

COLLEGE OF BASIC AND APPLIED SCIENCES

SCHOOL OF BIOLOGICAL SCIENCES

**UNDERSTANDING THE ACQUISITION AND KINETICS OF
NATURALLY ACQUIRED IMMUNITY TO *Pf*PRH5 COMPLEX
PROTEINS IN MALARIA**

BY

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**THIS THESIS IS SUBMITTED TO THE DEPARTMENT OF
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DECLARATION

I do hereby declare that this thesis is the product of my research work duly carried out under the supervision of Professors Gordon A. Awandare, Lars Hviid and Lea Barfod. I further declare that parts of this work or as a whole has not been submitted to any other institution for the award of a similar degree.

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ABSTRACT

Plasmodium falciparum causes the severe form of malaria affecting mainly children below the age of five years and pregnant women. In the last decade, there has been a global decline in the reported malaria case incidence. Despite the reduction in malaria-associated morbidity and mortality, the disease is still prevalent in many developing countries in the tropics. With emergence of insecticide resistance and drug resistance to the limited range of effective drugs currently available, malaria vaccines remain a critical component of any strategic plan to eliminate and eventually eradicate malaria. Individuals living in endemic regions do develop immunity to malaria after repeated exposure to the parasite and never achieve sterile immunity. Developing an effective malaria vaccine has been difficult mainly due to the complex parasite life cycle, antigenic variation and polymorphism of parasite ligands. Much of the vaccine studies have focused on the asexual blood stage of the parasite life cycle as the clinical symptoms associated with malaria are due to this stage. In spite of this, there is no blood stage vaccine.

P. falciparum reticulocyte binding-like protein homolog 5 (PfRH5) and its interacting proteins *P. falciparum* cysteine-rich protective antigen (PfCyRPA) and *P. falciparum* protein 113 (Pf113) have emerged as promising blood stage vaccine candidates due to the essential role of PfRH5 in merozoite invasion, its less polymorphic nature and the strain-transcending inhibitory effect of antibodies induced against the PfRH5 complex proteins. Nevertheless, not much is known about the role of PfRH5, PfCyRPA and Pf113 in naturally acquired immunity. In the present studies, the immunogenicity of PfRH5 complex proteins and the kinetics of antibodies induced against the PfRH5 complex proteins in natural infections were examined. In total, 206 Ghanaian children between

the ages of 1-12 years who were either symptomatic, asymptomatic or non-parasitemic and healthy were recruited at the Hohoe municipal hospital. Plasma levels of antigen-specific IgG antibodies among acute malaria patients on day of admission showed seroprevalence of PfRH5, PfCyRPA and Pf113 was low compared to other well-studied merozoite antigens. The predominant IgG subclass response to the PfRH5 complex proteins was IgG1 and IgG3. Plasma IgG levels were found not to correlate with protection from malaria, severity of disease, or parasitemia on day of admission. Following treatment, antibodies to the studied antigens rapidly declined suggesting plasma IgG levels to the PfRH5 complex proteins were markers of recent parasite exposure.

To better understand the mechanism of action of anti-PfRH5 and anti-PfCyRPA antibodies in naturally acquired immunity, human monoclonal antibodies were isolated from semi-immune Ghanaian adults and tested for functionality. The antibodies showed poor reactivity to PfRH5 and PfCyRPA recombinant proteins and did not exhibit inhibition against *P. falciparum in vitro*. In contrast, a panel of human monoclonal antibodies isolated from individuals vaccinated with viral vectored PfRH5 elicited strain-transcending inhibitory effects *in vitro*. Thus, knowledge gained from the functional and structural characterization of the human anti-PfRH5 monoclonal antibodies would greatly advance the rational design of PfRH5 based vaccines.

DEDICATION

I dedicate this work to my kids, Milena and Aubrey.

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TABLE OF CONTENTS

DECLARATION	ii
ABSTRACT	iii
DEDICATION	v
ACKNOWLEDGEMENTS	vi
LIST OF FIGURES	xi
LIST OF TABLES	xiii
LIST OF ABBREVIATIONS	xiv
CHAPTER ONE	1
1 INTRODUCTION.....	1
1.1 Aims.....	3
CHAPTER TWO.....	5
2 LITERATURE REVIEW.....	5
2.1 Epidemiology of Malaria.....	5
2.2 Clinical manifestations of malaria.....	6
2.2.1 Asymptomatic infection	7
2.2.2 Uncomplicated malaria	8
2.2.3 Severe malaria syndromes.....	8
2.2.3.1 Cerebral malaria (CM)	9
2.2.3.2 Severe malarial anaemia (SMA).....	10
2.2.3.3 Respiratory Distress (RD).....	10
2.2.4 Pregnancy-associated Malaria (PAM).....	11
2.3 Malaria diagnosis and treatment.....	11
2.4 The Life cycle of <i>Plasmodium falciparum</i>	15
2.5 Mechanism of merozoite invasion of RBC and molecular networks	16
2.5.1 Egress of Merozoites	17
2.5.2 Primary attachment to Erythrocyte Membrane	18
2.5.3 Apical reorientation of the Merozoite	19
2.5.4 Tight junction formation and parasite entry	20
2.5.5 Structure of the PfRH5 Protein complex.....	21
2.6 Antibody Structure	26
2.6.1 Immunoglobulin classification.....	28
2.7 Immunity to malaria	30

2.8	Longevity of malaria induced antibody levels	32
2.9	Immunological B cell memory to malaria	34
2.10	Malaria vaccines	36
2.10.1	Challenges in developing malaria vaccines	37
2.10.2	Pre-erythrocytic (Liver) stage vaccines.....	38
2.10.3	Transmission blocking vaccines (TBV).....	40
2.10.4	Blood stage vaccines	41
2.11	Towards epitope-based malaria vaccines.....	43
2.12	Human monoclonal antibodies from memory B cells	46
2.13	Growth inhibition assay (GIA) as a test for antibody function.....	48
CHAPTER THREE.....		50
3	MATERIALS AND METHODS	50
3.1	Ethical Approval.....	50
3.2	Study area	50
3.3	Study design and sampling	51
3.3.1	Inclusion criteria for children participants	53
3.3.2	Exclusion criteria for children participants.	54
3.3.3	Inclusion criteria for adult participants	54
3.3.4	Exclusion criteria for adult participants	55
3.4	Sample size	55
3.5	Parasitological detection by microscopy	55
3.6	Sample Preparation.....	56
3.7	Recombinant antigens.....	57
3.8	Plasma IgG reactivity and IgG subclass measurements by ELISA.....	57
3.9	Memory B-cell immortalisation and cloning.....	59
3.10	Recombinant monoclonal antibody	61
3.11	Reverse transcription PCR (RT-PCR) of single B cells	61
3.12	cDNA and immunoglobulin chain amplification.....	61
3.13	Transcriptionally active PCR (TAP).....	62
3.14	Transfection	65
3.15	Restriction digest of plasmid vector and variable region cDNA	65
3.16	Ligation of plasmid vector and variable region cDNA.....	66
3.17	Transformation of bacterial cells.	66

3.18	Thawing of parasites	67
3.19	<i>In vitro</i> parasite culture	67
3.20	<i>In vitro</i> growth inhibition assay	68
3.21	Statistical Analysis	68
CHAPTER FOUR		70
4	RESULTS	70
4.1	Clinical characteristics of study population	70
4.2	Seroprevalence and levels of IgG specific for PfRH5 complex proteins and other merozoite proteins.	71
4.3	Association of merozoite-specific IgG levels with clinical presentation	73
4.4	Kinetics of merozoite-specific IgG levels following <i>P.falciparum</i> malaria....	76
4.5	PfRH5-specific IgG and its role in clinical protection	78
4.6	Isolation of human anti-PfRH5 and anti-PfCyRPA monoclonal antibodies ...	80
4.7	Single-cell RT-PCR and IgG gene amplification of antigen specific polyclonal cell lines.....	83
4.8	Cloning of V _H /V _L gene pairs into expression vectors	88
4.9	Antibody production and screening for specificity	90
4.10	<i>In vitro</i> growth inhibition with human anti-PfRH5 monoclonal antibodies	91
CHAPTER FIVE		94
5	DISCUSSION	94
5.1	Conclusions	105
BIBLIOGRAPHY		107
APPENDICES		137

LIST OF FIGURES

Figure 2.1: Global distribution of malaria endemic countries from 2000 to 2016.....	6
Figure 2.2: Life cycle of <i>Plasmodium falciparum</i>	16
Figure 2.3: Three-dimensional (3D) structure of <i>P. falciparum</i> merozoite	17
Figure 2.4: Timeline of <i>P.falciparum</i> merozoite invasion of host RBC after schizont rupture.....	21
Figure 2.5: A proposed model for PfrH5 protein complex.....	23
Figure 2.6: Structure of PfrH5	24
Figure 2.7: A schematic representation of an IgG molecule depicting a Y shape.	27
Figure 3.1:Map of Ghana showing study sites (Asutsuare and Hohoe).....	51
Figure 3.2: Flow chart showing sampling time points for children recruited at Hohoe.	54
Figure 3.3 Flow chart showing sample preparation of blood samples collected from adults in Asutsuare.....	60
Figure 3.4: Schematic representation TAP PCR.....	63
Figure 4.1: Seroprevalence of merozoite specific IgG and IgG subclass responses to merozoite antigens in acute malaria children.	72
Figure 4.2: Association of IgG levels with clinical <i>P. falciparum</i> categories.....	74
Figure 4.3: Correlation of IgG levels with parasitemia on day of admission	76
Figure 4.4: Kinetics of merozoite specific IgG levels following <i>P.falciparum</i> infection.	77
Figure 4.5: Role of Rh5 specific IgG levels in clinical protection.....	79
Figure 4.6: PfrH5 specific IgG levels in semi-immune adults.....	80
Figure 4.7: ELISA screen of EBV immortalized memory B cell culture supernatants for specificity to PfrH5.....	82

Figure 4.8: ELISA screen of EBV immortalized memory B cell culture supernatants for specificity to PfCyRPA.....	83
Figure 4.9:Amplification of V_{γ} and V_{λ} genes from CyRPA specific polyclonal cell line 1b4E which was single cell sorted into 24 well plates.	85
Figure 4.10 Amplification of V_{γ} and V_{κ} genes from RH5 specific polyclonal cell line 2B which was single cell sorted into 24 well plates.	86
Figure 4.11:Amplification of V_{γ} genes from RH5 specific polyclonal cell line 6.8B which was single cell sorted into 48 well plates.....	87
Figure 4.12:Amplification of V_{κ} genes from RH5 specific polyclonal cell line 6.8B which was single cell sorted into 48 well plates.	88
Figure 4.13: TAP expression fragments generated from amplified V_H/V_{λ} gene pairs from cell line 1B4E.....	89
Figure 4.14: TAP expression fragments generated from amplified V_H/V_{κ} gene pairs from cell line 2B	89
Figure 4.15: TAP expression fragments generated from amplified V_H/V_L gene pairs from cell line 2B	90
Figure 4.16: Antibody secretion and reactivity.	91
Figure 4.17: Growth inhibition effects of panel of human monoclonal antibodies	92

LIST OF TABLES

Table 2.1: Major ligands in <i>P.falciparum</i> merozoite invasion.....	25
Table 3.1 Reaction setup for 5´and 3´TAP fragments.	64
Table 4.1: Clinical characteristics of study participants	71
Table 4.2: Correlations between merozoite antigen-specific IgG responses	73
Table 4.3: EC ₅₀ values of anti PfRh5 monoclonal antibodies against different clinical isolates	93

LIST OF ABBREVIATIONS

A.U	Arbitrary units
AC	Asymptomatic controls
ACT	Artemisinin-based combination therapy
ADCI	Antibody dependent cellular inhibition
ADP	Adenosine diphosphate
AMA	Apical membrane antigen 1
ANOVA	Analysis of variance
BSA	Bovine serum albumin
Ca	Calcium
CD	Cluster of differentiation
CDC	Complement dependent cytotoxicity
Cdna	Complementary DNA
CDR	Complementarity determining region
CH	Heavy chain constant region
CHMI	Controlled human malaria infection
CL	Light chain constant region
CM	Cerebral malaria
CSP	Circumsporozoite surface protein
CyRPA	Cysteine rich protective antigen
DMEM	Dulbecco's modified Eagle medium
DNA	Deoxyribonucleic acid
EBA	Erythrocyte binding antigen
EBL	Erythrocyte binding-like

EBL	Erythrocyte binding-like
EC ₅₀	50% inhibitory concentration
EDTA	Ethylenediaminetetracetic acid
EGF	Epidermal-growth-factor
ELISA	Enzyme-linked immunosorbent assay
EM	Erythrocyte membrane
FAB	Fragment antibody binding region of an immunoglobulin
FBS	Fetal bovine serum
FC	Fragment crystallisable region of an immunoglobulin
GIA	Growth inhibition assay
GLURP	Glutamate rich protein
GLURP-R2	C-terminal repeat region-2 of GLURP
GP	Glycoproteins
GPI	Glycosylphosphatidylinositol
H ₂ O ₂	Hydrogen peroxide
H ₂ SO ₄	Sulphuric acid
HC	Heavy chain
HC	Healthy controls
HF	High fidelity
HIV	Human immunodeficiency virus
HRP	Horse radish peroxidase
IE	Infected erythrocyte
IFN- γ	Interferon gamma
IgG	Immunoglobulin

IgG	Immunoglobulin G
IgM	Immunoglobulin M
IMC	Inner membrane complex
iRBC	Infected red blood cell
KCL	Potassium chloride
KH ₂ PO ₄	Potassium dihydrogen phosphate
LC	Light chain
MenB	Meningococcus B
MSP	Merozoite surface protein
MSP	Merozoite surface protein
NaCl	Sodium chloride
NaHPO ₄	Sodium hydrogen phosphate
O.D	optical density
OPD	Out-patient department
OPV	Oral polio vaccine
<i>P. falciparum</i>	Plasmodium falciparum
PAM	Pregnancy associated malaria
<i>PBMC</i>	<i>Peripheral blood</i> mononuclear cells
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEI	polyethylenimine
Pf113	Plasmodium falciparum 113 protein
PfAARP	Plasmodium <i>falciparum</i> apical asparagine rich protein
PfAMA-1	<i>Plasmodium falciparum</i> apical membrane protein

PfCYRPA	<i>Plasmodium falciparum</i> cysteine-rich protective antigen
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein 1
PfHRP	<i>Plasmodium falciparum</i> histidine-rich protein
PfMSP	<i>Plasmodium falciparum</i> merozoite surface protein
PfRH5	<i>Plasmodium falciparum</i> reticulocyte binding-like protein homolog
PfRIPR	<i>Plasmodium falciparum</i> Rh5 interacting protein
PfSERA	<i>Plasmodium falciparum</i> serine repeat antigen
PfSUB1	<i>Plasmodium falciparum</i> Subtilisin-like protease 1
PNPP	p-Nitrophenyl phosphate
PV	Parasitophorous vacuole
PvDBP	<i>Plasmodium vivax</i> duffy binding protein
RBC	Red blood cell
RD	Respiratory distress
RDT	Rapid diagnostic test
RESA	Ring-infected erythrocyte surface antigen
RIFIN	Repetitive interspersed family
RNA	Ribonucleic acid
RPMI medium	Roswell Park Memorial Institute medium
rSAP	Shrimp Alkaline Phosphatase
RT-PCR	reverse-transcriptase-polymerase chain reaction
SD	Standard deviation
SEM	Standard error of mean
SM	Severe malaria
SMA	Severe malaria anaemia

SNP	Single nucleotide polymorphism
TBE	Tris/borate /EDTA
TBV	Transmission blocking vaccine
uRBC	Uninfected blood cell
VH	Heavy chain variable region
VL	Light chain variable region
VSA	Variant surface antigens
WHO	World Health Organisation
α TNF	Alpha- tumour necrotic factor

CHAPTER ONE

1 INTRODUCTION

Malaria remains one of the most important infectious diseases in the world, with children under 5 years and pregnant women having the greatest risk. Due to the enormous challenge malaria poses socially and economically, extensive research and resources have been channelled into combating the disease. Interventions such as improved diagnostics, insecticide residual spraying, insecticide-treated bed nets and the use of artemisinin-based combination drug therapies have helped reduce the disease burden over the past decades, but have been unsuccessful in eliminating the disease from endemic regions. Drug resistant and insecticide resistant parasites partly contribute to our inability to effectively eliminate malaria (White *et al.*, 2014). The development of a malaria vaccine seems the most promising way to effectively control morbidity and mortality, by complementing existing interventions.

