability of *Pf* antigens to induce MBCs and start to dissect the effect of individual antigens versus the immune environment induced during *Pf*-malaria on the development and maintenance of MBCs. Establishing whether MBCs and LLPCs can develop to these antigens is an important first step in vaccine development. In the vaccine trials presented here, malaria-naïve individuals were vaccinated with two blood stage antigens, AMA1 and MSP1. We demonstrate that in this context, MBCs and Ab develop readily to these two antigens.

The design of this vaccine trial also provided an opportunity to address another question important to the development of immune memory, namely; can the development of B cell immunity be enhanced in human vaccination by adding a TLR

ligand to the vaccine? Of particular interest in the generation of memory is the role of TLR9, a pattern recognition receptor that initiates innate immune responses. TLR9 detects microbial DNA with hypomethylated CpG motifs and in humans is preferentially expressed by plasmacytoid dendritic cells (PDC) and B cells [reviewed in (124). The net effect of TLR9 activation is the differentiation of Th1 cells and the induction of IgG isotype switching and Ab secretion. In a hepatitis B vaccine clinical trial the addition of CPG 7909, a B-class CpG oligodeoxynucleotide (ODN), accelerated the acquisition of specific Abs and increased peak Ab titers (92-94). CpG also has the capability to activate B cells independently of their antigen-specificity through bystander activation (18, 30). However, the impact of CpG on the generation of MBCs in response to primary immunization has not been delineated. Here we describe the kinetics of antigen-specific MBC acquisition in malaria-naïve individuals in response to vaccination with malaria antigens and provide evidence that CPG 7909 enhances this process in malaria-naïve individuals. This analysis was carried out in the context of two separate clinical trials of two candidate malaria subunit protein vaccines formulated on aluminum hydroxide gel (Alhydrogel), with and without CPG 7909, given to healthy malaria-naïve adults (149) (Martin *et al*., unpublished, www.clinicaltrials.gov #NCT00320658) .

4.2 Results

4.2.1 The acquisition of MBCs in naïve individuals in response to vaccination

To determine the kinetics, magnitude and longevity of MBCs generated in response to primary immunization of malaria-naïve volunteers to individual *Pf* antigens, as well as the impact of TLR9 activation on this process, we examined the acquisition of *Pf*-specific MBCs in malaria-naïve individuals enrolled in two Phase 1 clinical trials of two malaria subunit protein vaccine candidates, AMA1-C1 and $MSP1_{42}$-C1. Both AMA1-C1 and $MSP1_{42}$-C1 were formulated on Alhydrogel with and without CPG 7909. Longitudinal samples collected over approximately eight months were analyzed from 40 individuals, 20 from each trial, and within each trial half were vaccinated with CPG 7909-containing vaccines. Individuals were vaccinated on days 0, 28 and 56, and peripheral blood samples were collected at the times shown in Table 4.1. For both trials the mean viability of PBMCs after thawing was similar in the CPG and non-CPG groups, 92.3% and 95.9%, respectively (p=0.165).

MBC ELISPOTs were performed on all the PBMC samples, with each individual's samples analyzed in parallel to reduce variability in assay conditions. As there is not currently a standard way to analyze MBC ELISPOT data, we examined the MBC ELISPOT data both as the percentage of antigen-specific MBCs of the total IgG^+ MBCs, and MBCs as a frequency/10^6 PBMCs', referring to the number of PBMCs after the five-day culture. We examined both, as if there are significant changes in the IgG^+ MBCs due to bystander

Table 4.1 Sample size and mean vaccine-specific MBC percentage by vaccine type, CpG group and study day.

	AMA1-C1[a]				$MSP1_{42}$-C1[b]			
	Without CPG 7909		With CPG 7909		Without CPG 7909		With CPG 7909	
Study Day	*n*	% MBC[c] (95% CI)	*n*	% MBC (95% CI)	*n*	% MBC (95% CI)	*n*	% MBC (95% CI)
0[d]	10	0.03 (−0.16, 0.22)	10	0.02 (−0.77, 0.80)	11	0.01 (−0.05, 0.07)	9	0.01 (−0.38, 0.39)
3[e]	10	0.02 (−0.21, 0.26)	8	0.04 (−0.74, 0.82)				
7	10	0.01 (−0.20, 0.21)	9	0.05 (−0.73, 0.83)	11	0.01 (−0.05, 0.07)	9	0.004 (−0.38, 0.39)
28[d]	10	0.02 (−0.19, 0.23)	9	0.09 (−0.76, 0.94)	10	0.04 (−0.02, 0.10)	9	0.07 (−0.32, 0.45)
31	8	0.03 (−0.18, 0.24)	9	0.54 (−0.27, 1.36)	11	0.04 (−0.01, 0.10)	9	0.06 (−0.32, 0.44)
35[e]	10	0.09 (−0.14, 0.32)	8	2.94* (2.12, 3.75)				
56[d]	10	0.13 (−0.09, 0.35)	8	1.79* (0.97, 2.60)	10	0.10* (0.04, 0.15)	9	0.97* (0.59, 1.35)
59	8	0.11 (−0.11, 0.33)	9	1.58* (0.72, 2.43)	10	0.08* (0.02, 0.14)	8	1.20* (0.80, 1.60)
63[e]	7	0.26 (0.04, 0.48)	8	3.45* (2.55, 4.35)				
84[e]	8	0.75* (0.51, 0.98)	7	1.52* (0.67, 2.37)				
140	10	0.59* (0.37, 0.81)	9	1.44* (0.62, 2.26)	11	0.13* (0.08, 0.19)	9	0.83* (0.45, 1.21)
236[e]	7	0.15 (−0.21, 0.50)	4	1.41* (0.45, 2.37)				

[a] Missing data due to technical error (4.6%), individual lost to follow-up (5.8%), or unavailability of sample (7.5%).
[b] Missing data due to unavailability of sample (2.9%).
[c] Percentage vaccine-specific MBC and 95% CIs are obtained by fitting GEE models described in *Materials and Methods*.
[d] Vaccination day.
[e] Samples collected for AMA1-C1 trial only.
*, $p < 0.05$ vs study day 0.

activation via CpG for example, or alterations in non-B cell proportions due to activation with vaccination that persist through six-day cultures, the results could differ analyzed in these two ways. In the AMA1-C1 trial, prior to vaccination (baseline) the mean percentage of IgG^+ MBCs that were AMA1-C1-specific was not significantly different from the irrelevant control antigen KLH (percentage: 0.02% AMA1-C1-specific MBCs vs. 0.07% KLH-specific MBC; p=0.44). Similar patterns were observed for antigen-specific MBCs expressed as a percent of total IgG^+ MBCs or as the frequency per 10^6 PBMC post-culture. Compared to baseline, individuals receiving AMA1-C1/Alhydrogel without CPG 7909 did not have a statistically significant increase in AMA1-C1-specific MBCs until 28 days after the second vaccination as measured by frequency, and 28 days after the third vaccination as measured by percent (day 56: average frequency 58.4 p=0.012 vs. baseline; day 84: average percent 0.75% p<0.001 vs. baseline; Fig. 4.1 A,C). AMA1-C1-specific MBCs remained above baseline at 0.59% 84 days after the third vaccination (p<0.001 vs. baseline), and although the frequency of AMA1-C1-specific MBCs remained

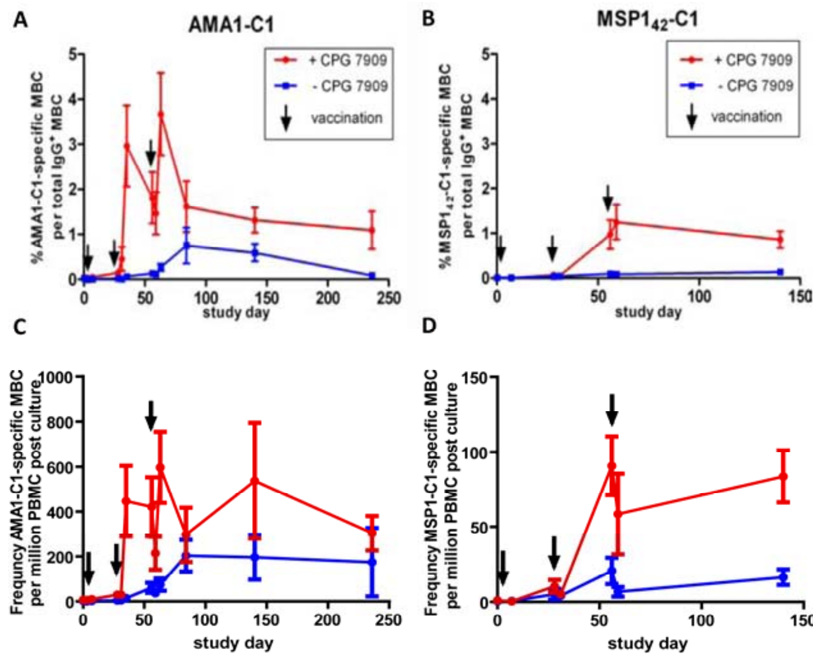


Figure 4.1 MBC are readily induced in malaria-naive individuals upon vaccination and CPG 7909 enhances the kinetics and magnitude of the AMA1-C1- and MSP1$_{42}$-C1-specific MBC response to vaccination. (A,C) Individuals vaccinated with AMA1-C1/Alhydrogel with CPG 7909 (red circles; n = 10) or without CPG 7909 (blue squares; n =10). **(B,D)** Individuals vaccinated with MSP1$_{42}$-C1/Alhydrogel with CPG 7909 (red circles; n = 9) or without CPG 7909 (blue squares; n = 11). Data are reported as mean percentage of IgG ± SEM. **(A,B)** or frequency per 10^6 PBMC ± SEM **(C,D)** The sample size at each time point is given in Table 4.1.

constant above baseline from 28 days after the third vaccination until the last timepoint measured (p=0.001 vs. baseline), AMA1-C1-specific MBCs decreased as a percentage of total IgG$^+$ MBCs to baseline levels approximately six months after the last vaccination (p=0.57 vs. baseline). At all other time points, the percentage of AMA1-C1-specific MBCs did not differ significantly from baseline levels. Thus, in response to vaccination with AMA1-C1/Alhydrogel (with or without CPG 7909) AMA1-C1-specific MBCs were generated, and these MBCs persisted at detectable levels six months after the last vaccination. In contrast, a dramatic increase in AMA1-C1-specific MBCs was observed in individuals vaccinated with AMA1-C1/Alhydrogel plus CPG 7909. In this group AMA1-C1-specific MBCs appeared in the peripheral circulation three days after the second vaccination and peaked three and seven days later as a percent and a frequency respectively, at 2.94% of all IgG$^+$ MBCs and 447/10^6 PBMC, and then decreased to 1.79% and 421/10^6 PBMC 21 days later on the day of the third vaccination (Fig. 4.1 A, C).

Seven days after the third vaccination the percentage peaked again at 3.45% and $596/10^6$ PBMC and then contracted to 1.41% and $303/10^6$ PBMC by the end of the study (236 days after the first vaccination), a rate of decline of approximately 0.4% per month. AMA1-C1-specific MBCs were significantly increased over baseline as a frequency at all time points beginning 28 days after the first vaccination and as a percent at all time points beginning seven days after the second vaccination through the end of the study period ($p<0.010$ vs. baseline). Compared to individuals who received AMA1-C1/Alhydrogel without CPG 7909, the mean percentage of AMA1-C1-specific MBCs was higher at all time points after vaccination, reaching statistical significance as a percentage on days 35, 56, 59 and 63 (all $p<0.010$) and marginal statistical significance on days 140 and 236 ($p=0.092$ and $p=0.061$, respectively). Similarly, as a frequency, AMA1-C1-specific MBCs reached statistical significance on days 35, 56, 63, 140 (all $p<0.040$) and marginal statistical significance on day 28 ($p=0.062$). Thus, the inclusion of CPG 7909 enhanced the kinetics and magnitude of the AMA1-C1-specific MBC response.

CPG 7909 had a similar impact on the acquisition of MBCs in response to vaccination with $MSP1_{42}$-C1/Alhydrogel (Fig. 4.1 B,D). Compared to the AMA-C1 trial, fewer PBMC samples were collected in the $MSP1_{42}$-C1 trial, namely, on the day of each vaccination (days 0, 28 and 56), seven days after the first vaccination, three days after the second and third vaccination, and on day 140 (Table 4.1). Prior to vaccination (baseline) the mean percentage of IgG^+ MBCs that were $MSP1_{42}$-C1-specific was not significantly different from KLH (0.01% $MSP1_{42}$-C1-specific MBCs vs. 0.07% KLH-specific

MBC; p=0.89). Vaccination with $MSP1_{42}$-C1/Alhydrogel without CPG 7909 did not generate statistically significant levels of $MSP1_{42}$-C1-specific MBCs as a percent and as a frequency, until 28 days after the second vaccination, reaching 0.10% of all IgG^+ MBCs (p=0.005 vs. baseline) and a frequency of $21/10^6$ PBMC (p=0.003 vs. baseline). The percentage and frequency of $MSP1_{42}$-C1-specifc MBCs remained greater than baseline at three and 84 days after the third vaccination (day three after third vaccination, 0.08% p=0.022 vs. baseline, $7/10^6$ PBMC p=0.005 vs. baseline; day 84 after third vaccination, 0.13%, p<0.001 vs. baseline, $16/10^6$ PBMC p=0.002 vs. baseline). Vaccination with $MSP1_{42}$-C1/Alhydrogel with CPG 7909, by contrast, generated a mean percentage of $MSP1_{42}$-C1-specific MBCs of 0.97% and frequency of $91/10^6$ PBMC on the day of the third vaccination (p≤0.001 vs. baseline) and remained at 1.20% and 0.83%, and a frequency of $59/10^6$ PBMC and $84/10^6$ PBMC, at three and 84 days after the third vaccination (p<0.001, p=0.001, p=0.002, and p<0.001 respectively, vs. baseline). Compared to individuals who received MSP1-C1/Alhydrogel without CPG 7909, the mean frequency of MSP1-C1-specific MBC/10^6 PBMC was higher at all time points starting 28 days after the first vaccination, reaching statistical significance on days 28, 56, 59 and 140 (all p<0.040) and marginal statistical significance on day 31 (p=0.090). Thus, the inclusion of CPG 7909 enhanced the kinetics and magnitude of both the AMA1-C1- and MSP1-C1-specific MBC responses. Because samples were not collected seven days after the second and third vaccination in the $MSP1_{42}$-C1 trial, we do not know whether the MBC percentage reached higher peak levels, as observed in the

AMA1-C1 trial. Thus, despite the difference in the ability of AMA1-C1 and $MSP1_{42}$-C1 to generate vaccine-specific MBCs in the absence of CPG 7909, the CPG 7909-containing vaccines resulted in a similar percentage of vaccine-specific MBCs at the end of each study, approximately 1% of total IgG^+ MBCs, compared with approximately 0.1% for the non-CPG 7909-containing vaccines. Frequencies of antigen-specific MBCs induced did, however, vary significantly between the AMA1-C1 and MSP1-C1 vaccines.

4.2.2 The acquisition of MBCs mirrors and predicts Ab responses in malaria-naïve vaccine recipients

In humans the role MBCs play in maintaining Ab titers and LLPCs remains unclear. The longitudinal design of this study allowed an examination of the relationship between MBCs and Ab titers in a manner not possible in cross-sectional analyses. In general we observed that vaccination with either AMA1-C1 or $MSP1_{42}$-C1 on Alhydrogel

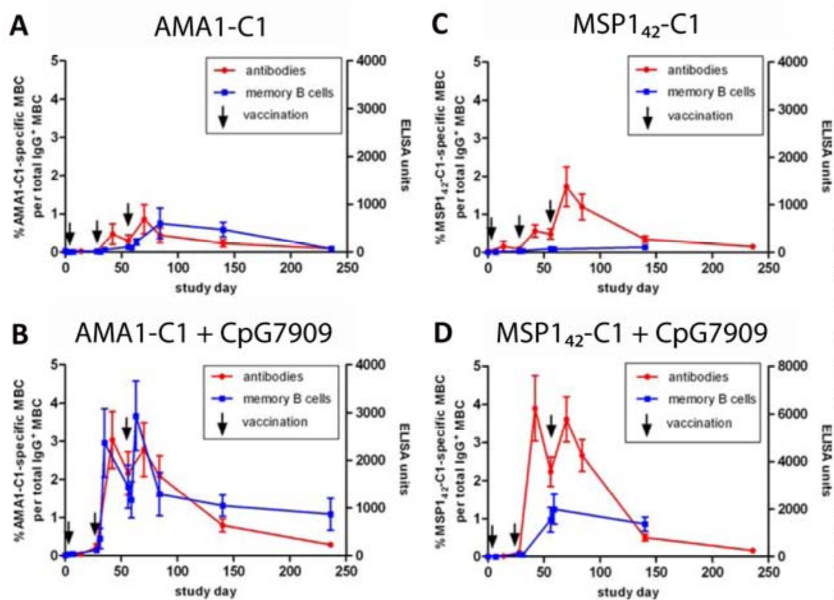


Figure 4.2 The AMA1-C1- and MSP142-C1-specific Ab response mirrors the corresponding MBC response and is enhanced by CPG 7909. The Ab levels (red circles) determined by ELISA are given for individuals vaccinated with AMA1-C1/Alhy-drogel **(A,B)** or MSP142- C1/Alhydrogel **(C,D)** without CPG 7909 **(A,C)** or with CPG 7909 **(B,D)**. Ab levels for each vaccine are the average of the 3D7 and FVO responses. The corresponding percentages of Ag-specific MBC are given for comparison (blue squares). Data are reported as mean percentage (MBC) or mean ELISA units (Ab) ± SEM. The sample size at each time point is given in Table 4.1.

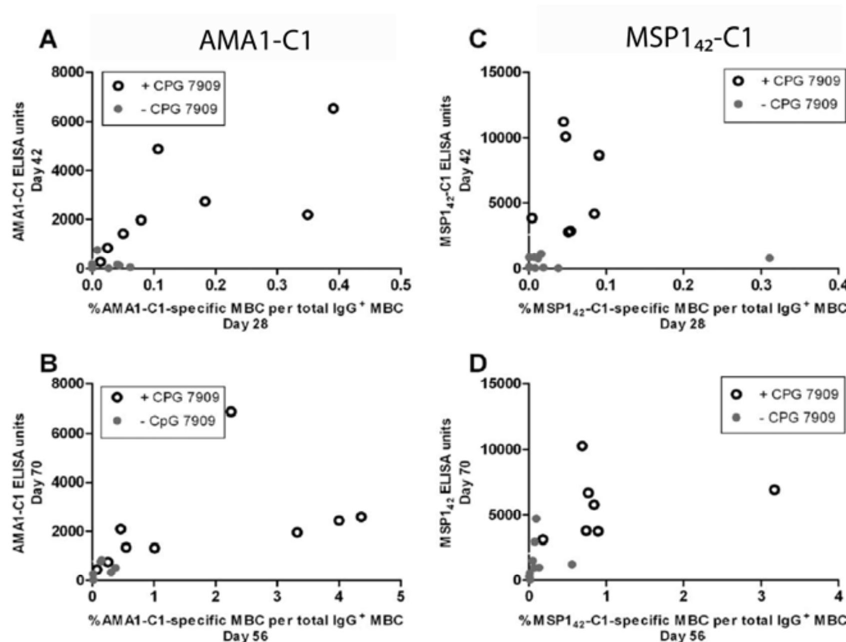
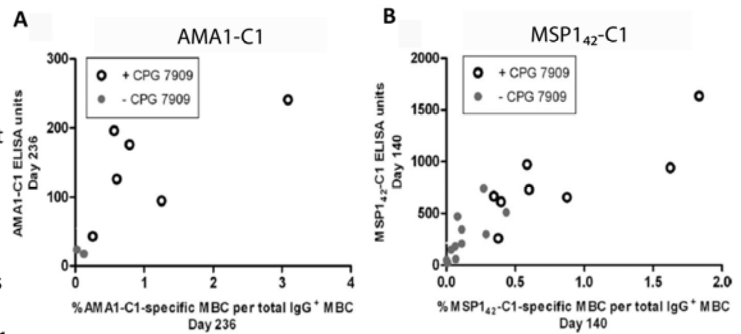


Figure 4.3 The level of Ag-specific MBC at the time of booster vaccination predicts the Ab response 14 days later. For AMA1-C1 the percentage of Ag-specific MBC at the time of the second and third vaccinations predicted the levels of AMA1-C1 Ab 14 days later **(A)** second vaccination, r = 0.70, p = 0.003 **(B)** third vaccination, r = 0.87, p < 0.001. A similar relationship was observed for the MSP142-C1 vaccine **(C)** second vaccination, r = 0.47, p = 0.057 **(D)** third vaccination, r = 0.83, p < 0.001. At these time points corresponding ELISPOT and ELISA data were available for 15 individuals in the AMA1-C1 trial and 17 individuals in the MSP142-C1 trial. Ab levels for each vaccine are the average of the 3D7 and FVO responses.

generated Ab levels that correlated with the vaccine-specific MBC response, and for both vaccines the inclusion of CPG 7909 induced higher levels of Abs and MBCs (Fig. 4.2). We also observed that the percentage of AMA1-C1-specific MBCs on the day of the second and third vaccination (days 28 and 56) was highly correlated with the levels of AMA1-C1 Abs 14 days later (days 42 and 70) (Fig. 4.3 A) [second vaccination, r=0.70, p=0.003; Fig. 4.3 B) third vaccination, r=0.87, p=<0.001)}. The majority of the Ab response likely represents the differentiation of MBCs into short-lived PCs given the rapid decline in titers that followed. In the $MSP1_{42}$-C1 trial we observed a similar relationship between MBCs at the time of revaccination and Ab titers 14 days later in the $MSP1_{42}$-C1 trial (Fig. 4.3 C second vaccination r=0.47, p=0.057; Fig. 4.3 D third vaccination r=0.83, p<0.001). To determine the relationship between antigen-specific

Figure 4.4 At steady-state, levels of Ag-specific MBC and Ab are highly correlated. To determine the correlation between Ag-specific MBC and Ab titers closer to steady-state, the last time point with corresponding ELISPOT and ELISA data **(A)** AMA1-C1 trial day 236 **(B)** MSP-1_{42}-C1 trial day 140 was analyzed in cross-section. A positive correlation between Ag-specific MBC and Ab titers was observed in both trials at steady-state (AMA1-C1, r = 0.80, p = 0.014; MSP1_{42}-C1, r = 0.86, p < 0.001). Corresponding ELISPOT and ELISA data were available for 9 individuals in the AMA1-C1 trial and 19 individuals in the MSP1_{42}-C1 trial at this timepoint. Ab levels for each vaccine are the average of the 3D7 and FVO responses.



MBCs and Ab titers at steady state (approximately three and six months after the last MSP1_{42}-C1 and AMA1-C1 vaccination, respectively), the last time point with corresponding MBC and ELISA data was analyzed in cross-section. We observed a positive correlation between antigen-specific MBCs and Ab titers in both trials [Fig. 4.4 A AMA1-C1, r=0.80, p=0.003; Fig. 4.4 B MSP1_{42}-C1, r=0.86, p<0.001). Since LLPCs are the likely source of Ab titers at this later time point, the correlation between MBCs and Ab titers suggests that the maintenance of LLPCs may be linked to MBCs.

4.2.3 Vaccination influences MBCs and PCs independently of antigen specificity

An examination of total IgG^+ MBCs circulating in the periphery showed that vaccination affected this population independently of antigen (Fig. 4.5). Vaccination with AMA1-C1/Alhydrogel plus CPG 7909 was associated with a decrease in the frequency of IgG^+ MBCs three days after each vaccination, followed by a gradual return to baseline (Fig. 4.5 B), although the decrease was only statistically significant after the third vaccination (p=0.012) At the same time points, the frequency of IgG^+ MBCs in those vaccinated with AMA1-C1/Alhydrogel without CPG 7909 did not show a consistent

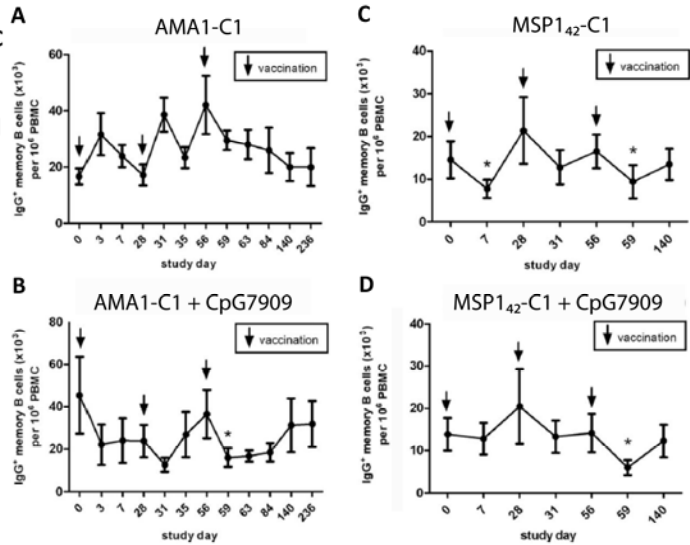


Figure 4.5 Vaccination appears to have an Ag-independent effect on the total IgG^+ MBC pool. Shown is the total IgG^+ MBC response expressed as the mean frequency for individuals immunized with AMA1-C1/Alhydrogel **(A,B)** or MSP1$_{42}$-C1/Alhydrogel **(C,D)** without CPG 7909 **(A,C)** or with CPG 7909 **(B,D).** Days on which there was a statistically significant ($p < 0.05$) decrease in the total IgG+ MBC frequency, as compared with the total IgG+ MBC frequency on the day of the preceding vaccination, are indicated with an asterisk. For AMA1-C1 there were 10 individuals in both the non-CPG 7909 and CPG 7909 groups. For MSP1$_{42}$-C1 there were 11 individuals in the non-CPG 7909 group and 9 individuals in the CPG 7909 group. Note that x-axis intervals are not proportional to the time intervals indicated.

response (Fig. 4.5 A). Vaccination with MSP1$_{42}$-C1 with or without CPG 7909, was associated with a decrease in the frequency of IgG^+ MBCs seven days after the first vaccination, and three days after the second and third vaccinations, followed by a return to baseline (Fig. 4.5 C, D). The decrease was statistically significant after the third vaccination in the MSP1$_{42}$-C1 with CPG 7909 group (p=0.018), and after the first and third vaccinations in the MSP1$_{42}$-C1 without CPG 7909 group (p=0.021 and p=0.037, respectively). Thus, MBCs appear to transiently leave the circulation after vaccination. To determine if there was a concomitant increase in total PC numbers indicating a polyclonal activation of MBCs, fresh PBMC from a subset of individuals in the AMA1-CI study were analyzed by flow cytometry (fresh PBMC from the MSP1$_{42}$-C1 trial were not available). Irrespective of the CPG 7909 status, three days after the second and third vaccination with AMA1-C1/Alhydrogel, there was an increase in $CD27^+CD38^{+++}$ PCs as a percentage of total $CD3^-CD19^+$ B cells which was statistically significant after the second vaccination in the CPG 7909 group (p<0.01). We have confirmed by direct ELISPOT on

freshly sorted cells that in our hands $CD19^{+}CD27^{+}CD38^{+++}$ cells secrete antibody while $CD19^{+}CD27^{+}$ MBCs, $CD19^{+}CD27^{-}$ naïve B cells, and $CD19^{-}$ non-B cells do not secrete antibody. Thus it appears that vaccination with TLR-containing vaccines might have the potential to polyclonally activate MBCs, independently of antigen, and that this activation may drive the differentiation of MBCs into PCs, according to one of the models we discussed in Chapter 1.5 (130).

4.3 Discussion

In this longitudinal study we examined the effect of *Pf*-subunit vaccination on MBC generation in malaria-naïve individuals and determined the impact of TLR9 activation on this process *in vivo*. MBCs and Abs developed reliably and robustly in response to AMA1 or MSP1 vaccination in malaria-naïve individuals, with antigen-specific MBCs persisting at least six months in the case of AMA1 and at least three months in the case of MSP1. This indicates that there are no overt intrinsic properties of these antigens which prevent a humoral response from developing. In support of this, another study found that vaccination with another blood-stage *Pf*-antigen, MSP3, also in malaria-naïve volunteers, induced an Ab response lasting at least one year (65). The results presented here offer new insights into the kinetics of the development of Abs and MBCs and provide evidence that the innate immune receptor TLR9 plays a significant role not only in the generation of MBCs in naïve individuals but may also affect the expansion of MBCs and the differentiation of existing MBCs into PCs. For the two protein subunit malaria vaccine candidates, AMA1-C1 and $MSP1_{42}$-C1, the inclusion

of CPG 7909 had a dramatic effect on malaria naïve individuals, resulting in a more rapid acquisition of vaccine-specific MBCs, in greater numbers. Antibody titers to MSP1 and AMA1 remained statistically significantly higher in individuals receiving vaccines with CPG 7909 versus without CPG 7909 at days 238 and 236 respectively (74, 149). Interestingly, using one of these, the AMA1-C1 vaccine, in a cohort of semi-immune adults in Mali, the addition of CPG 7909 did not enhance the acquisition of vaccine-specific MBCs either kinetically or numerically (190). The mechanistic meaning of this apparent refractoriness to TLR9 activation is of considerable interest for the purpose of vaccine design.

The longitudinal design of this study of the response of naïve individuals to vaccination permitted a detailed characterization of the kinetics of MBC generation and maintenance in response to primary and secondary vaccination. The capacity for a detailed characterization was most apparent in the analysis of the AMA1-C1 vaccine trial in which PBMC samples were collected at several time points after each vaccination. We observed that AMA1-C1-specific MBCs peaked in the peripheral circulation seven days after the second and third vaccinations, representing approximately 3-4% of the total IgG^+ MBC pool. Although it has been reported for diphtheria vaccination that the magnitude of the peak MBC response decreased with each booster immunization (150), we did not observe a significant difference between peaks in this study [day 35 CPG 7909 group, 2.94% (95% CI, 2.12-3.75) vs. day 63 CPG 7909 group, 3.45% (95% CI, 2.44-4.35); p=0.328]. The differences between the studies may be due to the length of time

between vaccination or the efficacy of the vaccines themselves. It is of interest that the second AMA1-C1 vaccination generated AMA1-C1-specific MBCs at levels comparable to those observed after influenza (173, 208) and smallpox (48) booster vaccination. Irrespective of CPG 7909 status, the rate of decline of AMA1-C1-specific MBCs was approximately 0.4% per month. If this rate held steady, within two years the level of AMA1-C1-specific MBCs would approach pre-immune levels in the CPG 7909 group. However, we do not know whether, or at what level, the antigen-specific MBC pool reaches equilibrium. In a cross-sectional study 18 months after smallpox vaccination, antigen-specific MBCs, as a percentage of the total IgG^+ MBCs, decreased to 0.1% from a peak of 1% 14 days after vaccination (48). Similarly, in individuals receiving influenza booster vaccinations, influenza-specific MBCs increased from low levels before vaccination to 8.2% of IgG^+ MBCs 14 days after vaccination, and then declined rapidly to <1% 80 days post-vaccination (208). Based on these observations, AMA1-C1-specific MBCs would be expected to reach equilibrium at ~0.3% within a year after the final vaccination.

Although TLR9 expression has been reported to be low in naïve B cells and constitutively high in MBCs (29), the impact of this differential expression on the *in vivo* responsiveness to CpG in humans at the cellular level is not known. As measured by the MBC response, we observed no effect of CPG 7909 on primary immunization with AMA1-C1 or $MSP1_{42}$-C1, suggesting that CPG 7909 had little effect on naïve B cells directly, or indirectly through TLR9-expressing PDCs, However, once generated by

primary immunization, TLR9-expressing antigen-specific MBCs responded dramatically to secondary immunization in the presence of CPG 7909. Although the relative impact of TLR9 activation in PDCs versus MBCs on the secondary response *in vivo* is not known, it is clear from the results of our *in vitro* experiments described in Chapter 3, that purified MBC differentiate into ASCs upon TLR9 activation, as has been shown by others (46).

Interestingly, the mechanisms underlying the apparent expansion and contraction of circulating antigen-specific MBCs still need to be elucidated. The contraction phase may represent migration of MBCs to lymphoid tissue where newly generated MBCs compete for limited homeostatic niches in the MBC compartment. Alternatively, in a manner analogous to T cell antigen-driven expansion and contraction, contraction may represent an activation-induced cell death phenomenon (96). Another possibility is the differentiation of a large portion of MBCs into SLPCs. What remains unknown is which factors control the magnitude of the peak response and the subsequent steady state level.

The results presented here also address the controversy surrounding the relationship between MBCs, LLPCs, and serum Ab levels. In general, we observed a positive correlation between the magnitude of the vaccine-specific MBC response and Ab titers. We also observed that the percentage of vaccine-specific MBCs present at the time of the second and third vaccinations predicted Ab titers two weeks later as has been observed. The majority of this Ab was likely produced by short-lived PCs given the rapid decline in titers that followed. Similar results were recently reported for infants

immunized with the serogroup C meningococcal conjugate vaccine in which the frequency of specific MBCs at the time of boosting correlated with post-vaccination titers (31). However, the Ab titers we observed closer to steady state (approximately three and six months after the last $MSP1_{42}$-C1 and AMA1-C1 vaccination, respectively) were likely produced by LLPCs, and thus the correlation between MBCs and Ab titers at steady state suggests that the maintenance of LLPCs may be linked to MBCs. The cellular and molecular nature of this relationship remains poorly understood.

In addition to the antigen-specific induction of MBCs, we observed an approximately two fold antigen-independent decrease in the frequencies of total IgG^+ MBCs in circulation three days after the majority of vaccinations. This drop may reflect the migration of MBCs into lymphoid tissues, apoptosis of MBCs, differentiation of MBCs into SLPCs or LLPCs, or a combination thereof. The concurrent increase in $CD27^+CD38^{+++}$ PCs we observed three days after the second and third vaccinations in the AMA1-C1/Alhydrogel study suggests that the decline in MBCs may be due in part to their differentiation into PCs. In a separate phase I study of AMA1-C1/Alhydrogel without CPG 7909, we observed a similar increase in PCs three days after vaccination (unpublished). The hypothesis that polyclonal activation can drive the differentiation of MBCs into PCs is supported by other studies that have examined the antigen-independent effects of vaccination on PCs. Bernasconi *et al.* observed an increase in ASCs directed against *Toxoplasma gondii* and measles six days after vaccination with tetanus toxoid (30). These authors attributed this to the polyclonal activation and differentiation of all MBCs

into PCs. Odendahl *et al.* also observed an increase in circulating ASCs of unknown specificity six days after vaccination with tetanus toxoid, but interpreted the ASCs to be LLPCs displaced from the bone marrow by newly generated tetanus-specific PCs that better competed for bone marrow PC niches (155). However, a recent study showed that up to one third of circulating ASCs appearing after influenza vaccination were not vaccine-specific, and had recently divided, ruling out the possibility that these were LLPCs displaced from the bone marrow (134). Collectively, these findings are consistent with a model in which the decrease in total IgG^+ MBCs (which we observed after vaccination) is due in part to their polyclonal activation and differentiation into PCs (130). The results of this study also provide an important baseline for our further investigation of the B cell response to malaria vaccine candidates and natural infection with *P. falciparum*.