The life cycle of malaria parasites involves a vertebrate host and an invertebrate vector. Malaria vaccine studies have aimed to target the different stages of the parasite life cycle (i.e. the pre-erythrocytic, the intra-erythrocytic, and the sexual stages), although majority of the studies have focused on the asexual blood stage, as the clinical symptoms associated with the disease are related to the asexual intra-erythrocytic stage of the parasite life cycle. Merozoite invasion of host red blood cells (RBCs) is a complex process involving a plethora of parasite ligands. Merozoite surface proteins are secreted and translocated to the merozoite surface shortly before erythrocyte invasion, and most of them play essential roles in merozoite invasion of erythrocyte. Antibodies against ligands known to play a role in invasion have been shown to exhibit *in vitro* growth inhibitory activity and antibody dependent cellular inhibition (Hodder *et al.*, 2001,

Yazdani *et al.*, 2006). Furthermore, antibodies to some blood stage antigens have been shown to be associated with protection from malaria in naturally acquired immunity (Hodder *et al.*, 2001, O'Donnell *et al.*, 2001). These blood stage antigens have thus been of interest to researchers as vaccine targets.

The well-studied and advanced asexual blood stage vaccine targets include merozoite surface protein (MSP) -1, -2, -3 and apical membrane antigen 1 (AMA-1) (Chauhan *et al.*, 2010). Several of these vaccine targets are in different phases of clinical trials; nonetheless, there is no licensed blood stage malaria vaccine. The only licensed malaria vaccine currently available is a pre-erythrocytic vaccine, RTS,S. A meta-analysis of cohort studies establishing a correlation between antibody levels of major merozoite antigens such as MSP-1 and AMA-1 and protection from *P. falciparum* malaria has been disappointing as these antigens do not produce effective protective immune responses (Fowkes *et al.*, 2010).

Developing malaria vaccines has been quite challenging mainly due to the complexity of the parasite life cycle, antigenic variation and the redundancy in parasite ligands, resulting in strain specific immune responses. To overcome this challenge, there is a need to identify vaccine targets that are relatively conserved but more importantly are essential in merozoite invasion.

P. falciparum reticulocyte-binding protein homolog 5 (PfRH5) is considered a promising blood stage vaccine target as preliminary studies have suggested it is a target of natural immunity, although seroprevalence of PfRH5 in endemic populations is low compared to that of other merozoite proteins (Douglas *et al.*, 2011, Tran *et al.*, 2013). It is still unclear how an antigen that is essential for merozoite invasion is able to evade the host immune system. The recently identified members of the PfRH5 complex; PfCyRPA and

Pf113 have also ignited some interest as vaccine targets but there are few studies on the immunogenicity of these antigens in natural malaria infections and whether they are targets of natural immunity.

In the present study, the immunogenicity and seroprevalence of PfRH5, PfCyRPA and Pf113 amongst Ghanaian children who had concurrent malaria infection and healthy children within the same community were examined. Human monoclonal antibodies to PfRH5 and PfCyRPA were generated, characterized and tested in growth inhibitory assay (GIA) against several *P. falciparum* strains. Antibodies were not generated against Pf113 due to challenges in producing the recombinant antigen during the study.

1.1 Aims

The study was aimed at assessing the potential of PfRH5, PfCyRPA and Pf113 as blood stage vaccine targets by examining the immunogenicity and seroprevalence of the antigens following natural infections, and generating human monoclonal antibodies to the antigens that inhibit merozoite invasion.

Hypothesis I: PfRH5, PfCyRPA and Pf113 are immunogenic in natural infections inducing antibody responses similar to other well-studied merozoite antigens. Antibody levels are associated with protection from malaria and severe disease.

Objective I: Examine the immunogenicity of the antigens and the seroprevalence among Ghanaian children with symptomatic malaria infection and asymptomatic children within the same community.

Objective II: Determine the role of merozoite antigen-specific IgG levels in clinical protection.

Objective III: Study the kinetics and longevity of antigen-specific IgG antibody levels following infection.

Hypothesis II: Semi-immune adults living in endemic regions who are seropositive to PfRH5 and PfCyRPA antigens have memory B cells that can be isolated and used to generate human monoclonal antibodies.

Objective IV: Generate a library of human anti-PfRH5 and anti-PfCyRPA monoclonal antibodies from semi-immune adults.

Objective V: Test the inhibitory abilities of the monoclonal antibodies generated.

CHAPTER TWO

2 LITERATURE REVIEW

2.1 Epidemiology of Malaria

Malaria is caused by obligate intraerythrocytic protozoan parasites of the *Plasmodium* genus. There are six different *Plasmodium* species (*P. falciparum*, *P. vivax*, *P. malariae*, *P. ovale*, *P. knowlesi*, and *P. cynomolgi*) (Knowles and Gupta, 1932, Schimdt *et al.*, 1961). capable of infecting humans with malaria through transmission by female Anopheles mosquitoes. *P. falciparum* is the main cause of severe malaria in humans. *P. vivax* also infects humans with moderate morbidity (Biryukov and Stoute, 2014, Cowman and Crabb, 2006).

Malaria is endemic in most tropical and subtropical regions of the world, predominantly in Sub-Saharan Africa, South-east Asia and the Eastern Mediterranean region (**Figure 2.1**). In 2016 an estimated 216 million cases of malaria and 445 000 deaths were reported globally. Out of these reported cases, the world health organisation (WHO) African region accounted for 90%, WHO South-east Asia 7%, and WHO Eastern Mediterranean region 2%. The number of deaths recorded follows a similar pattern with most deaths occurring in the WHO African region (91%) followed by WHO South-east Asia (6%) and then WHO Eastern Mediterranean region (1.8%). About 70% of the deaths recorded affected mainly children under 5 years (WHO, 2017).

In Ghana, malaria accounted for about 42% of all outpatient department (OPD) cases and 48% of all hospital admissions among children less than 5 years in the first quarter of 2017 (GHS, 2017). The parasite prevalence rate among children decreased from 47% in 2000 to 45% in 2010 (EPI, 2013). Similarly, malaria associated deaths in children under

five years also reduced from 14.4% to 0.6% from 2000 to 2012 (GHS, 2014). Malaria transmission intensity in Ghana is heterogeneous as transmission varies along different ecological regions (Awine *et al.*, 2017). Transmission is intense with a seasonal peak during the rainy season (June –October) in the northern part of the country. However, in forest zones and coastal ecological areas, parasite prevalence peaks twice in a year (Awine *et al.*, 2017). *P. falciparum* is the dominant circulating *plasmodia* specie with low-level infections of *P. malariae* and *P. ovale*.

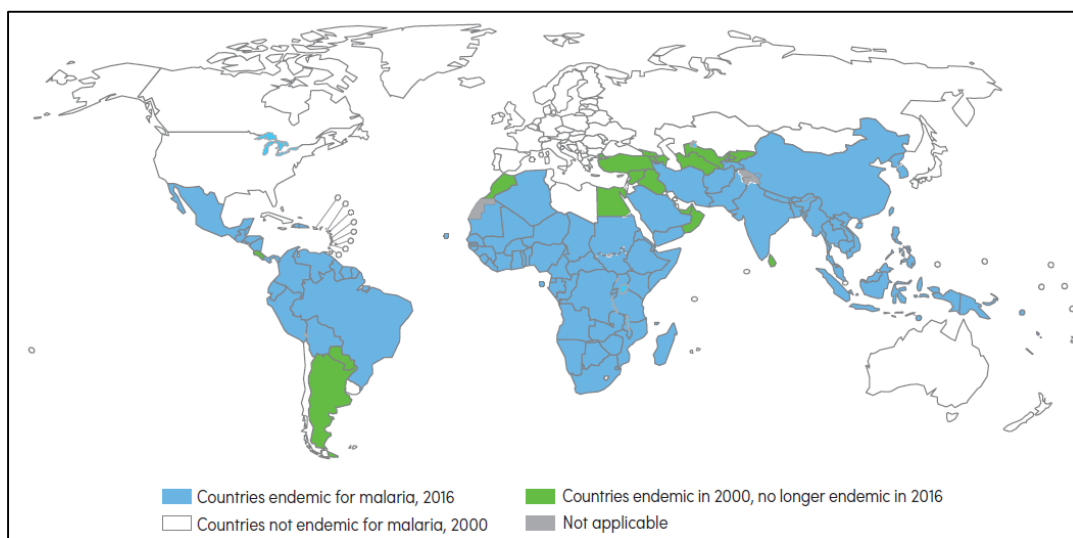


Figure 2.1: Global distribution of malaria endemic countries from 2000 to 2016.

Malaria endemic countries declined from 108 in 2000 to 91 in 2016. The blue areas represent countries still endemic for malaria in 2016. Green areas represents countries previously endemic for malaria in 2000 but not in 2016. White areas represent countries not endemic for malaria since 2000. Source: (WHO, 2016)

2.2 Clinical manifestations of malaria

Clinical manifestations of malaria are because of the cyclic asexual replication and invasion of the host erythrocytes by *P. falciparum* merozoites. Symptoms of malaria may range from fever, chills, nausea, vomiting to more severe symptoms such as coma and anaemia, acute renal failure and pulmonary problems (Trampuz *et al.*, 2003). The virulence of *P. falciparum* is attributed to the ability of the parasite to adhere to receptors on endothelial surfaces, thereby evading clearance of infected erythrocytes by the spleen.

Host immune responses such as the release of pro-inflammatory cytokines enhances parasite sequestration, further complicate disease, leading to coma or death.

The outcome of an infection with *P. falciparum* could be asymptomatic, uncomplicated or severe disease, which is influenced by factors such as the immune status of the infected individual, presence of antimalarial prophylaxis or previous treatment with antimalarial drugs and parasite density of the inoculum (Bartoloni and Zammarchi, 2012). Disease symptoms do not manifest immediately following an infection with *P. falciparum*. There is a pre-patent period (the period between sporozoite inoculation and detectable asexual blood-stage parasitemia) and an incubation period (time from sporozoite inoculation to clinical symptoms). In non-immune individuals infected with *P. falciparum* malaria, the median pre-patent period is 10 days (ranging from 9-13 days) (Trampuz et al., 2003).

2.2.1 Asymptomatic infection

Asymptomatic *Plasmodium* infection is a case where an individual is infected with *Plasmodium* parasites without showing any clinical symptoms. Majority of people living in endemic regions are asymptomatic in both high and low transmission settings (Galatas et al., 2015). A correlation is established between high endemicity and prevalence of asymptomatic patients in some parts of Africa (Dal-Bianco et al., 2007) and the Brazil Amazonian regions (Ana Lucia et al., 1995). Diagnosis of asymptomatic infection is difficult because of the absence of malaria-related symptoms and parasite densities being too low to be detected by microscopy (Laishram et al., 2012). Immunity is a major factor related to asymptomatic infection. As immunity develops, there is an increased ability to control parasite multiplication rates and consequently a decrease in parasite densities (Lindblade et al., 2013).

Studies have suggested a proportion of asymptomatic carriers could become symptomatic eventually, although the possibility of re-infections could not be ruled out (Lindblade *et al.*, 2013). It is still unclear how asymptomatic infections develop into symptomatic infections. A prospective study following asymptomatic Gabonese children until onset of clinical symptoms observed that children who were infected with new parasite clones developed clinical symptoms with increased parasitemia (Kun *et al.*, 2002). The clinical symptoms were attributed to the rapid multiplication of a clone different from clones present at enrolment, and which the immune system had not yet been exposed to (Lines and Armstrong, 1992, Kun *et al.*, 2002). Asymptomatic *Plasmodium* infection carriers are considered a threat to any elimination or eradication programme as there are major concerns they may serve as reservoirs during the dry season to seed transmission in the subsequent wet season (Laishram *et al.*, 2012, Lindblade *et al.*, 2013).

2.2.2 Uncomplicated malaria

The initial symptoms of uncomplicated malaria are nonspecific and are characterized by an increase in body temperature, malaise, headache, vomiting, muscle aches, mild anaemia and a palpable spleen after several days of infection (White *et al.*, 2014). Older children and adults living in endemic regions develop partial immunity with age; as such infection with *P. falciparum* in such semi-immune individuals usually result in morbidity but rarely leads to severe disease.

2.2.3 Severe malaria syndromes

In malaria endemic regions, most *P. falciparum* infections result in uncomplicated disease. Only a small proportion of malaria cases result in severe disease affecting mainly young children and pregnant women (Dondorp *et al.*, 2008). Several studies have shown that, as transmission intensities decrease, the median age for severe disease tends

to increase (Snow *et al.*, 1994, Snow *et al.*, 1997, Reyburn *et al.*, 2005). Severe malaria can result in potentially fatal conditions such as hyperparasitemia, severe malarial anaemia (SMA), cerebral malaria (CM), respiratory distress and metabolic acidosis. In lower transmission settings, adults may also be afflicted with severe disease with additional syndromes including jaundice, renal dysfunction, pulmonary oedema and shock (Schofield and Grau, 2005). Progression of an infection to severe syndromes can occur rapidly and patients may have one or several complications co-existing or rapidly evolving which could be fatal. Thus, patients must be quickly assessed for any of these symptoms which are indications of an increased risk of developing life-threatening complications (Trampuz *et al.*, 2003).

2.2.3.1 Cerebral malaria (CM)

In their recent report, WHO defines CM as presence of *P. falciparum* asexual parasites in the peripheral circulation with impaired consciousness or unarousable coma persisting more than one hour with a Blantyre coma score less than 3 or a Glasgow coma score less than 11, without other identifiable causes of coma such as meningitis, bacterial or fungal infection or cerebrovascular accidents (WHO, 2014). When left untreated, CM cases could result in fatal conditions, with a mortality rate of about 19% in sub Saharan-Africa (Murphy and Breman, 2001). Even with treatment, there have been reports of neurological sequelae among CM survivors such as speech impairment, cognitive impairment, cortical blindness, epilepsy and cerebral palsy (Molyneux *et al.*, 1989, Brewster *et al.*, 1990).

The molecular and cellular mechanisms underlying CM are complex involving both parasite derived factors and immune modulators. The sequestration of infected RBCs (iRBCs), leukocytes or platelets in the microvasculature can lead to vascular obstruction, increasing levels of pro-inflammatory cytokines and chemokines such as tumour necrosis

factor (TNF)- α and interferon (IFN)- γ , and decreasing levels of anti-inflammatory cytokines such as interleukin-10 all of which contribute to pathogenesis of CM (Beeson and Brown, 2002, Armah *et al.*, 2005).

2.2.3.2 Severe malarial anaemia (SMA)

In endemic areas, SMA is a severe condition affecting particularly young children. WHO defines SMA as haemoglobin levels less than 5g/dL or haematocrit levels less than 15% in children under 12 yrs and in adults' haemoglobin levels less than 7g/dL or haematocrit levels less than 20% (WHO, 2014). SMA involves destruction of iRBC and uninfected RBC (uRBC), sequestration of iRBC in the spleen, and insufficient erythropoiesis, which contribute to disease pathology (Perkins *et al.*, 2011, White *et al.*, 2014). In developing countries, malaria-induced anaemia may be worsened by other parasitic infections such as helminthic infections, tuberculosis and human immunodeficiency virus (HIV) infection (Bartoloni and Zammarchi, 2012).

2.2.3.3 Respiratory Distress (RD)

Respiratory distress (RD) in severe malaria is a clinical manifestation of underlying metabolic acidosis associated with lactate acidaemia (English *et al.*, 1996). The symptoms include nasal flaring and deep breathing or increased respiratory rate (English *et al.*, 1996, Taylor *et al.*, 2012). RD usually presents concurrently with SMA or CM and predicts mortality (Awandare *et al.*, 2006) which in some instances increases mortality rates by several folds (English *et al.*, 1997). Vascular obstruction, impaired tissue perfusion due to reduced deformability of uRBC and severe anaemia (Maitland and Marsh, 2004, Taylor *et al.*, 2012) as well as cytokine imbalance (Awandare *et al.*, 2006) may play a role in RD during severe disease.

2.2.4 Pregnancy-associated Malaria (PAM)

In endemic settings, studies have shown that pregnant women become more susceptible to *P. falciparum* infection than non-pregnant women do within the same environment (Lindsay *et al.*, 2000). Susceptibility to PAM seems to correlate with parity as malaria incidence and severity decreases in multigravidae women (Brabin, 1983). The phenomenon of PAM is attributed to the sequestration of iRBC in the intervillous space of the placenta. Sequestration is mediated by a parasite ligand VAR2CSA (Salanti *et al.*, 2004), a member of the *Plasmodium falciparum* erythrocyte membrane protein 1 family (PfEMP1) which binds to chondritin sulphate A on the host membrane (Fried and Duffy, 1996). PAM is associated with preterm birth, SMA in pregnant women, low birth weight and higher infant mortality (Rogerson *et al.*, 2007).