Chapter 5: The design of the longitudinal study in Kambila, Mali and cohort description.

5.1 Introduction

To address fundamental questions related to the generation and maintenance of MBCs and Abs specific for *Pf* malaria during natural infections, we conducted a year-long prospective study in a rural village of Mali that experiences an intense, sharply-demarcated six-month malaria season annually. In this chapter we describe the cohort study that we designed to address the questions of how Ab responses to *Pf* develop (Chapter 6), whether *Pf* infection generates MBCs specific for *Pf* blood stage antigens, and if so, what is the pattern of acquisition of these MBCs (Chapter 7), and whether there are any phenotypic changes in B cells in malaria-exposed individuals (Chapter 8). Here we describe the study design, cohort demographics, clinical outcomes and our analysis of confounding factors in this cohort. The seasonal transmission of *Pf* at our study site, the focusing of the cohort on young children who are in the process of developing immunity to malaria and the longitudinal nature of the study gave us a rare opportunity to study the natural acquisition of MBCs and Abs in response to infection rather than vaccination.

5.2 Results

5.2.1 Description of the cohort demographics

In May 2006 we initiated an observational cohort study in Mali to investigate the mechanisms underlying naturally-acquired malaria immunity. The study population was

an age-stratified, random sample representing 15% of all individuals living in a small, rural, well-circumscribed, non-migratory community where antimalarial drugs were provided exclusively by the study investigators. At this site, 237 individuals were screened, and 12 individuals were excluded based on one of the following criteria; hemoglobin level <7 g/dL, fever ≥37.5°C, acute systemic illness, use of antimalarial or immunosuppressive medications in the past 30 days, and pregnancy. During a two-week period one month prior to the abrupt onset of the six-month malaria season, we enrolled 225 individuals in four age groups: 2–4 years (n=73), 5–7 years (n=52), 8–10 years (n=51), and 18–25 years (n=49). Attendance at scheduled follow-up visits was >99% for children (2-10 years) and 82% for adults (18-25 years) during the one-year study period indicating a high degree of study awareness and participation. Table 5.1 shows baseline demographic and clinical characteristics according to age group. Overall, 115 (51.1%) of 225 subjects were female, and the predominant ethnic groups were Bambara (134 (59.6%)) and Sarakole (77 (34.2%)). The prevalence of asymptomatic

Table 5.1 Baseline clinical and demographic characteristics of entire cohort, according to age group.

Characteristic	Age group 2–4 years (*n* = 73)	5–7 years (*n* = 52)	8–10 years (*n* = 51)	18–25 years (*n* = 49)	All (*N* = 225)
Female sex	42 (57.5)	25 (48.1)	19 (37.3)	30 (61.2)	115 (51.1)
Ethnicity					
Bambara	48 (65.8)	26 (50.0)	27 (53.0)	33 (67.4)	134 (59.6)
Sarakole	21 (28.8)	23 (44.2)	20 (39.2)	13 (26.5)	77 (34.2)
Fulani	3 (4.1)	3 (5.8)	3 (5.9)	2 (4.1)	11 (4.9)
Malinke	1 (1.4)	0 (0.0)	1 (2.0)	1 (2.0)	3 (1.3)
Plasmodium falciparum smear					
Positive result at enrollment[a]	4 (5.5)	4 (7.7)	5 (9.8)	3 (6.1)	16 (7.1)
Parasitemia, geometric mean no. of parasites/μL (95% CI)	1438 (159–12,973)	3616 (1500–8715)	415 (134–1287)	953 (39–23,382)	1137 (579–2232)
GI helminth infection at enrollment[b]	9 (13.0)	4 (8.3)	4 (9.3)	0 (0.0)	17 (9.0)
Schistosoma haematobium in urine at enrollment[c]	0 (0.0)	0 (0.0)	2 (4.3)	9 (28.1)	11 (6.0)
Hemoglobin level at enrollment, mean ± SD, g/dL	11.2 ± 1.2	11.8 ± 1.0	12.3 ± 1.1	13.6 ± 1.4	12.1 ± 1.5
Distance of residence from clinic, mean ± SD, meters	393 ± 109	393 ± 135	378 ± 105	360 ± 92	382 ± 112
Bed net use[d]	21 (28.8)	16 (30.8)	9 (17.6)	15 (30.6)	61 (27.1)

NOTE. Data are no. (%) of subjects, unless otherwise indicated. CI, confidence interval; GI, gastrointestinal.
[a] All subjects were asymptomatic at enrollment.
[b] Data available for 190 subjects.
[c] Data available for 184 subjects.
[d] Nightly bed net use self-reported at the end of the malaria season.

parasitemia at enrollment was 7.1% (16 subjects) and did not vary significantly with age ($p=0.83$). Of note, asymptomatic parasitemia during the dry season is commonly observed in Mali (56, 58). Of 190 subjects with stool sample data available, 17 (9.0%) showed evidence of intestinal helminthes; of 184 subjects with urine sample data available, 11 (6.0%) showed evidence of *S. haematobium* infection. These prevalences were lower than expected, likely due to community-wide albendazole treatment prior to this study. The mean distance between the study clinic and subjects' residences was 382 meters (range, 127–881 meters), and 61 (27.1%) of 225 participants self-reported nightly bed net use during the rainy season.

5.2.2 Analysis of confounding factors

Age is well known as a covariate of immunity in malaria endemic areas. Genetic red blood cell (RBC) polymorphisms that are reported to affect susceptibility to *Pf* include HbS (sickle cell trait), HbC, α-thalassemia, glucose-6-phosphate dehydrogenase (G6PD) deficiency, as well as blood group O. Although the mechanisms of protection remain to be fully elucidated, individuals with the genotype HbAS, HbAC or HbCC have a reduced risk of clinical malaria compared to individuals with a HbAA genotype (77). These polymorphisms are common in the regions of Africa where studies of *Pf*-malaria are conducted. The prevalence of sickle cell trait (HbAS), for example, exceeds 25% in some areas (146). Although these RBC polymorphisms are associated with decreased risk of malaria, as measured by odds ratios (91, 101, 142, 168, 206) or incidence rate ratios (205), their impact on the time to first malaria episode is not known. In our

Table 5.2 Frequency of red blood cell polymorphisms, according to age group.

	Age group				
Polymorphism	2–4 years	5–7 years	8–10 years	18–25 years	All
Hemoglobin type					
AA	50/69 (72.5)	43/50 (86.0)	35/51 (68.6)	32/42 (76.2)	160/212 (75.5)
AS	8/69 (11.6)	4/50 (8.0)	5/51 (9.8)	5/42 (11.9)	22/212 (10.4)
AC	10/69 (14.5)	3/50 (6.0)	11/51 (21.6)	5/42 (11.9)	29/212 (13.7)
CC	1/69 (1.5)	0/50 (0.0)	0/51 (0.0)	0/42 (0.0)	1/212 (0.5)
*G6PD**A–					
Female					
Normal	32/42 (76.2)	23/25 (92.0)	15/19 (79.0)	23/26 (88.5)	93/112 (83.0)
Heterozygous	6/42 (14.3)	2/25 (8.0)	3/19 (15.8)	3/26 (11.5)	14/112 (12.5)
Homozygous	4/42 (9.5)	0/25 (0.0)	1/19 (5.3)	0/26 (0.0)	5/112 (4.5)
Male					
Normal	26/29 (89.7)	18/24 (75.0)	27/30 (90.0)	14/16 (87.5)	85/99 (85.9)
Hemizygous	3/29 (10.3)	6/24 (25.0)	3/30 (10.0)	2/16 (12.5)	14/99 (14.1)
α-thalassemia					
Normal	35/67 (52.2)	34/51 (66.7)	26/51 (51.0)	30/42 (71.4)	125/211 (59.2)
Heterozygous	32/67 (47.8)	15/51 (29.4)	25/51 (49.0)	12/42 (28.6)	84/211 (39.8)
Homozygous	0/67 (0.0)	2/51 (3.9)	0/51 (0.0)	0/42 (0.0)	2/211 (1.0)
ABO blood group					
O	20/67 (29.9)	15/49 (30.6)	16/48 (33.3)	12/26 (46.2)	63/190 (33.2)
A	27/67 (40.3)	13/49 (26.5)	11/48 (22.9)	7/26 (26.9)	58/190 (30.5)
B	16/67 (23.9)	15/49 (30.6)	14/48 (29.2)	5/26 (19.2)	50/190 (26.3)
AB	4/67 (6.0)	6/49 (12.2)	7/48 (14.6)	2/26 (7.7)	19/190 (10.0)

NOTE. Data are no. of subjects with a given trait/no. of subjects for whom data was available (%). There were 225 subjects enrolled: 73 in the 2–4 years group, 52 in the 5–7 years group, 51 in the 8–10 years group, and 49 in the 18-25 group.

cohort, HbAS and HbAC phenotypes were present in 22 (10.4%) and 29 (13.7%) of 212 individuals, respectively (Table 5.2). The prevalence of the *G6PD**A$^-$ genotype was 17.0% (19 of 112) among females (heterozygous and homozygous individuals) and 14.1% (14 of 99) among males (hemizygous individuals). $^-$α/αα and $^-$α/$^-$α genotypes were found in 84 (39.8%) and two (1.0%) of 211 individuals, respectively. Because of the low frequency of the $^-$α/$^-$α genotype, individuals with this genotype were excluded from further analyses. Blood groups O, B, A, and AB were identified in 63 (33.2%), 58 (30.5%), 50 (26.3%), and 19 (10.0%) of 190 individuals, respectively.

Table 5.3 Malaria outcomes for the entire cohort, by age group.

	Age group					
Outcome	2-4 years (n=73)	5-7 years (n=52)	8-10 years (n=51)	18-25 years (n=49)	All (n=225)	*P*
Malaria incidence, mean ± SD, no. of episodes[a]	1.99 ±1.25	1.94 ±1.21	0.98 ±1.05	0.08 ± 0.28	1.33 ± 1.30	<0.001
Severe malaria, no. of episodes[b]	4	1	0	0	5	
≥ 1 malaria episode, no. (%) of subjects	63 (86.3)	45 (86.5)	31 (60.8)	4 (8.2)	143 (63,6)	<0.001
Time to first malaria episode, median, days[c]	101	114	130	153	115	0.016
Parasitemia at first malaria episode, geometric mean no. of parasites/µl (95% CI)	34,374 (24,955-47,348)	15,687 (9,623-25,574)	10,433 (5,079-21,427)	8,816 (4,082-19,037)	19,625 (15,004-25,688)	0.036

[a] A malaria episode was defined as axillary temperature ≥ 37.5°C, asexual parasitemia ≥ 5000 parasites/µl, and a nonfocal physical examination by the study physician.
[b] In accordance with the World Health Organization definition of severe malaria (1).
[c] Days since study enrollment.

Importantly, several features of this study favored an unbiased detection of malaria episodes: (1) the study population was an age-stratified, random sample representing 15% of all individuals living in a well-circumscribed, non-migratory community, (2) follow-up at scheduled visits during the course of the study was >99% for children and 82% for adults, indicating a high degree of study awareness and participation, (3) antimalarial drugs were provided exclusively by the study investigators who were available at all times at the only easily accessible health care facility, and (4) the average distance of individuals' homes to the study clinic was 0.38 kilometers (range: 0.13 – 0.88 kilometers), minimizing geographic and logistic

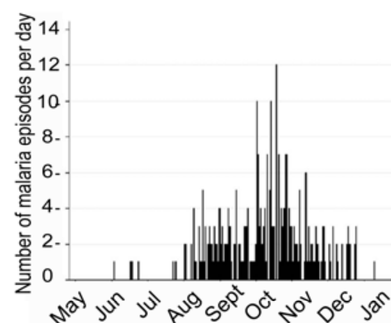


Figure 5.1 Number of clinical malaria episodes per day from May 2006 to January 2007 for the whole cohort. There were 298 clinical episodes defined as axillary temperature ≥ 37.5°C, *Pf* asexual parasitemia ≥5000 parasites/µLand a nonfocal physical examination by the study physician.

barriers to study participation. A record of the number of malaria cases per day from May 2006 to January 2007 (Fig. 5.1) illustrates the intense, seasonal malaria transmission at this site. During 495 clinic visits that occurred during the study period, 298 episodes of malaria were diagnosed. Table 5.3 summarizes malaria outcomes according to age group. As expected, malaria incidence decreased with age ($P < 0.001$), and the five cases of severe malaria were confined to children five years old or younger. Among those who presented with malaria, the median time to first malaria episode (as measured in days from study enrollment) increased with age (101 days for subjects two to four years old vs. 153 days for subjects 18–25 years old; p=0.016). The geometric mean parasite density per microliter at time of the first malaria episode decreased with age (p=0.036).

Because adult subjects had few episodes of malaria (Table 5.3), the analysis of RBC polymorphisms and malaria outcomes focused on children aged two to ten years. Time-to-event analysis (Fig. 5.2 A) showed that HbAS was associated with a significant delay in the time to first malaria episode (p=0.038 by log rank test). Among children who presented with malaria, HbAS was associated with a median 34-day delay to the first episode, compared with the non-HbAS group (median, 145 days for the HbAS group vs. 111 days for the non-HbAS group; p=0.017). HbAS was also associated with a 53% reduction in malaria incidence (mean malaria incidence, 0.82 episodes for the HbAS group [95% confidence interval {CI}, 0.48 –1.17] vs. 1.76 episodes for the non-HbAS group [95% CI, 1.56 –1.96]; p=.003). Although the geometric mean parasite densities at

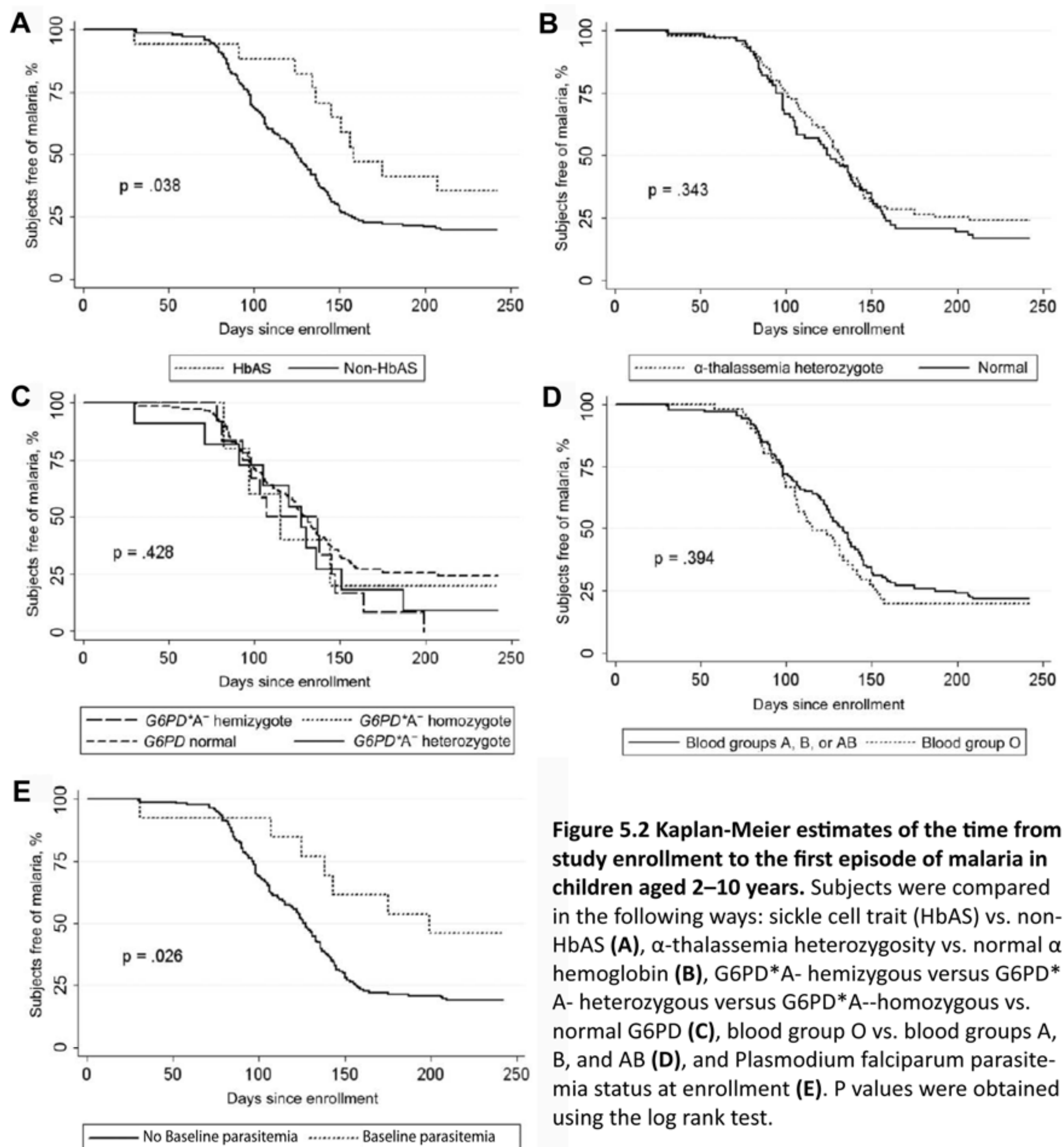


Figure 5.2 Kaplan-Meier estimates of the time from study enrollment to the first episode of malaria in children aged 2–10 years. Subjects were compared in the following ways: sickle cell trait (HbAS) vs. non-HbAS **(A)**, α-thalassemia heterozygosity vs. normal α hemoglobin **(B)**, G6PD*A- hemizygous versus G6PD*A- heterozygous versus G6PD*A--homozygous vs. normal G6PD **(C)**, blood group O vs. blood groups A, B, and AB **(D)**, and Plasmodium falciparum parasitemia status at enrollment **(E)**. P values were obtained using the log rank test.

the time of the first malaria episode were lower in HbAS children, this difference was not statistically significant (9,033 parasites/µL in the HbAS group [95% CI, 1,364–59,825] vs. 21,257 parasites/µL in the non-HbAS group [95% CI, 16,312–27,701]; p=0.83). The five cases of severe malaria occurred in non-HbAS children (3 HbAA children and two HbAC children). HbAC phenotype, *G6PD**A⁻ genotype (hemizygosity, heterozygosity,

and homozygosity), -α/αα genotype, and blood group O were not associated with a delayed time to first malaria episode (Fig. 5.2 B–D) or a decreased incidence of malaria.

To assess for potential confounding of the protective effect of HbAS, factors that might influence malaria risk were stratified by Hb type (Table 5.4). While there was a higher proportion of females in the HbAS group (p=0.022), HbAS children did not differ significantly from non-HbAS children with regard to age, weight, ethnicity, distance of residence from the clinic, or frequency of *G6PD**A$^-$ and -α/αα genotypes. The prevalence of asymptomatic parasitemia at enrollment was 9.4% (12 of 128) among HbAA children and 0% (0 of 41) among HbAC and HbAS children. However, asymptomatic parasitemia at enrollment was associated with a delayed time to first

Table 5.4 Univariate analysis of baseline characteristics of children aged 2-10 years, stratified by hemoglobin (Hb) type.

Characteristic	Hemoglobin type			
	HbAA (*n* = 128)	HbAC (*n* = 24)	HbAS (*n* = 17)	*P*[a]
Age, mean ± SD, years	5.6 ± 2.3	6.0 ± 2.8	5.1 ± 2.2	.382
Female sex	59 (46.1)	12 (50.0)	13 (76.5)	.022
Weight, mean ± SD, kg	17.9 ± 5.4	18.6 ± 6.8	18.1 ± 8.1	.718
Ethnicity				
Bambara	72 (56.3)	14 (58.3)	8 (47.1)	.453
Sarakole	48 (37.5)	8 (33.3)	8 (47.1)	.435
Fulani	6 (4.7)	2 (8.3)	1 (5.9)	ND
Malinke	2 (1.6)	0 (0.0)	0 (0.0)	ND
Distance of residence from clinic, mean ± SD, meters	386 ± 111	395 ± 59	381 ± 177	.163
Bed net use[b]	38 (29.7)	5 (20.8)	2 (11.8)	ND
Plasmodium falciparum smear positive at enrollment	12 (9.4)	0 (0.0)	0 (0.0)	ND
GI helminth infection at enrollment	10 (8.6)	3 (13.6)	2 (12.5)	ND
Schistosoma haematobium detected in urine at enrollment	1 (0.9)	1 (4.4)	0 (0.0)	ND
*G6PD**A–[c]	22 (17.9)	3 (12.5)	3 (17.6)	.99
α-thalassemia, heterozygous	50 (41.3)	15 (62.5)	6 (37.5)	.792
Blood group O	40 (31.3)	9 (37.5)	2 (11.8)	ND

NOTE. Data are no. (%) of subjects, unless otherwise indicated. GI, gastrointestinal; ND, not done.

[a] For the comparison of HbAS versus non-HbAS (i.e., HbAA and HbAC) children (statistical test not done if <3 in subgroup).

[b] Nightly bed net use self-reported at the end of the malaria season.

[c] Includes heterozygous, hemizygous, and homozygous individuals.

malaria episode (Fig. 5.2 E; p=0.026, by log rank test) and a trend toward decreased malaria incidence. Thus, HbAS children were not delayed in their time to first malaria episode as a result of the effect of asymptomatic parasitemia. There were too few HbAS children to make meaningful comparisons with respect to the remaining variables.

It is unlikely that differential mosquito exposure confounded the association between HbAS and the time to first malaria episode because HbAS individuals appeared to be randomly distributed in this small, well-circumscribed village, which lacks a dominant body of water (Fig. 5.3). Moreover, the frequency of bed net use was evenly distributed between HbAS and non-HbAS individuals. It is also unlikely that access to the study clinic played a significant role because the distance to the clinic from the individuals' residences did not differ by hemoglobin type.

Cox regression analysis revealed that greater age (HR, 0.87 [95% CI, 0.80–0.94];

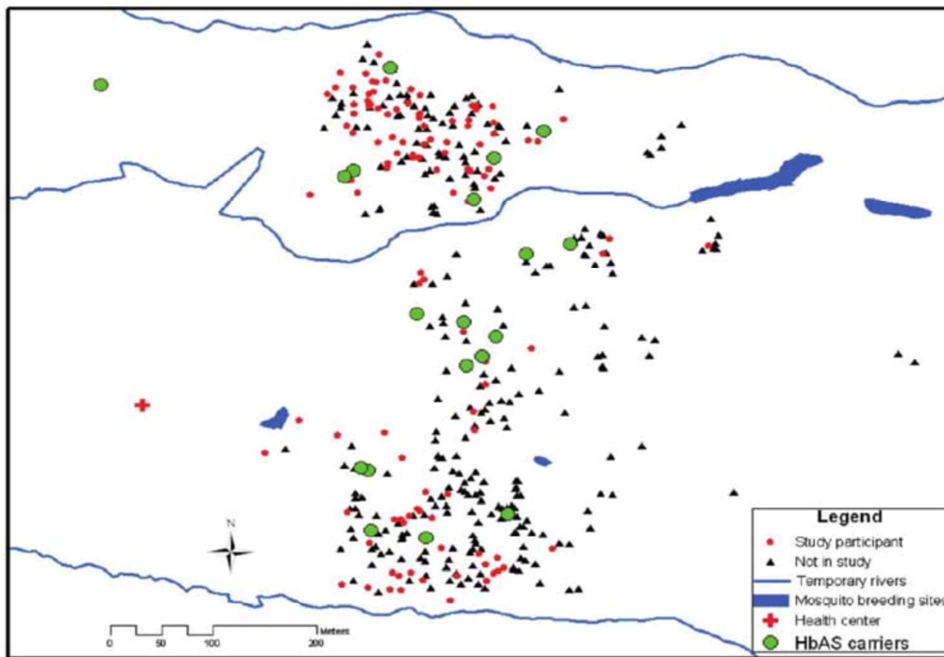


Figure 5.3 Distribution of study participants and sickle cell trait (HbAS) carriers who are study participants in the entire Kambila village population. Three houses include >1 HbAS carrier and symbols are superimposed on one another. The total number of HbAS carriers is 22.

p=0.001), HbAS phenotype (HR, 0.48 [95% CI, 0.26–0.91]; p=0.024), and asymptomatic parasitemia at enrollment (HR, 0.35 [95% CI, 0.14–0.85]; p=0.021) were associated with decreased malaria risk. Poisson regression analysis showed that greater age (incidence rate ratio [IRR], 0.90 [95% CI, 0.85– 0.95]; $P < 0.001$) and HbAS phenotype (IRR, 0.46 [95% CI, 0.27– 0.79]; p=0.005) were significant predictors of decreased malaria incidence, whereas asymptomatic parasitemia at enrollment was not ($P > 0.100$). Removal of the $^{-}\alpha/\alpha\alpha$ genotype from the analysis decreased the hazard ratio (HR, 0.44; p=0.020) and incidence rate ratio (IRR, 0.44; p=0.004) for HbAS, indicating negative epistasis between these polymorphisms. Factors that did not independently predict either measure of malaria risk (hazard ratio or incidence rate ratio) were sex, weight, distance of residence from clinic, bed net use, helminth infection, HbAC phenotype, _G6PD_*A$^-$ genotype (hemizygosity, heterozygosity, or homozygosity), $^{-}\alpha/\alpha\alpha$ genotype, and blood group O.

5.3 Discussion

Three of the confounding factors we analyzed, age, HbAS and asymptomatic parasitemia at enrollment, at the end of the dry season, were associated with clinical protection. RBC polymorphisms that are reported to affect susceptibility to *Pf* are common in the regions of Africa where studies of *Pf*-malaria are conducted, including HbS, HbC, α-thalassemia, G6PD deficiency, as well as blood group O. Although these RBC polymorphisms are associated with decreased risk of malaria, as measured by odds ratios (91, 101, 142, 168, 206) or incidence rate ratios (205), their impact on the time to

first malaria episode was not known. We found that HbAS was associated with a 34 day delay in the median time to first malaria episode, and this association remained statistically significant in multivariate Cox regression analysis. HbAS was unique in this regard as we found no association between HbAC, α-thalassemia, G6PD deficiency, or blood group O and a delayed time to first malaria episode. This observation along with the observation of increasing time to first malaria infection with increasing age has not been made previously to our knowledge, likely due to the paucity of prospective longitudinal studies of malaria to date. Our finding that HbAS was associated with a 34 day delay in the time to first malaria infection has direct applicability to vaccine trials, as the World Health Organization (WHO) recently recommended that the time to first malaria episode serve as the primary end point in phase III malaria vaccine trials. Given our findings it would be advisable for malaria vaccine trials and observational studies that use this end point to include Hb typing in the design of studies conducted in areas where HbAS is prevalent. As chronic asymptomatic parasitemia was found to be protective as well, it might be advisable to take this into consideration as well when initiating malaria vaccine trials. The other covariate of immunity in our study, age, is well known as such in malaria endemic areas.

The clinical outcomes we found in this cohort match general epidemiological observations with decreasing malaria risk and decreasing malaria incidence with increasing age. We observed no clusters of higher malaria incidence within the village, with malaria episodes spread fairly evenly geographically throughout the village. This

indicates that it is unlikely that exposure to *Pf* varies significantly across the cohort. We found the study population to be non-migratory (limiting exposure to increased or decreased *Pf* transmission), to live equidistant from the health center (medical care is equally accessible to all study participants), and to have a high degree of study awareness and participation. Overall this study site and cohort are well suited for the questions we aim to address.

Chapter 6: A prospective analysis of the Ab response to *P. falciparum* before and after the malaria season by protein microarray

6.1 Introduction

As discussed earlier it has been shown that purified IgG from malaria-immune adults, when transferred to children acutely ill with malaria, reduced fever and parasitemia (42), thus indicating that Abs against *Pf* proteins play a critical role in controlling the blood stage of the infection. However, which of the 5,400 possible *Pf* proteins (83) elicit the production of protective Abs is not known. A number of studies have been carried out in malaria endemic areas to identify a correlation between malaria immunity and an individual's *Pf*-specific Abs. These studies have been constrained to measuring Abs to the relatively few *Pf* proteins made available through traditional cloning methods (< 0.5% of the proteome) (198). Thus far, correlations between Abs to the relatively small number of *Pf* proteins that have been tested and malaria immunity have not been firmly established, suggesting either that Abs against these proteins do not play a role in protective immunity, or that Abs against single parasite proteins are insufficient to confer protection. To address these critical knowledge gaps we used *Pf* genome sequence data (83) and high throughput cloning and *in vitro* protein expression methods (52) to construct a protein microarray representing ~23% of the *Pf* proteome (1,204 known and hypothetical proteins). In a single assay, Abs against these proteins can be detected and quantified in the plasma of *Pf*-exposed individuals. In this study we analyzed 220 individuals from the Kambila

cohort (Described in Chapter 5.2.1) by probing plasma samples collected before and after the six-month malaria season to gain insight into the kinetics of the humoral immune response to *Pf* and to identify *Pf* -specific responses that are associated with protection from malaria.

6.2 Results

6.2.1 Validity and reproducibility of the protein microarray assay

To assess the validity of Ab reactivity against proteins expressed in the high-throughput translation system, we included three well-characterized, correctly folded *Pf* proteins being developed as malaria vaccine candidates on the same protein microarray chip—CSP, AMA1-C1, and MSP-2. Ab reactivity against these malaria vaccine candidates correlated with Ab reactivity against the same proteins expressed in the high-throughput system (CSP (r=0.77; $P < 0.001$), AMA-C1 (r=0.78; $P < 0.001$), and MSP-2 (r=0.96; $P < 0.001$); Fig 6.1). Each microarray chip also contained both positive and negative controls and an IgG standard curve to normalize data from arrays probed at different times (detailed in methods section 2.5.10.2). The reproducibility of the microarray assay was assessed by probing two microarray chips that had been printed on separate occasions with Ab against the 3' hemagglutinin tag. Reactivity against hemagglutinin on all spots for the two microarray chips was highly correlated (r=0.92; $P < 0.001$). To further assess the reproducibility of the microarray assay, we constructed a second smaller microarray chip containing the 49 proteins to which Ab reactivity was

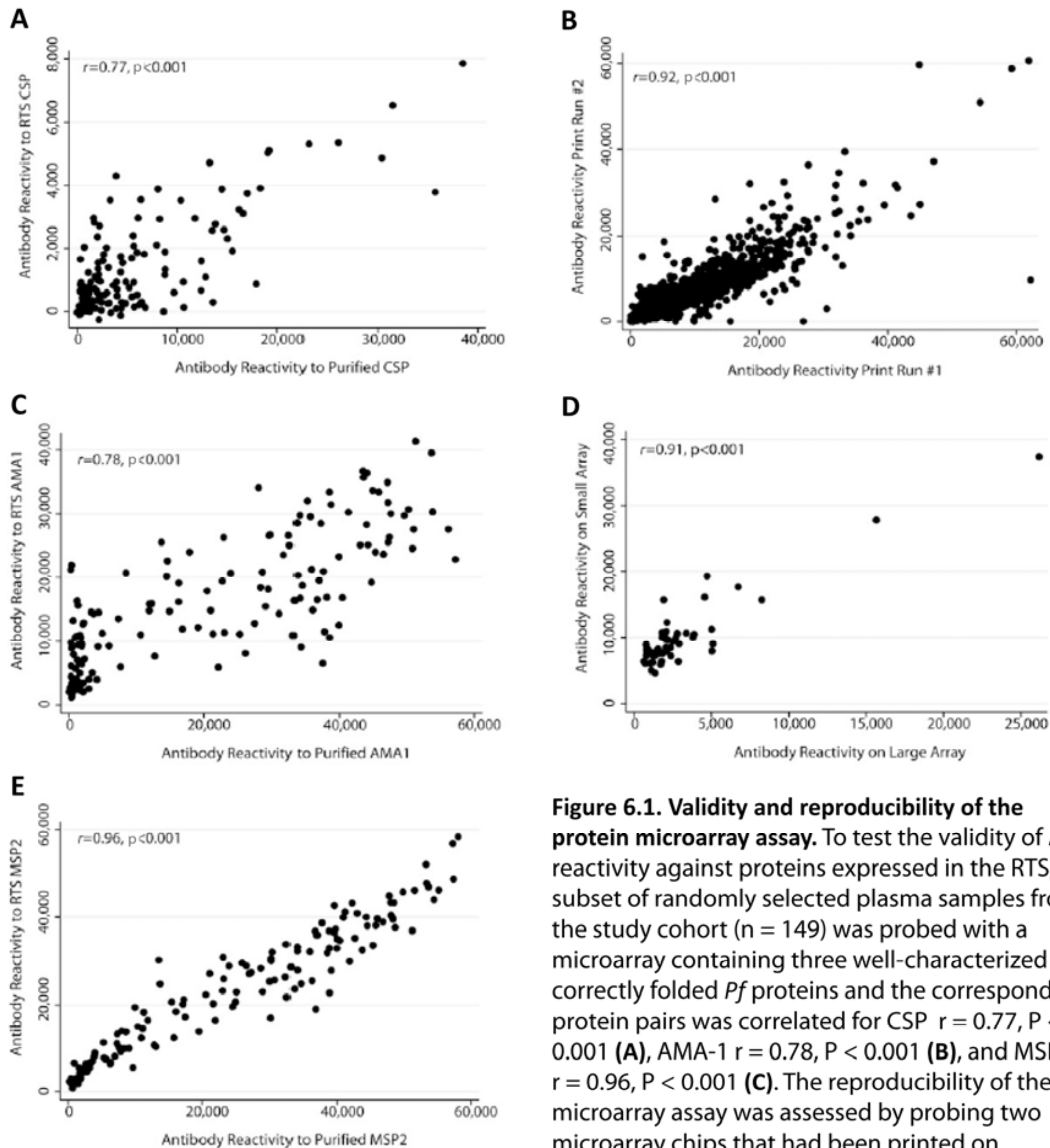


Figure 6.1. Validity and reproducibility of the protein microarray assay. To test the validity of Ab reactivity against proteins expressed in the RTS, a subset of randomly selected plasma samples from the study cohort (n = 149) was probed with a microarray containing three well-characterized correctly folded *Pf* proteins and the corresponding protein pairs was correlated for CSP r = 0.77, P < 0.001 **(A)**, AMA-1 r = 0.78, P < 0.001 **(B)**, and MSP-2 r = 0.96, P < 0.001 **(C)**. The reproducibility of the microarray assay was assessed by probing two microarray chips that had been printed on separate occasions with Ab against the 3′ hemagglutinin tag. Reactivity against hemagglutinin on all spots for the two microarray chips was highly correlated r = 0.92, P < 0.001 **(D)**. To assess the reproduc- ibility of the micro- array assay further, we constructed a second smaller microarray containing the 49 signature proteins identified on the larger microarray. A random subset of the original plasma samples (n = 149) was probed against this second array and showed that Ab reactivity against the 49 proteins for the same plasma samples tested on the smaller and larger microarrays was highly correlated r = 0.91, P < 0.001 **(E)**. Of note, the displacement in the intercept from zero in **(E)** is attributable to the difference in laser power.

associated with protection from malaria (described below in section 6.2.3). A random subset of the original plasma samples (n = 149) was probed against this smaller array.

The Ab reactivity against the 49 proteins for the same plasma samples tested on the smaller and larger microarrays was highly correlated (r=0.91; $P < 0.001$; Fig. 6.1 D).

6.2.2 Analysis of *Pf*-specific Ab profiles before and after the malaria season

We examined Ab reactivity to the 2,320 *Pf* proteins (representing 1,204 unique proteins) on the microarray in plasma collected before and after the six-month malaria season. Of the 225 individuals enrolled in the study, microarray data was available from 220 individuals before the malaria season and 194 individuals after the malaria season. Ab reactivity to 491 of the 2,320 proteins, measured before the malaria season, exceeded two SDs above the negative control (a rapid translation reaction into which an empty plasmid vector is placed). These 491 proteins are listed in Table S.1 and referred to here as immunogenic proteins. Based on mass spectrometry data (80) obtained from PlasmoDB (www.plasmodb.org), the life cycle stage at which these 491 proteins are maximally expressed is as follows: sporozoite (25.2%), merozoite (5.5%), trophozoite (16.8%), gametocyte (20.6%), and unknown (31.9%). Gene ontologic analysis indicated that ~40% of the immunogenic proteins are expressed in the membrane of the parasite or host erythrocyte, and that they are overrepresented in the biological process categories of "pathogenesis", "cytoadherence to microvasculature", "antigenic variation", and "rosetting" (Fig. 6.2, Table 6.1). In a study that used a smaller version of the same microarray platform (250 proteins), 32 'serodominant' proteins were identified in a study of *Pf*-exposed adults in Kenya (63), 26 of which were identified as immunogenic in this study (overlapping proteins are indicated by shading in Table S.1).

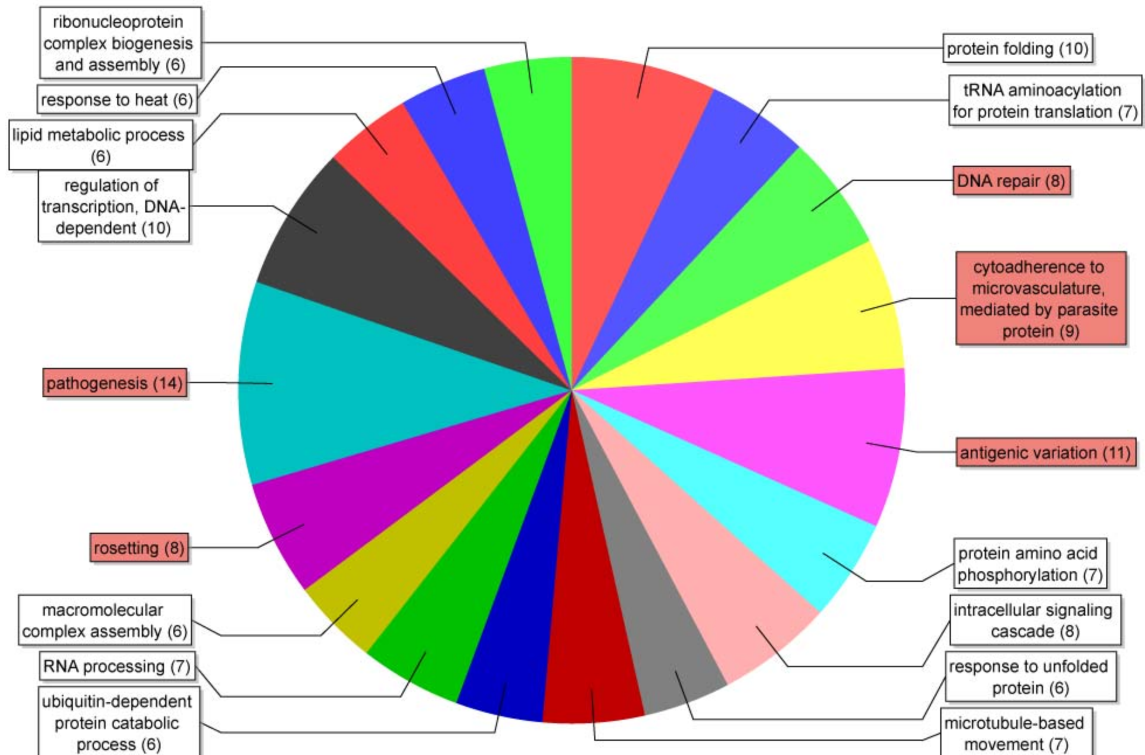


Figure 6.2 Gene ontology classification of the 491 immunogenic *Pf* proteins. Shown is the proportion of 491 immunogenic proteins expressed during the sporozoite and blood stages of the *Pf* life cycle based on mass spectrometry data obtained from PlasmoDB (www.plasmodb. org). Biological process gene ontology categories are shown. The size of each slice represents the number of proteins assigned to each category. Categories that are statistically significantly overrepresented relative to the entire microarray are indicated in red. Gene ontology categories with <5 assigned proteins were not included in the figure. The complete results of the gene ontology analysis for immunodominant proteins are given in Table 6.2.

Both the breadth and intensity of Ab reactivity to the 491 immunogenic proteins increased with age (Fig. 6.3 A), and from before to after the *Pf* transmission season (Fig. 6.3 B). The average number of proteins recognized by *Pf*-exposed individuals increased with age, both before ($P < 0.0001$; Fig. 6.3 C) and after ($P < 0.0001$; Fig. 6.3 C) the malaria season. The average number of proteins recognized by children in each age group increased from before to after the malaria season ($p<0.0001$ for each comparison; Fig. 6.3 C), whereas the number recognized by adults did not increase

Table 6.1. Gene ontology classifications overrepresented in the immunogenic *Pf* proteins relative to the entire *Pf* microarray.

GO Term	Name	FDR*	FWER†	single test p-value‡	# in test group	# in ref. group	# non annot test	# non annot ref. group
GO:0020002	host cell plasma membrane	7.21E-05	3.81E-05	1.13E-06	13	1	232	580
GO:0051809	passive evasion of immune response of other organism during symbiotic interaction	7.21E-05	5.72E-05	1.33E-06	11	0	234	581
GO:0020033	antigenic variation	7.21E-05	5.72E-05	1.33E-06	11	0	234	581
GO:0043657	host cell	7.21E-05	1.18E-04	3.54E-06	16	4	229	577
GO:0018995	host	7.21E-05	1.18E-04	3.54E-06	16	4	229	577
GO:0043245	extraorganismal space	7.21E-05	1.18E-04	3.54E-06	16	4	229	577
GO:0044419	interspecies interaction between organisms	7.21E-05	1.44E-04	3.90E-06	21	9	224	572
GO:0044403	symbiosis, encompassing mutualism through parasitism	7.21E-05	1.44E-04	3.90E-06	21	9	224	572
GO:0051704	multi-organism process	1.31E-04	3.27E-04	8.87E-06	21	10	224	571
GO:0044421	extracellular region part	1.31E-04	4.51E-04	1.12E-05	17	6	228	575
GO:0052564	response to immune response of other organism during symbiotic interaction	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0051805	evasion or tolerance of immune response of other organism during symbiotic	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0052173	response to defenses of other organism during symbiotic interaction	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0051834	evasion or tolerance of defenses of other organism during symbiotic interaction	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0051807	evasion or tolerance of defense response of other organism during symbiotic	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0051832	avoidance of defenses of other organism during symbiotic interaction	1.31E-04	5.25E-04	1.18E-05	11	1	234	580
GO:0050896	response to stimulus	1.31E-04	5.56E-04	1.32E-05	27	18	218	563
GO:0009405	pathogenesis	2.42E-04	0.001089	2.78E-05	14	4	231	577
GO:0020030	infected host cell surface knob	4.62E-04	0.002539	5.52E-05	8	0	237	581
GO:0007157	heterophilic cell adhesion	4.62E-04	0.002539	5.52E-05	8	0	237	581
GO:0020013	rosetting	4.62E-04	0.002539	5.52E-05	8	0	237	581
GO:0016337	cell-cell adhesion	4.62E-04	0.002539	5.52E-05	8	0	237	581
GO:0006952	defense response	4.63E-04	0.002657	5.64E-05	11	2	234	579
GO:0005576	extracellular region	4.75E-04	0.002848	6.48E-05	17	8	228	573
GO:0020035	cytoadherence to microvasculature, mediated by parasite protein	7.89E-04	0.005507	1.18E-04	9	1	236	580
GO:0044406	adhesion to host	7.89E-04	0.005507	1.18E-04	9	1	236	580
GO:0051825	adhesion to other organism during symbiotic interaction	7.89E-04	0.005507	1.18E-04	9	1	236	580
GO:0050839	cell adhesion molecule binding	7.89E-04	0.005507	1.18E-04	9	1	236	580
GO:0030430	host cell cytoplasm	0.001091	0.007877	1.66E-04	10	2	235	579
GO:0005539	glycosaminoglycan binding	0.001236	0.010144	1.90E-04	7	0	238	581

GO:0030246	carbohydrate binding	0.001236	0.010144	1.90E-04	7	0	238	581
GO:0030247	polysaccharide binding	0.001236	0.010144	1.90E-04	7	0	238	581
GO:0001871	pattern binding	0.001236	0.010144	1.90E-04	7	0	238	581
GO:0004872	receptor activity	0.001285	0.011184	1.94E-04	11	3	234	578
GO:0043656	intracellular region of host	0.001285	0.011184	1.94E-04	11	3	234	578
GO:0016020	membrane	0.002221	0.01979	4.48E-04	43	52	202	529
GO:0004197	cysteine-type endopeptidase activity	0.002873	0.026228	4.82E-04	9	2	236	579
GO:0051701	interaction with host	0.003069	0.028732	5.12E-04	12	5	233	576
GO:0004871	signal transducer activity	0.007197	0.069795	0.001269	11	5	234	576
GO:0060089	molecular transducer activity	0.007197	0.069795	0.001269	11	5	234	576
GO:0005488	binding	0.007197	0.07112	0.001324	155	300	90	281
GO:0006974	response to DNA damage stimulus	0.007316	0.077333	0.001371	8	2	237	579
GO:0006281	DNA repair	0.007316	0.077333	0.001371	8	2	237	579
GO:0009719	response to endogenous stimulus	0.007316	0.077333	0.001371	8	2	237	579
GO:0022610	biological adhesion	0.007447	0.080375	0.00138	10	4	235	577
GO:0007155	cell adhesion	0.007815	0.087743	0.00143	9	3	236	578
GO:0008234	cysteine-type peptidase	0.007815	0.087743	0.00143	9	3	236	578
GO:0031224	intrinsic to membrane	0.00843	0.098133	0.001885	16	12	229	569
GO:0016021	integral to membrane	0.00843	0.098133	0.001885	16	12	229	569
GO:0046872	metal ion binding	0.022674	0.25113	0.003881	38	51	207	530
GO:0043167	ion binding	0.022674	0.25113	0.003881	38	51	207	530
GO:0008270	zinc ion binding	0.024819	0.27586	0.004746	18	17	227	564
GO:0050794	regulation of cellular process	0.025929	0.29086	0.005116	21	22	224	559
GO:0006350	transcription	0.028185	0.31812	0.006073	15	13	230	568
GO:0046914	transition metal ion binding	0.028185	0.321402	0.006371	23	26	222	555
GO:0050789	regulation of biological process	0.029869	0.341892	0.007184	21	23	224	558
GO:0051056	regulation of small GTPase mediated signal transduction	0.039286	0.445501	0.007607	4	0	241	581
GO:0007265	Ras protein signal transduction	0.039286	0.445501	0.007607	4	0	241	581
GO:0046578	regulation of Ras protein signal transduction	0.039286	0.445501	0.007607	4	0	241	581
GO:0009966	regulation of signal	0.039286	0.445501	0.007607	4	0	241	581
GO:0006950	response to stress	0.045623	0.502748	0.009268	15	14	230	567
GO:0006351	transcription, DNA-dependent	0.045623	0.507235	0.009426	13	11	232	570

*FDR: corrected p-value by False Discovery Rate control.
†FWER: corrected p-value by Family Wise Error Rate.
‡Single Test p-Value: p-Value without multiple testing corrections.

significantly (Fig. 6.3 C). Similarly, the number of proteins recognized by at least 90% of individuals in each age group also increased with age and *Pf* transmission (Fig. 6.3 D). By the end of the malaria season, nearly all of the 491 immunogenic proteins were recognized by at least 50% of individuals eight years of age and older. *Pf*-naïve adults (n =27) recognized an average of 203 of the 491 immunogenic proteins (Fig. 6.3 C); however, the level of Ab reactivity against these proteins was relatively low, similar to

levels observed in two to four year old children before the malaria season (Fig. 6.3 C), but lower than that of individuals aged five years and older before the malaria season ($P < 0.01$ for each comparison) and lower than that of all children and adults after the malaria season ($P < 0.01$ for each comparison). Moreover, only 24 of the 491 immunogenic proteins (4.9%) were recognized by 90% of *Pf*-naïve adults (Fig. 6.3 D), in contrast to the 346 proteins (70.5%) recognized by 90% of *Pf*-exposed adults after the malaria season (Fig. 6.3 D). Of note, Ab reactivity to the 24 proteins recognized by malaria-naïve adults increased along with Ab reactivity to all 491 immunogenic proteins in *Pf*-exposed individuals (Fig. 6.3 E), suggesting that the low level of Ab reactivity in samples from *Pf*-naïve adults may be attributable, in part, to cross-reactivity of Abs generated in response to other protozoa of the phylum Apicocomplexa such as *Toxoplasma gondii*. (3), a relatively common infection in the U.S. (116), rather than nonspecific binding.

We also quantified the level of Ab reactivity to the 491 immunogenic proteins with age and in response to *Pf* transmission. For each study participant we calculated the average Ab reactivity to the 491 immunogenic proteins before and after the malaria season, and then plotted the mean of these values within each one year age group (Fig. 6.3 F). Two year-old children began the malaria season with Abs to the immunogenic proteins—presumably elicited in response to *Pf* exposure during the preceding malaria season. The mean level of Abs to these proteins increased during the subsequent malaria season in each age category, but increases only reached statistical significance

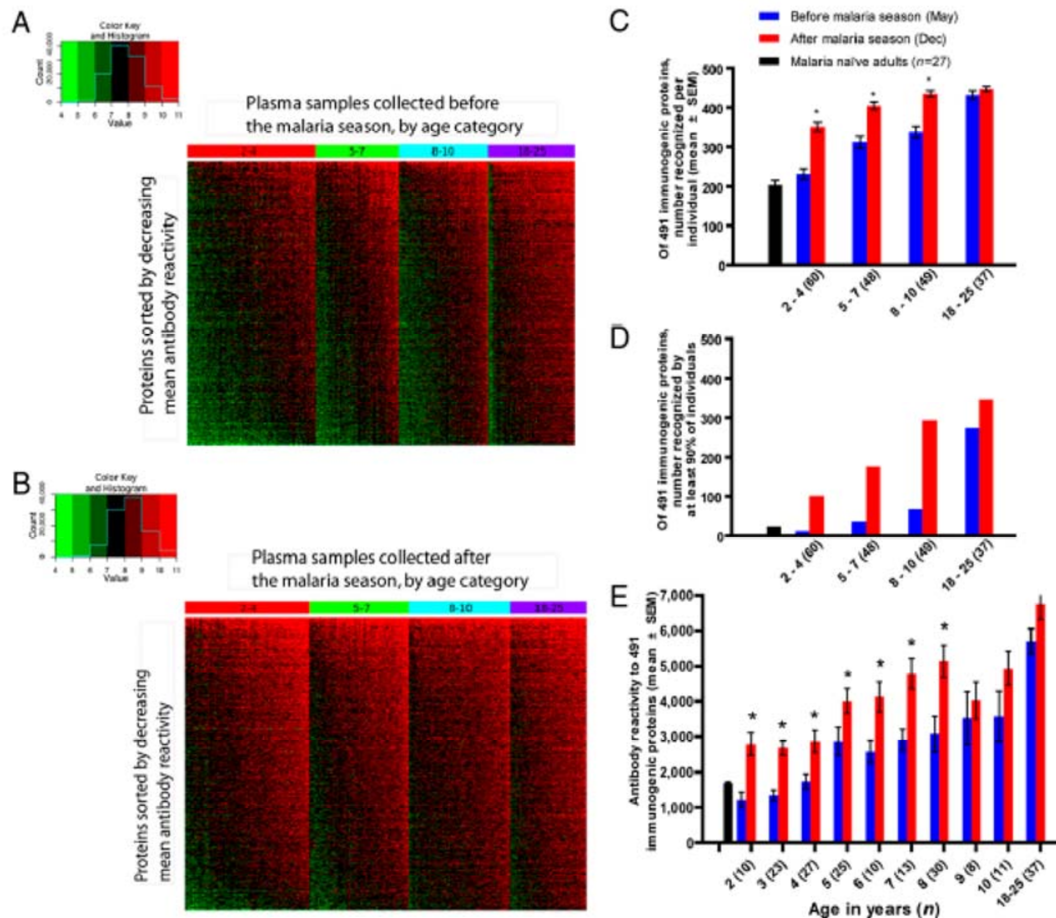


Figure 6.3 Impact of age and *Pf* transmission on *Pf*-specific Ab profiles. Heat maps of proteins analyzed for immunoreactivity across plasma samples collected before **(A)** and after **(B)** the malaria season show that the breadth and intensity of Ab reactivity increases with age and in response to *Pf* transmission. The 491 immunogenic proteins are represented in rows in descending order of immunoreactivity. Individual plasma samples are in columns and grouped by age in years. Within each age group, samples are sorted by increasing average immunoreactivity. Red indicates positive immunoreactivity, black indicates intermediate immunoreactivity, and green indicates no immunoreactivity. **(C)** Of the 491 immunogenic proteins, the average number recognized by *Pf*-exposed individuals increased with age both before (P<0.0001) and after (P<0.0001) the malaria season. Significant increases in the number of proteins recognized from before to after the malaria season within age groups are indicated by an asterisk (P < 0.0001 for all significant changes). **(D)** Number of proteins recognized by at least 90% of *Pf*-exposed individuals increased with age and *Pf* transmission. **(E)** Average level of Ab reactivity to the 491 immunogenic *Pf* proteins (reactivity tothenegative control subtracted) measured before the malaria season increased with age. In both children and adults, the level of Ab reactivity increased from before to after the malaria season. Statistically significant (P<0.05) increases are indicatedby an asterisk.

(p<0.05) in children aged two to eight years. In comparing the levels of Abs before the malaria season in aged two to three years, most of the increase in Ab levels from before to after the malaria season appeared to be short-lived—decreasing over the six month

dry season during which there was little to no *Pf* transmission. However, the level of the *Pf*-specific Abs before the malaria season was higher in children aged three years versus two years, suggesting that a small portion of the Abs acquired by two-year-old children over the malaria season persisted for six months in the absence of *Pf* exposure. This general pattern of rapid seasonal rise in Ab reactivities and gradual acquisition of persistent Ab responses continued to adulthood at which point Ab reactivity was substantial before the malaria season and only increased by a relatively small amount during the malaria season. Importantly, in children aged two to ten years, the combined Ab reactivity to the 491 immunogenic proteins measured before the malaria season was associated with decreased malaria risk (age adjusted OR 0.34 [95% confidence interval, 0.13 to 0.89]; p=0.03), whereas the combined Ab reactivity to the same proteins measured after the malaria season was not (age adjusted OR 2.15 [95% confidence interval, 0.59 to 7.89]; p=0.25).

6.2.3 Identifying *Pf*-specific Ab profiles before the malaria season that correlated with subsequent protection from uncomplicated malaria

To determine if Abs to particular *Pf* proteins within the 491 immunogenic proteins present before the malaria season correlated with subsequent protection from malaria, we compared Ab profiles of children who did not experience malaria (‘protected’) versus those who experienced at least one malaria episodes (‘susceptible’) during the eight-month study period. We focused on children aged eight to ten years for this analysis to avoid the confounding effect of age on malaria risk, and because the

majority of individuals above and below this age range were protected or susceptible, respectively (Fig. 5.3 C). Importantly, all of the eight to ten year old children classified as 'protected' had at least one blood smear positive for asexual *Pf* parasites at scheduled follow-up visits during the malaria season, indicating that protected children were not misclassified as such because of lack of *Pf* exposure. Furthermore, protected and susceptible children in this age group were similar in terms of factors potentially related to malaria risk and exposure including age, gender, ethnicity, distance of residence from the study clinic, mosquito bed net use, and prevalence of common red blood cell

Table 6.2 Baseline characteristics of children aged 8-10 years classified as susceptible (>1 malaria episodes) and protected (no malaria episodes).

Characteristic†	Susceptible (*n* = 29)	Protected (*n* = 12)	*P* Value‡
Age—yr	8.4 ±0.7	8.9 ±0.9	0.09
Male sex—no. (%)	19 (65.5)	6 (50.0)	0.49
Weight—kg	23.4 ±3.7	25.3 ±3.6	0.13
Ethnicity—no. (%)			
Bambara	15 (51.7)	6 (50.0)	1.00
Sarakole	10 (34.5)	6 (50.0)	0.46
Fulani	3 (10.3)	0 (0.0)	__
Malinke	1 (3.5)	0 (0.0)	__
Distance of residence from study clinic—meters	368 ±87	368 ±66	0.82
Bednet use—no. (%)§	6 (20.7)	2 (18.2)	__
Pf smear positive at enrollment—no. (%)¶	0 (0.0)	0 (0.0)	__
GI helminth infection at enrollment—no. positive/no. available (%)‖	2/25 (8.0)	1/9 (11.1)	__
Schistosoma haematobium detected in urine at enrollment—no. positive/no. available (%)‖	2/26 (7.7)	0/11 (0.0)	__
HbAS¶	0 (0.0)	0 (0.0)	__
*G6PD**A-	6 (20.7)	3 (25.0)	1.00
α-thalassemia, heterozygous	16 (55.2)	5 (41.7)	0.51
Blood group O	9 (31.0)	6 (50.0)	0.30

*****Plus-minus values are means ± s.d. GI, gastrointestinal; HbAS, hemoglobin AS phenotype; *G6PD**A, the mutation responsible for glucose-6-phosphate dehydrogenase in Mali, includes heterozygous, hemizygous, and homozygous individuals.

†Methods used to determine these characteristics are described elsewhere (14).

‡The characteristics of susceptible and protected individuals were compared with the use of Fisher's exact tests and Wilcoxon rank-sum tests for binary and continuous variables, respectively. _Statistical test not done if <3 in subgroup.

§Nightly bednet use self-reported at the end of the malaria season.

¶Because asymptomatic parasitemia before the malaria season and sickle cell trait were independently associated with decreased malaria risk in this cohort (14), these individuals were excluded from this analysis.

‖ The lower than expected prevalence of helminth infections was likely due to community-wide albendazole treatment prior to this study.

polymorphisms and helminth coinfection (Table 6.2). Individuals with asymptomatic *Pf* parasitemia before the malaria season or sickle cell trait were excluded from this analysis (n = 10), because these factors were independently associated with decreased malaria risk in this cohort (Chapter 5.2.1). Using plasma samples collected before the malaria season, we identified 49 proteins to which the mean Ab reactivity was higher among protected (n = 12) versus susceptible (n = 29) children ($\alpha = 0.05$, false discovery rate-corrected). Heatmaps of immunoreactivity against these 49 proteins demonstrate the difference between samples from protected and susceptible children (Fig. 6.4). Of the 491 immunogenic proteins, there were none to which Ab reactivity was significantly higher in the susceptible children. Of the 49 proteins associated with protection, four are being developed as malaria vaccines candidates—sporozoite threonine–asparagine-

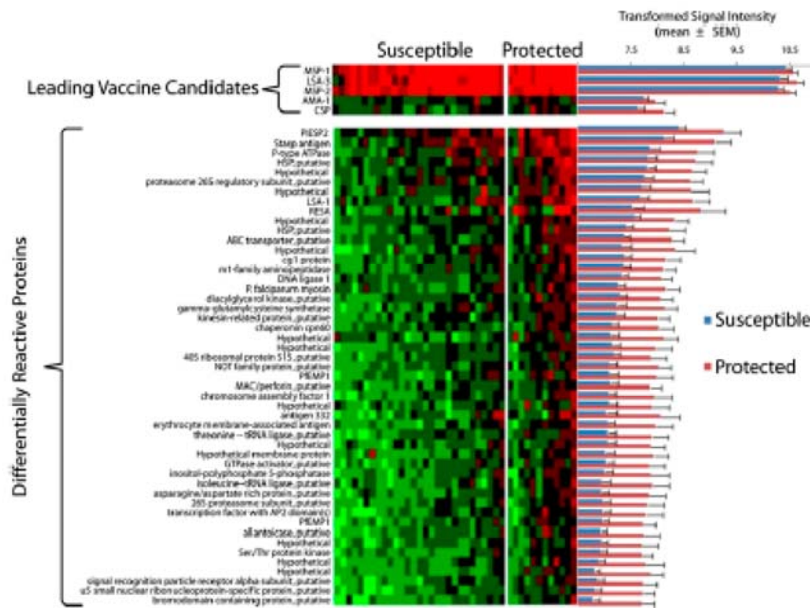


Figure 6.4 *Pf*-specific Ab profiles associated with protection from malaria. Heat map showing the difference in immunoreactivity measured before the malaria season against 49 proteins (in rows) in malaria-susceptible (Left, n = 29) and malaria-protected (Right, n = 12) children aged 8–10 years. (Right) Bar graph shows that the mean Ab reactivity against each of these proteins is higher in protected (red bars) vs. susceptible (blue bars) children ($\alpha = 0.05$, false discovery rate-corrected). (Upper) For comparison, Ab reactivity against 5 proteins expressed in the rapid translation system that did not discriminate between protected and susceptible children is shown. These 5 proteins correspond to the malaria vaccine candidates CSP, LSA-3, MSP-1, MSP-2, and AMA-1. For the heat map, red indicates positive immunoreactivity, black indicates intermediate immunoreactivity, and green indicates no immunoreactivity. For the bar graph, data are mean ± SEM.

Table 6.3 Proteomic features of *Pf* proteins associated with protection from uncomplicated malaria.

Gene ID	Protein Name	*P* *	Signal Peptide	# TM Domains	Mass Spec. Expression Evidence†	Affy Expression Evidence‡
PFI0510c	Hypothetical, conserved	0.00002	No	0	Spor	gam
PFD1060w	u5 small nuclear ribonucleoprotein-specific protein	0.0004	No	0	mero>spor>gam>troph	early troph
PFC0875w	ABC transporter	0.0005	No	13	unknown	early ring
PFA0510w	bromodomain containing protein	0.0005	No	0	gam>spor	early troph
PF14_0419	Hypothetical, conserved	0.0005	No	0	gam>spor	early schiz
PF14_0170	NOT family protein	0.0008	No	0	spor>mero	early schiz
PFL1545c	chaperonin cpn60	0.0008	Yes	1	iRBCm>trop=schiz>gam	early troph
PFA0110w	RESA	0.0009	No	0	mero=troph=schiz>iRBCm>gam	mero
MAL13P1.148	*Pf* myosin	0.001	No	0	troph>gam	early troph
PFL1620w	asparagine/aspartate rich protein	0.001	No	0	Spor	early troph
PF13_0179	isoleucine--tRNA ligase	0.001	No	0	mero>gam>troph	early troph
PF11_0232	Hypothetical, conserved	0.001	No	0	spor>gam	early troph
PF10_0356	LSA-1	0.002	Yes	1	liver(Zhu, 1991 #428)	mero
PF14_0384	Allantoicase	0.002	No	0	gam>troph	early troph
MAL13P1.278	Ser/Thr protein kinase	0.002	No	0	spor>troph>mero	gam
PF13_0350	signal recognition particle receptor alpha subunit	0.002	no	0	gam>mero>iRBCm	early troph
PF10_0177	erythrocyte membrane-associated antigen	0.002	Yes	1	Troph	gam
PFI0925w	gamma-glutamylcysteine synthetase	0.002	No	0	Mero	early ring
MAL7P1.138	Hypothetical, conserved	0.002	No	5	gam>spor	early ring
PFE0090w	chromosome assembly factor 1	0.002	No	0	troph>mero	early schiz
PF11_0507	antigen 332	0.002	No	0	iRBCm=troph=schiz	early troph
PFE0055c	HSP	0.003	No	1	iRBCm>gam>troph=schiz	mero
PF14_0344	Hypothetical, conserved	0.003	Yes	0	iRBCm>troph=schiz>mero>spor>gam	mero
MAL13P1.22	DNA ligase 1	0.003	Yes	0	troph>spor	early schiz
PF11_0008	PfEMP1	0.003	No	1	spor and iRBCm(Scherf, 2008 #462)	gam
PF11_0158	Hypothetical	0.004	No	0	mero>troph=gam>spor	gam
PF11_0175	HSP	0.004	Yes	1	troph=schiz>iRBCm>mero>gam>spor	mero
PF14_0632	26S proteasome subunit	0.004	No	0	iRBCm>mero=gam>spor	late troph
MAL13P1.323	Hypothetical, conserved	0.004	No	2	spor>gam>mero=troph	early ring
PF14_0681	diacylglycerol kinase	0.004	No	0	gam>mero	early schiz
PF13_0003	PfEMP1	0.004	No	0	spor and iRBCm(Scherf, 2008 #462)	early ring
MAL13P1.140	Hypothetical, conserved	0.004	No	0	Spor	gam
PFI0345w	GTPase activator	0.004	No	0	Gam	early ring
PFL0470w	Hypothetical, conserved	0.004	No	0	mero>troph	late schiz
PF07_0006	Starp antigen	0.004	Yes	0	spor(Fidock, 1994 #422)>gam	early ring
PF13_0190	Hypothetical, conserved	0.004	No	0	gam>mero>iRBCm	early troph
PFB0115w	Hypothetical, conserved	0.004	No	0	troph>spor	early troph
PF13_0285	inositol-polyphosphate 5-phosphatase	0.004	No	3	unknown	gam
PF11_0270	threonine -- tRNA ligase	0.004	Yes	0	mero>troph=schiz>iRBCm>gam>spor	early troph
PFI0855w	Hypothetical, conserved	0.004	No	0	Gam	late troph
PF07_0035	cg1 protein	0.004	Yes	0	iRBCm>spor	late troph

PFB0260w	proteasome 26S regulatory subunit	0.005	No	0	iRBCm>schiz=troph>mero>gam>spor	gam
PFL0545w	kinesin-related protein	0.005	No	0	spor>gam	gam
MAL13P1.56	m1-family aminopeptidase	0.005	No	1	mero>troph=schiz>gam>iRBCm>spor	early troph
PF07_0126	transcription factor with AP2 domain(s)	0.005	No	0	spor=troph>mero	early ring
PFL0590c	P-type ATPase	0.005	No	8	mero>spor>gam>troph	early troph
MAL6P1.131	SET-domain protein	0.005	No	0	mero>gam>spor	
PFE0060w	PIESP2	0.005	Yes	3	iRBCm>troph=schiz>gam	early troph
PFD0430c	MAC/perforin	0.005	Yes	0	Spor	gam

**P* value for the comparison of reactivity between protected and susceptible children. The Benjamini-Hochberg method was used to correct for the false discovery rate.

†From PlasmoDB, Sequest algorithm (www.plasmodb.org). Stage-specific expression evidence in this column that is not based on mass spectrometry evidence is noted. Abbreviations:

‡From PlasmoDB

rich protein (STARP), liver stage antigen-1 (LSA-1), ring-infected erythrocyte surface antigen (RESA), and antigen 332. There were also several highly conserved proteins such as an ATP-binding cassette (ABC) transporter (*PFC0875w*), PIESP2 (*PFE0060w*), and a protein with a membrane-attack complex/perforin (MACPF)-like domain (*PFD0430c*). Of the 25 *var* gene products (174) included on the microarray, two were associated with protection—*PF13_0003* and *PF11_0008*. There were also 12 hypothetical proteins. Table 6.3 summarizes the proteomic features of these 49 proteins. Notably, Ab reactivity against proteins representing the leading malaria vaccine candidates CSP, LSA-3, MSP1, MSP-2, and AMA1 did not discriminate protected and susceptible children (Fig. 6.4, top).

6.3 Discussion

In this study we used a protein microarray representing ~23% of the *Pf* proteome to gain insight into the kinetics of acquiring *Pf*-specific humoral immune responses and to identify Abs against known and hypothetical *Pf* proteins that may be associated with naturally-acquired protection from uncomplicated malaria. The approach described

here addresses two technical bottlenecks that have hindered studies seeking to correlate naturally-acquired malaria immunity with specific Ab responses in *Pf*-exposed populations: (1) the ELISA-based platform that is limited to evaluating one antigen at a time and (2) traditional cloning and protein expression and purification methods that have made <0.5% of the *Pf* proteome available for analysis. The many seroepidemiological studies that have evaluated this limited set of proteins have yet to establish a firm correlation between Ab responses and naturally-acquired malaria immunity (198). Although more recent studies have sought to correlate malaria immunity with Ab responses to as many as 18 *Pf* antigens by ELISA (158), (110) or protein microarray (88), these studies evaluated already characterized malaria vaccine candidates.