2.3 Malaria diagnosis and treatment

Timely and accurate diagnosis of malaria are an integral part of effective case management strategy. In areas of high endemicity, prompt diagnoses and treatment are critical particularly for vulnerable populations such as children and non-immune individuals amongst whom progression of the disease can be fatal. Presumptive treatment based on signs, symptoms and physical examination are employed in most malaria-endemic countries where health care resources and trained personnel are limited. However, malaria symptoms are unspecific and common to other fevers; therefore, diagnosis cannot be reliably made based on symptoms of fever. Clinical diagnosis based on just symptoms and physical examination may lead to indiscriminate use of antimalarial drugs and misdiagnosing of other potentially serious infectious diseases (Reyburn *et al.*, 2004, Polage *et al.*, 2006, Lubell *et al.*, 2008). In all cases of suspected malaria infection, WHO recommends diagnosis and confirmation by laboratory

diagnosis by microscopic examination of blood smears and/or the use of rapid diagnostic testing (RDT) of blood samples before antimalarial treatment is started.

Microscopic examination of Giemsa-stained blood films remains the gold standard for malaria diagnosis. The process allows the use of light microscopy for parasite detection in blood smear. This method is widely accepted due to its low cost, simplicity, ability to detect and identify the infecting *Plasmodium* species, all of which are important for the effective management and treatment of malaria (Tangpukdee *et al.*, 2009). Although widely accepted, microscopic diagnosis requires trained laboratory technologist with the ability to identify species in mixed infections or at low parasite densities. Microscopic diagnosis is labour intensive and time consuming with low sensitivity. Diagnosis by microscopy may be unavailable in rural communities with no electricity or proper medical facilities. In low transmission settings, microscopic confirmation in all suspected cases may be more costly for the detection of a small number of patients with malaria. In such instances, health personnel are to identify individuals with recent exposure to malaria and presence of fever without other known causes before performing microscopic examination.

RDT kits are immuno-chromatographic kits that allow detection of antigens or enzymes specific to the parasite, which could be either genus- or specie- specific. There are three *Plasmodium* enzymes that current malaria RDTs detect; *Plasmodium* histidine-rich protein 2 (pHRP-2) specific to *P.falciparum* or *P. vivax*, *Plasmodium* lactate dehydrogenase (pLDH) which is specific to *P.falciparum* or *P. vivax* or can be pan-specific and *Plasmodium* aldolase which is pan-specific (Wilson, 2012). RDTs provide a low-cost alternative for malaria diagnosis particularly in rural endemic regions where other malaria diagnostic tools such as microscopy are unavailable. There has been a

marked increase in the use of malaria RDTs especially in high transmission settings as noted by the WHO, 1.6 billion RDTs were handed out between 2010 and 2016 (WHO, 2017). Several studies have reported on the impact of RDT on the management of fevers and the use of antimalarials (Kyabayinze *et al.*, 2010, Ansah *et al.*, 2015, Boyce *et al.*, 2015) and antibiotics (Hopkins *et al.*, 2017). RDTs are very useful in individuals who have just received artemisinin treatment and thus do not have detectable parasites in blood smears. In patients, presenting symptoms compatible with severe malaria symptoms but a negative blood film examination, RDTs should be used together with blood films prepared and examined at 6-12 hr intervals.

Serological techniques like immunofluorescence antibody testing (IFA) can be used for diagnosis but is rarely used in the acute diagnosis of malaria (Tangpukdee *et al.*, 2009). IFA diagnosis is usually carried out for screening of blood donors or for diagnosing a recent malaria infection in non-immune individuals. The technique is based on detecting antibodies against the asexual blood stage parasites. IFA is specific, sensitive and reliable but is time consuming and subjective.

Malaria diagnosis can be made with other molecular techniques such as polymerase chain reaction (PCR), loop-mediated isothermal amplification (LAMP) and microarrays. PCR diagnosis is the most sensitive and specific diagnostic method, useful in detecting very low parasitemia and mixed infections (Morassin *et al.*, 2002). The technique has been widely used for accurate species identification, detecting drug resistance and treatment failure. The high cost of PCR based diagnosis and need for skilled personnel has restricted its use as a routine diagnostic method in field settings. The LAMP technique is a cheaper alternative to conventional PCR with comparable specificity and sensitivity. The method was originally based on the amplification of the 18S ribosomal RNA gene (Han *et al.*, 2007) with sensitivity of about 100 parasites/ μ l. Subsequent studies with

primers targeting the mitochondrial DNA showed an increase in sensitivity and specificity for other species such as *P. ovale* and *P. malariae* (Polley *et al.*, 2010).

The WHO recommends treatment of uncomplicated *P. falciparum* malaria amongst children and adults, excluding pregnant women, with artemisinin-based combination therapies (ACT) (WHO, 2015). An ACT consists of an artemisinin derivative, which is a fast acting drug, and a partner drug whose effect is longer lasting. The artemisinin component swiftly clears parasites from circulation within the 48hr cycle and has an effect on the sexual stages. The longer acting partner of the ACT clears any remaining parasites to help prevent the development of artemisinin resistance. In Ghana, the nationally recommended first line ACTs are artesunate+ amodiaquine, artemether + lumefantrine and dihydroartemisinin + piperaquine (GHS, 2014). An ACT course would usually be a 3-day regimen which spans two asexual cycles ensuring only a small proportion of parasites are available for the partner drug to act on. In the case of reinfection or recrudescence (which can be caused by treatment failure) within 28 days, the second line of recommended ACT should be used. However, treatment failure after 28 days is considered a new infection and should be treated with one of the first line ACTs (WHO, 2015).

Pregnant women suffering uncomplicated malaria are treated with oral quinine + clindamycin in their first trimester. ACTs can be safely used for the treatment of uncomplicated malaria in the second and third trimesters. Treatment of uncomplicated malaria amongst non-immune individuals travelling to non-endemic regions also requires ACTs.

2.4 The Life cycle of *Plasmodium falciparum*

Plasmodium species undergo a complex life cycle, which requires two hosts; a vertebrate host (human) and an invertebrate host (mosquito) (**Figure 2.2**). The life cycle begins with the injection of sporozoites from the salivary glands of a female *Anopheles* mosquito into the subcutaneous tissue of the human host during a blood meal (Farrow *et al.*, 2011). The sporozoites enter the blood stream within 5-8min (Guilbride *et al.*, 2012) and are transported to the liver where they invade hepatocytes. During the pre-erythrocytic liver phase, sporozoites are non-inflammatory and asymptomatic. The sporozoites undergo 4-5 cycles of schizogony and differentiate into infective merozoites which exit the hepatocytes into circulation to invade host RBCs marking the beginning of the erythrocytic stage (Farrow *et al.*, 2011).

Upon invasion of the RBCs by the merozoites, the intraerythrocytic parasites undergo asexual replication and go through different stages that is, rings, trophozoites and schizonts. The schizonts replicate to produce 12-32 merozoites, which are released upon rupture and invade new RBCs. Some of the merozoites escape the asexual replication and develop into sexual forms; male and female gametocytes. Signals that trigger sexual differentiation are not fully understood but may include metabolic stress in the schizonts, immune responses to parasites or antimalarial drugs (Baton and Ranford-Cartwright, 2005, Alano, 2007). After the mosquitoes feed, gametogenesis is triggered by the drop in temperature and xanthurenic acid from the insect. Macrogametocytes emerge from the erythrocytes as non-motile spherical female gametes, whereas male gametocytes undergo rapid replication to form eight motile microgametes. Fertilization of macrogametes by microgametes results in the formation of a diploid zygote in the mosquito gut. The zygote subsequently undergoes endomeiotic division giving rise to tetraploid zygote. The zygote gradually matures into an ookinete that moves by gliding motility and traverses the gut

epithelium. The ookinete encysts in the epithelial basal lamina to form an oocyst that undergoes mitotic divisions and subsequently differentiates into daughter sporozoites. The sporozoites then migrate to the salivary glands of the mosquito, ready to be released during a blood meal (Baton and Ranford-Cartwright, 2005, Vaughan, 2007).

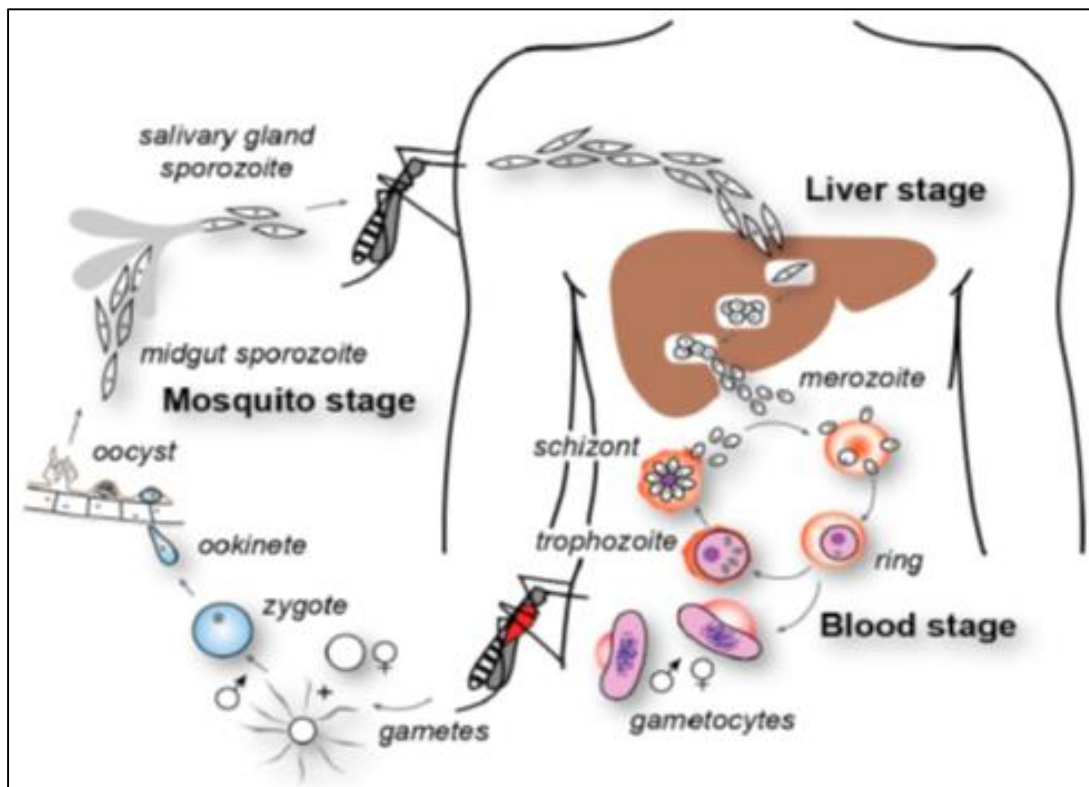


Figure 2.2: Life cycle of *Plasmodium falciparum*.

Sporozoites are injected into skin of the human host during a blood meal by a female *Anopheles* mosquito and subsequently invade the hepatocytes. Sporozoites develop within the hepatocytes producing merozoites which are released into circulation. The merozoites invade the host RBCs and undergo schizogony producing merozoites that reinvade the host RBCs. A minority of merozoite develop into the sexual forms, gametocytes which are ingested by the mosquito and fuse to form a zygote within the mosquito. The zygotes develop into sporozoites to begin the cycle. Adapted from (Cowman and Crabb, 2006)

2.5 Mechanism of merozoite invasion of RBC and molecular networks

Plasmodium falciparum merozoite is ovoid in shape (about 1.5 μ m long) and bound by three layers of membrane, which is termed pellicle. The pellicle consists of plasma membrane and underlying the plasma membrane is the inner membrane complex (IMC)

(Figure 2.3). The nucleus, mitochondrion and plastid organelle lies at the wider end. At the apical end, are three apical polar rings, secretory organelles (rhoptries and micronemes) and dense granular structures called exonemes (Yeoh *et al.*, 2007). The apical polar rings serve as the microtubule-organizing centre. The parasite cytoskeleton made up of two or three microtubules, lies between the IMC and the plasma membrane (Bannister and Mitchell, 2009, Baum *et al.*, 2009, Farrow *et al.*, 2011, Hanssen *et al.*, 2013). Invasion of RBCs by merozoites is a well-coordinated stepwise process that involves antigens secreted by the rhoptries and micronemes (**Figure 2.4**) (**Table 2.1**).

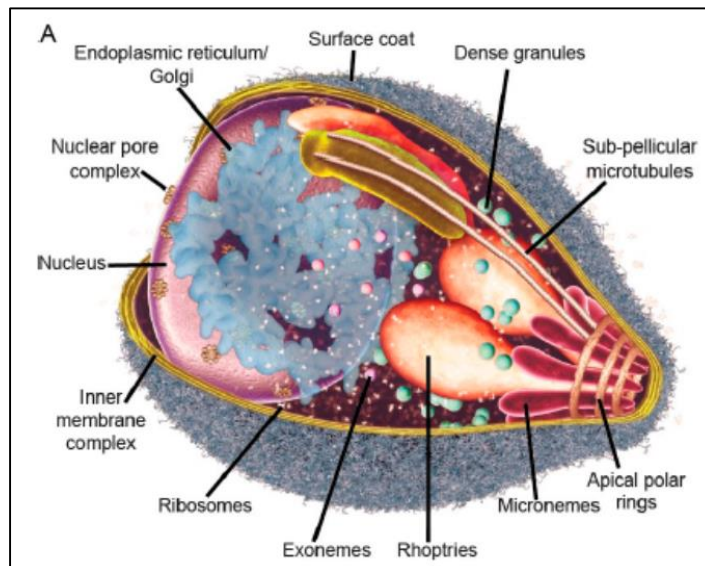


Figure 2.3: Three-dimensional (3D) structure of *P. falciparum* merozoite

The ovoid-shaped merozoite is bound by plasma membrane and an underlying IMC. The apical end harbours the rhoptries and micronemes, which are secretory organelles that secrete parasite ligands during merozoite invasion. At the wider end lies the nucleus, mitochondrion and plastid organelle (Cowman & Crabb 2006).

2.5.1 Egress of Merozoites

Erythrocyte invasion begins with egress of merozoites from iRBCs by disrupting the parasitophorous vacuole (PV) and host cell membrane. The signals that trigger merozoite egress are unknown in *P. falciparum*, but in another apicomplexan member *Toxoplasma gondii*, the phytohormone abscisic acid acts as a signal molecule inducing production of

cyclic adenosine diphosphate (ADP)-ribose, which causes an increase in intracellular calcium levels. Increased calcium levels in turn induce micronemal protein secretion (Nagamune *et al.*, 2008). As the parasite crosses the PV membrane as well as the host cell membrane, the parasite dehydrates the host RBC leading to an increase in size of PV compartment and poration of the erythrocyte membrane (EM) (Glushakova *et al.*, 2010). Subsequently, the EM curls out around the pore, buckles and turns itself inside out rapidly dispersing the merozoites and resulting in efficient detachment from the infected erythrocytes (Lew, 2011, Wirth and Pradel, 2012).

EM cytoskeleton consists of spectrin/actin scaffolds anchored by protein 4.1, which interacts with spectrin and actin. The parasite remodels the host RBC membrane by phosphorylation of protein band 3, causing it to dissociate from the sub-membranous skeleton. Other parasite proteins such as ring-infected erythrocyte surface antigen (RESA) are integrated into the submembranous cytoskeleton, deforming the EM and making it rigid. A subtilisin-like protease (PfSUB1) released from the exoemes (Yeoh *et al.*, 2007) cleaves PV merozoite proteins like serine repeat antigen (SERA) proteins and merozoite surface protein-related protein (MSRP 2) (Silmon de Monerri *et al.*, 2011). In addition to plasmodial proteases, the parasite also recruits a host protease, erythrocyte-derived calpain-1 for egress. calpain-1, is activated by intracellular Calcium and plays a role in digestion of cytoskeletal proteins (Chandramohanadas *et al.*, 2009).

2.5.2 Primary attachment to Erythrocyte Membrane

After mature merozoites emerge from ruptured schizonts, the merozoites bind to host EM. Primary contact between merozoite and EM is weak and mediated by the merozoite surface proteins (MSP). These proteins are not evenly spread on the merozoite, as some are more apically concentrated with a role in adhesion (Sanders *et al.*, 2005).

2.5.3 Apical reorientation of the Merozoite

Following attachment of the merozoite to host EM, the merozoite apically positions itself such that its apex, harbouring the rhoptries and microneme organelles, are positioned close to the erythrocyte membrane. *P. falciparum* apical membrane antigen 1 (PfAMA-1) is a major invasion adhesin with a role in reorientation and subsequent tight junction formation by forming a ring with the rhoptry neck protein complex (RON-2,-4,-5,-8). After the apical reorientation of the merozoite, secondary protein-protein interactions take place, which effectively activate the invasion process. The activation process involves interactions of parasite ligands at the apical end of merozoite binding to their receptors on the erythrocyte membrane (Cowman *et al.*, 2012).

Erythrocyte binding-like (EBL) and reticulocyte binding-like protein families (RH) are the two main protein families which play critical roles in invasion by binding their receptors on the erythrocyte membrane and activating downstream processes that successfully commit the parasite entry into the cell (Cowman *et al.*, 2012). The EBL and RH proteins are directly involved in inducing an internal signal that triggers rhoptry content release for the formation of a tight junction. The two protein families act cooperatively in invasion and show some degree of compensation for a loss of receptor by overexpression of another to ensure an efficient invasion. For instance, a disruption in *eba-175* gene resulted in an increase in transcription of *PfRH4* gene (Baum *et al.*, 2005, Stubbs *et al.*, 2005, Lopaticki *et al.*, 2011a). Furthermore, PfRH2b specific antibodies were found to inhibit more strongly, strains in which different *eb1* genes have been disrupted, an observation consistent with PfRH2b complementing EBL proteins (Lopaticki *et al.*, 2011b). With the exception of PfRH5 gene (see more below), all other RH and EBL genes can be disrupted but some studies have suggested a need for a

minimum expression of these ligands to ensure high affinity attachment and activation of the invasion machinery (Stubbs *et al.*, 2005).

2.5.4 Tight junction formation and parasite entry

After an irreversible attachment at the EM by the merozoite's apical end mediated by the EBL and RH ligands, there is a tight junction formation which serves as a bridge between the merozoite and erythrocyte, through which the parasite makes its entry into the host cell (Cowman *et al.*, 2012). The release of PfRH proteins triggers the release of the RON complex from the rhoptries, which is then inserted into the host cell membrane. AMA-1 binds RON2 forming a circumferential ring enclosing the apex of the parasite (Srinivasan *et al.* 2011). Tight junction formation triggers release of rhoptry bulb contents including proteins and lipids needed to form the PV. The PV membrane envelops invading merozoites and offers protection to the parasite (Cowman *et al.*, 2012).

The driving force for the migration of the tight junction towards the rear of the merozoite is provided by an acto-myosin motor complex. The complex includes type XIV myosin heavy chain (MyoA), myosin light chain (PfMLC) and two membrane associated proteins GAP45 and GAP50. The invasion motor machinery is well studied in *T. gondii* and well conserved across the *Apicomplexa* family as all apicomplexan parasites sequenced to date encode orthologs of MyoA, MTIP/MLC, GAP45 and GAP50. Actin filaments concentrate around the tight junction in a ring-like pattern to which myosin associates and provide the force drawing the parasite into the space created by the PV membrane. The tight junction is drawn into the space across the merozoite surface pulling with it the host membrane until the parasite is sealed off by the fusion of PV membrane and host membrane (Baum *et al.*, 2008, Farrow *et al.*, 2011, Sharma and Chitnis, 2013).

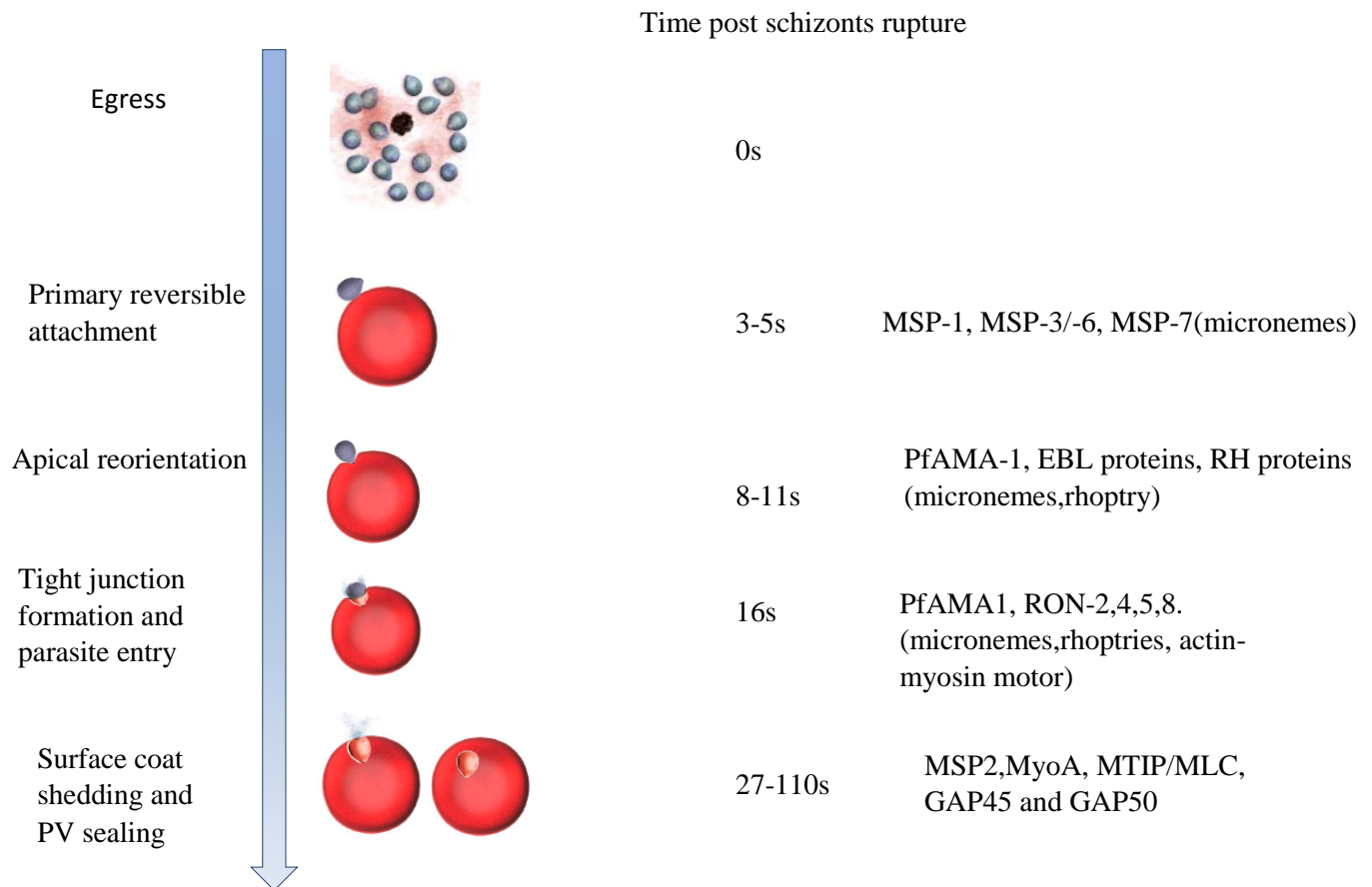


Figure 2.4: Timeline of *P.falciparum* merozoite invasion of host RBC after schizont rupture

Merozoite invasion begins with rupture of mature schizonts releasing merozoites. Merozoites attach to host RBC membrane and apically reorient. Secondary protein-protein interactions occur to induce the formation of a tight junction. Parasite pulls itself through tight junction and is enclosed in the PV, shedding off its surface coat proteins. Adapted from (Bannister and Mitchell, 2003, Cowman *et al.*, 2012)

2.5.5 Structure of the PfrH5 Protein complex

PfrH5 is a 63 kD protein secreted in the rhoptries and processed to a 45 kD fragment, which is translocated to the apical invasive end of the merozoite during invasion (Baum *et al.* 2009). PfrH5 complexes with an epidermal growth factor (EGF)-like protein *P. falciparum* RH5 interacting protein (PfriPR) (Chen *et al.*, 2011) and cysteine rich protective antigen (PfCyRPA) (Reddy *et al.*, 2015a) to form an invasion protein complex. PfCyRPA is a 43 kD protein located at the apical end of the *falciparum* merozoite (Dreyer *et al.*, 2012). Orthologs of PfCyRPA with about 40% sequence identity have been identified in other *Plasmodium* species including *P. knowlesi*, *P. cynomolgi* and *P.*

reichenowi (Favuzza *et al.*, 2017). Pf113 is different from PfrRH5 and PfCyRPA in the sense that, expression of Pf113 is not restricted to only the blood-stage parasites but it is also expressed during the liver stages (Florens *et al.*, 2002) and by the gametocytes (Silvestrini *et al.*, 2010). Pf113 was originally identified as a putative GPI-anchored merozoite protein through purification of detergent-resistant membranes from late blood-stage parasites (Sanders *et al.*, 2005). Pf113 is considered one of the abundant merozoite antigens (Sanders *et al.*, 2007) localized to the merozoite surface as tetramers (Galaway *et al.*, 2017).

Unlike other members of the PfrRH family; PfrRH5 is smaller and lacks a transmembrane domain and a cytoplasmic domain (Rodriguez *et al.*, 2008). The absence of a transmembrane domain by PfrRH5 requires the protein binds other proteins that are anchored to the merozoite surface. Initially, PfCyRPA was reported as a glycosylphosphatidylinositol (GPI) anchored protein that tethers PRH5 to the merozoite surface (Reddy *et al.*, 2015b). This report has been disputed and recent studies rather point to Pf113 as the GPI-linked protein that anchors PfrRH5 at the N terminus to the merozoite surface (Galaway *et al.*, 2017).

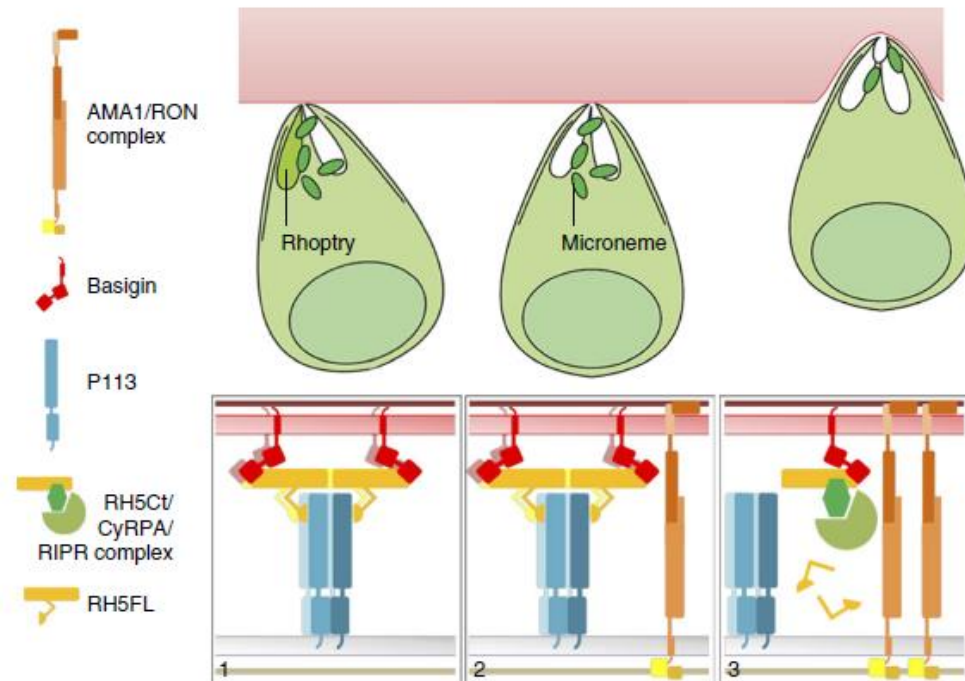


Figure 2.5: A proposed model for PfrH5 protein complex

After the merozoite apically reorients during merozoite invasion, there is release of rhoptry and micronemal proteins. PfrH5 is anchored onto the merozoite surface by Pf113 allowing PfrH5 to bind basigin (1). PfrH5 binding to basigin triggers the formation of a tight junction between AMA-1 and the RON complex (2). PfCyRPA-RiPR complex then binds PfrH5 causing its release from Pf113 by the proteolytic cleavage of PfrH5 amino terminal. RH5Ct denotes the processed 45kD PfrH5 lacking the N terminus. RH5FL denotes full length RH5. Figure adapted from (Galaway *et al.*, 2017).

A model for the PfrH5 complex has been proposed where at the point of invasion (**Figure 2.5**), Pf113 is clustered as tetramers on the merozoite surface. PfrH5 then binds Pf113 at the N terminal region and basigin at the C terminus. Subsequently, PfCyRPA-RiPR binds PfrH5 and triggers the release of PfrH5 from Pf113. The release of PfrH5 from Pf113 possibly involves an unknown protease which cleaves PfrH5 at the N terminus or mutually exclusive binding of PfrRiPR (Galaway *et al.*, 2017). Further insights have been gained into the structure of the PfrH5 complex proteins through binding to monoclonal antibodies. The PfrH5 protein adopts a kite-like structure (Wright *et al.*, 2014) (**Figure 2.6**). The crystal structure of PfCyRPA resolved at 2.5 Å reveals a β -sheet molecule stabilized by disulfide bonds with 6 β propeller blades burying the

disulfide and tryptophan residues (Favuzza *et al.*, 2017). The role of PfrH5 complex is emerging, as binding of PfCyPA-RiPR to PfrH5 triggers Ca^{2+} release into the RBC. This is believed to induce the irreversible formation of a tight junction between AMA-1 and RON2 (Volz *et al.*, 2016).

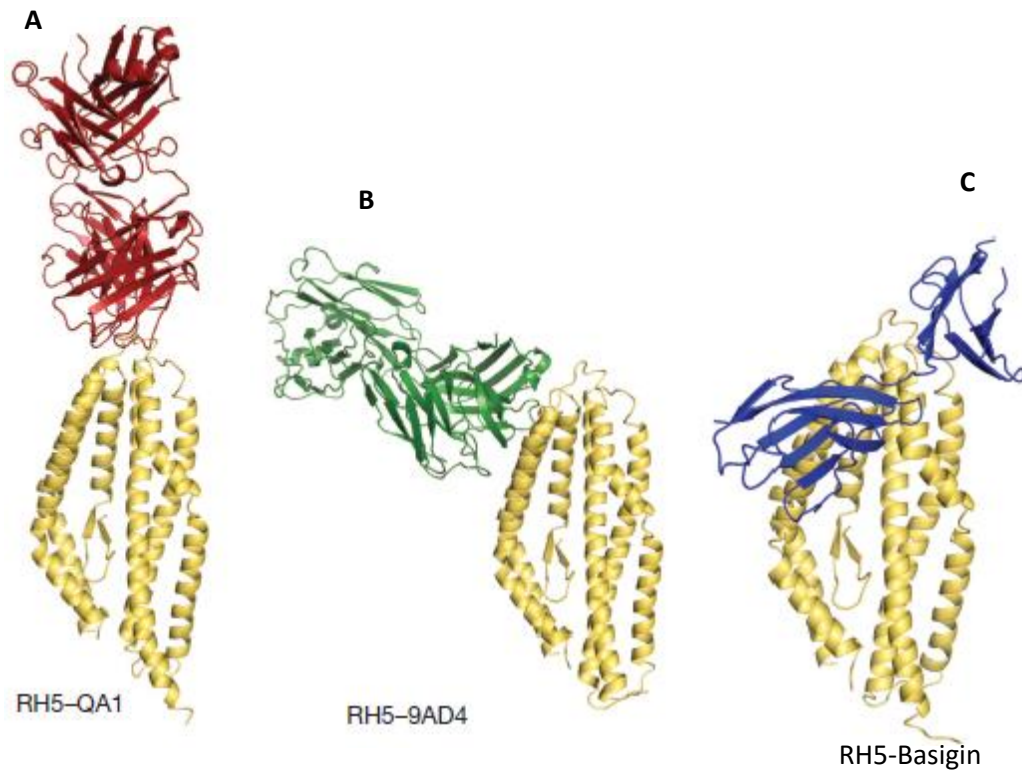


Figure 2.6: Structure of PfrH5

Structure of PfrH5 bound to inhibitory mouse monoclonal antibodies (QA1 and 9D4) and its receptor basigin. PfrH5 adopts a kite-like structure with basigin binding to the tip of PfrH5. QA1 and 9D4 bind distinguishable sites with QA1 binding loops at the tip of PfrH5. 9D4 binds helices 2 and 3 of PfrH5. Figure adapted from (Wright *et al.*, 2014)