We compared Ab profiles of children based on whether or not they had experienced malaria during the eight-month study period. We chose this study endpoint, rather than the incidence of symptomatic infection or time to first symptomatic infection, because it represents the best possible clinical outcome for malaria vaccine candidates targeting pre-erythrocytic and erythrocytic stages. Ab reactivity to 49 known and hypothetical *Pf* proteins was significantly higher in eight to ten year old children who were infected with *Pf* but did not experience symptoms of malaria. Four of these proteins are already being developed as malaria vaccine candidates—STARP, LSA-1, RESA, and antigen 332. STARP is a highly conserved protein expressed primarily on sporozoites, and to a lesser extent, during the liver and early

intra-erythrocytic stages of infection (78). STARP-specific IgG, whether acquired naturally or through irradiated sporozoite immunization, has been shown to inhibit sporozoite invasion of human hepatocytes (160). Although LSA-1 is expressed during the liver stage (215), where cell-mediated immunity likely predominates, LSA-1-specific IgG levels in individuals living in malaria endemic areas have been associated with protection against malaria (115). Furthermore, LSA-1-specific Ab responses have been detected in individuals vaccinated with irradiated sporozoites (127). However, in the only phase I/II trial conducted to date, vaccination with recombinant LSA-1 did not protect against *Pf* experimental sporozoite challenge (76). RESA is released by the merozoite upon erythrocyte invasion where it interacts with the spectrin network of the host cell membrane (81). A vaccine combining RESA, MSP1 and MSP-2 was the first blood-stage vaccine to show some efficacy, reducing parasitemia in children enrolled in a Phase I/IIb trial in Papua New Guinea (85), although the relative contribution of the three antigens to protection remains unclear. Antigen 332 is expressed in trophozoites and translocated to the erythrocyte membrane during the schizont stage (102). Abs against Pf332 have been shown to inhibit parasite grown *in vitro* (6) and have been associated with decreased parasitemia (200) and decreased malaria risk (5) in field studies.

We identified differential Ab reactivity against other highly conserved proteins that could be targeted as malaria vaccines. For example, *PFC0875w* is predicted to be an ABC transporter, and vaccination with ABC transporter proteins has been shown to

protect against pathogenic bacteria in animal models (84, 187). PIESP2 (*PFE0060w*) is a highly conserved protein of unknown function that is thought to be expressed on the surface of infected red blood cells (79). A protein with a MACPF-like domain (*PFD0430c*) is an intriguing target for both pre-erythrocytic and transmission blocking vaccines since it plays a role in the traversal of hepatic sinusoids by sporozoites (111) and the mosquito midgut epithelium by ookinetes (118), possibly by forming pores in the host cells' plasma membranes (119). Of the 25 *var* gene products (174) included on the microarray, two were associated with protection: *PF13_0003* and *PF11_0008*. Both belong to the Group A *var* cluster which is characterized by relative structural homogeneity (131) preferential expression in patients with severe malaria, and rapid induction of Abs (113). The analysis also identified 12 hypothetical proteins that were associated with protection, which highlights the potential value of this approach for identifying novel vaccine candidates. Further study of selected antigens identified by this strategy will be needed to define their potential as malaria vaccine candidates. In addition, with the inherent bias in any single epidemiological study, larger studies in other settings will be needed to validate this as an approach for identifying correlates of naturally-acquired malaria immunity.

Ab reactivity to several intracellular proteins was significantly higher in the protected group. Abs to intracellular proteins are typically viewed as markers of past infection, and not as evidence for vaccine potential; and it is possible that higher Ab reactivity against intracellular proteins among protected individuals is a marker of

enhanced parasite killing. However, intracellular proteins of other parasites have been shown to induce protective Abs. For example, immunization with heavy chain myosin induced protection against *Brugia malayi* challenge in a rodent model (197). Interestingly, in the present study, we found that Ab reactivity to *P. falciparum* myosin was associated with protection from malaria. Given how little is known about the infection biology of *P. falciparum* in humans, it may be premature to categorically dismiss immunogenic proteins as potential vaccine targets based solely on subcellular location.

Ab reactivity to the malaria vaccine candidates CSP, LSA-3, AMA1, MSP1 and MSP-2 did not discriminate protected and susceptible children (Fig. 6.4, top).This is consistent with recent clinical trials in which vaccination with AMA1 (171) or MSP1 (156) did not confer protective immunity, although reformulating these antigen constructs, adding novel vaccine adjuvants or simply vaccinating malaria-naïve individuals who have not yet imprinted an immune reaction to the given antigens in the context of *Pf* infection, may improve their efficacy. It is also possible that many of the highly immunogenic *Pf* antigens, including these, are expressed by the parasite as decoy antigens, and Abs to these does not confer protection. Abs to CSP did not correlate with protection from clinical disease in this study, consistent with seroepidemiological studies (104) and vaccine trials (RTS,S vaccine) (2, 27) which found no association between CSP-specific Ab levels and protection from clinical disease. However, some

studies have shown a relationship between CSP-specific Ab levels and protection from *Pf* infection (i.e. positive blood smear without malaria symptoms) (2, 10, 15).

An inherent drawback to the use of the *in vitro* transcription and translation system to produce *Pf* proteins is the possibility that not all proteins will be properly folded and display all possible antigenic epitopes. We observed a correlation between Ab reactivity to proteins expressed in this system and to the corresponding well characterized, correctly folded recombinant proteins spotted on the same array, but despite this correlation, it is likely that this method will fail to detect all potential Ab reactivities.

This study also sheds light on the interface between the complex, multistage *Pf* parasite and the host immune response by determining the life-cycle stage, sub-cellular location, and biological process of the *Pf* antigens recognized by *Pf*-exposed individuals. Further analysis to determine if patterns of Ab reactivity against individual antigens can be predicted by these, and other proteomic features, may inform malaria vaccine development.

This analysis also provided insight into the kinetics of naturally-acquired humoral responses to *Pf* in an area of intense seasonal malaria transmission. We found that Ab reactivity to *Pf* proteins rose dramatically in children during the malaria season; however, most of this response appeared to be short-lived based on the cross-sectional analysis at the end of the dry season which revealed only modest incremental increases in Ab reactivity with age. Because there is little to no Pf transmission at the study site

during the six-month dry season (Chapter 5.2.1 and(59)) and IgG has a half-life of approximately twenty-one days (180), we infer that *Pf*-specific Abs in circulation at the end of the dry season are generated by long-lived plasma cells (LLPCs), whereas the increase in Ab levels observed after the malaria season likely reflects the differentiation of naive and/or memory B cells (MBCs) into short-lived plasma cells (SLPCs) that disappear by the end of the next dry season. However, with increasing age there appears to be a gradual expansion of long-lived, *Pf*-specific plasma cells. Thus, it appears that the *Pf*-specific LLPC compartment gradually "fills" with repeated *Pf* exposure. In age-adjusted analysis, protection from malaria was associated with the overall level of *Pf*-specific Abs in circulation before the malaria season, whereas Ab levels after the malaria season were not associated with protection. Taken together, these data suggest that the delayed acquisition of immunity to uncomplicated malaria may be attributable, in part, to the gradual, step-wise acquisition of LLPCs, not conferring protection until a critical threshold of circulating Ab is exceeded. Our results also suggest that SLPCs derived from MBCs in response to acute *Pf* infection, a process that peaks six to eight days after antigen reexposure (30), at least in the case of vaccination, may not provide Abs rapidly enough to prevent the onset of malaria symptoms, which can occur as early as three days after the start of the blood stage infection (179). This model stands in contrast to the humoral immune response after reexposure to some viruses in which longer incubation periods allow virus-specific MBCs to differentiate into plasma cells that contribute to the control of viral replication before

symptoms develop. For example, follow-up studies of hepatitis B vaccinees have shown that protection can persist despite the decline of anti-hepatitis B Abs to undetectable levels (202), presumably because of the recall response of persistent MBCs. An alternative explanation for the maintenance of Ab levels through the six-month dry season is the persistence of low-grade parasitemia despite little to no *Pf* transmission at the study site. Although only seven percent of individuals were smear positive when they were enrolled just prior to the malaria season, this may underestimate the proportion of the cohort that experienced intermittent, low-grade parasitemia in the months prior to enrollment. At present, the molecular and cellular mechanisms that underlie the generation and maintenance of *Pf*-specific LLPCs and MBCs are not known. It is possible that the large number of *Pf* antigens overwhelms the immune system's capacity to select for and commit a sufficient number of MBCs and PCs specific for any given *Pf* antigen to a long-lived pool (169). If immunity to clinical malaria requires high levels of Abs to a large number of *Pf* proteins, the inability to commit large numbers of LLPCs specific for any given *Pf* antigen during any given infection may explain, in part, why malaria immunity is acquired slowly. An alternative, non-mutually exclusive explanation for the gradual acquisition of malaria immunity is the length of time needed to acquire Abs that cover the range of antigenic diversity in the parasite population as discussed in Chapter 1.7 (186).

The analysis described here also demonstrates how protein microarrays representing large portions of the *Pf* proteome can be used to probe the interface

between the parasite and the host immune response, and to identify Ab profiles against known and hypothetical proteins that are associated with naturally-acquired malaria immunity. This approach, if validated in larger studies and in other epidemiological settings, could prove to be a useful strategy for identifying malaria vaccine targets and for better understanding fundamental properties of the human immune response to *Pf*.

Chapter 7: ***P. falciparum*-specific MBCs and Abs increase gradually over years with cumulative exposure in an area of intense seasonal *Pf* transmission.**

7.1 Introduction

In this chapter we present data addressing whether *Pf* infection generates MBCs specific for *Pf* blood stage antigens, and if so, whether they accumulate with age and cumulative *Pf* exposure, and also whether their frequency correlates with protection from malaria. In addition, we determine whether acute, symptomatic *Pf* infection results in an increase in the number of *Pf*-specific MBCs and the levels of *Pf*-specific Abs, and if so, whether this increase remains stable over a six-month period of markedly reduced *Pf* transmission. By taking advantage of the tetanus immunization schedule in Mali in which infants and women of child-bearing age are vaccinated, we compare the relative efficiencies of the acquisition of tetanus toxoid (TT)- and *Pf*-specific MBCs and Abs, and also test three hypotheses: 1) that growth of the MBC compartment depends on immunological experience rather than age, 2) that *Pf* infection induces non-specific activation of bystander B cells (62, 89), and 3) that polyclonal activation during heterologous immune responses is a general mechanism for maintaining MBCs and LLPCs (30).

7.2 Results

7.2.1 Malaria immunity is acquired gradually despite intense exposure to the *Pf* parasite.

For the MBC analysis reported in this chapter, a subset of 185 individuals was randomly selected within each of the four age categories from those in the Kambila

Table 7.1 Baseline characteristics of MBC subset by age group.

	Age group, years				All
	2-4	5-7	8-10	18-25	
Gender, % female (no.)	66.1 (39)	48.7 (18)	33.3 (14)	61.7 (29)	54.1 (100)
Ethnicity, % (no.)					
Bambara	62.7 (37)	51.4 (19)	54.8 (23)	66.0 (31)	59.5 (110)
Sarakole	32.2 (19)	43.2 (16)	35.7 (15)	27.7 (13)	34.1 (63)
Fulani	3.4 (2)	5.4 (2)	7.1 (3)	4.3 (2)	4.9 (9)
Malinke	1.7 (1)	0.0 (0)	2.4 (1)	2.1 (1)	1.6 (3)
Hemoglobin AS, % (no.)[a]	13.6 (8)	8.1 (3)	7.1 (3)	10.6 (5)	10.3 (19)
P. falciparum smear positive at enrollment, %	6.8 (4)	10.8 (4)	11.9 (5)	6.4 (3)	8.7 (16)
Parasitemia if smear positive at enrollment, parasites/microliter [geometric mean (95% CI)]	1438 (159–12973)	3616 (1500 – 8715)	415 (134–1287)	953 (39-23381)	1137 (579–2232)
GI helminth, % positive at enrollment (no.)[c]	14.6 (8)	8.3 (3)	11.8 (4)	0 (0)	9.7 (15)
Urine schistosomiasis, % positive at	0 (0)	0 (0)	5.3 (2)	29.0 (9)	7.4 (11)
Distance lived from clinic, meters (mean ±SD)	395	408	365 (±83)	359 (±91)	382
Bed net use, % (no.)[e]	27.3 (15)	41.2 (14)	17.1 (7)	39.5 (15)	30.4 (51)

[a]Data available for 177 subjects.
[b]All subjects were asymptomatic at enrollment.
[c]Data available for 154 subjects; GI=gastrointestinal.
[d]Data available for 148 subjects.
[e]Nightly bednet use self-reported at the end of the malaria season.

cohort who had complete sets of PBMC samples over the entire study period. The baseline demographic and clinical characteristics of this subset (Table 7.1) did not differ significantly from the entire cohort. In this subset during the one-year study period there were 380 unscheduled clinic visits, during which 219 cases of malaria were diagnosed (similar to the 495 unscheduled clinic visits and 298 cases of malaria in the entire cohort (Table 5.3)), and included the five cases which met the WHO criteria for severe malaria (1). Malaria episodes were defined as an axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000 parasites/μL, and a non-focal physical exam by the study physician. As expected in this region of Mali, all malaria cases were confined to a six-month period that began in July, peaked in October, and ended by January (Fig. 7.1 A). The incidence

of malaria and the proportion of individuals experiencing at least one malaria episode decreased with age, whereas the time to the first malaria episode increased with age (Fig. 7.1 B). Thus, despite intense annual *Pf* transmission at this study site, immunity to malaria is acquired slowly.

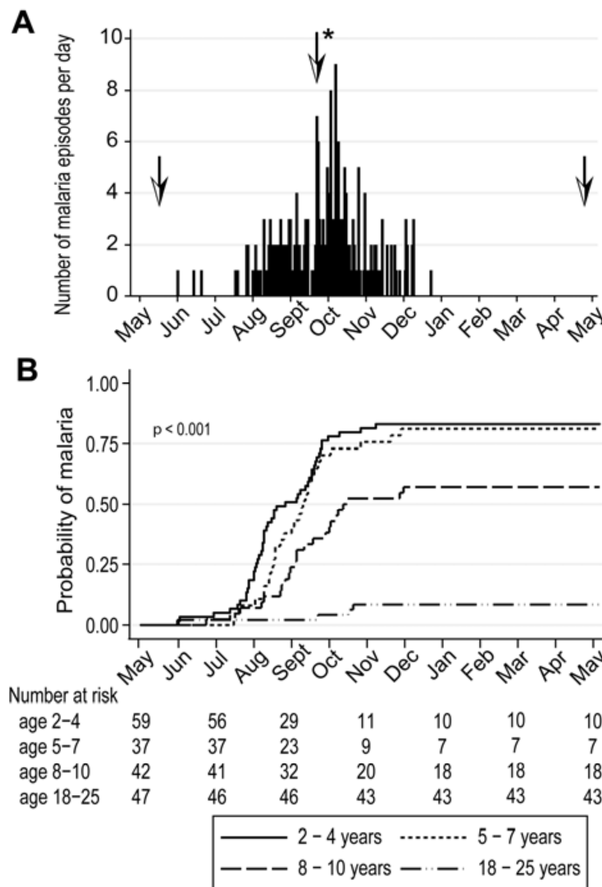


Figure 7.1 Malaria immunity is acquired gradually despite intense exposure to the *Pf* parasite. There were 219 clinical episodes defined as axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000 parasites/μL and a nonfocal physical examination by the study physician. **(A)** To track the B-cell response to acute malaria, and after a period of reduced *Pf* exposure, PBMCs and plasma compared in Fig. 7.3 were collected at points indicated by the arrows: before the malaria season, two weeks after the first malaria episode (arrow with asterisk indicates the mean time to first malaria episode, 132 days from enrollment), and six months after the end of the malaria season. **(B)** Kaplan-Meier estimates of the cumulative probability of malaria over the study period, according to age category. The number of individuals at risk over the study period is shown below the graph. The P value was obtained using the log rank test.

7.2.2 Analysis of *Pf*-specific and TT-specific MBCs and Abs in *Pf*-uninfected children and adults before the malaria season

We first established baseline levels of IgG^+ AMA1-, MSP1- and TT-specific MBCs and Abs in *Pf*-uninfected, healthy children and adults in May just before the malaria season, a point at which there had been little to no *Pf* transmission for five months. For this analysis we excluded individuals with asymptomatic *Pf* parasitemia (8.7% of cohort; Table 7.2), because they showed a decreased risk of malaria and

tended toward higher frequencies of AMA1- and MSP1-specific MBCs and levels of Ab, which could obscure age-related differences in MBC and Ab acquisition. Because of the small number of individuals with asymptomatic parasitemia at baseline and the age range of these individuals, there were too few to analyze as a comparator group. In addition, the meaning of persistent parasitemia relative to immunity is not clear-cut as this persistent parasitemia could be due to a property of the parasite rather than the human immune system and could result in increased immunity due to prolonged infection with a less virulent parasite.

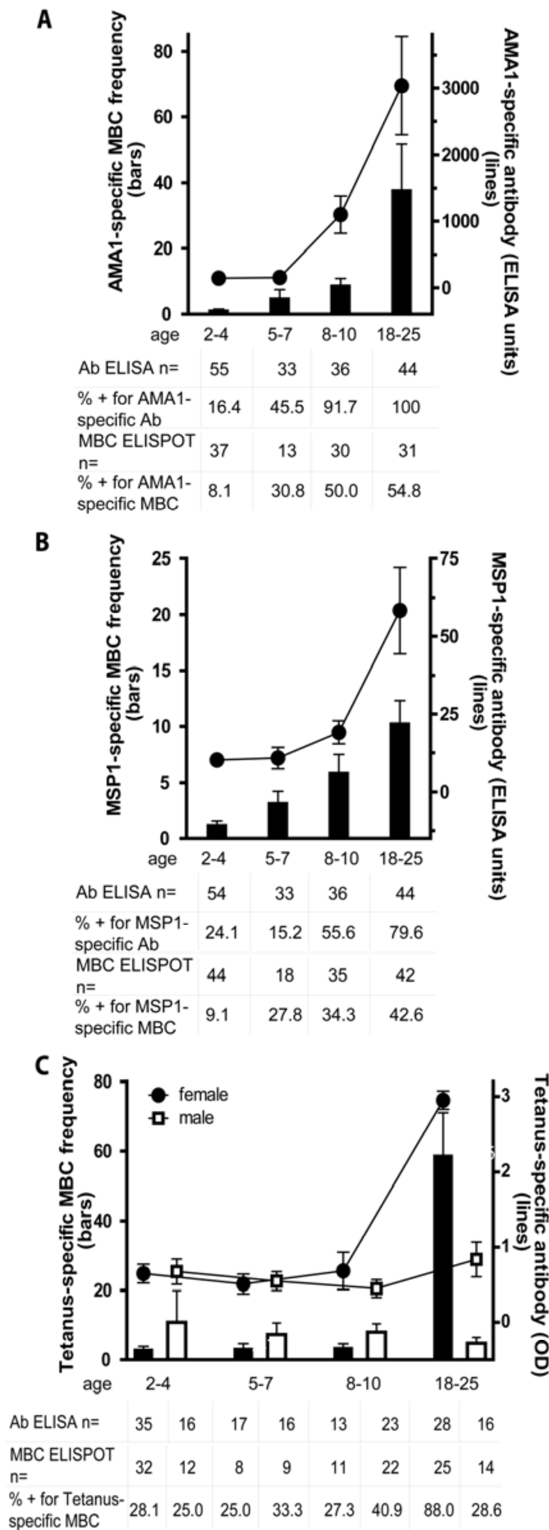
We focused our analyses on MBCs and Abs specific for *Pf* blood-stage antigens because humoral responses are known to be critical to blood-stage immunity (42). We examined the response to the same two blood stage proteins, AMA1 and MSP1, that we studied the MBC and Ab responses to in vaccination of *Pf*-naïve individuals in Chapter 4 (45). This afforded the opportunity to compare the acquisition of B cell memory to the same antigens after vaccination versus natural *Pf* infection. Because there was high

Table 7.2 Malaria outcomes for the MBC subset, by age group.

	Age group, years				
	2-4 (n=59)	5-7 (n=37)	8-10 (n=42)	18-25 (n=47)	All (n=185)
Malaria incidence, mean (±SD)[a]	1.86 (±1.28)	1.81 (±1.17)	0.95 (±1.08)	0.09 (±0.28)	1.19 (±1.27)
Severe malaria incidence, no.[b]	4	1	0	0	5
At least one malaria episode, % (no.)	83.1 (49)	81.1 (30)	57.1 (24)	8.5 (4)	57.8 (107)
Time to first malaria episode, days (median)[c]	101	121	124	153	118
Parasitemia at first malaria episode, parasites/microliter [geometric mean (95% CI)]	39084 (30579 – 49954)	26417 (19440 – 35896)	20561 (15683 – 26956)	8816 (4082 – 19037)	28678 (24334 – 33799)

[a]Malaria episode defined as T $\geq$37.5°C, asexual parasitemia $\geq$ 5000/microliter, and non-focal physical examination.
[b]WHO definition of severe malaria[1].
[c]Days since study enrollment.

intra-individual variability in the frequency of IgG^+ MBCs during this study, these antigen-specific data were analyzed using the more stable parameter of PBMC post culture rather than as a percentage of IgG^+ MBCs. In the present study, the mean frequency of AMA1-specific MBCs per 10^6 PBMCs increased with age (Fig. 7.2 A; 2-4 yr: 1.2 [95% CI: 0.45-1.9]; 5-7 yr: 5.0 [95% CI: -0.2-10.1]; 8-10 yr: 8.9 [95% CI: 4.9-12.9]; 18-25yr: 37.8 [95% CI: 10.4-65.3]; p<0.001), as did the proportion of individuals with detectable AMA1-specific MBCs (2-4 yr: 8.1%; 5-7 yr: 30.8%; 8-10 yr: 50.0%; 18-25yr: 54.8%; p<0.001). Similarly, AMA1-specific Ab levels and the proportion of individuals seropositive for AMA1-specific Abs increased with age (Fig. 7.2 A; p<0.001 for both comparisons). There was a positive correlation between the frequency of AMA1-specific MBCs and Ab levels (Spearman's correlation coefficient = 0.43; p<0.001). We observed a similar age-associated increase in the frequency of MSP1-specific MBCs,although the overall frequency was lower than that for AMA1-specific MBCs (Fig. 7.2 B; 2-4 yr: 1.2 [95% CI: 0.55-1.9]; 5-7 yr: 3.2 [95% CI: 1.2-5.2]; 8-10 yr: 5.9 [95% CI: 2.9-9.0]; 18-25yr: 10.3 [95% CI: 6.3-14.3]; p<0.001). Likewise, the proportion of individuals who had detectable MSP1-specific MBCs (2-4 yr: 9.1%; 5-7 yr: 27.8%; 8-10 yr: 34.3%; 18-25yr: 47.6%; p=0.001) was similar to that for AMA1. MSP1-specific Ab levels and the proportion of individuals seropositive for MSP1-specific Abs also increased gradually with age (Fig. 7.2 B; p<0.001 for both comparisons). There was a positive correlation between the frequency of MSP1-specific MBCs and Ab levels (Spearman's correlation coefficient = 0.33; p<0.001). Remarkably, despite exposure to 50-60 infective



age	2-4	5-7	8-10	18-25
Ab ELISA n=	55	33	36	44
% + for AMA1-specific Ab	16.4	45.5	91.7	100
MBC ELISPOT n=	37	13	30	31
% + for AMA1-specific MBC	8.1	30.8	50.0	54.8

age	2-4	5-7	8-10	18-25
Ab ELISA n=	54	33	36	44
% + for MSP1-specific Ab	24.1	15.2	55.6	79.6
MBC ELISPOT n=	44	18	35	42
% + for MSP1-specific MBC	9.1	27.8	34.3	42.6

age	2-4		5-7		8-10		18-25	
Ab ELISA n=	35	16	17	16	13	23	28	16
MBC ELISPOT n=	32	12	8	9	11	22	25	14
% + for Tetanus-specific MBC	28.1	25.0	25.0	33.3	27.3	40.9	88.0	28.6

Figure 7.2 The *Pf*-specific MBC and long-lived antibody compartments expand gradually with age. Shown are the MBC frequencies per 10^6 PBMCs post culture (bars, left axis) and antibody levels in ELISA units (AMA1, MSP1) or as OD (TT) (lines, right axis) specific for AMA1 **(A)** and MSP1 **(B)** by age category; and TT **(C)** by age category and gender; before the malaria season in *Pf*-uninfected individuals. The frequency of AMA1- and MSP1-specific MBCs increased with age ($p<0.001$ for both), as did the level of AMA1- and MSP1-specific antibodies ($p<0.001$ for both). There were no significant differences by gender for the AMA1- and MSP1-specific responses. To determine if the expansion of *Pf*-specific MBCs with age was driven by exposure to antigen or simply a function of age, we measured the TT-specific MBC and antibody response with age. In Mali, infants are vaccinated with TT, and females receive a TT booster around the age of 15 years to prevent neonatal tetanus. In contrast to AMA1 and MSP1, the frequency of TT-specific MBCs and the level of TT-specific antibodies for males did not change significantly from age 2 to 25 years ($p=0.80$ and $p=0.44$, respectively). However, the frequency of TT-specific MBCs and the level of TT-specific antibodies was higher in female adults compared to female children ($p<0.001$ for both comparisons). MBC frequencies were determined by ELISPOT and are expressed per million PBMC. The number of individual samples assayed and the percent of individual samples that exceeded the limit of detection (i.e. those considered positive) is indicated below the graph. p values were obtained by the Kruskal-Wallis test. Data are shown as mean ± s.e.m.

mosquito bites per month at the peak of each malaria season in this area (59), only approximately half of adults had detectable MBCs specific for AMA1 and MSP1, even though most had detectable AMA1- and MSP1-specific Abs. Of the 72 individuals without detectable AMA1-specific MBCs before the malaria season, 64 (88.9% [95% CI 79.3 - 95.1]) did not have detectable MSP1-specific MBCs, suggesting that failure to generate MBCs to one *Pf* antigen is associated with failure to generate MBCs to other *Pf* antigens.

To understand if the expansion of *Pf*-specific MBCs with age was driven by repeated exposure to *Pf* antigens or simply a function of age, we determined the frequency of MBCs specific for an unrelated antigen, tetanus toxoid (TT), with age. In Mali, a single TT vaccine is administered to infants less than six months of age and a second TT vaccine is administered to females around 15 years of age to prevent neonatal tetanus. Thus, we measured TT-specific Ab and MBC responses at least 18 months after TT vaccination for children and at least three years after booster vaccination for adults, a point at which the TT-specific response is likely to be at steady state. In contrast to what was observed for AMA1- and MSP1-specific MBCs, the frequency of TT-specific MBCs among males did not change significantly from age two to 25 years (Fig. 7.2 C) (2-4 yrs: 10.8 [95% CI -7.4-29.0], 5-7 yrs: 7.3 [95% CI 0.7-13.9], 8-10 yrs: 8.0 [95% CI 3.1-12.8], 18-25 yrs: 4.7 [95% CI 1.4-8.1]; p=0.80). Similarly, the proportion of male adults who were positive for TT-specific MBCs did not differ significantly from male children (2-4 yrs: 25.0%, 5-7 yrs: 33.3%, 8-10 yrs: 40.9%, 18-25

yrs 28.6%; p=0.80). The slightly higher frequency of TT-specific MBCs in male versus female children was not statistically significant. However, the frequency of TT-specific MBCs was significantly higher in female adults compared to female children (Fig. 7.2 C; mean frequency of TT-specific MBCs per million PBMC by age group (2-4 yrs: 2.9 [95% CI 1.1-4.7], 5-7 yrs: 3.2 [95% CI 0.2-6.1], 8-10 yrs: 3.4 [95% CI 1.1-5.7], 18-25 yrs: 58.7 [95% CI 34.2-83.3]; p<0.001) presumably the result of booster vaccination. Likewise, the proportion of female adults who were positive for TT-specific MBCs was significantly higher as compared to female children (2-4 yrs: 28.1%, 5-7 yrs: 25.0%, 8-10 yrs: 27.3%, 18-25 yrs 88.0%; p<0.001). For both females and males, TT-specific Ab levels mirrored MBC frequencies (Fig. 7.2 C)—clearly increasing from female children to female adults (p<0.001), while not changing significantly by age in males (p=0.44). Overall, TT-specific Ab levels and MBC frequencies correlated (Spearman's correlation coefficient = 0.48; p<0.001). The observation that *Pf*-specific MBCs increased with age while TT-specific MBCs in individuals who received no booster vaccine did not increase and tended to decrease slightly with age indicates that the increase in *Pf*-specific MBCs is driven by

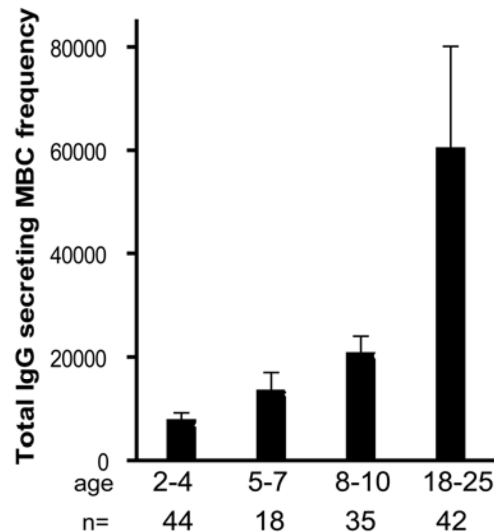


Figure 7.3 The size of total IgG⁺ MBC comparment expands gradually with age. The frequency of IgG⁺ MBCs per million PBMCs measured before the malaria season increased with age (P<0.001). The number of individuals in each age category is indicated. The P value was obtained by the Kruskal-Wallis test. Data are shown as mean ± s.e.m

repeated antigen exposure and is not simply a function of age. Of note, the size of the total IgG^+ MBC compartment, as reflected in the peripheral blood, increased with age (Fig. 7.3; $p<0.001$), consistent with the maturation of the total MBC compartment with immunological experience.

7.2.3 Longitudinal analysis of the *Pf*- and TT-specific MBC and Ab responses two weeks after acute malaria and after a prolonged period of decreased *Pf* exposure

To assess the *Pf*-specific MBC and Ab responses to acute malaria, and to determine the stability of this response during a period of little to no *Pf* transmission, we measured the frequencies of MBCs and Ab levels specific for AMA1 and MSP1 14 days after the first episode of malaria (convalescence), and in a cross-sectional survey at the end of the following dry season (month 12), and compared these frequencies to the pre-malaria season baseline (month 0; as detailed above). Malaria episodes were defined as an axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000 parasites/µL, and a non-focal physical exam by the study physician. Because few adults experienced malaria (Table 7.2), this analysis only included children aged two to ten years (see Fig. 7.4 for sample sizes at each timepoint). The mean frequency of AMA1-specific MBCs in children aged two to ten years increased from month 0 to convalescence (Fig. 7.4 A; month 0: 4.7 [95% CI: 2.8-6.6]; convalescence: 13.4 [95% CI: 2.7-24.1; p=0.006] and then decreased from convalescence to month 12 (Fig. 7.4 A; month 12: 5.9 [95% CI: 2.4-9.4]; p=0.93 versus convalescence) to a point just above the frequency at month 0 (Fig. 7.4 A; p=0.021, month 0 vs. month 12). Likewise, the level of AMA1-specific Abs increased from month 0 to convalescence (Fig. 7.4 A; month 0: 422.8 [95% CI: 228.7-617.0];

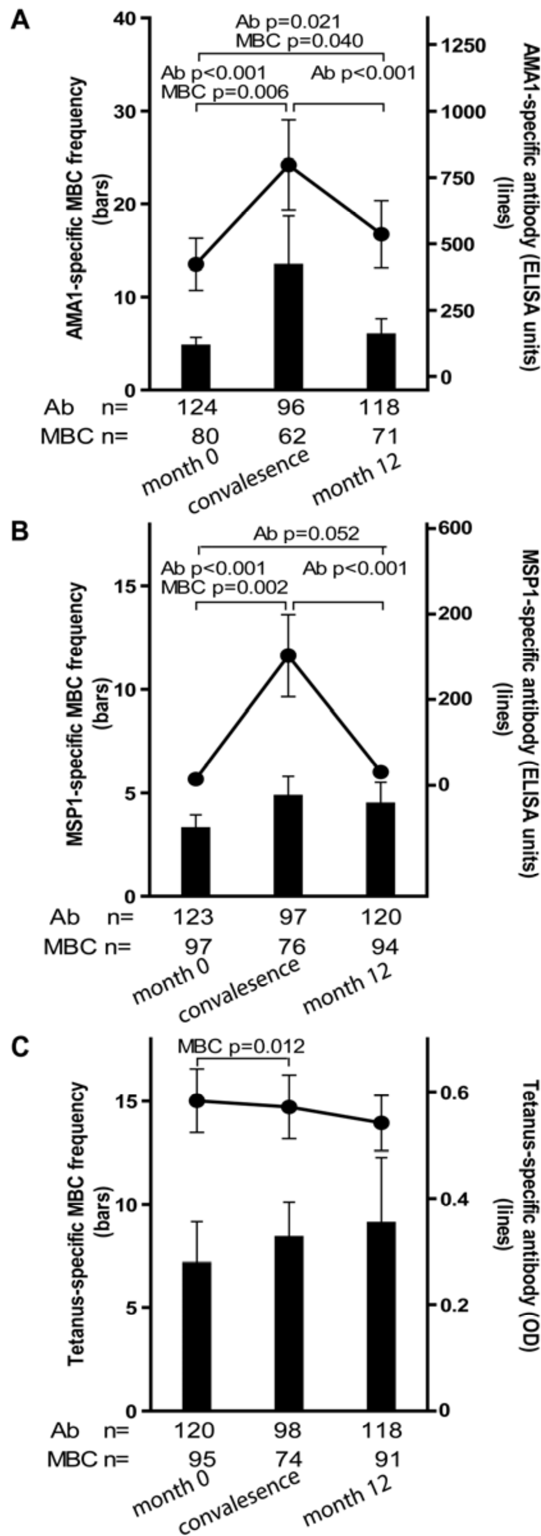


Figure 7.4 Longitudinal analysis of the *Pf*- and TT- specific MBC and Ab response. Compared to month zero, the MBC frequencies and antibody levels specific for AMA1 **(A)** and MSP1 **(B)** increased two weeks after the first episode of malaria and then contracted to a point slightly higher than pre-infection levels after a six-month period of decreased *Pf* exposure. Compared to month zero, there was a small but statistically significant increase in TT-specific MBC two weeks after the first episode of malaria **(C)**, whereas the level of TT-specific antibodies did not change. The number of individuals in each age category is indicated. Only statistically significant P values are shown. P values were obtained by the Wilcoxon matched-pairs signed-rank test. Data are shown as mean ± s.e.m.

convalescence: 797.2 [95% CI: 460.0-1134.7; p<0.001] , and then decreased from convalescence to month 12 (Fig. 7.4 A; month 12: 535.5 [95% CI: 283.8-787.2]; p<0.001 versus convalescence], to a point just above month 0 levels (Fig. 7.4 A; p=0.040, month 0 vs. month 12).