Table 2.1: Major ligands in *P.falciparum* merozoite invasion

Organelles	ligand	Probable function	Structural features
Microneme	PfAMA-1	Binds RON complex to form tight junction. Phosphorylation of cytoplasmic tail may trigger signalling	Plasminogen, apple, nematode (PAN) motifs.
	PfEBA-175	Binds glycophorin A and activates invasion machinery through signaling	EBL family with DBL domains.
	PfEBA-140	Binds glycophorin C on erythrocytes	EBL family with DBL domains
	PfEBA-1	Binds glycophorin B on erythrocytes	EBL family with DBL domains
	PfEBA-181	Binds an unknown erythrocyte receptor	EBL family with DBL domains
	PfRIPR	Binds to PfrH5	10 EGF domains
	PfCyRPA	Binds to PfrH5	C-terminal GPI-anchor motif twelve cysteine residues
	MTRAMP	Motor-associated protein	Thrombospondin-like domains
Rhoptery neck	PfRH5	Binds an unknown basigin on EM	PfRh family
	PfRH4	Binds complement receptor 1. Cytoplasmic tail required for merozoite invasion	PfRh family
	PfRH1	Binds an unknown receptor Y on erythrocyte	PfRh family
	PfRH2a	Binds an unknown receptor Z on erythrocyte	PfRh family
	PfRH2b	Binds an unknown receptor Z on erythrocyte	PfRh family
	PfRON2	Inserts into RBC membrane and forms tight junction with AMA-1	Multipass transmembrane protein
	PfRON4	Inserts into RBC membrane and binds to RON2	
Rhoptery neck	PfRON5	Complexes RON2 proteins and AMA1 at tight junction	
GPI-anchored surface proteins	MSP-1	Putative band 3 ligand, mediates attachment to host membrane.	Two C-terminal domains packed side by side.
	MSP-2	Structural role as surface coat.	Unordered repetitive structure
	MSP-4	Unkown.	C-terminal EGF domain
	MSP-5	Unkown.	C-terminal EGF domain
	Pf113	Binds RH5	

Table adapted from (Cowman and Crabb, 2006)

2.6 Antibody Structure

Antibodies or immunoglobulins are glycoproteins produced by plasma cells that function to protect the human body against pathogens such as viruses, bacteria, protozoans and other infectious agents. Immune responses elicited by antibodies are very complex and specific. Antibodies function through direct binding to antigens from the pathogens, and in some instances; antigen binding may not have any biological effect. Elimination of the pathogen may involve secondary interactions of antibodies with effector molecules such as complement system (referred to as complement dependent cytotoxicity, CDC). Antigens are usually complex and present numerous epitopes thus antibodies produced to antigens can be from different B lymphocyte clones (polyclonal antibodies) or from a single B lymphocyte clone (monoclonal). Monoclonal antibodies have become very useful in diagnostics and therapeutic applications as well as research tools. Currently about 30 monoclonal antibodies have been licensed by the United States Food and Drugs Authority for the treatment of several diseases including cancer, infectious diseases and immunological defects (Nelson *et al.*, 2010). Monoclonal antibodies have been produced by hybridoma technology by fusing mouse spleen cells with myeloma cells. The antibodies generated by hybridomas are generally from mice and as such not appropriate for therapeutic purposes.

The immunoglobulin molecule is folded into a compact globular structure with four polypeptide chains consisting of two heavy (H) chains (50-70kD) which are glycosylated with oligosaccharide moieties, and two non-glycosylated light (L) chains (23kD), taking a Y shape (**Figure 2.7**). Within the antibody structure are inter- and intra-chain disulphide bonds that hold the subunits together. Inter-chain disulphide bonds within a flexible region called the hinge region, and other non-covalent interactions hold the two heavy

chains together. The light chains are also joined to the heavy chains by disulphide bonds. Each polypeptide chain has intra-chain disulphide bonds (Schroeder & Cavacini 2010)

Both the heavy and light chains can further be divided based on the amino acid composition into a variable (V) and constant (C) regions. Within the immunoglobulin molecules are repeating homology sequences of about 110 amino acids residues denoted immunoglobulin domains which are characteristic of immunoglobulin gene superfamily members. The light chain has two domains V_L and C_L with 110 amino acids each, whereas the heavy chains have one V_H and three or four C_H domains. The variability of amino acid sequences in the variable regions between antibody molecules provides the diversity needed by the immune system to fight foreign particles (Janeway, 2001).

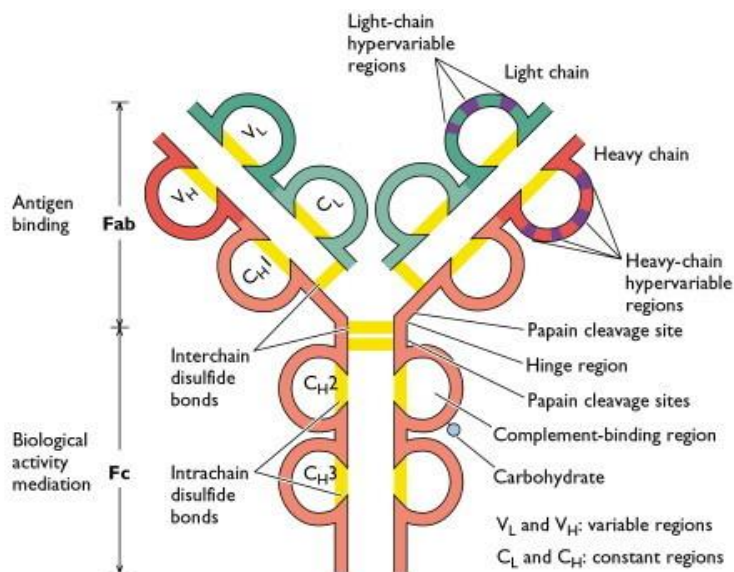


Figure 2.7: A schematic representation of an IgG molecule depicting a Y shape.

An antibody molecule is made up two heavy chains (H) and two light chains (L) held together by disulphide bonds. The heavy and light chains is divided into constant (C) and variable (V) regions. The Fab fragments are the antibody binding domains consisting of the light chain and V_H and C_{H1} of the heavy chain. The Fc portion binds effector molecules. Adapted from <http://www.microbiologyinfo.com/antibody-structure-classes-and-functions/>

By comparing amino acid sequences in the variable regions of immunoglobulin molecules, it has been found that the variability occurs in a specific region called the

hypervariable region or complementarity-determining region (CDR). Intervening regions between CDRs of the variable region show little variability and are called framework region. Sections of CDR with the greatest variability are around residues 30, 55 and 95. Antibodies with same specificity have similar CDRs whilst antibodies of different specificities have different CDRs. There are three CDRs from the amino terminal of an antibody, denoted CDR1, CDR2 and CDR3. The variable regions fold in a manner that exposes the CDR on the surface of the chain. Both the heavy and light chain CDRs join together to form a cleft that serves as the antigen-binding site of an immunoglobulin molecule.

CDRs also determine the specificity of an antibody molecule as they specify the shape and ionic properties of an antibody (Janeway, 2001).

Proteolytic digestion of an antibody with papain in the presence of reducing agent yields three fragments, two of which are identical. The two identical fragments consist of the light chain and the V_H and C_{H1} of the heavy chain referred to as Fab fragments (for **f**ragment **a**ntibody **b**inding) as it harbors the site of antigen binding and are monovalent. The third fragment does not have antigen binding activity and is referred to as the Fc fragment (for **f**ragment **c**rystallizable) consisting C_{H1} and C_{H2} domains. The Fc regions defines the effector functions of antibody molecules (Schroeder and Cavacini, 2010).

2.6.1 Immunoglobulin classification

There are five antibody classes expressed in humans which differ in the amino acid sequences in the constant region of the heavy chain, size, and their solubility *in vitro*, referred to as isotypes (Vidarsson *et al.*, 2014). The different isotypes are immunoglobulin G (IgG), immunoglobulin M (IgM), immunoglobulin A (IgA), immunoglobulin E (IgE), and immunoglobulin D (IgD). Although there are different

immunoglobulin classes, they all have the same basic structural units with slight differences.

Immunoglobulin G, IgG is the most abundant serum protein, accounting for 10-20% of protein in human serum. IgG is further divided into four sub-classes IgG1, IgG2, IgG3 and IgG4. Nomenclature of IgG subclass is based on the decreasing abundance level in the plasma (IgG1>IgG2>IgG3>IgG4). IgG1 and IgG3 are primarily induced by soluble or membrane bound protein antigens. Antibody responses to bacterial polysaccharide antigens are mainly dominated by IgG2 and to a lesser extent IgG1. Non-infectious allergens would induce IgG4 antibody responses. The main mechanism of action of IgG1 and IgG3 is activation of complement system and opsonisation of pathogens which leads to their clearance by effector cells such as macrophages and phagocytes. IgG3 has shorter half-life is more effective at activating complement than IgG1 (reviewed in Hjelholt *et al.*, 2013, Vidarsson *et al.*, 2014)

In a typical immune response following an exposure to new antigen, a specific antibody response is developed within weeks. A primary antibody is secreted by short lived plasma cells after interaction of naïve B cells with antigen specific T helper cells. The primary antibody response is of low affinity and is transient. Some of the B and T cells move into the lymphoid follicles and form germinal centres. B cells then undergo affinity maturation and class switching giving rise to plasma antibody-secreting cells and memory B cells. The antibodies secreted by plasma cells within the germinal centres undergo appropriate class switching and are of high affinity (Jacob *et al.*, 1991). Memory B cells do not secrete antibodies but code for the antibody. Upon reinfection with the same antigen, a secondary response that is rapid and of high affinity is mounted as memory B cells rapidly differentiate into plasma cells. Consequently, as the infection is repressed, the memory B cells and plasma populations begin to contract. The plasma

cells migrate to the bone marrow and continue secreting antibodies which can persist for years and are referred to as long lived plasma cells (Smith *et al.*, 1996).

2.7 Immunity to malaria

It has long been observed that children living in areas of intense and stable transmission of *P. falciparum* experience severe malaria with associated mortality, whereas adults within the same communities may occasionally experience clinical symptoms but hardly any deaths (Hviid, 2005). This phenomenon clearly demonstrates acquisition of some form of immunity following natural infections. Naturally, acquired immunity (NAI) to malaria is developed after repeated exposures and may take several years to develop although this immunity is not sterile.

Immunity to malaria is known to take three forms i) anti-disease where individuals are protected against malaria associated clinical symptoms, ii) anti-parasite where an individual is protected against high parasite densities and iii) pre-munition where one prevents new infections by maintaining low parasitemia below a certain threshold and thus is protected against disease (Doolan *et al.*, 2009). In holoendemic regions with perennial infection, young children acquire anti disease immunity against severe disease first and rapidly, before acquiring the more slowly developing anti-parasite immunity.

Despite our limited understanding of the mechanisms of NAI, early studies have shown that IgG antibodies play a major role in naturally acquired immunity to malaria (Cohen *et al.*, 1961a). In such classical studies, the passive transfer of antibodies from immune adults to symptomatic children resulted in the resolution of febrile illness and a reduction in parasitemia. The magnitude and breadth of antibody responses are critical in NAI as individuals with a wider antibody response have a lower risk of developing clinical

disease (Osier *et al.*, 2008, Crompton *et al.*, 2010a). Plasma cells secrete serum antibodies that mediate protection by mainly targeting the asexual blood stage parasites. The asexual blood stage targets include antigens expressed on the merozoite surface during invasion (Cowman and Crabb, 2006) and the large clonally variant surface antigens (VSA) which are expressed on the iRBC membranes (Fowkes *et al.* 2016;Hviid *et al.* 2015). The most well-characterized VSA family is *P. falciparum* erythrocyte membrane protein (PfEMP1) family encoded by the *var* genes, and which mediates cytoadhesion of infected erythrocytes to host vasculature. The mechanism of antibody function in NAI involves opsonization of iRBC for phagocytosis by macrophages (Osier *et al.*, 2014a). The antibodies can inhibit merozoite invasion by disrupting interactions between merozoite antigens and their receptors (Persson *et al.*, 2013, Douglas *et al.*, 2015) or by preventing sequestration of IE (Fried *et al.*, 1998), activating complement lysis (Biryukov and Stoute, 2014) and intracellular killing of IE referred at as antibody dependent cellular inhibition (ADCI) (Bouharoun-Tayoun *et al.*, 1990). The slow process of acquiring NAI to *P. falciparum* suggests the parasite has evolved ways to evade the host immune systems. These strategies include clonal antigenic variation which presents the host immune system with an array of antigens overwhelming the immune system, antigen redundancy and polymorphism (Chan *et al.*, 2014). It is also evident *P. falciparum* is able to weaken the host immune system by modulating the maturation of antigen presenting cells (APC) (Urban *et al.*, 1999) and coating of PfEMP-specific IgG epitopes on iRBCs with IgM (Barfod *et al.*, 2011). Repeated exposure to *P. falciparum* is needed to maintain NAI for protection. Evidence from studies with children in endemic settings have shown asymptomatic children with parasites are much more protected than children without parasites (Males *et al.*, 2008). It is still unclear how much exposure is needed to fully acquire immunity but it is believed to be largely dependent on transmission

intensity. In medium to high transmission settings, a higher degree of immunity is attained by age 10 as opposed to low transmission settings where there is a shift in age to teenage years (Mwangi *et al.*, 2005, Marsh and Kinyanjui, 2006, Carneiro *et al.*, 2010). Acquisition of NAI is not only limited to age and transmission intensity; factors such as genetic traits of the host may affect risk of infection. Parasite diversity in particular settings also influences development of immunity. Immunity would be developed faster under conditions with less genetic diversity (Beeson *et al.*, 2016).

2.8 Longevity of malaria induced antibody levels

Several immuno-epidemiological studies examining the longevity of antibodies to *P. falciparum* infections amongst children and adults in endemic regions have largely concluded that antibody responses are short lived and require continual parasite exposure to maintain antibody levels (Cavanagh *et al.*, 1998, Fonjungo *et al.*, 1999, Giha *et al.*, 1999, Kinyanjui *et al.*, 2007, Akpogheneta *et al.*, 2008). After an acute infection, antibody levels peak and begin to decline several weeks or months following treatment. In areas of seasonal intense transmission, children produce antibodies to a wide range of *P. falciparum* antigens, but these contract in terms of the breadth and magnitude during the subsequent dry season. The antibodies secreted during the peak season are secreted by short-lived plasma cells (Crompton *et al.*, 2010a). This phenomenon in NAI is attributed to the large number of parasite antigens exposed to the host immune system that overwhelms the immune system such that it hampers its ability to commit significant proportions of antigen specific B cells to long-lived memory following a single episode of malaria. In agreement with this, it is observed that *P. falciparum* specific antibody levels are acquired with age in a gradual manner (Crompton *et al.*, 2010a, Weiss *et al.*,

2010) and antibody decay rates are slower in adults compared with children (Akpogheneta *et al.*, 2008) .

It should be noted that whilst the studies outlined above show a decline in antibody levels in the absence of parasite exposure, there have also been studies showing malaria specific antibodies persisting in the absence of an infection for months and even years (Torres *et al.*, 2008, Wipasa *et al.*, 2010, Fowkes *et al.*, 2012, Ayieko *et al.*, 2013). Longitudinal studies with pregnant women living in an area of stable malaria transmission have revealed VAR2CSA-specific memory B cells persist for several years, and capable of differentiating into antibody-secreting cells upon reactivation (Ampomah *et al.*, 2014). Previously immune migrants have been shown to have *P. falciparum* specific antibodies that persist for several years although the mean antibody titres may be lower compared to immune individuals living in endemic regions, supporting the hypothesis of long-lived antibody response (Moncunill *et al.*, 2013).

The differences in the longevity of malaria-specific antibodies between several studies could be attributed to differences in the sampling times on the antibody response curve (Fowkes *et al.* 2016). Following an initial stimulus by an antigen, there is a swift increase in antibody levels which peaks and subsequently begins to decay in a biphasic manner (Fowkes *et al.*, 2016). There is an initial decay of the increased antibody and then a much slower decay which can persist for long periods depending on the antigen. Estimation of merozoite specific antibody half-lives amongst Kenyan children following antibody increase (i.e the beginning of the biphasic decay) gave antibody half-lives ranging from 3-12 days, which is lower than that reported for IgG catabolic decay (21 days). Similar studies with asymptomatic children in the Gambia during the dry season showed half-lives of 2-8 weeks (Akpogheneta *et al.*, 2008). However, studies estimating half-lives during the second phase of decay have reported much higher half-lives for antibodies to

P. falciparum and *P. vivax* merozoite antigens in low transmission settings (Wipasa *et al.*, 2010, Fowkes *et al.*, 2012). Other factors contributing to the differences include variation in transmission intensities, sampling methods, methodology and antigens studied (Fowkes *et al.*, 2016).

Although antibody levels amongst immune migrants do not completely wane off, migrants do develop clinical malaria albeit the disease is less severe and parasitemia is better controlled than naïve non-immune individuals (Moncunill *et al.*, 2013). The observation that migrants lose some form of immunity overtime as they move away from endemic regions, together with the preceding short duration of *P. falciparum* induced antibody levels have led to a debate and suggestion that developing and maintaining memory to malaria specific antigens may be impaired (Portugal *et al.*, 2011, Fowkes *et al.*, 2012).

2.9 Immunological B cell memory to malaria

Evidence demonstrating memory B cell development in malaria infection comes from the rapid boosting of *P. falciparum* specific antibody levels upon reinfection after the dry season or extended periods of low transmission (Akpogheneta *et al.* 2010; de Souza *et al.* 2002). Data from controlled human malaria infection (CHMI) studies support effective malaria specific B cell memory development as previously exposed aparasitemic individuals have a much more robust antibody level in comparison to naïve non-exposed individuals when challenged with *P. falciparum* sporozoites (Obiero *et al.* 2015),

Although several studies have showed malaria specific memory B cells are acquired slowly over repeated infections (Ndungu *et al.* 2012; Dorfman *et al.* 2005), antibody

levels and memory B cells do not always correlate. Some studies have reported detectable antigen-specific antibodies without evidence of memory B cells (Dorfman *et al.*, 2005). Multigravid Ghanaian women were found not to have VAR2CSA specific antibodies at the onset of pregnancy yet they had detectable VAR2CSA-specific memory B cells (Ampomah *et al.* 2014). The presence of *P. falciparum*-specific memory B cells have been detected in Kenyan children long after exposure although plasma antibody levels were lower (Ndungu *et al.* 2012). Thus, measuring plasma antibody levels may not reflect the true *P. falciparum*-specific memory development in previously exposed individuals.

The role of memory B cells in naturally acquired immunity is not fully understood as well as the relative contributions of long-lived and short-lived plasma cells in clinical immunity. Several immunological studies have associated serum antibody levels with protection from clinical malaria, emphasizing the need for long-lived plasma cells (Cavanagh *et al.* 2004; Egan *et al.* 1996). It is argued this might be critical in immunity against sporozoites or merozoites where antibodies have a very short time frame within which to act (Hviid *et al.* 2015). However for other parasite antigens such as the VAR2CSA PfEMP1 which mediates adhesion of parasites to the intervillous lining of the placenta (Salanti *et al.* 2004), memory B cells may play a dominant role (Hviid *et al.* 2015). VAR2CSA-expressing parasites fail to survive in non-pregnant women or women who have never conceived. In multigravid non-pregnant women, VAR2CSA-specific plasma IgG levels are markedly lower than in *P. falciparum*-exposed pregnant women at time of delivery. This is in contrast to tetanus toxoid vaccine, a prophylactic vaccine administered during pregnancy, which strongly persists several months after delivery (Ampomah *et al.* 2014). In spite of this, boosting of VAR2CSA-specific antibodies occurs subsequently with malaria infection during pregnancy (Fowkes *et al.* 2012). It is thus, argued that clinical protection from PAM is driven by memory B cells whereas NAI

to other PfEMP1 antigens on the iRBC membrane relies on long-lived antibody levels (Hviid *et al.* 2015).

For other merozoite antigens such as PfEBA-175, PfAMA-1 and PfRH5, it is not fully understood why antibody responses to these antigens are transient. It is proposed that there is an age-driven transition from short-lived plasma cells to long-lived plasma cells. This suggestion is supported by studies in an area of seasonal intense malaria, where there was an age-dependent incremental increase in acquisition of antibodies (Crompton *et al.* 2010a). Another plausible explanation is that induction of long-lived plasma cells requires a higher amount of antigen than that required for the induction of short-lived plasma cells (Hviid *et al.* 2015).

2.10 Malaria vaccines

In the wake of emerging parasite resistance to drugs, coupled with emergence of insecticide resistant mosquitoes, malaria control programmes face an imminent threat. Developing a malaria vaccine is a critical intervention to complement existing control strategies to help eliminate malaria from endemic regions. The motivation to develop a malaria vaccine emanates from successes achieved with vaccines to other infectious diseases. The case of eradicating smallpox with vaccines and polio elimination with vaccines both demonstrate the ability of vaccines to help in the elimination and eradication of infectious diseases. For this reason, there have been extensive research into developing an effective malaria vaccine that would at least prevent malaria-associated morbidity and mortality amongst the most vulnerable populations. The primary aim of a malaria vaccine strategy is to induce an immunological memory response that is protective ahead of an infection, such that protection is offered upon exposure to the parasite. In spite of the extensive research and knowledge gained over

the past three decades, developing an effective malaria vaccine has been quite challenging. The challenges in developing malaria include our limited understanding of the interactions between the parasite and the host immune system (Langhorne *et al.*, 2008), and antigenic variation by the parasite as a mechanism to evade the host immune system (Crompton *et al.*, 2010b). The various vaccine strategies aim at targeting different stages of the parasite life cycle i.e transmission blocking vaccines, pre-erythrocytic stage vaccines and blood stage vaccines.

2.10.1 Challenges in developing malaria vaccines

Plasmodium parasites have a complex multistage life cycle, involving two hosts; a vertebrate and an invertebrate host. Progression from one stage to another stage in the life cycle is characterized by the expression of different parasite-specific antigens, each of which triggers an immune response. Although much knowledge is emerging about the malaria life cycle (Cowman *et al.*, 2012), the complex parasite biology remains a challenge for vaccine development. Malaria vaccine development is further complicated by the evolutionary ‘arms race’ between the human host and parasite (Takala and Plowe, 2009). Malaria parasites have evolved ways to overcome immune pressures exerted by the human host, by introducing extensive genetic variability particularly in antigens expressed on the parasite surface, most of which have been studied as vaccine targets. The effects of antigenic variation are demonstrated in vaccine studies where allele specific immune responses are induced. Additionally, malaria parasites have developed strategies that ensure rapid selection and expansion of mutations that favour evading the immune system.

The infective stages of the parasite are all haploid and the intracellular replication of parasites which are protected from the immune system allows for rapid expression and expansion of advantageous mutations (Mackinnon and Marsh, 2010). Our limited

understanding of the interactions between the parasite and the host immune system (Langhorne *et al.*, 2008) poses another level of difficulty as investigators continue to identify immunodominant epitopes which are targets of NAI. In spite of the challenges, extensive work has been undertaken in malaria vaccine development.

2.10.2 Pre-erythrocytic (Liver) stage vaccines

The liver stage of the *Plasmodium sp.* life cycle provides a critical point early in the parasite life cycle at which to arrest an infection either by using drugs or vaccines (Arama and Troye-Blomberg, 2014). Due to the high replication rate of sporozoites, an ideal liver stage vaccine must be highly efficacious to arrest the parasite growth at the liver stage. A single sporozoite escaping the liver stage can replicate to generate merozoites that progress to the blood stage and could lead to life-threatening disease, particularly in non-exposed individuals.

Attempts to develop a liver stage vaccine started several decades ago with studies in mice injected with X-irradiated *Plasmodium berghei* sporozoites (Nussenzweig *et al.*, 1967, Rieckmann *et al.*, 1974). It is evident from these early studies, that vaccination with irradiated sporozoites confer protection in mice following a sporozoite challenge compared to the non-vaccinated control. Subsequent studies in humans with irradiated sporozoites showed very high levels of protection in vaccinated volunteers (Rieckmann *et al.*, 1974). The underlying mechanism for protection is that irradiated sporozoites are capable of invading the hepatocytes but growth is arrested and they do not develop into late liver stages, thus presenting antigens to the host immune system without developing any blood stage infection (Hill, 2011). Although the sporozoite vaccine approach is highly efficacious, the approach requires repeated exposure to thousands of bites from irradiated mosquitoes which may be impractical for a vaccine intended for public use.

To overcome this challenge, Sanaria, a biotechnology company, has isolated thousands of sporozoites from the salivary glands of mosquitoes which are then purified, irradiated and cryopreserved in liquid nitrogen to preserve their viability (Hoffman *et al.*, 2010). Phase I/II clinical trials with the cryopreserved sporozoites administered by needle and syringe showed high level of protection in vaccinated individuals following *P. falciparum* sporozoite challenge (Epstein *et al.*, 2011, Seder *et al.*, 2013). As to whether the route of administration using a needle and syringe can replace mosquito gut with its salivary gland content in inducing sufficient immune response is an area that is not fully understood. Logistically, cryopreserving sporozoites in liquid nitrogen for deployment in developing countries is another issue to contend with. Besides, sporozoite challenge has only been with homologous strains with no data on heterologous strains.

The only licensed and moderately effective liver stage vaccine is the subunit vaccine RTS,S. RTS,S is a recombinant protein vaccine formulated in an adjuvant called AS01. RTS,S targets the circumsporozoite surface protein (CSP), a major surface coat protein on the sporozoite associated with protection from irradiated sporozoites (Gwadz *et al.*, 1979). Clinical trials with an initial CSP vaccine construct which spanned the central tandem repeat with the amino acid sequence NANP has shown the CSP protein to be poorly immunogenic (Ripley Ballou *et al.*, 1987). Building on previous works, a CSP hybrid vaccine was designed by fusing the central repeat (**R**) of CSP to the C terminal region containing the T cell epitopes (**T**) which is in turn fused to hepatitis B surface antigen (**S**) and the unfused **S** protein to obtain RTS,S vaccine (Stoute *et al.*, 1997). In this key clinical trial study, vaccinees immunized with RTS,S formulated with the immunostimulants mono- phosphoryl A and QS21 showed a higher protective efficacy than groups vaccinated with RTS,S formulated with mono- phosphoryl A plus Alum or RTS,S in an oil in water emulsion.

Subsequent studies of RTS,S formulated with adjuvant AS01_B or AS02_A have shown sterile efficacy of 30-50% following sporozoite challenge in healthy adults. Furthermore, a proportion of those protected showed an increase in the period before appearance of blood stage parasites (Kester *et al.*, 2009). There have been multiple clinical trials examining the efficacy of RTS,S/AS01 and RTS,S/AS02 and the duration of anti-circumsporozoite antibodies across different study sites in Africa with varying transmission intensities (Bejon *et al.*, 2008, Guinovart *et al.*, 2009, Kester *et al.*, 2009, Owusu-Agyei *et al.*, 2009, Aide *et al.*, 2010, Olotu *et al.*, 2011). The reported vaccine efficacy is between 33-50% and wanes off substantially with time (Olotu *et al.*, 2016, Greenwood *et al.*, 2017, Han *et al.*, 2017). There are concerns about the RTS,S vaccine schedule and its feasibility in the real world as it requires four vaccine doses. In spite of the seemingly low vaccine efficacy, RTS,S vaccine represents a major step in the long search for a malaria vaccine and forms a critical component of the limited arsenal of tools available to fight, control and eradicate malaria.

2.10.3 Transmission blocking vaccines (TBV)

Transmission-blocking vaccine strategies aim at inducing immunity against the sexual stages of the parasite that develops within the mosquito thus reducing the infectivity of the mosquito and preventing further spread of the disease (Saxena *et al.*, 2007). TBVs do not directly benefit the vaccinated individual but seeks to protect a population against infection with malaria through herd immunity. In addition, unlike vaccines targeting the parasite in the human stage where vaccines must be administered in childhood, TBV vaccination would be across all age groups. TBVs could also help prevent the emergence and spread of drug-resistant parasites or parasites that would be resistant to the other vaccine candidates (Carter *et al.*, 2000).

Target antigens of TBVs can be grouped into 2 main classes; pre-fertilization and post-fertilization antigens (Saxena *et al.*, 2007). The pre-fertilization antigens include Pfs48/45, Pfs230, Pfs40 and parasite chitinase PfCMT1 (Templeton and Kaslow, 1999, Pradel, 2007, Sauerwein and Bousema, 2015). Antisera against recombinant Pfs230 (Williamson *et al.*, 1995) and naturally acquired antibodies to gamete antigens significantly reduce infectivity of *P. falciparum* malaria parasites to mosquito (Drakeley *et al.*, 2006).

The post-fertilization target antigens are surface proteins expressed on the fertilized zygote and ookinete. The well-studied members of this class include Pfs25 and Pfs28. However, the soluble Pfs25 is poorly immunogenic. There are several studies developing adjuvants and delivery platforms to enhance the immunogenicity of Pfs25 and *Plasmodium vivax* (Pvs25) (Wu *et al.*, 2008, Shimp *et al.*, 2013, Talaat *et al.*, 2016).

Natural boosting following vaccinations with TBV vaccines such as Pfs25 and Pfs28 may be challenging. This is because these zygote surface antigens are expressed post fertilization in the mosquito midgut and are not naturally expressed on the circulating gametocytes in the human host (Carter *et al.*, 2000, Arama and Troye-Blomberg, 2014). Communities that are vaccinated with TBV could be at risk of a malaria epidemic if the transmission-blocking immunity induced by the TBV vaccine wanes off (Arama and Troye-Blomberg, 2014). Nonetheless, TBVs would be an essential part of strategies to eliminate or eradicate malaria.

2.10.4 Blood stage vaccines

Blood stage vaccines are important as components of a multistage malaria vaccine or standalone vaccines. Unlike TBVs, blood stage vaccines directly benefit the vaccinee by reducing morbidity as clinical symptoms of malaria are solely due to the asexual blood

stage. Blood stage vaccines that are highly effective could help avert any life-threatening clinical diseases. Furthermore, blood stage vaccines have the advantage of natural boosting after repeated infection, at the same time allowing for the development of NAI. The broad objective of designing blood stage vaccines is to induce high titres of functional antibodies that confer protection by inhibiting erythrocyte invasion or intra-erythrocytic parasite growth and possibly prevent sequestration of iRBC.

Most blood stage vaccine designs have been recombinant proteins of key merozoite antigens known to be the targets of antibodies in NAI. These subunit vaccines are administered in different adjuvant formulations, but there have been other vaccine platforms such as virosomes, peptide vaccines, whole blood stage parasites, DNA and viral vectored vaccines (Richards and Beeson, 2009). There have been many blood stage vaccine candidates that have progressed from the preclinical to the clinical phase. The well studied vaccine candidates include PfAMA-1, PfMSP-1, PfMSP-2, PfMSP-3, PfGLURP, PfSERA5, PfRESA and PfEBA175 (Ellis *et al.*, 2010b, Terheggen *et al.*, 2014, Beeson *et al.*, 2016, Sirima *et al.*, 2016), all of which have been known to be targets of protective antibodies in NAI.

Field studies with PfAMA-1 based vaccines in children (Sagara *et al.*, 2009, Ouattara *et al.*, 2010, Thera *et al.*, 2011) resulted in strain-specific immune responses with no protective efficacy against malaria. Similarly, challenge studies in naïve adults following vaccinations with AMA-1/AS01_B or AMA-1/AS02_A have been disappointing as there was no significant difference in the prepatent period or parasitemia between vaccinated and control groups (Spring *et al.*, 2009).

MSP-1 has also been the focus of blood stage vaccines for many years. Several challenge studies have shown that vaccination with MSP-1 vaccines induce protective immunity in

non-human primates (Christian *et al.*, 2005, Singh *et al.*, 2006, Lyon *et al.*, 2008). However, a phase-Ia study in malaria-naïve adults vaccinated with two allelic (3D7 and FVO) forms of MSP1₄₂ despite the absence of strain-specific immune responses, the biological effects of the antibodies as tested by GIA was very minimal (Malkin *et al.*, 2007).

As vaccines with single merozoite antigens induced strain-specific responses with limited efficacy in phase I/II trials, combining more than one antigen in a multi-antigen vaccine seems a theoretically appropriate strategy to exploit. GMZ22 is a combination vaccine consisting of PfMSP-3 and PfGLURP that has been tested in Phase IIb trials across several sites in Africa (Sirima *et al.*, 2016). The vaccine efficacy reported was very low to warrant the use of such vaccine in the field. It is possible that enhancing the immunogenicity of GMZ2 or using a more potent adjuvant will increase vaccine efficacy. Until now, clinical trials with combination vaccines have provided little evidence that combination vaccines utilizing antigens such as AMA1, MSP1 and CSP are more effective than single antigen vaccines (Ockenhouse *et al.*, 1998, Malkin *et al.*, 2008, Thompson *et al.*, 2008). Combination vaccines may be more effective vaccines when the individual antigens themselves are very efficacious. Identifying an efficacious blood stage vaccine candidate may be a step in the right direction.

2.11 Towards epitope-based malaria vaccines

Conventionally, the basic principle underlying vaccine development is to expose the immune system to weakened forms of a pathogen such that vaccination does not cause disease but primes the immune system to respond appropriately upon encounter with the active pathogen. Historically, vaccinology probably began in medieval China by administering dried scabs from smallpox lesions to healthy individuals with the aim of

protecting them against the infection (Rappuoli, 2001, Donati and Rappuoli, 2013). This was however unpopular until Edward Jenner formally introduced the term ‘vaccines’ in the Western world by administering an infectious material from cowpox lesions into humans for protection against smallpox. Centuries later, when it became known infections were caused by microorganisms and viruses, Louis Pasteur pioneered the rational development of vaccines. Pasteur’s approach to developing a vaccine was to ‘isolate, inactivate and inject’ the microorganism. This approach was followed for many years by vaccinologists to successfully develop vaccines against poliovirus, mumps, rubella, diphtheria, tetanus toxoid and so on. the infectious agent (Worboys, 2007).