The MSP1-specific MBC and Ab responses followed a similar pattern. The mean frequency of MSP1-specific MBCs in children aged two to ten years increased from month 0 to convalescence (Fig. 7.4 B; month 0: 3.3 [95% CI: 2.0-4.6];

convalescence: 4.8 [95% CI: 2.9-6.8; p=0.002] and then decreased from convalescence to month 12 (Fig. 7.4 B; month 12: 4.5 [95% CI: 2.4-6.6]; p=0.71 versus convalescence) to a point just above the frequency at month 0 (Fig. 7.4 B; p=0.156, month 0 vs. month 12). Likewise, the level of MSP1-specific Ab increased from month 0 to convalescence (Fig. 7.4 B; month 0: 14.6 [95% CI: 10.5-18.6]; convalescence: 302.6 [95% CI: 111.7-493.4; p<0.001], and then decreased from convalescence to month 12 (Fig. 7.4 B; month 12: 31.1 [95% CI: 5.5-56.6]; p<0.001 versus convalescence], to a point just above month 0 levels (Fig. 7.4 B; p=0.052, month 0 vs. month 12).

To determine if malaria induces non-specific activation of 'bystander' MBCs, we compared the frequencies of TT-specific MBCs and Ab levels before the malaria season (month 0) to that 14 days after acute malaria (convalescence). We observed a small, but statistically significant increase in the frequency of TT-specific MBCs from month 0 to convalescence (Fig. 7.4 C; month 0: 7.1 [95% CI: 3.1-11.2]; convalescence: 8.4 [95% CI: 5.0-11.8; p=0.012) that did not change significantly at month 12 (month 12: 9.1 [95% CI: 3.2-15.4]; p=0.974 versus convalescence]. In contrast, TT-specific Ab levels decreased slightly from month 0 to convalescence, and again from convalescence to month 12, although neither decline was statistically significant (Fig. 7.4 C; month 0: 0.58 [95% CI: 0.5-0.7]; convalescence: 0.57 [95% CI: 0.5-0.7; p=0.063]; month 12: 0.54 [95% CI: 0.4-0.6]; p=0.525 versus convalescence). Collectively these results indicate that malaria infection results in an increase in the frequencies of both *Pf*-specific, and bystander

MBCs. However, malaria selectively induces *Pf*-specific Ab production but does not appear to drive the differentiation of bystander naïve and memory B cells into PCs.

7.2.4 AMA1- and MSP1-specific MBC frequencies and Ab levels and malaria risk

We determined prospectively whether AMA1- or MSP1-specific Ab levels or MBC frequencies measured just prior to the six month malaria season were associated with the subsequent risk of malaria. For this analysis a malaria episode was defined as an axillary temperature ≥37.5°C, *Pf* asexual parasitemia ≥5000 parasites/µL, and a non-focal exam by the study physician. Because the incidence of malaria was very low in adults during the study period (Table 7.2), they were excluded from this analysis. Three measures of malaria risk were analyzed: 1) whether or not malaria was experienced, 2) the incidence of malaria, and 3) the time to the first malaria episode. In the corresponding multivariate regression models (logistic, Poisson, and Cox regression) which controlled for age, sickle cell trait, and concurrent asymptomatic *Pf* parasitemia, we found no correlation between malaria risk and AMA1- or MSP1- specific Ab levels or MBC frequencies. As discussed below, this finding was not unexpected based on the observation that the malaria vaccine candidates AMA1 and MSP1 did not confer protection against malaria in clinical trials (156, 171).

7.3 Discussion

In this year-long prospective study of children and adults in an area of intense, annual, sharply demarcated *Pf* transmission, we show that MBCs specific for *Pf* can be acquired, but did so gradually in a stepwise fashion over years of repeated *Pf* exposure. MBCs specific for two *Pf* antigens, AMA1 and MSP1, increased in frequency in response to acute *Pf* infection, and then contracted during a six-month period of decreased *Pf* exposure to a point slightly above pre-infection levels. Cross-sectional analysis of individuals aged two to twenty-five years just before the malaria season indicated that this step-wise, incremental increase in *Pf*-specific MBCs with each malaria season contributes to the gradual expansion of the *Pf*-specific MBC compartment with cumulative *Pf* exposure. By comparison, the stable frequency of TT-specific MBCs with age after immunization in infancy indicates that growth of antigen-specific MBC compartments does not simply occur with age, but requires repeated antigen exposure. Although infants have immature immune systems and have responses of lower magnitude than do older children and adults, studies have shown that vaccination in infancy can result in immunity lasting 18 years (50, 212). With regard to TT vaccination in infancy, a study in Cameroon found no difference in the responses of children recorded to have one or three TT vaccinations in infancy, all before the age of six months, and 55% and 62%, respectively, of individuals re-vaccinated at age 14-15 and at age 10-11 responded with titers above the seropositivity threshold set at 0.10 UI/ml (163). This is relatively similar to our data, with low responses evident after vaccination

in infancy, and the majority of individuals responding to vaccination with Ab and MBC levels higher than those of un-boosted individuals. The suggestion of these data that some individuals might not have persisting responses from vaccination in infancy and could be experiencing a primary response at the age 15 “booster” vaccination rather than a secondary response makes the contrast with the incremental response to *Pf* antigens even more remarkable. The ultimate comparison between humoral responses to a vaccine and to infection is difficult to make, as these are very different immune stimuli. What is clear, however, is that these individuals are able to mount a response and a recall response, indicating no long-term immunosuppressive effect of *Pf*. Data indicating that children aged five have comparable immunogenicity to adults following vaccination (13), further indicates that although infants have a disadvantage in mounting lasting immune responses, relatively young children can have competent immune responses. In spite of this finding, but in keeping with our data, there are differences in the ability of malaria-naïve children and adults to respond to malaria. Malaria-naïve adults are initially more susceptible to severe malaria than malaria-naïve children, but adults develop protective immunity and antibody faster than do children (20, 106). The implications of this with regard to the mechanisms required to develop protective immunity are not clear, but could be related to a more anti-inflammatory profile in children, preventing severe disease, the increased levels of lymph node homing receptors (90) or inhibitory receptors (145) on regulatory T-cells in children versus adults, or possibly impaired responses to TLR ligands documented in neonates

(136) persist into childhood, as we have shown TLR ligands can affect MBC and Ab development (Chapter 4). We do not formally know if the gradual gain in *Pf*-specific MBCs is in fact due to an increase in long-lived MBCs, or whether those MBCs require *Pf*-stimulation and would be lost if *Pf* transmission did not resume after the six-month dry season. Recent studies in mouse models are revealing multiple, phenotypically and functionally distinct populations of MBCs (14, 60) and it will be of interest to further characterize the MBCs described here.

The study described here provides a rare view of the acquisition and maintenance of human B cell memory. Most prospective studies of human B and T cell immunological memory have evaluated responses to vaccination rather than natural infection, in part because of the difficulty of predicting who within a population will be infected with a given pathogen at a given time. In response to a single vaccination, several studies have described an expansion and contraction of vaccine-specific MBCs (19, 208) and $CD8^{+}$ memory T cells (141). In one of the few longitudinal studies of MBC responses to natural infection, Harris *et al*. examined antigen-specific MBC responses in patients after presenting with acute *Vibrio cholerae* infection, a pathogen that elicits long-term protection against subsequent disease in endemic areas (97). In contrast to our results, they observed that the majority of patients acquired IgA and IgG MBCs specific for two *Vibrio cholerae* antigens and that these increased from day two to day 30 after presentation with cholera, and persisted at this level up to one year after infection.

Whereas MBCs mediate recall responses to reinfection by rapidly expanding and differentiating into PCs, in one model of a topic still debated LLPCs residing in the bone marrow constitutively secrete Ab in the absence of antigen and thus provide a critical first line of defense against reinfection (87). Logistical constraints precluded the direct measurement of circulating PCs in this study. However, we took advantage of the discrete six-month dry season, a period of little to no *Pf* transmission, to infer the relative contributions of SLPCs and LLPCs to the *Pf*-specific IgG response based on a serum IgG half-life of approximately twenty-one days (147). Two weeks after acute malaria, AMA1- and MSP1-specific Ab levels increased significantly and then decreased over a six-month period to a point just above pre-infection levels, indicating that the majority of PCs generated in response to acute *Pf* infection were short-lived. This observation is consistent with previous studies that described rapid declines in *Pf*-specific Abs within weeks of an acute malaria episode (36, 122). We infer that the small net increase in *Pf*-specific Abs at the end of the six-month dry season represents the acquisition of *Pf*-specific LLPCs. Because *Pf* transmission resumes after the six-month dry season, we cannot estimate the long-term decay rate of *Pf*-specific Abs or these apparently LLPCs in the absence of reinfection. It remains to be seen whether long-term decay rates of *Pf*-specific Abs are comparable to rates of Ab decay after exposure to common viral and vaccine antigens such as mumps and measles which elicit Abs with half-lives exceeding 200 years (12). The small incremental gains in AMA1- and MSP1-specific Abs in response to acute malaria mirrors the gradual exposure-related increase

in *Pf*-specific MBCs, consistent with the long-lived Abs being the products of LLPCs derived from MBCs. It may be that repeated exposure to the parasite is necessary to 'fill' the LLPC compartment to the point where basal levels of circulating Abs to any given *Pf* antigen reach a protective threshold. From these data *Pf* appears to induce a relatively high SLPC-to-LLPC ratio.

In addition, a remarkably high proportion of adults in the present study did not have detectable AMA1- or MSP1-specific MBCs despite annual exposure to fifty to sixty infective mosquito bites per person per month at the height of the malaria season (59), similar to what Dorfman *et al.* observed in a cross-sectional study in Kenya (64). In contrast, most female adults had detectable TT-specific MBCs three to ten years after a single TT booster vaccine in adolescence. In Chapter 4.2 we discussed our results of vaccination In *Pf*-naïve U.S. adults where following just two vaccinations AMA1- and MSP1-specific MBCs were reliably generated (45). Taken together, these observations indicate that the relatively inefficient generation and/or maintenance of *Pf*-specific MBCs in response to natural *Pf* infection cannot be ascribed entirely to inherent deficiencies in the antigens themselves.

In multivariate analysis we found no correlation between the frequency of MBCs and levels of Abs specific for AMA1 or MSP1 and malaria risk. This is not necessarily unexpected in light of recent clinical trials that showed that vaccination with either AMA1 or MSP1 did not confer protection (156, 171). Furthermore, we suspect that the frequency of MBCs *per se* may not reliably predict clinical immunity to malaria

regardless of antigen specificity. Malaria symptoms only occur during the blood stages of *Pf* infection and can begin as early as three days after the blood stage infection begins (179).Because the differentiation of MBCs into PCs peaks approximately six to eight days after re-exposure to antigen (30), there may not be sufficient time for MBCs specific for *Pf* blood stage antigens to differentiate into the Ab-secreting cells that would prevent the onset of malaria symptoms. In contrast, the longer incubation period of other pathogens allows MBCs to differentiate into protective Ab-secreting cells before symptoms develop. For example, follow-up studies of hepatitis B vaccinees have shown that protection can persist despite the decline of hepatitis B-specific Abs to undetectable levels (202), presumably due to the recall response of persistent MBCs. Thus, protection against the blood stages of malaria may depend on achieving and maintaining a critical level of circulating Ab that can rapidly neutralize the parasite. MBCs may contribute to the gradual acquisition of protective immunity by differentiating into LLPCs with each *Pf* infection.

It is also possible that *Pf* infection disrupts the immune system's ability to generate or maintain MBCs or LLPCs. The differentiation of B cells into long-lived MBCs depends to a great extent on the affinity of their BCRs for antigen. Recently, evidence was presented that affinity maturation of B cells may fail to occur in the absence of adequate Toll-like receptor (TLR) stimulation (55). As we discussed in Chapter 4.3, based on trials using the same AMA1-C1 CpG vaccines, Malian adults appear refractory to the TLR9 agonist CpG, relative to malaria-naïve U.S. vaccinees (190), raising the possibility

that the slow acquisition of MBCs observed here may be due to a failure of B cells to undergo affinity maturation during *Pf* infection. Although our data do not directly address the role of apoptosis in the gradual acquisition of *Pf*-specific MBCs, it is worth noting that we found no evidence of *Pf*-induced ablation of *Plasmodium*-specific MBCs, as was observed in mice four days after *Plasmodium yoelii* infection (209). The relatively inefficient response to natural *Pf* infection also does not appear to be due to a persistent, *Pf*-induced general immunosuppression as the frequency of TT-specific MBCs increased significantly in most adult females in response to a single TT booster vaccination, an increase that appeared to be maintained for years. In an experimental model of lymphocytic choriomeningitis virus (LCMV) infection, a high antigen-to-B cell ratio disrupted germinal center formation and the establishment of B cell memory (214). It is plausible that a similar mechanism is at play during the blood stage of *Pf* infection when the immune system encounters high concentrations of parasite proteins. Indeed, germinal center disruption is observed in mice infected with *P. berghei* ANKA (35) and *P. chabaudi* (4). It is also possible that specific parasite products selectively interfere with the regulation of B cell differentiation (175) or with the signals required for sustaining LLPCs in the bone marrow (154). It is also conceivable that the high level of class-switched SLPCs we observed in response to *Pf* infection arises from pre-diversified $IgM^+IgD^+CD27^+$ (marginal zone) B cells—analogous to the rapid protective response against highly virulent encapsulated bacteria that do not elicit classical T-dependent responses (201). These and other hypotheses could be tested by applying

systems biology methods (164) and targeted *ex vivo* and *in vitro* assays to rigorously conducted prospective studies of *Pf*-exposed populations.

Here we also provide evidence concerning the mechanism by which MBCs and LLPCs are maintained. We observed a modest but statistically significant increase in TT-specific MBCs two weeks after acute malaria, in support of the hypothesis that MBCs are renewed by polyclonal or 'bystander' activation (30). The stable frequency of TT-specific MBCs with age suggests that the rate of loss of TT-specific MBCs is balanced by the small increases in TT-specific MBCs over time, possibly due in part to *Pf*-induced polyclonal activation. It is of general interest to determine which parasite products are responsible for the polyclonal activation of MBCs observed here. Studies *in vitro* suggest that *Pf* drives polyclonal MBC activation by the cysteine-rich interdomain regions 1α (CIDR1α) of the *Pf* erythrocyte membrane protein 1 (PfEMP1) (61, 62), but it is conceivable that *Pf*-derived TLR agonists (125, 159) or bystander T cell help (103, 107, 117) also contribute to MBC proliferation in the absence of BCR triggering (139).

Similarly, it has been hypothesized that non-specific polyclonal stimulation maintains long-lived Ab responses by driving MBCs to differentiate into SLPCs or LLPCs (30). It has also been proposed that *Plasmodium* infection generates large amounts of non-specific Ig (49) through polyclonal B cell activation (62, 89). However, despite the presence of TT-specific MBCs and their expansion following *Pf* infection, we did not observe a concomitant increase in TT-specific IgG. This finding is consistent with recent human studies that demonstrate a lack of bystander IgG production after heterologous

vaccination or viral infection (12, 57); as well as studies in mice that demonstrate PC persistence after MBC depletion (7), and the failure of MBCs to differentiate into PCs *in vivo* upon TLR4 and 9 activation (166). This finding does not represent an overt inability of TT-specific MBCs to differentiate into PCs, since adult females in this study had a sharp increase in tetanus IgG after a single tetanus booster. It is possible that bystander MBCs specific for antigens other than TT differentiate into PCs after *Pf* infection, but based on the results of this study we hypothesize that the preponderance of IgG produced in response to malaria is specific for the ~2400 *Pf* proteins expressed during the blood-stage of infection (80), and that increases in 'non-specific' IgG reflect boosting of cross-reactive B cells (51, 213). From a basic immunology perspective, these data support a model in which non-specific stimuli contribute to MBC self-renewal, but not to the maintenance of LLPCs. Studies of other Ab specificities and isotypes before and after malaria and other infections would test this hypothesis further. Although a recent mouse study showed that MBCs do not proliferate *in vivo* after immunization with an irrelevant antigen (28), this may reflect the difference in requirements for MBC maintenance in mammals with relatively short life spans.

Animal models have provided important insights into the immunobiology of *Plasmodium* infection (210), but ultimately, despite obvious experimental limitations, it is critical to investigate the human immune response to *Pf* in longitudinal studies since findings from animal models do not always mirror human biology or pertain to the clinical context (53, 138). Key challenges for future studies will be to determine the

molecular basis of the inefficient generation of MBCs and LLPCs in response to *Pf* infection and to determine the longevity of these cells in the absence of *Pf* transmission over longer periods of time. Greater insight into the molecular and cellular basis of naturally-acquired malaria immunity could open the door to strategies that ultimately prove useful to the development of a highly effective malaria vaccine.

Chapter 8: Atypical memory B cell expansion in individuals in the Kambila cohort

8.1 Introduction

Data presented in Chapter 7 provides evidence that what appear to be normal *Pf*-specific MBCs are ultimately acquired in individuals living in malaria endemic areas. Here we address the question: is there, in addition, any evidence for an abnormal or atypical expansion of any MBC population in malaria exposed individuals?

This is an important factor to consider as the development of sub-optimal memory B cells due to cellular exhaustion or anergy, or the induction of an immuno-regulatory B cell type could be related to the slow development of *Pf*-specific MBCs that we have observed. During normal MBC or PC development, B cells could be diverted into a dysfunctional phenotype, removing these cells from the functional MBC repertoire, resulting in the development of an altered B cell phenotype. Alternately B cells with a non-traditional B cell function, akin to regulatory B cells, could alter the normal development of MBCs by cytokine secretion interrupting normal MBC development and thus contributing to the slow acquisition of *Pf*-specific MBCs. Ultimately this could affect the ability to mount a parasite-clearing immune response, and contribute to the slow acquisition of immunity to malaria. Both human and murine malarias induce regulatory T cells (176). As *Pf* has already demonstrated several properties which lend to evading the immune system, it seems likely that more mechanisms could exist. The ability of pathogens to impair B cell function has not been as widely studied as has been the effect on T cells, but there is evidence to suggest that

B cell impairment is induced during HIV (144), herpes simplex infection (24), and in animal models, in EBV (185). In a murine model malaria infection decreased the IgG response to vaccination as compared to uninfected mice (140), and taken together with data we have presented in Chapters 6 and 7 and the studies discussed in Chapter 1.7 which show that *Pf*-specific Ab is short-lived, and inconsistently generated, it could very well be that there is an underlying cellular dysfunction in MBC development in response to malaria.

One of the more interesting possibilities of altered B cell function in this regard is that initially described by Ehrhardt *et al.* (71), where a morphologically and functionally distinct human MBC population was found in tonsil, defined by the expression of FCRL4, a member of a recently identified family of FcR like proteins. FCRL4^{+} MBCs were found almost exclusively in lymphoid tissues near epithelial surfaces. Most MBCs in humans express CD27, a member of the TNF family, and have somatically mutated V genes and switched Ig isotypes. The expression of the classical marker for human MBCs, CD27, is much reduced on FCRL4^{+} MBCs, but these B cells have undergone isotype switching and somatic hypermutation. FCRL4^{+} MBCs express the activation markers CD69, CD80 and CD86 and are functionally distinct from CD27^{+} FCRL4^{-} MBCs, as FCRL4^{+} MBCs proliferate and secrete high levels of immunoglobulins in response to cytokines and CD40 ligand (CD40L) but fail to proliferate in response to BCR crosslinking or treatment with Staphylococcus aureus Cowen (SAC). Recent transcriptome analyses of FCRL4^{+} and FCRL4^{-} MBCs showed that these two populations differentially express genes in several

categories including cell-cycle regulators, adhesion molecules, homing receptors and signal transduction intermediates (70). Although a distinct function has not yet been attributed to FCRL4^{+} MBCs *in vivo,* their exclusive location in epithelial associated lymphoid tissues and their activated phenotype suggest to Ehrhardt et al. that they may play a normal role in mucosal defense against invading pathogens.

Recently, Moir *et al.* (143) showed that in the peripheral blood of HIV patients with high viremia, an atypical population of FCRL4^{+}CD20^{hi}CD27^{-}CD21^{lo} MBCs was greatly expanded, representing on average 19% of total B cells, compared to less than 4% in healthy individuals. These atypical MBCs in HIV-infected individuals had undergone somatic hypermutation and class switching albeit to lower levels as compared to CD27^{+} MBCs. Compared to naive B cells and classical MBCs, the atypical MBCs in the peripheral blood of HIV-infected individuals proliferated less to BCR-crosslinking and/or CD40L and the TLR agonist, CpG, and showed a decreased ability to differentiate into Ab secreting cells in response to CpG and SAC. The atypical MBCs in HIV-viremic individuals expressed relatively high levels of inhibitory receptors and a profile of homing receptors similar to that described for tissue-based FCRL4^{+} MBCs (70, 143) and for exhausted CD8^{+} T cells during chronic viral infection (203). Because of the overall hypo-responsiveness of these atypical MBCs, their altered expression of inhibitory and homing receptors that together are signatures for virus-induced exhaustion of T cells (54, 191, 203), Moir *et al.* coined these atypical MBCs 'exhausted MBCs'. HIV-specific MBCs were found to be increased in the exhausted MBC compartment as compared to the classical MBC

compartment, in contrast, influenza-specific MBCs were more prevalent in the classical MBC compartment (143). Importantly, exhausted MBCs were found in normal levels in peripheral blood of individuals treated early to reduce viremia, and decreased by approximately half in individuals with high levels of atypical MBCs after their viremia was brought down by antiretrovirals. The expanded level of atypical MBCs in these individuals may be maintained by viral spiking (S. Moir, unpublished observations). These authors proposed that chronic HIV stimulation of B cells may lead to their premature exhaustion, contributing to the poor Ab responses in HIV-infected individuals. As an increased prevalence of these atypical MBCs may contribute to (if these cells have a regulatory function), or be a side effect of (if these cells are dysfunctional cells derailed on their way to becoming functional MBCs) the delayed acquisition and short-lived nature of immunity, MBCs and/or LLPCs in response to *Pf* malaria, in this chapter we report on analyses to determine whether B cells phenotypically and functionally similar to these atypical MBCs are expanded in individuals chronically exposed to *Pf*. A more thorough exploration of B cell phenotypes in malaria-exposed individuals could reveal important aspects of the B cell response to *Pf* infection and inform the design of malaria vaccines that go beyond the traditional empiric approach and address *Pf*-specific modulation of the immune response.

8.2 Results

8.2.1 Atypical MBCs are greatly expanded in individuals in malaria endemic areas

An age-stratified subset was randomly selected from the Kambila study cohort (peak season: n-87, pre-season: n=109) that, based on baseline characteristics of study subsets and malaria outcomes, did not differ significantly from the entire cohort. The B cells in the peripheral blood of the volunteers, before the start of the malaria transmission season and at the peak of the malaria transmission season in October were characterized by flow cytometry using a panel of fluorophore conjugated Abs specific for CD19, CD27, CD21, CD10 and CD20 that allowed the identification of immature B cells ($CD19^+$ $CD10^+$), naïve B cells ($CD19^+$ $CD27^-$ $CD21^{hi}$ $CD10^-$), classical MBCs ($CD19^+$ $CD27^+$ $CD21^{hi}$ $CD10^-$), activated MBCs ($CD19^+$ $CD27^+$ $CD21^{lo}$ $CD20^{hi}$ $CD10^-$), atypical MBCs ($CD19^+$ $CD27^-$ $CD21^{lo}$ $CD20^{hi}$ $CD10^-$) and plasma cells or plasma blasts ($CD19^+$ $CD27^+$ $CD21^{lo}$ $CD20^-$). IgD expression was not examined, but would be expected to be in $CD19^+$ $CD27^-$ $CD21^{hi}$ $CD10^-$ naïve B cells. Fig. 8.1 shows the gating strategy used to identify these B cell subpopulations and a representative example of an individual from the U.S. as compared to an individual in Mali.

A comparison of the proportion of B cells in each subpopulation in the peripheral blood at the peak of malaria transmission is given as a percent of total $CD19^+$ B cells (Fig. 8.2 A-F). The percent of $CD19^+$ B cells per PBMC did not vary significantly between individuals before and at the peak of the malaria transmission season (before season: mean 12.16% [95% CI, 11.36-12.97] vs. peak season: mean 11.71% [95% CI, 10.86-12.57]

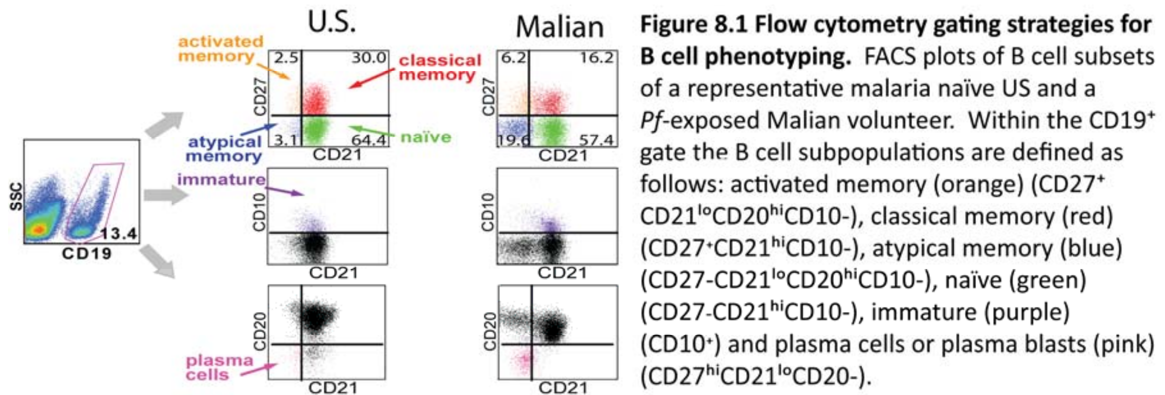


Figure 8.1 Flow cytometry gating strategies for B cell phenotyping. FACS plots of B cell subsets of a representative malaria naïve US and a *Pf*-exposed Malian volunteer. Within the CD19$^+$ gate the B cell subpopulations are defined as follows: activated memory (orange) (CD27$^+$ CD21^{lo}CD20^{hi}CD10-), classical memory (red) (CD27$^+$CD21^{hi}CD10-), atypical memory (blue) (CD27-CD21^{lo}CD20^{hi}CD10-), naïve (green) (CD27-CD21^{hi}CD10-), immature (purple) (CD10$^+$) and plasma cells or plasma blasts (pink) (CD27^{hi}CD21^{lo}CD20-).

p=0.451). The relative proportions of all the B cell subpopulations analyzed per total B cells for each age group are also shown as stacked plots in Fig. 8.2 G. The percent of plasma cells/plasma blasts (Fig. 8.2 B) was similar in both U.S. and Malian adults and children. The percent of naïve B cells, and immature B cells had a tendency to decrease with increasing age in Malians and for these subsets, U.S. adults appeared similar to Malian children (Fig. 8.2 A, C). As compared to U.S. adults, Malian adults had a similar percent of classical MBCs (Fig. 8.2 D). Malian children in both the two to seven and eight to ten year age groups had a smaller percent of classical MBCs as compared to either U.S. or Malian adults, likely an age-related phenomenon unrelated to malaria. The increase with age of classical MBCs is consistent with the increase in total IgG$^+$ MBCs we observed using the MBC ELISPOT assay (Fig. 7.3). Strikingly, the percent of atypical MBCs was significantly higher in Malian adults and children compared to U.S. adults, with a trend of increasing atypical MBCs with increasing age among the Malian donors (Malian adults: mean 15.5% [95% CI, 9.7-21.2] vs. U.S. adults: mean 1.6% [95% CI, 1.0-2.2]; p<0.001; Malian children: mean 9.8% [95% CI, 8.2-11.3]; p<0.001 vs. U.S. adults) (Fig. 8.2 E). The percent of B cells with an activated MBC phenotype was significantly

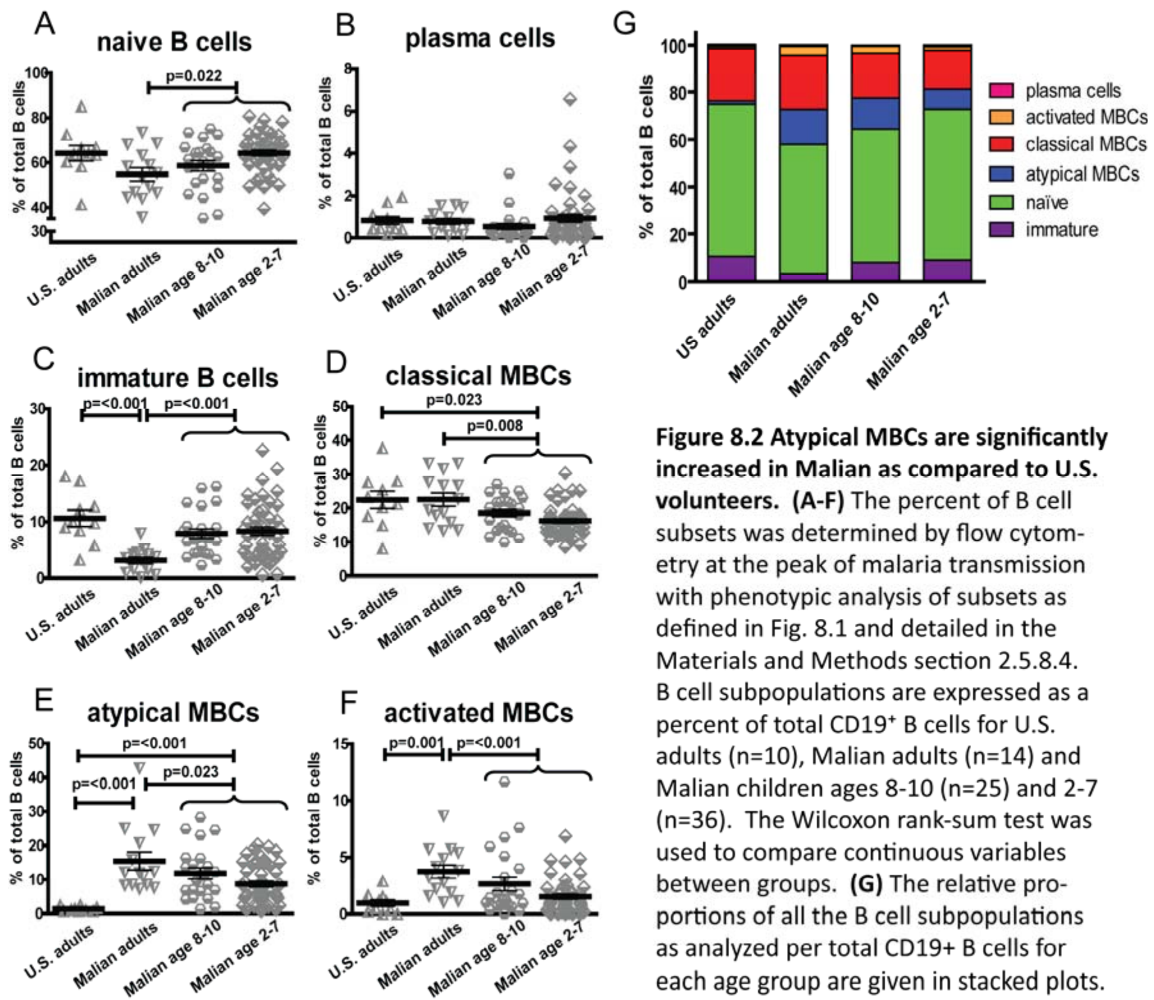


Figure 8.2 Atypical MBCs are significantly increased in Malian as compared to U.S. volunteers. **(A-F)** The percent of B cell subsets was determined by flow cytometry at the peak of malaria transmission with phenotypic analysis of subsets as defined in Fig. 8.1 and detailed in the Materials and Methods section 2.5.8.4. B cell subpopulations are expressed as a percent of total $CD19^+$ B cells for U.S. adults (n=10), Malian adults (n=14) and Malian children ages 8-10 (n=25) and 2-7 (n=36). The Wilcoxon rank-sum test was used to compare continuous variables between groups. **(G)** The relative proportions of all the B cell subpopulations as analyzed per total CD19+ B cells for each age group are given in stacked plots.

higher in Malian adults as compared to Malian children and U.S. adults (Malian adults: mean 3.7% [95% CI, 2.5-5.0] vs. U.S. adults: mean 1.3% [95% CI, 0.6-2.1]; p=0.001; Malian children: mean 1.9% [95% CI, 1.4-2.4]; p<0.001 vs. Malian adults) (Fig. 8.2 F). As with the atypical MBCs, there was a trend of increased activated MBCs with increased age among the Malian donors. For both atypical MBCs and activated MBCs the largest differences were between U.S. and Malian adults.

We further characterized atypical MBCs, classical MBCs and naïve B cells from peripheral blood of a randomly selected subset of Malian adults (n=6) and children age

two to four (n=6) to determine the expression level of several inhibitory and homing receptors which are characteristic of both tissue-based (70) and exhausted MBCs (143) (Fig. 8.8). FCRL4, the cell-surface marker that defines tissue-based MBCs and is a characteristic of exhausted MBCs was expressed at significantly higher levels on atypical MBCs compared to classic MBCs and naïve B cells. The expression pattern of inhibitory and homing receptors on atypical MBCs was similar for Malian children and adults, and comparable to that observed in HIV viremic individuals and in FCRL4^{+} tonsilar MBCs (70, 71, 143). Atypical MBCs showed increased expression of the inhibitory receptors CD85j and CD22. No differences were observed between classical MBCs and atypical MBCs in the expression of the inhibitory receptor LAIR1, although as compared to naïve B cells both subpopulations expressed less LAIR1. CD11c and CXCR3 levels were increased on atypical MBCs as compared to either classic MBCs or naïve B cells. CXCR4, CD62L, CXCR5 and CCR7 expression was decreased on atypical MBCs and little difference between subpopulations was observed in the expression of CCR6 (Fig. 8.3) or CD72. The phenotypes of both the naïve B cells and classical MBCs were similar to that described for U.S. individuals. Overall these cells closely match the phenotype of the atypical MBCs characterized in HIV (143), and the receptor expression fits the profile of cells that are refractory to BCR-mediated activation, with the increased levels of inhibitory receptors, and unlikely to participate in germinal center reactions, with the decreased levels of lymph-node homing receptors and increased levels of tissue homing receptors.