By the end of the 20th century, vaccines to many infectious diseases that plagued human had been developed. The vaccine composition were traditionally based on whole pathogens that had been inactivated or attenuated either by chemical, heat or radiation or by serial passaging *in vitro* to reduce virulence (Delany *et al.*, 2013). The ineffective methods of inactivating the pathogens raised major safety concerns with the use of whole pathogens, which may revert to their pathogenic form. A classical example is the oral polio vaccine (OPV), which comprises three live attenuated serotypes and was used for several years by the WHO to disrupt poliovirus transmission. Vaccination with OPV was found to result in rare vaccine-induced poliomyelitis in some vaccinees due to reversion of the attenuated form of the virus to a virulent form (Macadam *et al.*, 1989, Kew *et al.*, 2005). The inability to culture certain infecting agent *in vitro* also made it difficult to develop vaccines against such diseases. Parasite diversity on a global scale also affects the vaccine efficacy of such whole organism vaccines in different geographical locations (Delany *et al.*, 2013).

The onset of the genomic era and advances in recombinant DNA techniques revolutionised vaccine development beyond Pasteur’s approach in what is termed reverse

vaccinology (Rappuoli, 2001). As the genome sequences of microorganisms became available, vaccinologists are able to predict vaccine targets based on the genome of an organism without the need for *in vitro* culturing. The first vaccine developed using reverse vaccinology is the Meningococcus B (MenB) vaccine. Developing a vaccine against MenB using the conventional whole pathogen approach has been unsuccessful as the outer membrane protein is vastly polymorphic and the capsular polysaccharide is poorly immunogenic because of its similarity to a host self-antigen. By employing reverse vaccinology approach, a vaccine against MenB (Bexsero®) has been licensed for use in humans (Hwang *et al.*, 2012, Delany *et al.*, 2013).

Currently, vaccines against most infectious diseases are designed to induce antibody responses that neutralize the pathogens (Plotkin, 2008). As much insight is gained into host-pathogen interactions, vaccinologists are adopting the reductionist approach of making vaccine compositions simpler as subunit vaccines and highly focused in inducing appropriate antibody responses. Pathogens have co-evolved strategies over extended periods to evade host immunity, which makes vaccine development challenging with issues such as polymorphism and low immunogenicity of antigens.

It has become evident conventional vaccine strategies are unsuccessful against pathogens such as HIV and *Plasmodium*. In recent years, vaccinologists are adapting structure and epitope-based strategies in developing vaccines (Sette and Fikes, 2003). Structure-based vaccinology refers to the rational design of vaccines by combining the structural information of proteins and bioinformatics to select for potent immunogens that induce appropriate immune responses (Dormitzer *et al.*, 2008). Epitope-based vaccinology utilizes the epitopes of protective antibodies isolated from infected individuals. The advantages of using the epitope-based approach in vaccine design include fine-tuning the immune response. During an infection, not all antigens induce a protective immune

response, thus identifying few specific epitopes that induce protective responses would be useful in improving vaccine efficacy. Epitope based vaccines could also help eliminate undesired immune responses. Not all antibody responses induced by epitopes are beneficial to the host, as some non-protective antibodies could interfere with the actions of neutralizing antibodies (Gershoni *et al.*, 2007, Thomas and Luxon, 2013, Oscherwitz, 2016).

In the field of malaria, rational design of vaccines based on structure and epitope of antigens is gradually gaining grounds. Few studies have sought to characterize the neutralizing epitopes of the existing malaria vaccine candidates such as PfAMA-1 (Coley *et al.*, 2007), PFRH5 (Wright *et al.*, 2014), PfCyRPA (Favuzza *et al.*, 2017), and a leading *P. vivax* vaccine, *P. vivax* duffy binding protein (PvDBP) (Chen *et al.*, 2016). The first step in epitope-based vaccine approach is to isolate monoclonal antibodies from humans or immunized mice. The monoclonal antibodies are then tested for functionality. In the present study, the *in vitro* growth inhibitory assay was used in examining the neutralizing ability of the isolated monoclonal antibodies. The epitopes of the monoclonal antibodies with the desired properties are subsequently mapped and reconstituted into vaccine immunogen (Ahmad *et al.*, 2016). Thus, the present study sought to generate human monoclonal antibodies to well conserved and non-redundant *P.falciparum* antigens, PFRH5 and PfCyRPA. Mapping the epitopes and structural features of neutralizing and non-neutralizing monoclonal antibodies would provide further insights into the rational design of PFRH5 based vaccine.

2.12 Human monoclonal antibodies from memory B cells

Originally, monoclonal antibodies were generated from murine hybridomas (Kohler and Milstein, 1975) which made them inappropriate for use in humans. To overcome this

challenge, several methods have been devised, including human-mouse chimeric monoclonal antibodies and humanized monoclonal antibodies from transgenic mice (Nelson *et al.*, 2010). Other methods include EBV-immortalization of B cells and phage display libraries of IgG genes isolated from plasma cells. In the present study, we generated monoclonal antibodies by immortalizing memory B cells from malaria immune adults.

EBV is a γ herpesvirus that infects B-lymphocytes by binding of viral envelope glycoproteins gp350 and gp32 to CD21 receptor on B cells and human leukocyte antigen (HLA) class II molecules respectively (Price and Luftig, 2014). Of particular interest is the ability of EBV to transform resting B-lymphocytes into lymphoblastoid cell lines, providing an invaluable *in vitro* system that has been employed in generating human monoclonal antibodies from memory B cells. There are several advantages in using memory B cells as opposed to antibody-secreting plasma cells. Antibody-secreting plasma cells generated in response to an infection can be obtained in high numbers during the peak of an immune response, which usually is between 6-10 days. The plasma cells then migrate to the bone marrow and develop into long-lasting plasma cells. The narrow time frame to isolate the plasma cells can be exploited in experimental immunizations. However, in natural infections it is generally impossible to predict or determine the exact time frame to isolate plasma cells. In addition, isolating peripheral plasma cells does not select for high responder individuals. Thus in making human monoclonal antibodies from plasma cells, it is ideal to clone and express the human IgG genes. This can be done by phage display or single-cell RT-PCR.

In contrast, memory B cells can persist for long periods of time and comprise all the antibody specificities that an individual has acquired in their lifetime following

vaccinations or natural infections. These cells can be isolated at any point in time and do not require booster immunizations or recent exposure to an infecting agent. Unlike plasma cells which are terminally differentiated, memory B cells maintain considerable growth potential and can be transformed by EBV into antibody-secreting cells (Lanzavecchia *et al.*, 2007). The aforementioned factors informed the decision to use the EBV immortalization method.

2.13 Growth inhibition assay (GIA) as a test for antibody function

The GIA assay provides an *in vitro* system to examine the neutralizing abilities of antibodies against blood stage antigens. The GIA assay has been used in both pre-clinical (Douglas *et al.*, 2011, Dreyer *et al.*, 2012) and clinical studies to prioritize parasite antigens for consideration as blood stage vaccine targets (Malkin *et al.*, 2007, Roestenberg *et al.*, 2008, Ellis *et al.*, 2010a).

The underlying principle of GIA is to measure the change in parasite multiplication rate over one or two merozoite invasion cycles. Various techniques have been used to measure the endpoint activity in GIA including microscopic reading of blood smears, flow cytometry with DNA dyes and parasite derived lactate dehydrogenase (pLDH) activity. In the current study, pLDH assay was used in examining the inhibitory effects of the monoclonal antibodies. The pLDH assay is a biochemical assay that measures the enzymatic activity of the parasite lactate dehydrogenase enzyme and by so doing measures the parasite viability which comprises both parasite invasion and growth. The GIA assays does not truly reflect physiological conditions as the assay set up does not include other components of naturally acquired immunity such as monocytes or complement system. Thus, GIA examines the ability of antibodies to block or interfere with merozoite invasion and or growth, which cannot be attributed to ADCC or CDC as,

observed in antibody-mediated NAI. Rather GIA to some extent models what happens with vaccine induced unnatural immunity.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Ethical Approval

The ethical approval for the recruitment and collection of samples for the study was obtained from the institutional review board of Noguchi Memorial Institute for Medical Research, Ghana (026/13-14) (CPN 010 /12) and the Ethical Review Committee of the Ghana Health service (Ministry of Health file GHS-ERC 08/05/14). Informed consent was sought from study participants before recruitment, and for children below the age of 12 years, consent was obtained from parents or other legal guardians before recruitment. Antimalarial treatment was given to patients in accordance with Ghana Health Service guidelines.

3.2 Study area

The study was conducted in two separate demographic locations; Hohoe which is the capital of Hohoe municipal assembly, Volta Region, and Asutsuare, a town within the Shai-Osudoku district, Greater Accra Region. Asutsuare and Hohoe are located about 120 km and 220 km northeast of Accra (**Figure 3.1**), respectively. Both sites are characterized by semi-deciduous forest vegetation. Climate patterns in the two study areas are similar with two main seasons: dry season (December–March) and rainy season (April–September). The main occupations of the inhabitants of Asutsuare are fishing and farming whereas farming is the major activity at Hohoe. Malaria transmission at both areas is reported to be holoendemic with two seasonal peaks: major season April- July and minor season September-November (Kweku *et al.*, 2008, Adu *et al.*, 2012). The dominant circulating malaria specie within the study areas is *P.falciparum* with a few

cases of *P.ovale* and *P.malariae* reported (Kweku *et al.*, 2008). The majority of the population at Asutsuare is of the Ga-Adangbe ethnic group and the minor ethnic groups include Ewes, Hausas and Akans. The major ethnic group in Hohoe is the Ewes interspersed with other minority groups like Akans and other ethnic groups from Northern Ghana.



Figure 3.1: Map of Ghana showing study sites (Asutsuare and Hohoe)

3.3 Study design and sampling

To examine the immunogenicity and seroprevalence of PfRH5 complex proteins in natural infections, children suffering from malaria were recruited at Hohoe. A total of

110 children with ages ranging from 1 to 12 years reporting to the Hohoe Municipal hospital with fever were screened by RDT (First Response®, Transnational Technologies, UK) and confirmed by microscopy for malaria before being recruited for the study. Sampling at Hohoe was carried out between May and September, 2014 during the peak of the malaria season. The first day of recruitment was considered day 0 of sampling serving as the baseline. The children were subsequently grouped as either malaria-positive by being both RDT- and microscopy-positive and as febrile controls (fever but RDT- and microscopy-negative). The malaria-positive children were further grouped into severe malaria (SM) and uncomplicated malaria (UM). SM was defined as the presence of one or more of the following symptoms without any other known causes i) impaired consciousness (Blantyre coma score ≤ 3) lasting longer than 30 minutes, (ii) acidosis, defined as respiratory distress (rapid, deep, and labored breathing), (iii) hypoglycemia (blood glucose < 2.2 mM), (iv) hemoglobin concentration < 5 g/dL, (v) > 2 convulsions within the last 24 h, (vi) shock, and/or (vii) hyperparasitemia ($> 500,000/\mu\text{L}$). Malaria patients not showing any of these symptoms were then classified as UM. In the present study, protection as a function of antigen-specific plasma IgG levels was defined as absence of clinical symptoms of malaria at the time of recruitment. After recruitment, the malaria-positive children were followed-up on day 14 by which the infection would have cleared and day 42 by which time the immune responses would have stabilized (Fig 3.2) to study the longevity and kinetics of merozoite antigen-specific plasma IgG levels after natural infections. To study the protective role of PfRH5-, PfCyRPA- and Pf113-specific plasma IgG levels in NAI, healthy children within Hohoe community were further recruited into the study. The healthy children were classified as either asymptomatic control (presence of parasites with no clinical symptoms) or as healthy controls (no detectable parasites by microscopy). Blood samples were obtained from

anonymous 40 Danish blood donors to serve as non-exposed samples for standardizing ELISA experiments. Written informed consent was obtained from donors who were mainly residents of greater Copenhagen.

To generate human monoclonal antibodies to PfRH5 and PfCyRPA, semi-immune adults were recruited from Asutsuare. Blood samples were collected from adults because a higher volume of blood was required to be able to isolate sufficient B cells for immortalization assays. A total of 79 healthy adults from Asutsuare who were exposed to malaria but were malaria negative by RDT were recruited at the end of the April-June malaria season during the first year (2014) of sampling. In the following year of sampling (2015), 15 individuals who were sero-reactive to PfRh5 in the previous year were recruited.

3.3.1 Inclusion criteria for children participants

For the clinical patients, all children between the ages of 1-12 years who were RDT and microscopy positive for *P. falciparum* infection and oral temperature $> 37.5^{\circ}\text{C}$. Parents or guardians must consent to participate in study. For non-clinical participants, children between the ages of 1-12 years who were without symptoms of malaria and without detected alternative cause of febrile illness.

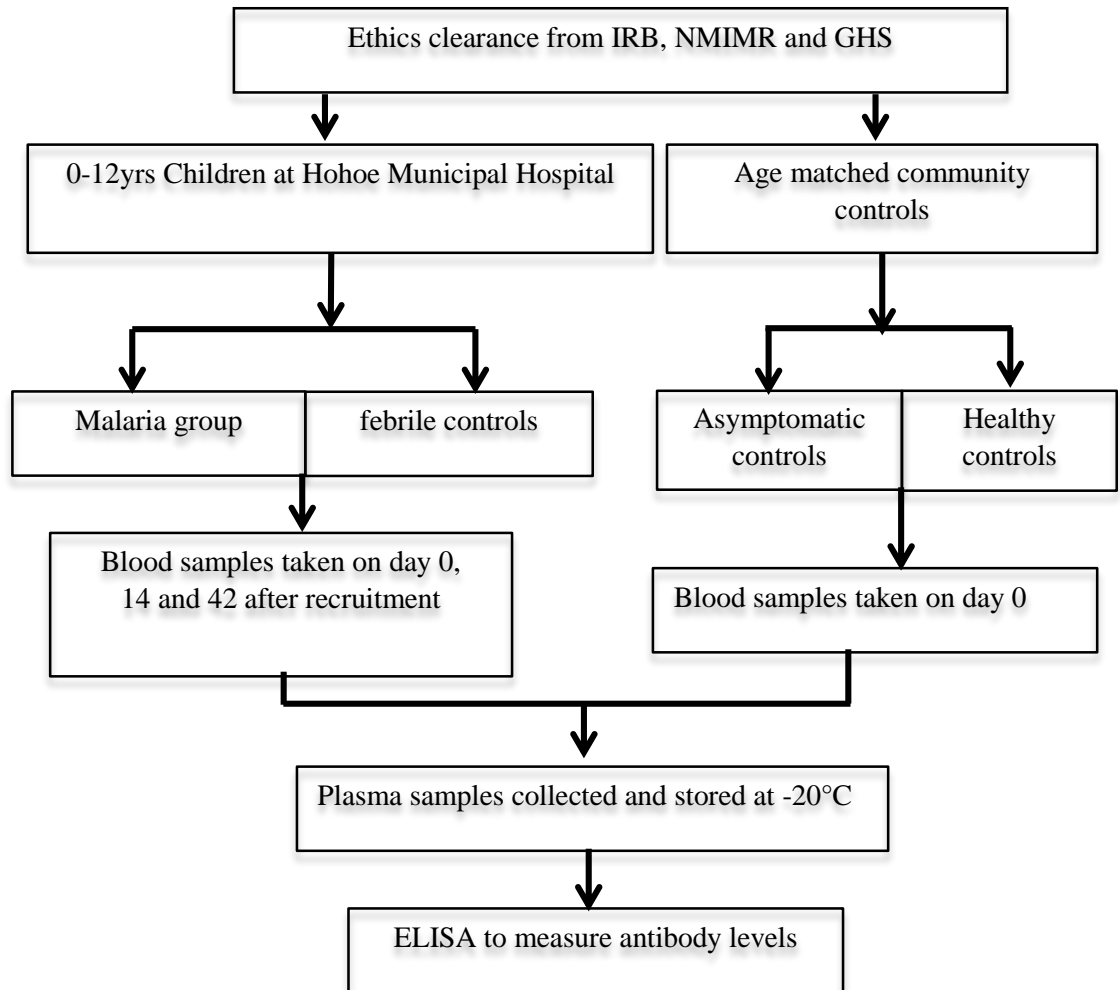


Figure 3.2: Flow chart showing sampling time points for children recruited at Hohoe

3.3.2 Exclusion criteria for children participants.

Refusal to consent to participate in study. Children with major medical condition, sickling genotypes and parasitemia $\leq 2500/\mu\text{l}$ were excluded.