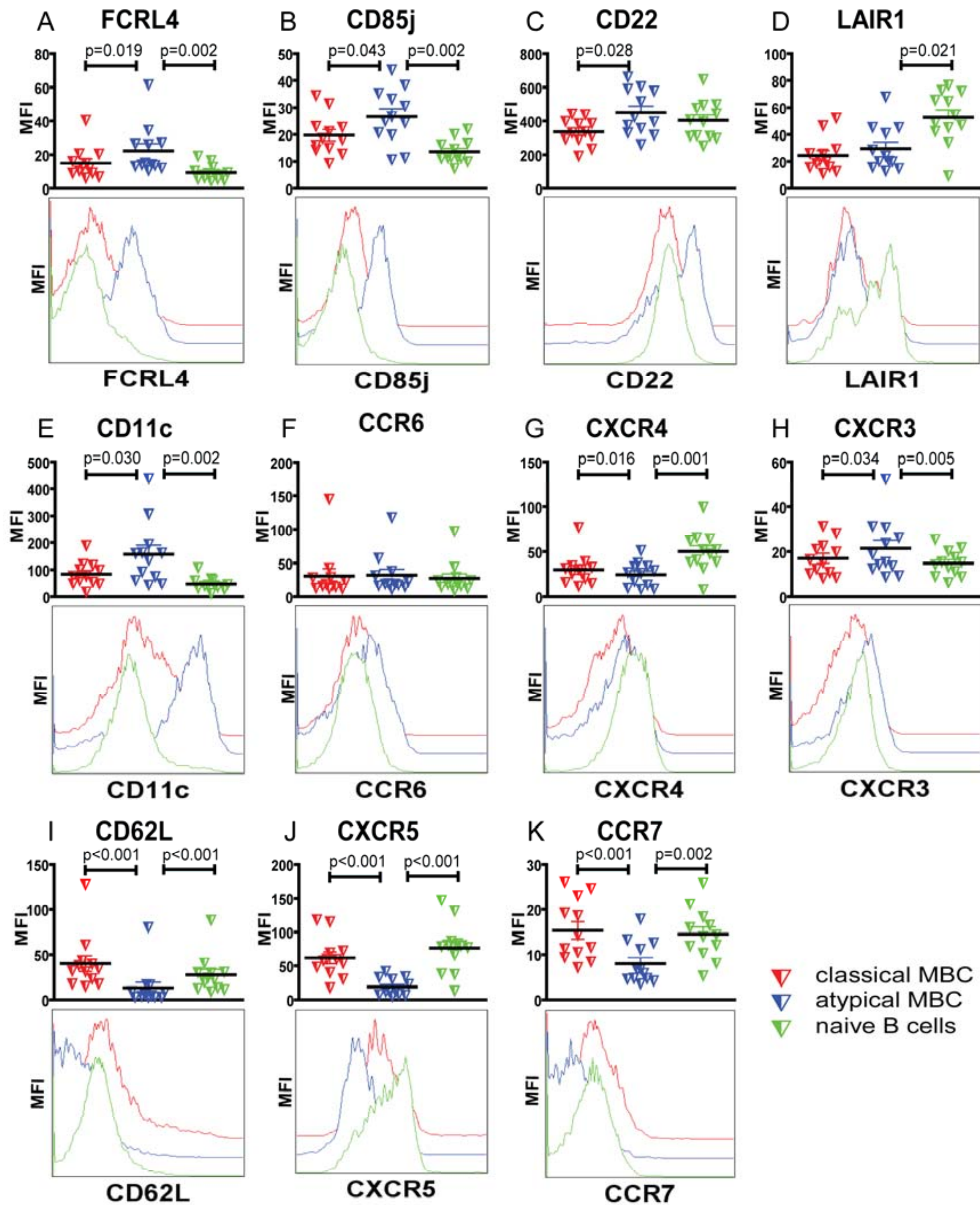


Figure 8.3 Inhibitory and tissue-homing receptor expression is increased and lymph node homing receptor expression is decreased on atypical MBCs relative to classical MBCs. FACS analysis of the expression of inhibitory and homing receptors on naïve B cells (green), atypical MBCs (blue) and classical MBCs (red) on a subset of 12 Malian individuals: 6 children age 2-4 and 6 adults. For each panel **(A-F)** the top plots show individual MFI values for each cell subpopulation of each individual, as well as the average MFI and standard deviation. Underneath are histograms of the MFI for each subpopulation of a representative individual. The expression of inhibitory receptors **(A-D)**, tissue homing receptors **(E-H)** and lymph node homing receptors **(I-K)** is given for classical MBCs, atypical MBCs and naïve B cells (as defined in Fig. 1) using appropriately labeled Abs specific for CD19, CD27, CD21 and the particular inhibitory and homing receptors indicated. The Wilcoxon matched pair test was used for this comparison.

To determine if these atypical MBCs are present at the end of the dry season, as opposed to only during the malaria transmission season, we examined the percentage of atypical MBCs at the end of the dry season and found comparable percentages in both children and adults (Malian adults aged 18-25 years: 14.8% [95% CI: 11.0-19.1], Malian children aged two to ten years: 10.2% [95% CI: 8.7-11.8]). At this timepoint, with increasing age, and as a percentage of total $CD19^+$ B cells we observed a decrease in immature B cells ($p<0.001$) and naïve B cells ($p=0.047$) and an increase in resting IgG^+ MBCs ($p<0.001$) and activated IgG^+ MBCs ($p<0.001$). Overall there was little difference in the total B cell subset composition before the malaria transmission season and at the peak of transmission, and the age-related patterns were the same.

To determine if atypical MBCs were expanded in individuals in other, geographically distinct malaria endemic settings in a small collaborative study we analyzed adults living in an area of low *Pf* transmission in Peru. In this region of Peru,

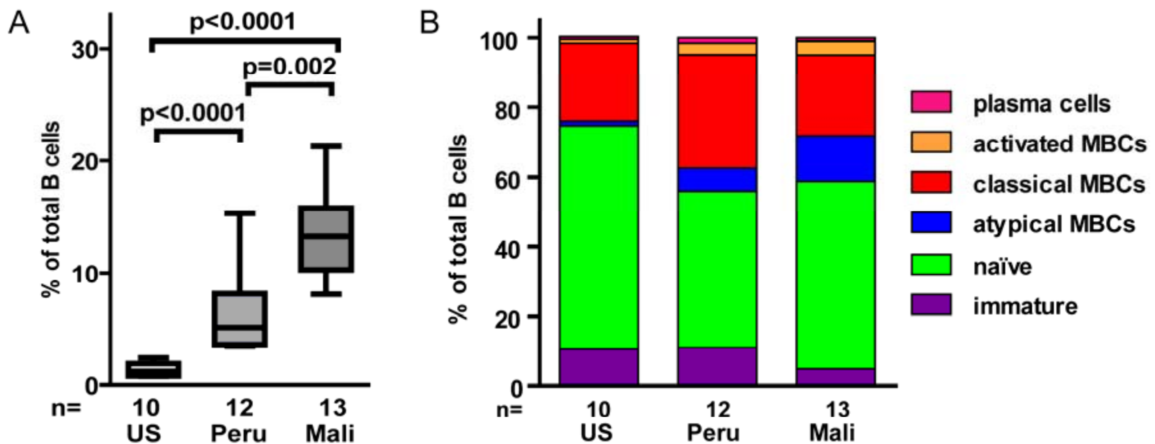


Figure 8.4 Atypical MBCs increase with increased *Pf* transmission in individuals in two endemic transmission settings. Comparison of atypical MBC frequencies as a percent of total B cells in healthy U.S. blood bank controls, Peruvian adults with prior reported *Pf* infection and current *Pf* parasitemia (Day 0), and Malian adults with prior reported *Pf* infection and current *Pf* parasitemia **(A)**. Total B-cell subset analysis **(B)**. Groups are as described in part **(A)** with subsets defined within $CD19^+$ cells as in Fig. 8.1

individuals receive approximately one to two infectious bites per year as compared to the 50-60 infectious bites per month at the height of the malaria transmission season in Kambila. To make the most direct comparison possible between high and low transmission settings we selected adults in Peru with *Pf* parasitemia who have a history of prior *Pf*-malaria, excluding those with no prior history of *Pf*-malaria, and we selected adults in Mali only at their first positive *Pf* parasitemia of the transmission season, as many have positive *Pf* parasitemia at multiple timepoints, and all adults in the Kambila cohort have a history of *Pf*-malaria (Fig.8.4). However, individuals in Peru had symptoms of malaria, while individuals in Mali were asymptomatic. In comparing B cell subsets in individuals from Peru and Mali, we observed no differences in the percentages of plasma cells, activated MBCs, classical MBCs, or naïve B cells. Compared to individuals in Peru, individuals in Mali had a lower average percentage of immature B cells (Peru: 11.0%, Mali: 4.8%; p=0.011) and increased percentage of atypical MBCs (Peru: 6.5%, Mali: 13.1%; p=0.002) (Fig. 8.4). Compared to healthy

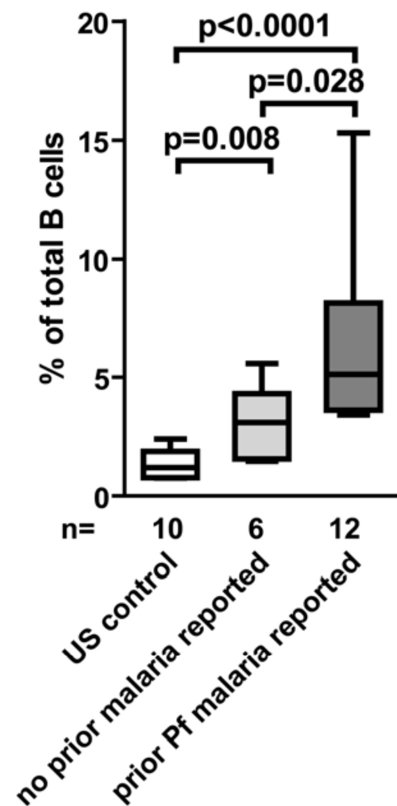


Figure 8.5 Comparison of atypical MBC percentages in Peruvian individuals separated by reported prior *Pf*-malaria. Comparison of atypical MBC frequencies as a percent of total B cells in healthy US blood bank controls, Peruvian individuals with no prior reported *Pf* infection and current *Pf* parasitemia, and Peruvian individuals with one or more prior reported *Pf* infection and current *Pf* parasitemia.

U.S. donors, individuals in Peru and Mali had a lower average percentage of naïve B cells (U.S.: 64.2% Peru: 45.0%, Mali: 54.0%; U.S. vs. Peru p=0.003; U.S. vs. Mali p=0.013), and a greater average percentage of activated MBCs (U.S.: 0.9%, Peru: 3.5%, Mali: 4.0%; U.S. vs. Peru p=0.002; U.S. vs. Mali p=0.0003) (Fig. 8.4 B), and atypical MBCs (U.S.: 1.4%, Peru: 6.5%, Mali: 13.1%; U.S. vs. Peru p<0.0001; U.S. vs. Mali p<0.0001) (Fig.8.4). Compared to healthy U.S. donors, individuals in Peru also had a greater average percentage of classical MBCs (U.S.: 22.4% Peru: 32.6%, p=0.038), and individuals in Mali had a lower average percentage of immature B cells (U.S.: 10.6% Mali: 4.8%, p=0.003).

We also compared individuals in Peru presenting with *Pf* parasitemia with no prior history of malaria (n=6) and those with one or more prior cases of *Pf*-malaria (n=12). Individuals presenting with *Pf* parasitemia with no prior history of malaria had higher percentages of atypical MBCs than malaria-naïve U.S. volunteers, but lower percentages of atypical MBCs as compared those with one or more prior cases of *Pf*-malaria (U.S. control: 1.4%, no prior malaria: 3.1%, prior *Pf*-malaria: 6.6%; U.S. vs. Peru no prior malaria: p=.008, Peru no prior vs. prior malaria p=0.028) (Fig. 8.5). These data suggest that atypical MBCs increase with increased *Pf* exposure. We cannot, however, rule out the possibility that atypical MBCs are a phenomenon related to race rather than malaria exposure as we do not have access to the ethnicity of U.S. blood bank donors. It is a possibility that Africans are genetically predisposed to atypical MBC development and that Peruvians also have a genetic predisposition to atypical MBC development to a lesser extent than do Africans. Considering the increase in atypical MBCs from Peruvian

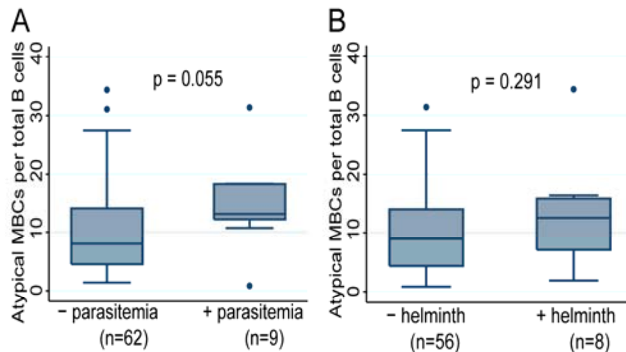


Figure 8.6 The percent of atypical MBCs is larger in children with persistent asymptomatic *P.falciparum* parasitemia as compared to parasite-free children. Shown is the percent of atypical MBCs per total B cells in children aged 2-10 years **(A)** with (n=9) or without (n=62) *Pf* parasitemia at the end of the dry season or **(B)** with (n=8) or without (n=56) helminth infection. The Wilcoxon rank-sum test was used to compare continuous variables between groups.

adults with no prior history of malaria to Peruvian adults with prior history of malaria within the same ethnic group does suggest, however, that this expansion is related to malaria exposure rather than being solely a genetic phenomenon, or resulting from exposure to other pathogens which is unlikey to differ significantly in these groups.

To attempt to determine if the increase in atypical MBCs in the Kambila cohort is related to exposure to *Pf*, and to get the cleanest comparison of exposure versus non-exposure in this study we compared the percentage of atypical MBCs in children with or without asymptomatic *Pf* parasitemia at the end of the six-month dry season. Since little or no parasite transmission occurs over the dry season (59), *Pf* parasitemia at this time reflects a chronic infection persisting from the previous year's transmission season. Conversely, aparasitemic individuals at this time have likely been aparasitemic for months. We felt that this would be the optimal timepoint to make this comparison as during the transmission season partially-immune individuals typically have asymptomatic parasitemias and recent exposure to *Pf* is more difficult to determine. The percent of asymptomatic parasitemic individuals was similar across all age groups and ranged between 3-5% (Table 5.1). There was a trend toward a higher percentage of

atypical MBCs in children age two to ten years with asymptomatic *Pf* infection (n=9) as compared to those without infection (n=62) (Fig. 8.6 A; with *Pf* parasitemia: mean 14.7% [95%CI, 2.7-21.0]; without *Pf* parasitemia: mean 9.9% [95%CI, 8.1-11.7]; p=0.055). In multivariate regression analysis that included age as a covariate, this association did not reach statistical significance. Intestinal helminth infection was not associated with a significant change in the percentage of atypical MBCs in children aged two to ten years (Fig. 8.6 B); with helminth infection: n=8 mean 13.4% [95%CI, 5.2-21.6]; without helminth infection: n=56 mean 9.9% [95%CI, 8.1-11.7]; p=0.291), although the sample size may be too small to detect a significant difference. Of note, neither the percentage of atypical MBCs before the malaria season nor the percentage at the peak of the malaria season was associated with malaria risk, as defined by malaria incidence or time to the first malaria episode (not shown).

8.2.2 Class switching in atypical MBCs in individuals in malaria endemic areas

Class switching occurs during MBC maturation, simultaneous with somatic hypermutation, with both processes dependent on AID expression. The proportion of IgG^+ $FCRL4^+$ B cells in Ehrhardt et al's study was 63%, followed by IgA at 28%, with the IgM isotype accounting for only 11% of $FCRL4^+$ B cells. The proportion of IgG^+ atypical MBCs in HIV positive individuals which responded to stimulation was approximately half, with IgM accounting for the other half, and IgA nearly undetectable (143). To assess class-switching in the MBC subpopulations we analyzed cell surface IgG expression of atypical and classical MBCs in our Malian cohort. Overall the pattern of

IgG-expression was similar for classical and atypical MBCs (Fig. 8.7) with the proportion of IgG$^+$ MBCs increasing with age in both classical and atypical MBCs. In each age group the proportion of atypical MBCs that were IgG$^+$ was slightly higher than the proportion of classical MBCs that were IgG$^+$. We conclude that isotype switching is similar in the different MBC subpopulations and that the high proportion of IgG$^+$ atypical MBCs indicates that it is likely these cells have undergone somatic hypermutation.

In the Peruvian cohort we also examined the percentage of atypical MBCs that were IgG$^+$ While individuals in the U.S. had an average of ~10% IgG$^+$ atypical MBCs, the percentage of IgG$^+$ atypical MBCs was dramatically increased in *Pf*-infected individuals from Peru with a history of prior *Pf*-malaria and further increased in individuals from Mali (U.S.: 9.11%, Peru: 49.6%, Mali: 59.3%; U.S. vs. Peru p=0.003; U.S. vs. Mali p=0.003; Peru vs. Mali p=0.008) (Fig. 8.8 A). Individuals in Peru with *Pf* parasitemia and no prior history of malaria had greater numbers of IgG$^+$ atypical MBCs than did individuals from the U.S., and although there was a trend toward a greater percentage of atypical MBCs in individuals with a prior history of *Pf*-

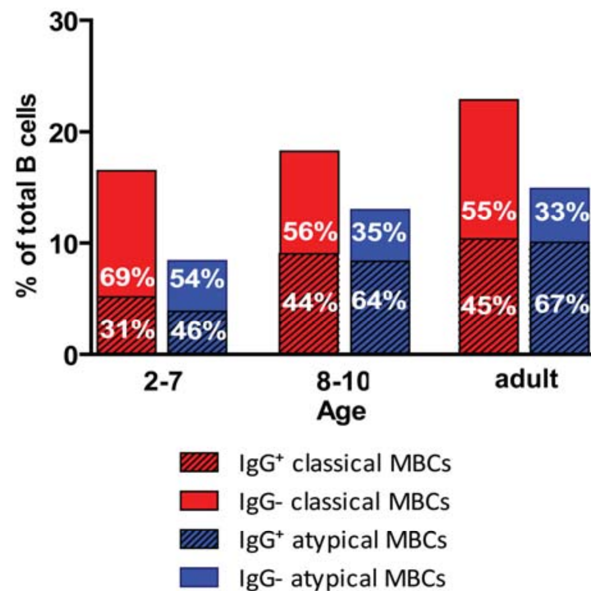


Figure 8.7 The IgG expression of atypical and classical MBCs is similar. Stacked plots showing the percentage of IgG$^+$ and IgG- MBC for both the classical MBC sub-population and the atypical MBC subpopulation.

malaria, the difference was not statistically significant (Fig.8.8 B).

Exhausted MBCs in HIV-viremic individuals were hypo-responsive in their ability to differentiate into Ab secreting cells (ASC) *in vitro* in response to polyclonal stimulation with a combination of CpG and SAC (143). In preliminary studies, we separated peripheral blood B cells from Malian adults into atypical MBCs ($CD19^+$ $CD27^-$ $CD21^{lo}$), classical MBCs ($CD19^+$ $CD27^+$ $CD21^{hi}$) and naïve B cells ($CD19^+$ $CD27^-$ $CD21^{hi}$) as described (143). When stimulated with CpG and SAC atypical MBCs failed to produce any ASCs (n=1; classical MBCs 34,900 ASC/10^6 at end of culture [SD ± 2,000]; atypical MBCs 0 ASC/10^6; naïve B cells 1,300 ASC/10^6 [SD ± 200]). As compared to classical MBCs,

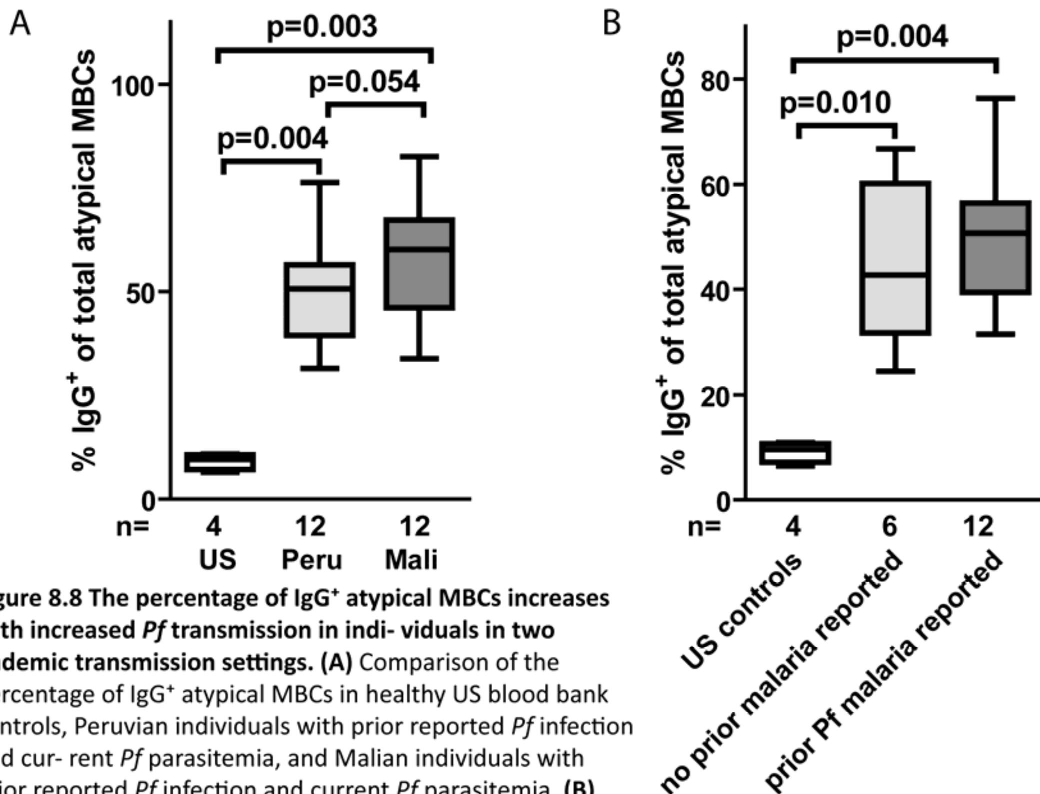


Figure 8.8 The percentage of IgG⁺ atypical MBCs increases with increased *Pf* transmission in indi- viduals in two endemic transmission settings. (A) Comparison of the percentage of IgG⁺ atypical MBCs in healthy US blood bank controls, Peruvian individuals with prior reported *Pf* infection and cur- rent *Pf* parasitemia, and Malian individuals with prior reported *Pf* infection and current *Pf* parasitemia. **(B)** Comparison of IgG⁺ atypical MBC percentages in US controls, Peruvian individuals with no prior reported *Pf* infection and current *Pf* para- sitemia, and Peruvian individuals with one or more prior reported *Pf* infection and current *Pf* parasitemia.

atypical MBCs responded poorly to the combination of pokeweed mitogen, SAC, CpG and IL-10, as we showed in Chapter 3, a combination that is more efficient in inducing the differentiation of MBCs into ASCs (n=2; classical MBCs 102,800 ASC/10^6 at end of culture [SD ± 18,200]; atypical MBCs 2,600 ASC/10^6 [SD ± 1,200]; naïve B cells 8,250 ASC/10^6 [SD ± 2,000]). Naïve B cells, as predicted (46), responded weakly to both stimulation cocktails. By these criteria, the atypical MBCs in Malian volunteers are hypo-responsive to stimuli that activate classical MBCs, and thus phenotypically and possibly functionally resemble exhausted MBCs.

8.2.3 Longitudinal profiling of B cell subsets in children before and after acute malaria

We investigated the impact of acute malaria on the relative proportion of B cells in each subset in children aged two to ten years. Compared to the pre-malaria season baseline (month 0), there were no significant changes in the percent of lymphocytes that were $CD19^+$ 14 days after acute malaria. Within the $CD19^+$ B cell population there were no significant changes in the percent of immature B cells, naïve B cells, or classical MBCs, after acute malaria. However, we observed a decrease in the percentage of total atypical MBCs (Fig. 8.9 A; month 0: 10.9% [95% CI: 9.4-12.4], convalescence: 8.7% [95% CI: 7.3-10.2]; p=0.027), and an increase in activated MBCs following acute malaria (Fig. 8.9 B; month 0: 1.6 [95% CI: 1.2-2.0], convalescence: 1.9 [95% CI: 1.4-2.4]; p=0.09). Within the activated and atypical MBC subset there was a significant decrease in the proportion that were IgG^+ (Fig. 8.9 C; activated MBC; month 0: 60.5% [95% CI: 56.7-64.3], convalescence: 36.7% [95% CI: 29.3-44.0]; p<0.0001; Fig. 8.9 D; atypical MBC;

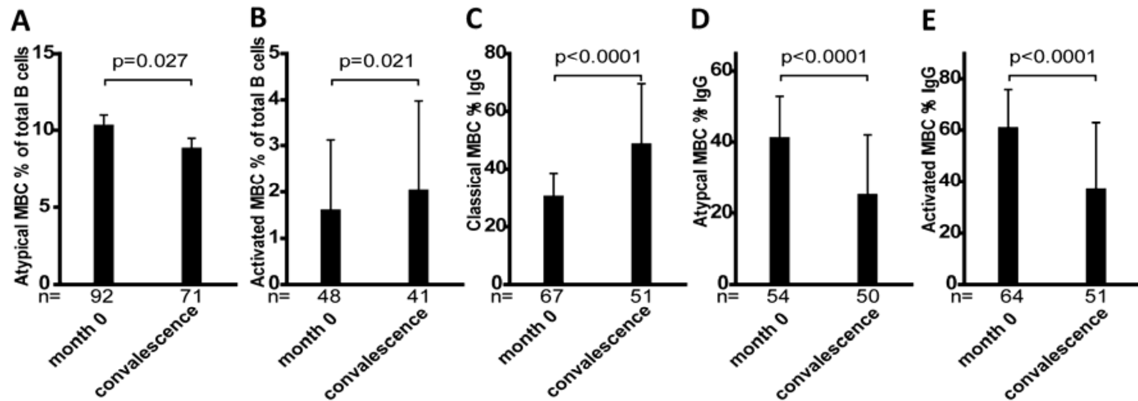


Figure 8.9 Acute malaria alters B cell subset composition in the periphery during convalescence. As a percentage of $CD19^+$ B cells, atypical MBCs decreased **(A)** and activated MBC increased **(B)** 14 days after acute malaria in children aged 2-10 years compared to the percentage before the malaria season. The percentage of classical MBC that are IgG^+ increases **(C)** and the percentage of atypical MBC **(D)** and activated MBC **(E)** that are IgG^+ decreased in this same age group 14 days after acute malaria. Data shown as mean ± s.e.m.

month 0: 39.2% [95% CI: 35.8-42.5], convalescence: 24.9% [95% CI: 20.0-29.7]; p<0.0001) and an increase in the proportion of classical MBCs that were IgG^+ (Fig. 8.9 E; month 0: 30.0% [95% CI: 28.0-32.1], convalescence: 48.2% [95% CI: 42.2-54.1]; p<0.0001). The decrease in the proportion of atypical MBCs in the peripheral blood suggests that this subpopulation may be trafficking out of the circulation into tissues in response to acute malaria. The increase in activated MBCs could be expected in response to infection and is likely due to activation of *Pf*-specific classical MBCs, but we cannot rule out the possibility at this point that they could result from atypical MBCs. As activated MBCs increase as a percent but decrease in percent IgG^+, the majority of MBCs that are activated during malaria are not IgG^+. The observed increase in IgG^+ MBCs is consistent with ELISPOT results and suggests that this compartment is gaining *Pf*-specific IgG^+ MBCs during malaria, most of which seem to be short-lived (Fig. 7.4). The decrease in both total atypical MBCs and the %IgG^+ atypical MBCs indicates that

IgG^{+} MBCs could be selectively migrating out of peripheral blood to sites in tissue, or could be selectively differentiated to a different phenotype over non-IgG^{+} atypical MBCs. *Pf*-mediated isotype switching of atypical or activated MBCs from IgG to IgE or IgA is another interesting possibility, but further studies would be required to address these questions.

8.3 Discussion

The finding we report here of an expanded, atypical MBC subpopulation in malaria-exposed individuals is the first description of a phenotypic alteration of MBCs in individuals exposed to *Pf*. At present, the factors that cause the expansion of the atypical MBCs are not known. In HIV infections the virus appears to play a role in driving B cells into the exhausted MBC subpopulation, although this might not be a direct effect of the virus itself. HIV causes massive depletion of memory T cells in the gut and results in damage to the intestinal epithelial microenvironment (177), and increased translocation of microbial products (33) such as bioactive LPS, which correlates with levels of immune activation in HIV infected humans and SIV infected rhesus and is absent in non-pathogenic SIV infection of sooty mangabeys (34). The effect of these high levels of bioactive microbial products is not fully known, but has been proposed to be a major component in causing the systemic immune activation observed in HIV and this could be the force driving the expansion of exhausted MBCs in HIV. In individuals with untreated HIV infections, the exhausted MBC subpopulation had a greater proportion of HIV-specific MBCs than did the classical MBC subpopulation (143) and in

patients whose viral loads were reduced to levels below detection by antiretroviral therapy, the number of exhausted MBCs decreased to about half over a period of six months. However, the exhausted MBC subpopulation in treated patients remained statistically greater than that in healthy donors, presumably due to viral spiking (unpublished observation). Due to the small cell numbers we were able to obtain in our Malian cohort, especially from young children, we were unable to carry out similar assays to directly determine if *Pf*-specific MBCs were differentially represented in the atypical and classical MBC populations. Finding that persistent *Pf* infection may be associated with a greater degree of expansion of atypical MBCs, suggests that parasite antigens or other parasite products may be responsible for driving the B cells into the atypical MBC subpopulation. Other factors could account for the expanded atypical MBCs in Malian individuals, such as genetic background or environmental factors associated with *Pf* transmission that were not assessed in our study, for example, malnutrition or the increased seasonal exposure to other pathogens. Although we did not test for HIV, it is unlikely that HIV is responsible for the expanded atypical MBC compartment in our study population since the prevalence of HIV in Mali is extremely low (1.5%) and based on demographic data we would expect HIV prevalence be lower than the country average in our study population (157).

Isotype switching occurs during memory B cell differentiation, simultaneous with somatic hypermutation and the presence of a significant proportion of IgG^+ atypical MBCs indicates that these cells have likely undergone somatic hypermutation and are

likely MBCs by this classification. The proportion of atypical MBCs that were IgG$^+$ increased with increasing age, and with increased cumulative exposure to *Pf.* Peruvian adults with no prior reported malaria episodes (i.e. the measured episode reported here is hypothetically their first malaria episode) had comparable levels of IgG$^+$ atypical MBCs at this first reported episode as did two to seven year old Malian children, who have likely had many malaria episodes, even though the average percentage of total atypical MBCs in Malian children age two to seven was more than twice that of Peruvian adults, indicating that the dramatic change, from approximately 10% IgG$^+$ in malaria naïve individuals to approximately 50% IgG$^+$ in malaria-exposed individuals, is associated with the expansion of atypical MBCs. This apparently rapid shift in the proportion of IgG$^+$ atypical MBCs with expansion of this population is consistent with a model where atypical MBCs are generated as a byproduct of a dysfunctional or altered germinal center reaction during which class switching occurs, or a model where atypical MBCs are generated from existing classical MBCs, as the proportion of IgG$^+$ cells was the same within an individual in these two populations, increasing in parallel with increasing age.

The role of atypical MBCs in the context of malaria remains unclear. It has been suggested that FCRL4$^+$ MBCs resident in mucosal lymphoid tissue play a role against invading pathogens, possibly through their influence on other cells, either directly or indirectly through the secretion of cytokines (70, 71). Moir *et al.* (143) concluded that the HIV-associated exhaustion of B cells may play a role in the diminished HIV-specific Ab responses in infected individuals as these cells are enriched in HIV-specificity and

reticent to differentiate into ASCs in vitro, with an output similar to naïve B cells with stimulation. By analogy it may be that the atypical MBCs in *Pf*-exposed individuals play a similar role in the inefficient acquisition of *Pf*-specific MBCs and the predominance of a short-lived, rather than long-lived Ab and MBC response to *Pf*, as our preliminary results suggested that atypical MBCs in malaria exposed individuals were hypo-responsive to polyclonal B cell activation, with a readout of ASC differentiation less than that for naïve B cells. Hypothetically, *Pf*-specific classical MBCs in the periphery at some point during *Pf* infection or convalescence could switch to the hyporesponsive atypical MBC phenotype, leaving a minority of *Pf*-specific MBCs as fully functional classical MBCs. In this model the "short-lived MBCs" we observe 14 days after presentation with malaria could be altered either by subsequent malaria episodes or by persisting *Pf* antigen. Alternatively atypical MBCs could result from a diversion of classical MBC development during initial differentiation in germinal centers, preventing development of a significant proportion of *Pf*-specific classical MBCs. In this model, the short-lived MBCs we observe could be differentiating into PCs in a productive response to infection, or could be yet another (abnormally short-lived) population resulting from an altered germinal center reaction. Collectively, these observations could reflect the generation of atypical MBCs in lieu of the normal generation of long-lived MBCs or PCs in response to *Pf* infection.

As suggested by Ehrhardt et al., atypical MBCs could be functional, and the expansion of atypical MBCs in *Pf* infection may in some way benefit the host, reflecting the unique relationship between the parasite and the host that allows the

asymptomatic persistence of the parasite within an otherwise functional immune system in individuals who have acquired clinical immunity. Given that the human and *Plasmodium* genomes have co-evolved (148), it is possible the *Pf* has shaped immune mechanisms which allows chronic and recurrent infections to occur. Presumably the persistence of the parasite has some benefit to the host as indicated, for example, by our finding that asymptomatic *Pf* infection is associated with protection against clinical disease (Chapter 5.2.1). It is possible that atypical MBCs play a beneficial role in protecting the host from clinical disease by modulating immune responses, for example, through the secretion of cytokines to control inflammation.

It will be important to determine if individuals with expanded atypical MBCs can be effectively vaccinated to produce long term *Pf*-specific memory responses as the expansion of this population could indicate an altered immune response to *Pf* that will be maintained through subsequent exposure to *Pf* antigens. As discussed in Chapter 4.3, we have observed that compared to U.S. adults, Malian adults appear to respond less well to the same candidate malaria vaccine in Phase I clinical trials as measured by the generation of antigen-specific MBCs. Future studies will be needed to determine whether the expansion of atypical MBCs represents a protective response or an immune evasion strategy of *Pf*, and if the latter, whether it can be overcome by vaccination that specifically addresses this mechanism.

Chapter 9: Discussion

Here I present data focused on humoral immunity to *Pf* malaria, a major world health concern in the present day. As a result of studies showing short-lived and inconsistently generated Ab responses, and incomplete protection from malaria, there has been doubt in the malaria research community as to whether true immunologic memory to *Pf* develops or can be maintained. I present data addressing the development of Abs and MBCs in response to subunit malaria vaccines in malaria-naïve individuals (Chapter 4) in contrast to the development of Abs and MBCs to natural malaria infection (Chapter 7), the presence of large numbers of atypical MBCs in individuals in malaria endemic areas (Chapter 8), and the identification of the specificity of protective Ab responses (Chapter 6). In addition my data allows me the opportunity to comment on general questions regarding the mechanisms of MBC and LLPC development and maintenance.

Overall I found the efficiency and magnitude of acquisition of *Pf*-specific Abs and MBCs to depend heavily on the context of exposure. In the context of subunit vaccination given to malaria naïve adults, *Pf*-specific Abs and MBCs are efficiently generated with three vaccinations and maintained for at least three to six months. This is in sharp contrast, however, with the response to *Pf*-malaria in which *Pf*-specific Abs and MBCs are gradually acquired over years of repeated infections, with incremental increases each malaria season. In analysis of 491 immunogenic *Pf* proteins, both the breadth and intensity of Ab reactivity increased with age, asymptomatic *Pf* infection,

and with malaria transmission. The increase from before to after the *Pf* transmission season is less pronounced with increasing age. The majority of the Ab response to *Pf*-exposure measured by both protein microarray and ELISA was short-lived with an incremental increase with each year of age in the titer of Ab maintained through the six-month dry season. Considering serological studies reporting a rapid decline of *Pf*-specific Ab in the light of my longitudinal data, it is plausible that these studies measured the sharp decline of the short-lived Ab response after acute malaria, rather than the long-lived component which is the minority of the Ab response. The same pattern was observed for *Pf*-specific MBCs, although the potential heterogeneity in this population, i.e. whether activated MBCs are contributing to the MBC ELISPOT readout during convalesence, as opposed to only resting MBCs reading out in the assay (as is the case during steady state) is not clear. This is an important point to address in future research. This inefficient development of Abs and MBCs seems to be specific to *Pf*-specific Abs and MBCs, and I found no evidence that *Pf* causes a general lasting immune-suppression as individuals in this cohort had efficient and stable responses to tetanus vaccination. Interestingly, in addition to the incremental acquisition of *Pf*-specific Abs and MBCs, in adults with a lifetime of exposure to malaria, the magnitude of the MBC response was dramatically lower than that seen in vaccinated malaria-naïve individuals. In addition the magnitude of the MBC response to a particular antigen might be determined by *Pf* infection and whether this can be subsequently overcome by vaccination remains to be determined. As the vaccinations done in semi-immune

Malian adults using the AMA1-C1 versus AMA1-C1 with CPG vaccines was done at the height of the malaria transmission season (190), it is not clear whether the MBC response to these antigen is set by *Pf* infection or whether individuals were unable to respond more robustly because of concurrent exposure to the parasite.

The two prior reports of *Pf*-specific MBCs in adults in malaria endemic areas indicate that a relatively high percentage of individuals do not have detectable MBCs to *Pf* antigens, and I found a similar pattern, with approximately half of adults in our cohort without detectable MBCs to the *Pf* antigens I tested. Although much can be learned from studies of vaccination, the relative complexity of infection clearly plays a role in MBC development in the case of *Pf* malaria. This inefficient acquisition of *Pf*-specific MBCs in response to natural infection contrasts not only with responses to these same antigens in a different population (malaria-naïve vaccine recipients), and the response to a different antigen in the same population (TT), but also contrasts to the one other disease where MBC development has been studied longitudinally to date, namely cholera. In response to cholera there is rapid acquisition of stable MBC levels (97, 112, 120) which differs dramatically from the incremental increase and high ratio of short-lived to long-lived MBCs resulting from *Pf* malaria. Collectively these data indicate significant impairment in the ability to develop MBCs and LLPCs in the context of *Pf* infection. As *Pf* transmission resumes after a six-month hiatus, I cannot comment definitively on the ultimate longevity of the Ab and MBC responses to *Pf.* However based on the empirical observation that semi-immune adults will become susceptible to

malaria over a one to two year period of non-exposure it seems probable that the LLPCs that result from *Pf* malaria have either an extremely short half-life, or are not acquired far in excess of the threshold required for maintaining neutralizing antibody. These semi-immune adults rarely succumb to fatal malaria, however, likely due to maintained *Pf*-specific MBCs differentiating into PCs upon antigen re-exposure, thus allowing symptoms but preventing fatal malaria. These individuals also re-acquire clinical immunity (again based on empirical observation) more rapidly than a malaria naïve individual would acquire immunity, suggesting a potential memory component in the response. This supports further the model I propose for independent homeostatic regulation of MBCs and LLPCs, by indicating that LLPCs are ultimately derived from MBCs, but antigen stimulation (or cross-reactivity of a certain affinity) is required for PC differentiation.

Another interesting finding in the light of this apparent impairment is the expansion of atypical MBCs in *Pf*-exposed individuals. The role and origin of these cells is still unclear, perhaps they have an immunosuppressive role, a protective role, or they may be non-functional. Atypical MBCs might result from deferred classical MBC development in a defective germinal center reaction or arise from alterations to existing MBCs. I hypothesize that these cells are largely non-functional with regard to antibody production and result from altered germinal center reactions. Atypical MBCs express a pattern of increased inhibitory receptors relative to classical MBCs, are phenotypically comparable to the hyporesponsive ‘exhausted MBCs’ described in HIV patients and are

also functionally hyporesponsive in their ability to differentiate into ASCs (per my preliminary data). In addition atypical MBCs in HIV patients have levels of somatic hypermutation that fall between naïve B cells and classical MBCs. With regard to their origin, in a study comparing spleens from fatal malaria, fatal sepsis, and normal control cases, researchers found that in malaria cases there was a severe depletion of B cells in the marginal zone where memory B cells typically are found and there were fewer germinal centers in malaria cases than in controls. Although activated B cells (and PCs) can migrate into the red pulp and differentiate in foci to secrete Abs, B cell numbers were lower in the red pulp of malaria cases than in controls (194). As a relatively high proportion of FCRL4$^+$ B cells were found in healthy tonsils (71), indicating that the expression of this inhibitory receptor is probably normal on certain B cells during a stage of germinal center development (likely to tightly regulate activation and differentiation resulting from BCR stimulation during positive selection), the interruption of germinal center organization that occurs during malaria could result in B cells at this stage of development exiting to the periphery rather than completing maturation into memory B cells. It is not clear either whether the large numbers of apparently short-lived MBCs observed during convalescence could be contributing to the atypical MBC pool, possibly upon re-entering a germinal center reaction to undergo further somatic hypermutation / affinity maturation after cycling through the periphery and receiving a signal or failing to receive a signal which results in their development into atypical MBCs. Studies done in our lab have shown that expression of FCRL4 on Ramos cells decreases BCR-mediated

activation and both initial and sustained calcium flux in response to either soluble or membrane-bound antigen (H. Won Sohn, unpublished observations). While tonic signaling through the BCR is known to be a requirement for survival of B cells, FCRL4 expression disrupts activation through the BCR without interrupting the tonic signal necessary for survival. Thus FCRL4 inhibits BCR activation by virtue of its expression alone without interrupting the tonic signaling through the BCR required for B cell survival, allowing these cells to survive in a hyporesponsive form. Whether these cells survive as a nonfunctional anomaly from a germinal center reaction or whether these cells represent another piece of immunity by having a non-traditional B cell function remains to be determined. The long term persistence of these cells and their decrease in peripheral blood during convalescence following malaria could indicate that they are maintained to perform a function and might ultimately be protective to the host, possibly through the secretion of cytokines, antigen presentation or another non-traditional B cell role, thus filling a role akin to an innate immune cell. As the BCR seems largely non-functional in these cells and preliminary microarray data indicates cytokine expression (S. Moir, unpublished observations), I hypothesize that these cells are influencing the immune environment via cytokine production. Alternately, the expression of FCRL4 could be reversible, allowing these cells to enter a form of stasis and then later resume a traditional B cell function, differentiating into PCs or classical MBCs. In short, together with the evidence for altered germinal center reactions in malaria, the decreased level of somatic hypermutation and the comparable level of IgG

class-switching in atypical versus classical MBCs, and the incremental acquisition of *Pf*-specific classical MBCs, I hypothesize that *Pf* alters germinal center reactions, resulting in the diversion of B cells into atypical MBCs in lieu of classical MBCs. These atypical MBCs are likely hyporesponsive in their ability to differentiate into antibody secreting cells, but influence immune responses via cytokine secretion. The alterations in germinal center reactions and the process of atypical MBC development could be driven by massive immune activation, high antigen numbers (as over 3,000 proteins are transcriptionally active during blood stage infection), high antigen levels (as tens of thousands of merozoites exit the liver and proceed to replicate exponentially), the pattern of antigen exposure (as more than 75% of proteins expressed during the blood stage of infection are transcriptionally active once causing a continuous cascade of gene expression), or repeated antigen exposure (192). I feel the most likely of these possibilities is that these changes result from large doses of antigen in combination with massive immune activation resulting from parasite products in the plasma. In support of this there are bioactive microbial products in the plasma in the case of HIV (33) and large doses of bioactive parasite products in the case of *Pf,* and both diseases cause widespread immune activation. These commonalities could underlie the development of atypical MBCs in these two diseases. Atypical MBCs could also result from classical MBCs going through abnormal differentiation into PCs. Ultimately it could be important to determine the role of atypical MBCs and whether they are generated in lieu of the normal development of long-lived MBCs or PCs in response to *Pf* infection, as well as to

ascertain whether atypical MBCs contribute to the apparent refractoriness of Malian adults to respond to *Pf* subunit vaccination. It will be important to determine if individuals with expanded atypical MBCs can be effectively vaccinated to produce long term *Pf*-specific memory responses as the expansion of this population could indicate an altered immune response to *Pf* that will be maintained through subsequent exposure to *Pf* antigens.

With regard to protection, since recent clinical trials show that vaccination with either AMA1 or MSP1 does not confer protection, I did not expect AMA1- and MSP1-specific MBCs to correlate with protection, which they did not. Based on the kinetics of MBC to PC differentiation which requires approximately six to eight days (30), and the more rapid kinetics of *Pf* replication to the point of clinical symptoms (i.e. malaria) which requires approximately three days once blood-stage infection begins (179), I suspect that the frequency of MBCs *per se* may not reliably predict clinical immunity to malaria regardless of antigen specificity but MBC to protective antigens might predict future Ab titers that would be protective as well as protection from fatal malaria. This collectively gives rise to a model of the development of protective humoral memory to *Pf* malaria, where *Pf*-specific Abs and MBCs of protective specificities increase following each malaria infection, expanding and contracting, but with a stepwise incremental gain in the long-lived component. Due to the kinetics of MBC differentiation into PCs, the relatively high levels of Ab required for protection versus the relatively low levels of Ab that are long-lived with each exposure, and the speed with which the parasite

Model for the gradual acquisition of humoral immunity to blood stage *P. falciparum*

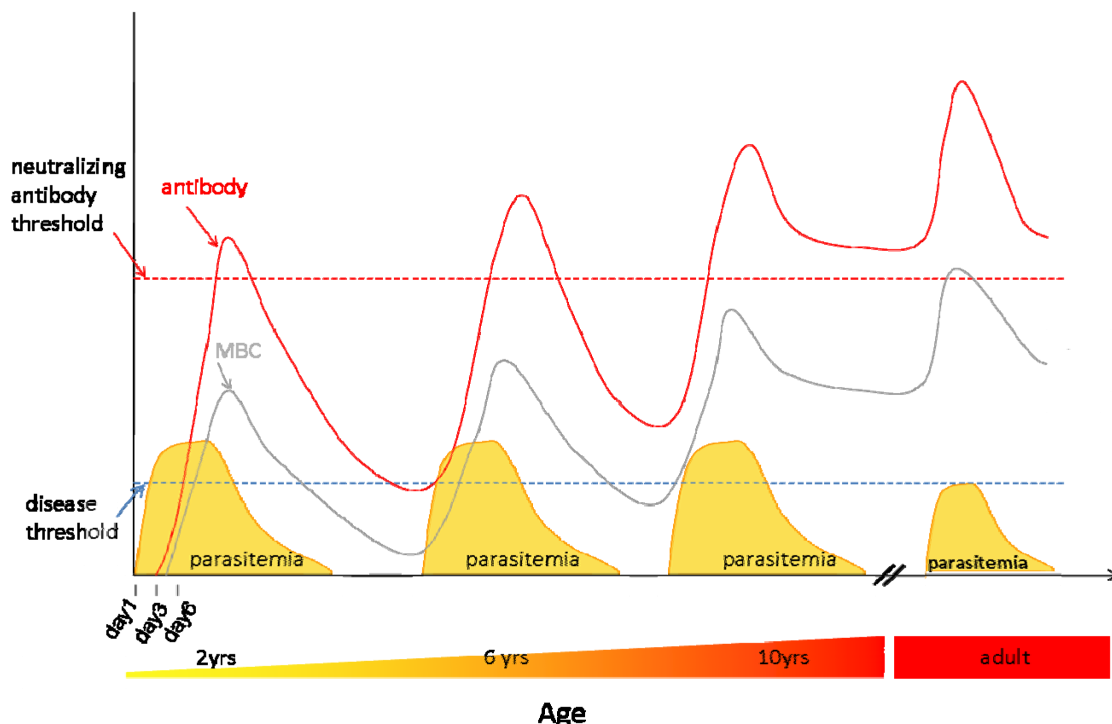


Figure 9.1 Model for the gradual acquisition of humoral immunity to blood stage *P. falciparum*. Individuals remain susceptible to malaria for years as titers of protective Ab and correlating MBCs increase in a gradual stepwise fashion, with the majority of the response dedicated to SLPCs rather than LLPCs. Malaria episodes continue to occur until Ab of protective specificities exceeds a neutralizing threshold. MBCs do not directly correlate with protection as the time required to differentiate into PCs is greater than that required by the parasite to cause illness, thus only the Ab present at the time of infection is protective against disease.

replicates, the individual continues to have malaria episodes until the long-lived Ab exceeds a neutralizing Ab threshold (Figure 9.1). The specificity and levels of Ab present in plasma at the time of infection would, however, be immediately protective, and considering the complexity of the parasite's life cycle within the host, it is likely that Ab to multiple *Pf* antigens will be required to induce clinical immunity.

In a protein microarray analysis of age-matched protected (children with no malaria episodes in spite of positive *Pf* parasitemia) and susceptible (children who had one or more episodes of malaria) I found a panel of 49 known and hypothetical proteins

to which Ab reactivity correlated with clinical protection. As Ab reactivity to some of the known proteins of this panel have been shown either to inhibit sporozoite invasion of human hepatocytes (160), to inhibit parasite growth *in vitro* (6), to be associated with decreased malaria risk or parasitemia in field studies (5, 115, 200), or induced by a multiple-antigen vaccine which reduced parasitemia in children (85), the protein microarray is likely an effective tool to identify novel vaccine candidates. Of the proteins I identified those Abs that are ultimately protective will need to be determined in future studies, possibly by further microarray profiling of Ab responses in larger prospective longitudinal cohort studies in various endemic settings.

Although the *Pf* antigens I tested clearly do not solely account for the suboptimal Ab and MBC responses measured in malaria endemic areas, as malaria naïve individuals rapidly developed high levels of Abs and MBCs to these proteins, the antigen seems to have some role in determining the magnitude of the Ab and MBC response as a similar pattern was observed with Abs and MBCs consistently higher to AMA1 than to MSP1 in both Kenya and Mali with exposure to the parasite, and in the U.S. in response to subunit vaccination. The quantity of protein given was less important in determining the resulting Ab and MBC responses in these trials, as no difference was observed in AMA1-specific Ab titers and MBC levels whether given the same dose as that given in the MSP1 trial or one quarter of this dose. Thus the pattern of greater Abs and MBCs to AMA1 versus MSP1 persisted across three study populations (Kenya and Mali following malaria and U.S. following vaccination) regardless of antigen dose, and regardless of

whether the antigen exposure was via vaccine or during acute infection. It would be interesting to further the studies that have already suggested some properties of MSP1 which might contribute to this (132), (100), as this could shed light on what ultimately determines the "immunogenicity" of a protein for humans.

One thing that does stand out with regard to the ability of Abs and MBCs to develop was the addition of CpG to subunit vaccines. TLR9 plays a significant role not only in the generation of MBCs in naïve individuals but also in controlling the behavior of existing MBCs. For the two protein subunit malaria vaccine candidates, AMA1-C1 and $MSP1_{42}$-C1, the inclusion of CPG 7909 had a dramatic effect on malaria naïve individuals, resulting in a more rapid acquisition of vaccine-specific Abs and MBCs, in greater number/titer. CPG 7909 had no effect on primary immunization with AMA1-C1 or $MSP1_{42}$-C1, suggesting that CPG 7909 had little effect on naïve B cells directly, or indirectly through TLR9-expressing PDCs. However, once generated by primary immunization, TLR9-expressing antigen-specific MBCs responded dramatically to secondary immunization in the presence of CPG 7909. Interestingly, the addition of CPG 7909 did not enhance the acquisition of vaccine-specific MBCs either kinetically or numerically when the AMA1-C1 vaccine ±CpG was tested in a cohort of semi-immune adults in Mali (190). The mechanistic meaning of this apparent refractoriness to TLR9 activation is of considerable interest for the purpose of vaccine design.

The development of an effective malaria vaccine would be a critical step toward the control and eventual elimination of this disease. Malaria has thus far proved

resistant to attempts at reliably inducing long-lived protection through vaccine efforts. This could be due to properties of the parasite or the immune environment induced during infection, and a better understanding of these processes could be instrumental in informing vaccine design. To date, most licensed vaccines are for pathogens that induce long-lived protective Abs after a single infection. In contrast, immunity to malaria is only acquired after repeated infections, is typically dependent on boosting by regular infections, and usually short-lived (129), as discussed earlier. For these reasons an effective malaria vaccine would expedite and streamline, rather than mimic, the natural course of the development of immunity in the context of *Pf* infection. To date malaria vaccines have at best provided only partial protection. It is worth noting, however, that only approximately 0.5% of the *Pf* genome has been explored for subunit vaccine potential, and rarely have individual proteins been used in combination. I have begun to address some of the major questions that remain for vaccine development including which antigen specificities confer protection, whether MBCs to *Pf* develop and can be maintained and what mechanisms might underlie the inefficient acquisition of immunity to malaria. In addition to these, questions that still remain for vaccine development include which Ab specificities confer protection, the affinity of these Abs required, the titre of these Abs required, whether these titers can be induced in malaria-experienced individuals or whether children must be vaccinated while still malaria-naïve, and whether these titers can then be maintained regardless of *Pf* exposure. With regard to affinity the possibility also exists that *Pf* causes increased AID expression ultimately

resulting in decreased affinity of Abs to *Pf* proteins, another important avenue to explore. In conclusion I hypothesize that a successful vaccine is probable if the correct antigens are identified, the Ab titers required for protection are defined, and individuals are vaccinated with a vaccine regimen sufficient to induce neutralizing Ab titers to these antigens prior to *Pf* exposure. This last point seems equally critical to the others as semi-immune adults in Mali, with prior exposure to *Pf,* responded to vaccination with a magnitude of MBCs comparable to the magnitude of MBCs which resulted from infection with *Pf.* This is in contrast to the much greater magnitude of MBCs generated by malaria-naïve adults in response to this same vaccine. This suggests that the magnitude of the response to *Pf* antigens is determined during *Pf* infection and once this is determined it cannot be overcome with subsequent vaccination. Further studies will be required, however, to address these questions.

My work has also given me the opportunity to address some fundamental questions regarding the development and maintenance of humoral memory in humans. *Pf*- and TT- specific MBCs increased with cumulative exposure to antigen, albeit at different rates, while TT-specific MBCs remain constant in the absence of further exposure, indicating that the increase in antigen-specific MBCs is driven by repeated antigen exposure and is not simply a function of age. Consistent with this, the size of the total IgG^+ MBC compartment, as reflected in the peripheral blood, increased with age and immunological experience. Regarding maintenance, unlike PCs, which are terminally-differentiated, MBCs may be maintained through homeostatic proliferation

(133), possibly through exposure to polyclonal stimuli (30). In support of this, TT-specific MBCs were slightly increased following malaria, possibly due to polyclonal activation. The stability of TT-specific MBCs that I observed until the age of twenty-five following vaccination in infancy is also striking in comparison to studies done in non-malaria exposed western populations. This difference in the ability to maintain MBCs could reflect the difference in exposure to infections and the subsequent polyclonal expansion of MBC populations. If the maintenance of MBCs in the human immune system does depend on polyclonal activation, it would be in-tune with the higher degree of pathogen exposure that humans have evolved with up until the last one-hundred years or so with the progressively more pathogen-free environments that have been created in recent human history. I hypothesize that the steady level of TT-specific MBCs I observed here reflects the maintenance of MBCs as it has naturally evolved resulting in a steady state throughout life achieved by frequent pathogen exposure, while the decline of these MBC populations observed in more pathogen-free environments is a reflection of the subsequent decrease in polyclonal activation these MBC populations are experiencing throughout time. The correlation between Abs and MBCs at steady state that I observed in both the U.S. vaccine trial and the Kambila cohort study suggest that MBCs are ultimately responsible for replenishing PCs. The non antigen-specific increase of PCs that I observed after the second and third vaccinations in the U.S. vaccine trials suggest that this process occurs by polyclonal activation of MBCs, but these are weak data, as these could be antigen-specific or cross-reactive PCs responding

to the vaccine, and better support for this model has been presented by others (134). My TT-specific data from the Kambila cohort study makes a much stronger case for the model I propose, that MBCs and PCs are maintained by different mechanisms, and polyclonal activation only has a role in maintaining MBC numbers while PC differentiation requires antigen-specific stimulation. It seems unlikely that the differentiation of MBCs into PCs would occur with any reasonable efficiency in the absence of antigen-specific stimulation as this could easily result in large amounts of irrelevant Ab or hypergammaglobulinemia following any infection, and the expense of resources for a terminally differentiated cell that is not assisting in eliminating the current infection. The apparent discrepancies in these data could, however, simply be a result of the differential effects of alum, which activates via the inflammosome (72), the TLR ligand CpG, and the massive impact of malaria on the immune system. The positive correlation I observed between MBCs and Abs in both the U.S. trials and the Kambila cohort study suggest that MBCs are ultimately responsible for replenishing PCs, although, again, this correlation likely depends on the point at which the sample is taken. The mechanism driving this is not yet clear. If signaling through the BCR is required it could be accomplished through relevant antigen stimulation or cross-reactivity, or alternatively this process could be driven by certain innate triggers which might not be activated during malaria. In all three studies the correlation between MBCs and Ab titers at steady state suggests that the long-term maintenance of LLPCs may be linked to MBCs. However, the cellular and molecular nature of this relationship

remains to be fully elucidated. Collectively these data support a model where MBC and PC are regulated through different mechanisms. I hypothesize that MBC numbers are maintained by polyclonal activation, and although PCs arise from MBCs it is via stimulation through the BCR with relevant or cross-reactive antigen. In this model the correlation of MBC numbers and PC numbers would depend on the point at which the sample is obtained. During and immediately after vaccination regimens, or relevant antigen exposure, one would expect a better correlation between MBCs and PCs of a given specificity. At timepoints further from vaccination or relevant antigen exposure correlation may not occur due to polyclonal activation of MBCs following other infections or PC numbers declining over time without being replenished by relevant antigen stimulation. Empirical observation that semi-immune adults who become susceptible to malaria over a one to two year period of non-exposure but rarely succumb to fatal malaria indicates that PCs to *Pf* might be short-lived, however, MBCs seem to be maintained and are capable of differentiating into PCs upon antigen re-exposure, thus allowing symptoms but preventing fatal malaria. This supports further the model I propose for independent homeostatic regulation of MBCs and LLPCs, by indicating that LLPCs are ultimately derived from MBCs, but antigen stimulation (or cross-reactivity of a certain affinity) is required for PC differentiation.

The studies described here provide a rare view of the acquisition and maintenance of human B cell memory. The ability to compare Ab and MBC responses to the same antigens in the context of vaccination of naïve individuals and in the

pathogenic context of an acute infection has given me valuable information about the development of humoral memory both in general and specifically in the case of *Pf.* These studies lend new insights into the observations of short-lived Ab responses to *Pf* antigens, and the delayed acquisition of malaria immunity. The findings I report here of gradual acquisition of *Pf*-specific Abs and MBCs over years of exposure, and the expansion of atypical MBCs in malaria-exposed individuals are the first descriptions of MBC development in response to malaria, and a phenotypic alteration of MBCs in individuals exposed to *Pf*. These findings have opened two new areas of research, namely the mechanisms underlying the slow acquisition of MBCs and the generation and function of atypical MBCs. An understanding of the cellular and molecular mechanisms involved could open the door to strategies for the design of a malaria vaccine that go beyond the traditional empiric approach and address *Pf*-specific interaction with the human host.

Table S1. ***Pf*** **proteins identified as immunogenic by protein microarray.***

Gene ID	Gene ID Annotated†	Protein Name	Normalized Antibody Reactivity
PFB0915w	PFB0915w-e2s1	liver stage antigen 3	152615.9595
PFI1475w	PFI1475w-s2	merozoite surface protein 1, precursor	139468.2384
PFB0300c	PFB0300c	merozoite surface protein 2 precursor	133931.7351
PF08_0140	PF08_0140e2s1	erythrocyte membrane protein 1 (PfEMP1)	65360.0092
PF14_0102	PF14_0102e1s1	rhoptry-associated protein 1, RAP1	57753.94791
PFB0310c	PFB0310c-e1	merozoite surface protein 4	52544.25246
PFL1930w	PFL1930w-s5	hypothetical protein	47988.93143
PF08_0137	PF08_0137e2s1	hypothetical protein	47631.15621
MAL7P1.77	MAL7P1.77e1s1	hypothetical protein	46226.5337
PF10_0348	PF10_0348e1s1	hypothetical protein	43263.7235
PF11_0226	PF11_0226-s1	hypothetical protein	40014.55264
PFD0105c	PFD0105ce1s1	SURFIN, surface-associated interspersed gene	39439.98399
PF10_0138	PF10_0138-s1	hypothetical protein	37236.96525
PFE0060w	PFE0060w-e2	hypothetical protein	36412.02639
PFD0310w	PFD0310w	sexual stage-specific protein precursor	31195.23728
PF10_0323	PF10_0323e1s1	hypothetical protein	31065.8283
PF07_0006	PF07_0006-e2	starp antigen	30620.62844
PFD0995c	PFD0995ce2s1	erythrocyte membrane protein 1 (PfEMP1)	30553.43811
PF14_0170	PF14_0170e1s5	hypothetical protein	30130.45474
MAL6P1.252	MAL6P1.252-e2	erythrocyte membrane protein 1 (PfEMP1)	28422.84341
PFI1475w	PFI1475w-s1	merozoite surface protein 1, precursor	27935.5303
PFE1590w	PFE1590w	early transcribed membrane protein	27477.9251
PF07_0129	PF07_0129e1s1	ATP-dept. acyl-coa synthetase	27309.74706
PF14_0461	PF14_0461-s1	hypothetical protein	26790.47396
MAL7P1.14	MAL7P1.14e1s1	hypothetical protein	26467.55614
PF11_0302	PF11_0302e1s1	hypothetical protein	25443.45625
PF14_0228	PF14_0228e1s1	hypothetical protein	25078.08075
PF07_0128	PF07_0128-e1s2	erythrocyte binding antigen	24634.38884
PFE1025c	PFE1025ce1s1	hypothetical protein	23416.01979
PF10_0138	PF10_0138-s2	hypothetical protein	23144.72091
PFL0795c	PFL0795ce1s1	hypothetical protein	23012.23767
PF14_0495	PF14_0495-s1	hypothetical protein	22767.07431
PF13_0003	PF13_0003e2s1	erythrocyte membrane protein 1 (PfEMP1)	22705.2586
PFL1410c	PFL1410c-s1	hypothetical protein	22607.42377
PFA0110w	PFA0110we2s2	ring-infected erythrocyte surface antigen precursor	22604.21633
PFA0410w	PFA0410w-s3	hypothetical protein	22482.71261

MAL6P1.252	MAL6P1.252-e1s3	erythrocyte membrane protein 1 (PfEMP1)	22358.28408
PFD0225w	PFD0225w-s5	hypothetical protein	22186.71471
PF14_0631	PF14_0631e1s2	hypothetical protein	22144.20458
PF11_0037	PF11_0037e2s1	hypothetical protein	22046.00363
PFE1600w	PFE1600we2s1	hypothetical protein	21806.60566
PFL2610w	PFL2610we2s1	STEVOR	21521.41892
PFL0590c	PFL0590ce1s1	p-type ATPase, putative	21334.59847
PF14_0407	PF14_0407-s2	hypothetical protein	21060.49502
PF07_0007	PF07_0007e1s1	hypothetical protein	21048.89483
PF08_0141	PF08_0141e2s1	erythrocyte membrane protein 1 (PfEMP1)	20913.80716
PF11_0008	PF11_0008e2s1	erythrocyte membrane protein 1 (PfEMP1)	20899.51859
PF07_0048	PF07_0048e2s1	erythrocyte membrane protein 1 (PfEMP1)	20576.3133
PFE0055c	PFE0055ce3s1	heat shock protein, putative	20452.9307
PF11_0507	PF11_0507e1s6	antigen 332, putative	20322.17949
PFI0580c	PFI0580c-e2	hypothetical protein	20253.37777
PF10_0124	PF10_0124-e1s2	hypothetical protein	20239.99899
PF11_0226	PF11_0226-s3	hypothetical protein	19976.16813
PF10_0401	PF10_0401e2s1	RIFIN	19588.49889
PF10_0356	PF10_0356-e1	liver stage antigen, putative	19551.3558
PFL0470w	PFL0470we1s1	hypothetical protein	19168.9945
PF07_0053	PF07_0053e1s4	hypothetical protein	19132.58053
PF14_0495	PF14_0495-s2	hypothetical protein	18946.66363
PF13_0190	PF13_0190e1s1	hypothetical protein	18772.03871
PFL0445w	PFL0445w-s1	hypothetical protein	18728.51992
PFI0240c	PFI0240c-e1s1	E1-E2_ATPase/hydrolase, putative	18525.67022
MAL7P1.92	MAL7P1.92e5s1	cysteine repeat modular protein 2 homologue	18402.28181
PFB0260w	PFB0260w-e2	proteasome 26S regulatory subunit, putative	18264.03101
PFL0015c	PFL0015ce2s1	RIFIN	18259.93569
PF14_0626	PF14_0626e5s2	dynein beta chain, putative	18203.65258
PFL2505c	PFL2505ce8s2	hypothetical protein	18060.60157
PFD0380c	PFD0380ce2s2	hypothetical protein	17969.72993
PFA0410w	PFA0410w-s2	hypothetical protein	17947.48879
MAL13P1.234	MAL13P1.234-e1s4	hypothetical protein	17788.92229
PF10_0079	PF10_0079-s1	hypothetical protein	17694.83855
MAL13P1.176	MAL13P1.176e1s2	Plasmodium falciparum reticulocyte binding protein 2 homolog b	17477.14642
PF08_0107	PF08_0107e2s1	erythrocyte membrane protein 1 (PfEMP1)	17397.95244
PF07_0016	PF07_0016-s1	hypothetical protein	17357.62457
PFA0410w	PFA0410w-s1	hypothetical protein	17338.00845
PF10_0075	PF10_0075e1s2	asparagine-rich antigen	17275.58967

PF10_0322	PF10_0322-s2	S-adenosylmethionine decarboxylase-ornithine decarboxylase	17141.1087
PFL0185c	PFL0185c-e3	nucleosome assembly protein 1, putative	16979.40771
PFA0430c	PFA0430ce1s1	hypothetical protein	16942.61442
PF10_0356	PF10_0356-e2s2	liver stage antigen, putative	16749.97001
PFI0260c	PFI0260c-s6	hypothetical protein	16722.36594
PF11_0507	PF11_0507e1s1	antigen 332, putative	16637.26818
MAL13P1.234	MAL13P1.234-e1s3	hypothetical protein	16633.18697
PFB0305c	PFB0305c-e1	merozoite surface protein 5	16538.4958
PFI1735c	PFI1735ce2s1	hypothetical protein	16354.26532
PFD0385w	PFD0385we1s2	hypothetical protein	16062.89978
PF13_0275	PF13_0275e2s1	hypothetical protein	15892.22657
PF08_0018	PF08_0018-s2	translation initiation factor-like protein	15872.85894
PFB0915w	PFB0915w-e2s2	liver stage antigen 3	15538.43009
PF14_0690	PF14_0690-e1s2	histone deactylase, putative	15507.8785
PFL0625c	PFL0625c-s1	eukaryotic translation initiation factor 3 subunit 10, putative	15282.34127
PF14_0751	PF14_0751-e1	fatty acyl coenzyme A synthetase-1, putative	15228.91766
PFI0460w	PFI0460w	hypothetical protein	15223.94654
PF13_0197	PF13_0197	Merozoite Surface Protein 7 precursor, MSP7	14869.03924
PF11_0358	PF11_0358-e2s2	DNA-directed RNA polymerase, beta subunit, putative	14501.87999
PF10_0224	PF10_0224e1s3	dynein heavy chain, putative	14277.26022
MAL6P1.252	MAL6P1.252-e1s2	erythrocyte membrane protein 1 (PfEMP1)	14260.29746
PFE0380c	PFE0380ce1s1	hypothetical protein, conserved	14193.51008
PF14_0170	PF14_0170e1s4	hypothetical protein	14130.47682
PF14_0699	PF14_0699e2s1	hypothetical protein, conserved	13962.52894
PFE0465c	PFE0465c-e1s1	RNA polymerase I	13834.50984
PF11_0351	PF11_0351e1s1	heat shock protein hsp70 homologue	13812.06223
MAL13P1.107	MAL13P1.107-s2	hypothetical protein	13670.71395
PFE0090w	PFE0090we1s1	hypothetical protein	13521.96267
PF10_0124	PF10_0124-e1s1	hypothetical protein	13519.23215
PFC0810c	PFC0810ce3s1	hypothetical protein	13463.04597
PF11_0479	PF11_0479-e3s1	hypothetical protein	13420.26677
PF14_0170	PF14_0170e1s3	hypothetical protein	13378.4088
MAL8P1.23	MAL8P1.23-s8	ubiquitin-protein ligase 1, putative	13266.74682
PF10_0251	PF10_0251e2s3	hypothetical protein	13222.4801
PF14_0315	PF14_0315-e2s5	hypothetical protein	13158.96852
PFI0370c	PFI0370ce1s1	subunit of proteaseome activator complex, putative	13101.74581
PF14_0344	PF14_0344e1s1	hypothetical protein	13095.46259
MAL13P1.140	MAL13P1.140e1s3	hypothetical protein	13025.29436
PFE1120w	PFE1120w-e4s2	hypothetical protein	12987.79414

PFI0170w	PFI0170we1s1	hypothetical protein	12968.09916
PF10_0214	PF10_0214-e2	hypothetical protein	12845.00108
PFE0465c	PFE0465c-e1s3	RNA polymerase I	12816.88091
MAL13P1.107	MAL13P1.107-s1	hypothetical protein	12759.1721
PF11_0129	PF11_0129-e1	hypothetical protein	12757.79701
PFC0085c	PFC0085ce2s1	hypothetical protein, conserved	12756.32375
PFL1605w	PFL1605w-s2	hypothetical protein	12690.90642
PFL1745c	PFL1745ce2s1	clustered-asparagine-rich protein	12634.56312
PF11_0161	PF11_0161e1s1	falcipain-2 precursor, putative	12612.23384
PF13_0153	PF13_0153e1s1	hypothetical protein	12577.80154
PF11_0374	PF11_0374-e2s1	hypothetical protein	12573.26126
PFE1120w	PFE1120w-e3s5	hypothetical protein	12529.9278
PFL1925w	PFL1925we1s1	cell division protein FtsH, putative	12486.0198
PFL0275w	PFL0275we1s2	hypothetical protein	12472.92094
PF10_0369	PF10_0369e2s1	helicase, putative	12456.50741
PF10_0242	PF10_0242e2s2	hypothetical protein	12391.59892
MAL13P1.234	MAL13P1.234-e1s1	hypothetical protein	12386.41008
PFC0170c	PFC0170ce1s1	dihydrolipoamide acyltransferase, putative	12381.67288
MAL6P1.201	MAL6P1.201-s2	leucyl-tRNA synthetase, cytoplasmic, putative	12297.49564
MAL8P1.23	MAL8P1.23-s4	ubiquitin-protein ligase 1, putative	12262.62503
PF07_0020	PF07_0020-e1s2	hypothetical protein	12219.01571
PF14_0327	PF14_0327e1s1	methionine aminopeptidase, type II, putative	12195.72241
PF13_0210	PF13_0210e1s3	hypothetical protein	12120.05022
MAL13P1.285	MAL13P1.285-e2s1	hypothetical protein	12099.54207
PFC0875w	PFC0875w-e1s1	transporter, putative	12096.44852
PFL1135c	PFL1135c-e1	hypothetical protein	12046.92063
PF10_0041	PF10_0041e1s2	U5 small nuclear ribonuclear protein, putative	11988.36178
PF07_0029	PF07_0029-e2	heat shock protein 86	11981.88784
PF08_0020	PF08_0020e1s2	hypothetical protein	11901.73488
PF14_0433	PF14_0433-e4	hypothetical protein	11868.9163
MAL13P1.218	MAL13P1.218-e2	UDP-N-acetylglucosamine pyrophosphorylase, putative	11850.69599
PFL2390c	PFL2390ce1s3	hypothetical protein	11805.22185
PF14_0188	PF14_0188e1s2	hypothetical protein	11798.15192
PF14_0515	PF14_0515-s1	hypothetical protein	11675.49099
PF08_0034	PF08_0034e1s2	histone acetyltransferase Gcn5, putative	11667.89814
PF13_0327	PF13_0327-e1	hypothetical protein	11638.1447
PFE1010w	PFE1010we1s1	protein phosphatase 2c, putative	11618.39136
PF10_0013	PF10_0013e2s1	hypothetical protein	11571.38037
PFE1120w	PFE1120w-e3s1	hypothetical protein	11570.53989

MAL13P1.123	MAL13P1.123e1s1	hypothetical protein	11541.44537
PFL1255c	PFL1255c-e1	hypothetical protein	11537.0175
PF13_0044	PF13_0044e2s1	carbamoyl phosphate synthetase, putative	11510.27053
PF14_0633	PF14_0633e1s1	hypothetical protein	11490.95815
PFB0265c	PFB0265c-s2	DNA repair endonuclease, putative	11476.24344
PF13_0012	PF13_0012	hypothetical protein	11441.50774
PF08_0089	PF08_0089e1s1	hypothetical protein	11414.95098
PF11_0175	PF11_0175e4s1	heat shock protein 101, putative	11407.66206
PF10_0215	PF10_0215e2s1	hypothetical protein	11240.78214
PFE1120w	PFE1120w-e3s6	hypothetical protein	11240.23131
MAL13P1.19	MAL13P1.19e1s1	hypothetical protein	11201.98804
PF11_0240	PF11_0240e2s3	dynein heavy chain, putative	11175.20979
PFC0150w	PFC0150we2s2	hypothetical protein, conserved	11168.62587
PFE1120w	PFE1120w-e4s3	hypothetical protein	11163.70034
PF11_0049	PF11_0049e1s1	hypothetical protein, conserved	11086.99732
PFD0225w	PFD0225w-s3	hypothetical protein	11046.32962
PFI0260c	PFI0260c-s5	hypothetical protein	11042.2394
PF14_0370	PF14_0370e2s2	RNA helicase, putative	11011.62255
PF14_0736	PF14_0736-e2	hypothetical protein	10997.62592
PFE0335w	PFE0335w-e1	hypothetical protein	10984.92669
MAL6P1.131	MAL6P1.131-e1s5	SET-domain protein, putative	10852.78571
PF14_0626	PF14_0626e4s2	dynein beta chain, putative	10827.30706
PF08_0140	PF08_0140e1s1	erythrocyte membrane protein 1 (PfEMP1)	10819.77022
PF10_0264	PF10_0264e2s1	40S ribosomal protein, putative	10793.12246
MAL6P1.146	MAL6P1.146-s2	P. falciparum PK4 protein kinase	10786.19724
PF11_0512	PF11_0512-e2	ring-infected erythrocyte surface antigen 2, RESA-2	10784.61191
PFI0010c	PFI0010ce2s1	RIFIN	10718.69697
PF07_0035	PF07_0035e1s1	cg1 protein	10633.55099
MAL13P1.148	MAL13P1.148e3s2	P. falciparum myosin	10619.39392
MAL8P1.139	MAL8P1.139-e2s2	hypothetical protein	10613.69831
PFC0120w	PFC0120we1s1	Cytoadherence linked asexual protein, CLAG	10589.42442
PF07_0128	PF07_0128-e1s1	erythrocyte binding antigen	10574.12949
MAL7P1.146	MAL7P1.146e5s1	hypothetical protein	10515.18301
MAL8P1.23	MAL8P1.23-s3	ubiquitin-protein ligase 1, putative	10495.06254
PF07_0087	PF07_0087	hypothetical protein	10446.86388
PFA0555c	PFA0555ce4s1	UMP-CMP kinase, putative	10419.34334
PF11_0356	PF11_0356-s2	hypothetical protein	10412.55787
PF14_0664	PF14_0664-e1s4	biotin carboxylase subunit of acetyl CoA carboxylase, putative	10401.24851
PFE0655w	PFE0655we2s3	hypothetical protein	10366.124

PFI0925w	PFI0925we1s2	gamma-glutamylcysteine synthetase	10339.66882
PFL1010c	PFL1010ce1s3	hypothetical protein conserved	10287.4824
PFC0210c	PFC0210c	circumsporozoite (CS) protein	10213.11895
PFB0115w	PFB0115we1s2	hypothetical protein	10151.08831
PFD0265w	PFD0265we2s1	pre-mRNA splicing factor, putative	10140.18259
PFI1265w	PFI1265we1s1	hypothetical protein	10132.87288
PF11_0008	PF11_0008e1s1	erythrocyte membrane protein 1 (PfEMP1)	10121.03625
PF13_0044	PF13_0044e2s2	carbamoyl phosphate synthetase, putative	10092.33613
PFC0440c	PFC0440ce1s3	helicase, putative	10077.89511
PF10_0078	PF10_0078-s2	hypothetical protein	10077.72497
PF14_0632	PF14_0632-e1s2	26S proteasome subunit, putative	10075.79315
PF10_0374	PF10_0374e6s2	gene 11-1 protein precursor	10062.15984
PFC0425w	PFC0425we1s3	hypothetical protein	10060.47542
PF14_0419	PF14_0419e9s2	hypothetical protein	10041.06512
PF13_0182	PF13_0182-s1	hypothetical protein	10028.29203
PF14_0316	PF14_0316e1s1	DNA topoisomerase II, putative	10003.98082
MAL13P1.56	MAL13P1.56e1s1	m1-family aminopeptidase	9986.474334
PFI0410c	PFI0410ce18s1	hypothetical protein	9971.239257
PF10_0153	PF10_0153e2s1	hsp60	9970.623807
PF08_0108	PF08_0108e1s1	pepsinogen, putative	9915.439661
PF11_0509	PF11_0509-e2s1	ring-infected erythrocyte surface antigen, putative	9874.110117
PFE0570w	PFE0570w-s3	hypothetical protein	9842.924739
PFE0565w	PFE0565w	hypothetical protein	9815.036893
PFE1085w	PFE1085we1s1	DEAD-box subfamily ATP-dependant helicase, putative	9792.772047
PF11_0422	PF11_0422e1s2	hypothetical protein	9781.596283
MAL7P1.29	MAL7P1.29-e1s2	hypothetical protein	9746.119092
PF14_0690	PF14_0690-e1s3	histone deactylase, putative	9740.39546
PF07_0020	PF07_0020-e1s1	hypothetical protein	9731.804696
PF11_0162	PF11_0162e1s1	falcipain-3	9719.1029
PF14_0392	PF14_0392e5s1	Ser/Thr protein kinase, putative	9714.572442
PF14_0345	PF14_0345-e2	hypothetical protein	9689.222157
PF14_0013	PF14_0013e2s1	hypothetical protein	9683.310742
MAL7P1.102	MAL7P1.102-s1	hypothetical protein	9676.546177
PFC0760c	PFC0760c-s3	hypothetical protein	9669.811735
PFC0430w	PFC0430we1s1	hypothetical protein	9643.566148
PF13_0062	PF13_0062e1s1	clathrin-adaptor medium chain, putative	9632.774166
PFE1120w	PFE1120w-e3s7	hypothetical protein	9626.195176
PF13_0157	PF13_0157e1s1	ribose-phosphate pyrophosphokinase, putative	9623.44828
PF07_0118	PF07_0118-s6	hypothetical protein	9621.942345

PF10_0092	PF10_0092-e3	hypothetical protein	9618.732457
MAL7P1.29	MAL7P1.29-e2	hypothetical protein	9598.588302
MAL6P1.201	MAL6P1.201-s1	leucyl-tRNA synthetase, cytoplasmic, putative	9592.588034
MAL13P1.22	MAL13P1.22e2s1	DNA ligase 1	9523.455581
PF10_0211	PF10_0211e1s3	hypothetical protein	9519.204339
PF10_0143	PF10_0143e1s3	ADA2-like protein	9513.65552
PF11_0270	PF11_0270e1s2	threonine -- tRNA ligase, putative	9497.656597
PFE1355c	PFE1355ce2s1	ubiquitin carboxyl-terminal hydrolase, putative	9494.149277
MAL13P1.176	MAL13P1.176e1s1	Plasmodium falciparum reticulocyte binding protein 2 homolog b	9425.328954
PF14_0144	PF14_0144e1s1	mRNA capping enzyme, putative	9417.252814
PFI0355c	PFI0355ce1s1	ATP-dependent heat shock protein, putative	9335.069157
PF11_0507	PF11_0507e1s4	antigen 332, putative	9324.467488
PF11_0395	PF11_0395e1s4	guanylyl cyclase	9317.207068
PFB0465c	PFB0465c-e2	hypothetical protein	9303.523031
PFI0630w	PFI0630we2s1	26S proteasome regulatory subunit, putative	9282.183577
PFL1395c	PFL1395ce1s4	hypothetical protein	9269.789113
PF14_0681	PF14_0681e2s1	diacylglycerol kinase, putative	9221.430289
PF10_0356	PF10_0356-e2s1	liver stage antigen, putative	9216.504126
PFL2440w	PFL2440w-e2s2	DNA repair protein rhp16, putative	9194.252968
MAL8P1.156	MAL8P1.156	hypothetical protein	9182.803714
PFL0440c	PFL0440ce1s1	hypothetical protein	9172.914086
PF13_0080	PF13_0080e1s3	hypothetical protein	9160.237244
PFB0150c	PFB0150ce2s2	protein kinase, putative	9149.570828
PF10_0079	PF10_0079-s3	hypothetical protein	9134.986366
PFL2120w	PFL2120we1s2	hypothetical protein	9087.52173
PFE0830c	PFE0830c-s2	unknown protein, mb2	9060.089014
PF14_0620	PF14_0620-e1	hypothetical protein	8996.705803
PF11_0008	PF11_0008e1s3	erythrocyte membrane protein 1 (PfEMP1)	8996.598515
PFL0085c	PFL0085c-e2	hypothetical protein	8989.089925
PFL1545c	PFL1545c-e1	chaperonin cpn60	8974.854239
PF11_0156	PF11_0156-e1	hypothetical protein	8972.449275
PF13_0161	PF13_0161e1s2	hypothetical protein	8918.935635
MAL13P1.230	MAL13P1.230e1s1	hypothetical protein	8905.901169
PF14_0170	PF14_0170e1s1	hypothetical protein	8888.355673
PF10_0132	PF10_0132-e1s2	phospholipase C-like, putative	8888.25613
PF14_0546	PF14_0546e1s1	hypothetical protein, conserved	8846.010759
PFL0545w	PFL0545w-e3s1	kinesin-related protein, putative	8758.925775
PFB0150c	PFB0150ce1s1	protein kinase, putative	8748.209963
PFL1880w	PFL1880we1s1	long-chain-fatty-acid--CoA ligase, putative	8732.729075

MAL8P1.142	MAL8P1.142e2s1	proteasome beta-subunit	8715.296014
PFI0315c	PFI0315ce1s1	hypothetical protein	8701.278482
PFI0550w	PFI0550w-e1s2	hypothetical protein	8689.103799
MAL6P1.237	MAL6P1.237-e6s1	hypothetical protein, conserved	8680.178562
MAL8P1.139	MAL8P1.139-e1s1	hypothetical protein	8678.778016
PF11_0294	PF11_0294e1s1	ATP-dependent phosphofructokinase, putative	8670.818707
MAL13P1.323	MAL13P1.323e1s2	hypothetical protein	8644.168464
PF14_0588	PF14_0588e1s1	hypothetical protein	8638.221052
PFI1425w	PFI1425w-e2	hypothetical protein	8630.049101
PFL2430c	PFL2430c	eukaryotic translation initiation factor 2b, subunit 2, putative	8626.481636
PF11_0245	PF11_0245-e1	translation elongation factor EF-1, subunit alpha, putative	8621.729026
PFB0150c	PFB0150ce2s3	protein kinase, putative	8609.636772
PFL2335w	PFL2335w-s2	hypothetical protein	8606.613155
PFL1070c	PFL1070ce1s1	endoplasmin homolog precursor, putative	8605.53858
PFB0640c	PFB0640c-e1s2	hypothetical protein, conserved	8586.666626
PFL0555c	PFL0555ce1s1	hypothetical protein	8581.190497
PF14_0096	PF14_0096e1s1	hypothetical protein	8567.335236
PF13_0003	PF13_0003e1s1	erythrocyte membrane protein 1 (PfEMP1)	8517.087752
PFL2190c	PFL2190ce1s2	hypothetical protein	8498.106985
PF11_0358	PF11_0358-e2s1	DNA-directed RNA polymerase, beta subunit, putative	8482.605802
PFC0635c	PFC0635ce1s1	translation initiation factor E4, putative	8470.186074
PF14_0668	PF14_0668e1s3	hypothetical protein	8468.381669
PFL1135c	PFL1135c-e2s1	hypothetical protein	8452.723083
PF13_0214	PF13_0214e2s1	elongation factor 1-gamma, putative	8447.128945
MAL13P1.39	MAL13P1.39e4s1	hypothetical protein	8441.19673
PF11_0165	PF11_0165e1s1	falcipain 2 precursor	8411.044537
PF07_0004	PF07_0004e2s1	hypothetical protein	8377.692877
PF10_0177	PF10_0177e1s1	erythrocyte membrane-associated antigen	8365.436223
PFB0315w	PFB0315w-s1	41 kDa antigen	8357.280331
PF14_0145	PF14_0145-s2	hypothetical protein	8340.915733
PF10_0320	PF10_0320e1s1	hypothetical protein	8337.194876
PF11_0344	PF11_0344	Apical membrane antigen 1 precursor, AMA1	8311.627487
PF11_0158	PF11_0158e1s2	hypothetical protein	8304.1304
MAL6P1.131	MAL6P1.131-e1s3	SET-domain protein, putative	8291.353271
MAL8P1.23	MAL8P1.23-s1	ubiquitin-protein ligase 1, putative	8261.708136
MAL8P1.11	MAL8P1.11-e2s1	hypothetical protein	8256.965595
PFE0055c	PFE0055ce4s1	heat shock protein, putative	8243.5595
PF11_0395	PF11_0395e1s3	guanylyl cyclase	8218.042431

PFL0635c	PFL0635ce1s2	bromodomain protein, putative	8204.965078
PFE0090w	PFE0090we1s2	hypothetical protein	8198.602302
PF14_0678	PF14_0678e3s1	exported protein 2	8175.532522
PF11_0240	PF11_0240e2s5	dynein heavy chain, putative	8174.641458
PF14_0631	PF14_0631e1s3	hypothetical protein	8173.479269
PF10_0085	PF10_0085e1s1	nucleolar protein NOP5, putative	8169.333665
MAL6P1.146	MAL6P1.146-s3	P. falciparum PK4 protein kinase	8147.889101
PF10_0214	PF10_0214-e3	hypothetical protein	8117.898368
PF10_0133	PF10_0133-s1	hypothetical protein	8106.783834
MAL7P1.138	MAL7P1.138e1s1	hypothetical protein	8058.196747
PF14_0664	PF14_0664-e1s1	biotin carboxylase subunit of acetyl CoA carboxylase, putative	8050.768398
PFB0405w	PFB0405w-s1	transmission-blocking target antigen s230 precursor	8036.013201
PF14_0515	PF14_0515-s2	hypothetical protein	8025.653714
MAL8P1.60	MAL8P1.60e4s1	hypothetical protein	8006.553977
MAL8P1.139	MAL8P1.139-e2s3	hypothetical protein	8003.091653
PFI1520w	PFI1520w	hypothetical protein	7991.128636
PF10_0143	PF10_0143e1s2	ADA2-like protein	7978.69891
PF14_0589	PF14_0589e1s2	valine - tRNA ligase, putative	7976.03119
PFI0240c	PFI0240c-e1s2	E1-E2_ATPase/hydrolase, putative	7962.420863
PF14_0664	PF14_0664-e1s3	biotin carboxylase subunit of acetyl CoA carboxylase, putative	7942.1471
PFE0935c	PFE0935ce1s1	RNA-binding protein, putative	7933.062828
PF13_0179	PF13_0179e1s1	isoleucine--tRNA ligase, putative	7884.268227
PF14_0338	PF14_0338e1s1	hypothetical protein	7874.18954
PF14_0593	PF14_0593e1s1	hypothetical protein	7858.143511
PFE1120w	PFE1120w-e3s3	hypothetical protein	7840.523849
PFL1300c	PFL1300ce1s1	hypothetical protein	7788.462024
PF08_0035	PF08_0035	hypothetical protein	7785.933475
PF08_0107	PF08_0107e1s2	erythrocyte membrane protein 1 (PfEMP1)	7736.62189
PF08_0054	PF08_0054	heat shock 70 kDa protein	7718.534823
PF11_0270	PF11_0270e1s1	threonine -- tRNA ligase, putative	7712.830563
PF14_0412	PF14_0412-e2	hypothetical protein	7674.124986
PFB0115w	PFB0115we1s1	hypothetical protein	7670.748794
PF07_0047	PF07_0047e1s1	cell division cycle ATPase, putative	7654.640199
PF11_0069	PF11_0069e1s1	hypothetical protein	7650.543302
PF14_0456	PF14_0456e2s2	hypothetical protein, conserved	7640.631399
PF08_0089	PF08_0089e1s2	hypothetical protein	7624.764323
PF07_0048	PF07_0048e1s2	erythrocyte membrane protein 1 (PfEMP1)	7619.90559
PF14_0377	PF14_0377-e1	vesicle-associated membrane protein, putative	7607.277771

PF10_0133	PF10_0133-s2	hypothetical protein	7602.093407
PF08_0018	PF08_0018-s1	translation initiation factor-like protein	7537.255186
PFL0405w	PFL0405we3s1	hypothetical protein	7536.74763
PF13_0339	PF13_0339e1s1	hypothetical protein	7533.131233
PFC0720w	PFC0720we1s1	hypothetical protein	7516.473682
MAL6P1.252	MAL6P1.252-e1s1	erythrocyte membrane protein 1 (PfEMP1)	7514.532873
MAL13P1.323	MAL13P1.323e1s3	hypothetical protein	7506.040184
MAL6P1.131	MAL6P1.131-e1s1	SET-domain protein, putative	7500.418405
PF13_0262	PF13_0262e2s1	lysine--tRNA ligase	7482.752188
PF13_0320	PF13_0320-e9	protein with aminophospholipid-transporting P-ATPase and guanyl cyclase domains	7474.502005
PFE0270c	PFE0270c-e1s1	DNA repair protein, putative	7474.215012
PF11_0048	PF11_0048e1s1	casein kinase II beta chain, putative	7467.822509
PFB0895c	PFB0895ce1s1	replication factor C subunit 1, putative	7464.714781
PF14_0370	PF14_0370e2s1	RNA helicase, putative	7442.984447
PF14_0494	PF14_0494e1s2	hypothetical protein, conserved	7436.278116
PF13_0210	PF13_0210e1s1	hypothetical protein	7435.281903
PF11_0053	PF11_0053-e1s2	PfSNF2L	7433.57798
PFI0510c	PFI0510ce1s2	hypothetical protein	7432.658531
MAL7P1.146	MAL7P1.146e5s2	hypothetical protein	7426.605798
PF13_0285	PF13_0285	hypothetical protein, conserved	7424.703323
MAL7P1.32	MAL7P1.32	hypothetical protein	7396.672225
PFI1730w	PFI1730w-e2	cytoadherence linked asexual protein 9(CLAG9)	7382.409075
PF14_0419	PF14_0419e6s1	hypothetical protein	7370.837308
PFB0340c	PFB0340ce2s1	cysteine protease, putative	7368.607055
PFD0430c	PFD0430ce3s1	hypothetical protein	7349.266825
PFI0235w	PFI0235we2s1	replication factor A-related protein, putative	7347.834265
PF14_0463	PF14_0463e1s4	chloroquine resistance marker protein	7341.372167
MAL13P1.78	MAL13P1.78e1s2	hypothetical protein	7311.694931
MAL13P1.133	MAL13P1.133-e3	hypothetical protein	7304.046282
PFC1065w	PFC1065we2s1	hypothetical protein	7278.659314
PF14_0647	PF14_0647-e3s2	hypothetical protein	7257.637323
PFC0425w	PFC0425we1s4	hypothetical protein	7254.322439
PF13_0040	PF13_0040-e2	DNA-directed RNA polymerase alpha chain, putative	7233.989381
PF14_0649	PF14_0649-e2s1	hypothetical protein	7233.309779
PFL1385c	PFL1385ce1s1	Merozoite Surface Protein 9, MSP-9	7231.525098
PFC0805w	PFC0805we1s2	DNA-directed RNA polymerase II, putative	7197.784259
PFA0170c	PFA0170ce1s1	hypothetical protein, conserved	7188.848384
PFB0635w	PFB0635we1s1	T-complex protein 1, putative	7185.067723
PF14_0649	PF14_0649-e2s2	hypothetical protein	7179.630575

PFD0445c	PFD0445c-s1	hypothetical protein	7162.72283
PFI1500w	PFI1500w-e2s2	hypothetical protein	7154.844139
PFL1620w	PFL1620we1s4	asparagine/aspartate rich protein, putative	7152.892601
PFI0345w	PFI0345we1s2	hypothetical protein	7146.854222
PFA0125c	PFA0125ce1s2	Ebl-1 like protein, putative	7090.66901
PFL1680w	PFL1680we1s2	splicing factor 3b, subunit 3, 130kD, putative	7088.068113
PF08_0012	PF08_0012e1s3	hypothetical protein	7053.298595
PF10_0379	PF10_0379e1s1	phospholipase, putative	7045.705137
PF13_0278	PF13_0278	hypothetical protein	7042.086823
PF10_0161	PF10_0161e1s1	hypothetical protein	7021.729422
MAL7P1.23	MAL7P1.23-e1s2	hypothetical protein	6987.412158
PF10_0224	PF10_0224e1s4	dynein heavy chain, putative	6976.407841
MAL8P1.132	MAL8P1.132e3s1	hypothetical protein	6969.24144
PF11_0407	PF11_0407e1s1	adrenodoxin reductase, putative	6933.424346
PF08_0012	PF08_0012e1s2	hypothetical protein	6926.642328
MAL13P1.201	MAL13P1.201e1s1	hypothetical protein	6919.964191
PF14_0632	PF14_0632-e1s1	26S proteasome subunit, putative	6909.752127
PFA0510w	PFA0510we1s3	hypothetical protein	6855.385223
MAL13P1.323	MAL13P1.323e1s1	hypothetical protein	6847.775256
PF11_0240	PF11_0240e2s2	dynein heavy chain, putative	6839.855305
PFL0730w	PFL0730we1s1	hypothetical protein	6834.613094
PFL1010c	PFL1010ce1s2	hypothetical protein conserved	6763.585876
PFL0305c	PFL0305ce2s1	hypothetical protein	6762.696782
MAL13P1.178	MAL13P1.178-e2	hypothetical protein	6702.31818
PFA0430c	PFA0430ce1s2	hypothetical protein	6692.494869
PF14_0463	PF14_0463e1s3	chloroquine resistance marker protein	6679.654276
PF11_0225	PF11_0225e1s1	PfGCN20	6675.064184
PFL1395c	PFL1395ce1s1	hypothetical protein	6659.357974
PF11_0395	PF11_0395e1s5	guanylyl cyclase	6658.733808
PF14_0326	PF14_0326e2s4	hypothetical protein	6644.021506
PF11_0404	PF11_0404e2s3	malaria antigen	6630.080343
PFI1620c	PFI1620c-s1	hyptohetical protein	6615.075509
PF10_0140	PF10_0140e2s1	hypothetical protein	6607.140105
PF14_0370	PF14_0370e1s1	RNA helicase, putative	6604.855744
PF07_0126	PF07_0126e1s2	hypothetical protein	6567.422807
PF08_0137	PF08_0137e2s2	hypothetical protein	6561.787212
PF14_0722	PF14_0722-e3s3	hypothetical protein	6556.433562
PF14_0470	PF14_0470-e2s1	hypothetical protein	6540.770424
PF14_0031	PF14_0031e4s2	hypothetical protein	6522.379348
PFL1085w	PFL1085we1s2	hypothetical protein	6516.321628
PF11_0232	PF11_0232e1s1	hypothetical protein	6486.300885
MAL7P1.134	MAL7P1.134e2s2	hypothetical protein	6483.238074

PF08_0127	PF08_0127e1s2	hypothetical protein	6482.116591
PF13_0121	PF13_0121-e3	dihydrolipoamide succinyltransferase, putative	6472.5763
PFI0410c	PFI0410ce17s1	hypothetical protein	6454.623098
PFE1120w	PFE1120w-e3s2	hypothetical protein	6450.243928
PF13_0153	PF13_0153e1s2	hypothetical protein	6437.075703
PFD0995c	PFD0995ce1s2	erythrocyte membrane protein 1 (PfEMP1)	6431.265995
PF14_0198	PF14_0198e1s1	glycine -- tRNA ligase, putative	6425.666746
PF08_0102	PF08_0102e1s2	asparagine-rich antigen Pfa55-14	6421.481626
PF11_0341	PF11_0341-e1s2	hypothetical protein	6393.491532
PFL1620w	PFL1620we1s3	asparagine/aspartate rich protein, putative	6366.527628
PF11_0395	PF11_0395e1s2	guanylyl cyclase	6359.654067
PF11_0307	PF11_0307e1s2	hypothetical protein	6335.347216
PFB0335c	PFB0335ce1s1	cysteine protease, putative	6334.054889
MAL7P1.134	MAL7P1.134e2s3	hypothetical protein	6333.2921
PF13_0173	PF13_0173e1s1	hypothetical protein	6323.246411
PFL1410c	PFL1410c-s3	hypothetical protein	6310.707611
PFE1320w	PFE1320w-s1	hypothetical protein	6298.410444
PFC0130c	PFC0130ce1s1	hypothetical protein, conserved	6291.954798
PF11_0158	PF11_0158e1s1	hypothetical protein	6281.779664
MAL6P1.131	MAL6P1.131-e1s4	SET-domain protein, putative	6280.254743
PFC0590c	PFC0590c-e2s1	hypothetical protein	6279.400678
MAL8P1.69	MAL8P1.69-e3	14-3-3 protein homologue, putative	6248.184309
PF13_0248	PF13_0248e1s1	pf47	6244.690582
PF14_0384	PF14_0384e1s1	hypothetical protein	6244.418351
MAL13P1.114	MAL13P1.114e3s1	hypothetical protein	6218.991029
PF14_0597	PF14_0597e2s1	cytochrome c1 precursor, putative	6197.582496
PF08_0135	PF08_0135e1s1	hypothetical protein, conserved	6153.27283
PF14_0338	PF14_0338e9s1	hypothetical protein	6141.637337
PF10_0143	PF10_0143e1s1	ADA2-like protein	6137.525035
PFD1060w	PFD1060we1s3	u5 small nuclear ribonucleoprotein-specific protein, putative	6135.557867
PFL0130c	PFL0130c-e1	hypothetical protein, conserved	6107.476554
PFB0645c	PFB0645c-e2	Ribosomal protein L13, putative	6106.553861
PF14_0315	PF14_0315-e2s3	hypothetical protein	6104.017873
PF10_0211	PF10_0211e1s2	hypothetical protein	6103.63962
MAL13P1.234	MAL13P1.234-e1s6	hypothetical protein	6061.10284
PFC0710w	PFC0710w-e3	inorganic pyrophosphatase, putative	6051.359702
PFC0180c	PFC0180ce4s1	membrane skeletal protein, putative	6036.993061
PF13_0350	PF13_0350	signal recognition particle receptor alpha subunit, putative	6030.116609
PFD0295c	PFD0295ce4s1	hypothetical protein	6028.296022

PF11_0008	PF11_0008e1s2	erythrocyte membrane protein 1 (PfEMP1)	6024.490463
PF14_0196	PF14_0196	hypothetical protein	6016.360122
PF14_0345	PF14_0345-e1s1	hypothetical protein	6005.765993
PFA0725w	PFA0725we1s1	SURFIN, surface-associated interspersed gene	5974.526097
PF14_0401	PF14_0401e2s1	methionine -- tRNA ligase, putative	5973.28568
PF08_0121	PF08_0121e1s1	peptidyl-prolyl cis-trans isomerase precursor	5967.693013
PFE0780w	PFE0780we6s1	hypothetical protein	5966.141173
PF13_0014	PF13_0014-e1	40S ribosomal protein S7 homologue, putative	5961.691164
MAL13P1.278	MAL13P1.278e1s5	Ser/Thr protein kinase	5959.922208
PFI0855w	PFI0855we1s1	hypothetical protein	5955.222393
PF14_0334	PF14_0334e1s1	NAD(P)H-dependent glutamate synthase, putative	5930.477035
PFI0265c	PFI0265ce7s1	rhoptry protein, putative	5926.144945
PFA0510w	PFA0510we1s2	hypothetical protein	5910.967246
MAL8P1.60	MAL8P1.60e15s1	hypothetical protein	5902.159318
PFI0345w	PFI0345we1s1	hypothetical protein	5894.30382
PF14_0249	PF14_0249	hypothetical protein	5863.357196
PFE0040c	PFE0040ce2s1	Mature parasite-infected erythrocyte surface antigen (MESA) or PfEMP2	5856.782515
PFI1000w	PFI1000w-s2	hypothetical protein	5855.942334
MAL8P1.23	MAL8P1.23-s9	ubiquitin-protein ligase 1, putative	5855.890597
PF13_0320	PF13_0320-e13s2	protein with aminophospholipid-transporting P-ATPase and guanyl cyclase domains	5835.057415
MAL6P1.237	MAL6P1.237-e6s2	hypothetical protein, conserved	5833.335879
MAL7P1.102	MAL7P1.102-s2	hypothetical protein	5828.9705
MAL13P1.346	MAL13P1.346e1s2	DNA repair endonuclease	5824.172003
PF13_0021	PF13_0021e1s1	small heat shock protein, putative	5819.944835
PFB0540w	PFB0540we1s2	hypothetical protein	5810.287972

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