3.3.3 Inclusion criteria for adult participants

Adults 18 years and over who consented to participate and were RDT and microscopy negative for *P. falciparum* infection.

3.3.4 Exclusion criteria for adult participants

Refusal to consent to participate in study. Individual with major medical condition or sickling positive. Pregnant women were excluded.

3.4 Sample size

Basic sample size calculation was built upon a case-control design where the objective was to demonstrate a difference in the proportion of antibody positivity from 20% (unprotected) to 50% (protected) carriage between severe and uncomplicated malaria cases. This led to a sample size of 45 per group and accounting for a 10% loss a total of $50+50 = 100$ well characterized patients. (EPI-info, stat-calc set at 1:1 design, 95% confidence and 80% power). For the purpose of community control samples a similar sample size calculation was relevant, that is, 100 community controls was required during the sample collection period.

3.5 Parasitological detection by microscopy

Parasitological detection by microscopy was carried out by preparing thick and thin blood smears on microscope slides. Thick smears were prepared by placing a drop of blood on the slide and evenly spreading it out in a circular motion. Thin smears were prepared by placing a drop of blood at one end of the slide and thinly spreading the blood out using a second slide held at an angle of 45° . The slides were air-dried, fixed with 100% methanol (VWR, USA) and subsequently stained with 10% Giemsa stain (Merck, Germany) for 15 min. The slides were then washed gently under running tap, air dried and viewed under oil with x100 objective lens of a light microscope. Parasitemia was estimated as the number of parasites per 200 white blood cells. Patients who consented

to participate in the study were grouped into severe and uncomplicated malaria according to the WHO guidelines on clinical and laboratory criteria(2014).

3.6 Sample Preparation

For the children, about 5 mL of venous blood was drawn into heparinized vacutainers and another 5 mL into EDTA vacutainers on days 0, 14 and 42 from the malaria group. For the other patient categories, venous blood was drawn into heparinized vacutainers only on day 0. The blood samples were centrifuged (800×g, 5min) to separate the plasma from the cells. The plasma was stored at -20°C until use. The RBC pellet from heparinized blood samples obtained from the malaria control group on day 0 was processed to freeze down iRBC. The RBC pellet containing the iRBC was washed twice with incomplete parasite medium (RPMI supplemented with 25mM HEPES and 50 µg/mL gentamicin sulfate (Sigma-Aldrich, Germany). The iRBCs were frozen using glycerolyte 57 (Fenwal, USA) as cryoprotectant. In brief, the freezing medium was mixed with the RBC pellet in a volume ratio of 5:1, which was added in two steps. One volume of the freezing medium was added dropwise to the RBC pellet whilst the tube was swirled and allowed to stand for 15 mins. Four volumes were then added in the second step. The mixture was aliquoted into cryovials, placed in mr.frosty containers (Thermofisher Scientific, UK) and frozen at -80°C overnight before transferring into liquid nitrogen (-196°C) until ready to use.

For the adults samples from Asutsuare, 20mL of venous blood samples were collected in 2014 into heparinized vacutainers and the plasma was separated and stored as described above. A higher volume of blood samples (60mL) were drawn from the 15 individuals who were recruited in 2015 to provide enough PBMC for immortalization assays to the antigens of interest.

Peripheral blood mononuclear cells (PBMC) were separated with Lymphoprep (Stem-cell Technologies, Germany) using Leucosep (VWR, USA) tubes and following the manufacturer's protocol. The PBMC were frozen using a controlled gradient-freezer (Hviid *et al.*, 1993) and stored in liquid nitrogen until ready to use.

3.7 Recombinant antigens

All the recombinant antigens used were kind gifts from other collaborating labs. Recombinant PfRH5 was expressed in a *Drosophila melanogaster* Schneider 2 stable cell line system as previously described by . The full length ectodomain of Pf113 and PfCyRPA used in the study were expressed in transient transfections of HEK 293 cells and described elsewhere (Galaway *et al.*, 2017). The recombinant PfGLURP antigen used was produced in *Escherichia Coli* as described previously (Theisen *et al.* 1995). GLURP R0 consist amino acids 24-489 represents the nonrepetitive amino terminal and GLURP R2 comprise amino acids 705-1178 representing the carboxy-terminal repeat region of GLURP. PfEBA-175 was recombinantly expressed in Baculovirus and comprises the receptor binding domain (Liang *et al.* 2000).

3.8 Plasma IgG reactivity and IgG subclass measurements by ELISA

Plasma IgG reactivity to PfRh5, PfCyRPA, GLURP R0, GLURP R2 and EBA175 recombinant proteins were determined by ELISA as described by others (Ampomah *et al.* 2014). Immunogenicity and seroprevalence of GLURP R0 (nonrepetitive amino terminal), GLURP R2 (carboxy terminal repeat region) and EBA175 have been extensively studied and were included in the ELISA assays as control merozoite antigens. In summary, 96-well flat-bottomed plates (Nunc, Thermofisher scientific, Denmark) were coated with the recombinant protein in Dulbecco's PBS (pH 7.2, 4°C, overnight)

and blocked for 1h at room temperature with dilution buffer pH 7.3 (10 g BSA, 0.2 g KCl, 0.2 g KH₂PO₄, , 29.2 g NaCl, 0.92g Na₂HPO₄, 10 mL, Triton-X100, 1 mL Phenol Red, 1 L distilled water . Plasma samples diluted 1:100 were added to the antigen-coated plate in duplicate wells and incubated for 1h. Wells were washed with washing buffer (dilution buffer without BSA), and horseradish peroxidase-conjugated rabbit-anti-human IgG (Abcam, USA) (1:3000) was added to detect bound antibody. The enzymatic reaction was measured using O-phenylene diamine (Sigma-Aldrich, Copenhagen, Denmark) substrate system and the optical density (OD) read at 490nm. Plasma samples from Danish non-exposed individuals were used as negative controls and for PfRh5 and PfCyRPA ELISAs, supernatants from polyclonal antibody-producing B cell-lines were used as positive controls. A Ghanaian hyperimmune serum was used as positive control for EBA 175, GLURP R0 and GLURP R2. Plate-to-plate and inter-assay variations were standardised using the positive controls. The cut-off for seropositivity was calculated as the mean + 3SD of the OD values obtained with the negative controls.

Immune reactivity to Pf113 was measured by ELISA following the procedure described by (Douglas *et al.* 2011). Nunc microtiter plates were coated with 2ug/mL Pf113 overnight and blocked with Casein solution (PerBio Science, UK) for 1h at room temperature. Plates were subsequently washed with 0.05 % tween in PBS (PBS/tween). Plasma samples diluted 1:100 in Casein solution were added to plates and incubated for 2h at room temperature. Alkaline phosphatase conjugated goat anti-human IgG (γ -chain) (Sigma Aldrich, UK) was added to detect bound antibody and incubated for 1h. The enzymatic reaction was measured using *p*-nitrophenylphosphate substrate (pNPP, Sigma Aldrich, UK) diluted in diethanolamine buffer (Thermofisher Scientific, UK) and OD read at 405nm.

For IgG subclass ELISA, plates were coated and blocked as described above. Native human IgG1, IgG2, IgG3 and IgG4 (AbD serotec, UK) were also coated on the plates to serve as positive controls. Plates were washed with 0.05% PBS/tween, plasma samples diluted 1:100 were added to the plates in duplicates and incubated for 2h at room temperature. After incubation, plates were washed and secondary antibodies: mouse anti-human IgG1 F_c biotin conjugated (Thermofisher Scientific, UK), biotin-mouse anti-human IgG2 (Thermofisher Scientific, UK), monoclonal anti-human IgG3-biotin (Sigma Aldrich, UK), monoclonal anti-human IgG4-biotin (Sigma Aldrich, UK) diluted 1:1000 was added to the corresponding plate and incubated for 1hr at room temperature. Subsequently, plates were washed again and incubated with ExtrAvidin-AP (Sigma Aldrich, UK) diluted at 1:5000 for 30 min. The enzymatic reaction was measured using *p*-nitrophenylphosphate substrate (pNPP, Sigma Aldrich, UK) diluted in diethanolamine buffer (Thermofisher Scientific, UK) and OD read at 405nm.

3.9 Memory B-cell immortalisation and cloning

PBMC from positive responders from the ELISA screening described above were immortalised as previously described (Traggiai *et al.*, 2004, Barfod *et al.*, 2007b). Immortalization of the B cells was done by infecting the B cells with Epstein-Barr virus. PBMCs were thawed and memory B cells were initially isolated by positive selection using CD22 isolation kit II (Miltenyi Biotec, Sweden) following manufacturer's protocol. The isolated memory B cells from three of the donors who were seropositive to PfRH5 were immortalized *in vitro* with EBV to generate antibody-secreting B cell clones. The cells were then plated out in 96-well round-bottomed plates at 100 cells/well in the presence of CpG oligonucleotide and irradiated PBMC (1×10^6 cells/well). Cell culture supernatants were screened after 2-3 weeks by ELISA for PfRH5- and PfCyRPA-

specific antibody production. Initially, pools of culture supernatants from four adjacent columns on each cell culture plate were tested in the first ELISA screen. Positive wells were retested in a second screen where the individual wells from the original plates are tested in an ELISA. Positive cultures were cloned into monoclonal antibodies initially by limiting dilution and subsequently as recombinant monoclonal antibodies.

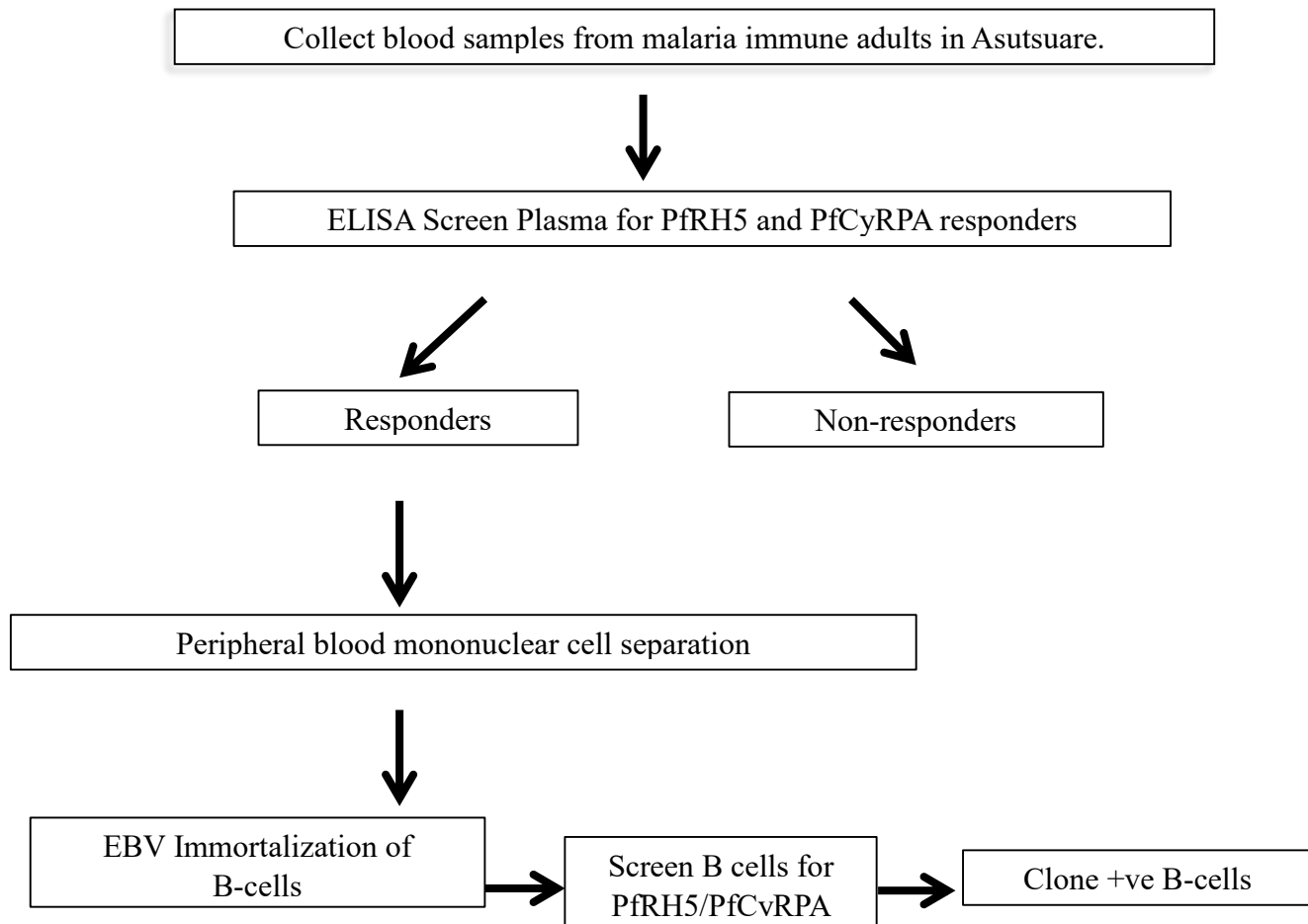


Figure 3.3: Flow chart showing sample preparation of blood samples collected from adults in Asutsuare.

3.10 Recombinant monoclonal antibody

Previously immortalized B cells were labelled with Sytox green nucleic acid stain (Thermofisher Scientific, UK) to distinguish between live and dead cells. Single B cells were sorted using Moflo cell sorter (Dako, Denmark) excluding duplets populations into 96-well plates containing 10mM Tris HCL buffer and an RNase inhibitor, RNAsin (Promega, UK). Plates were immediately frozen on dry ice before transferring to -80°C freezer and stored at -80° until plates were ready to be used.

3.11 Reverse transcription PCR (RT-PCR) of single B cells

In the RT-PCR, reverse transcriptase and specific primers convert mRNA from the leader sequence of HC and LC open reading frame to the 5' end of CH or CL to cDNA. Total RNA from the single-sorted B cells were reverse transcribed to cDNA using a sensiscript RT enzyme kit (Qiagen, UK) and random hexamers (appendix Table 1). The PCR reaction mastermix was prepared following the manufacturer's protocol consisting of sensiscript RT enzyme, 10X buffer, 5mM dNTP mix and nuclease-free water. The cycling conditions of the PCR were 37°C (40 min), 43°C (10min) and 50°C (10min) and PCR was performed on a Lab tech G Storm PCR machine (G-storm direct, UK). The cDNA samples were stored at -20 °C until used for cDNA amplification.

3.12 cDNA and immunoglobulin chain amplification

The variable regions of the HC or LC were then amplified using degenerate primers developed by Tiller *et al.* (2008) in two nested PCRs. The primer set was designed to cover the allotype diversity found at the annealing sites. In the first PCR (PCR1), cDNA was amplified to generate enough templates for the more specific subsequent PCR (PCR2). The cDNA templates were amplified using the same RT primer set (appendix

Table 1)) and high fidelity (HF) Phusion polymerase (New England Biolabs, UK). The amplification PCR (PCR1) reaction contained 1 μ L cDNA template, HF Phusion mastermix and water in a total volume of 20 μ L. The cycling conditions were 98°C (30s), 15 cycles (98°C for 10s, 72°C for 10min), 15 cycles (98°C for 10s, 68°C for 30s, 72°C for 1min), 72°C for 10min. For the next PCR step, (PCR2), the amplified cDNA was diluted 1:100 and used as the template for PCR 2. In PCR 2, primers were also degenerate and covered all variable exon diversities. Different primer sets were used to amplify the V_H and V_L gene transcripts (appendix Table 1) together with the HF Phusion polymerase. The amplicons were run on 1% Agarose gels using Tris/acetate /EDTA buffer. Molecular weights of the amplicons were estimated using Generuler DNA 1kb ladder (Thermofisher Scientific, UK). The positive bands were cut out and purified using the QIAquick gel extraction kit (Qiagen, UK) following manufacturer's protocol. The DNA was eluted in water and subsequently used in transcriptionally active PCR (TAP)

3.13 Transcriptionally active PCR (TAP)

It was anticipated that cloning of the amplified variable region gene pairs would involve several cloning reactions. The widely used approach of making transcriptionally active genes by cloning the genes into plasmid vectors, transforming and growing bacteria to purify plasmids would be time-consuming and labour-intensive. Hence, to cut down on the cost and save some time, an approach was adapted to clone the V_H/V_L gene pairs into transcriptionally active fragments in a method known as transcriptionally active PCR (TAP) (**Figure 3.4**) as described by others (Liang *et al.* 2002). The first step in the TAP PCR utilizes primer sequence specific to the gene of interest and universal TAP ends (**Figure 3.4**). The 5' universal TAP fragment sequence is designed complementary to the

CMV promoter. The 3' universal end is designed to overlap with DNA fragment containing SV40 terminator sequence.

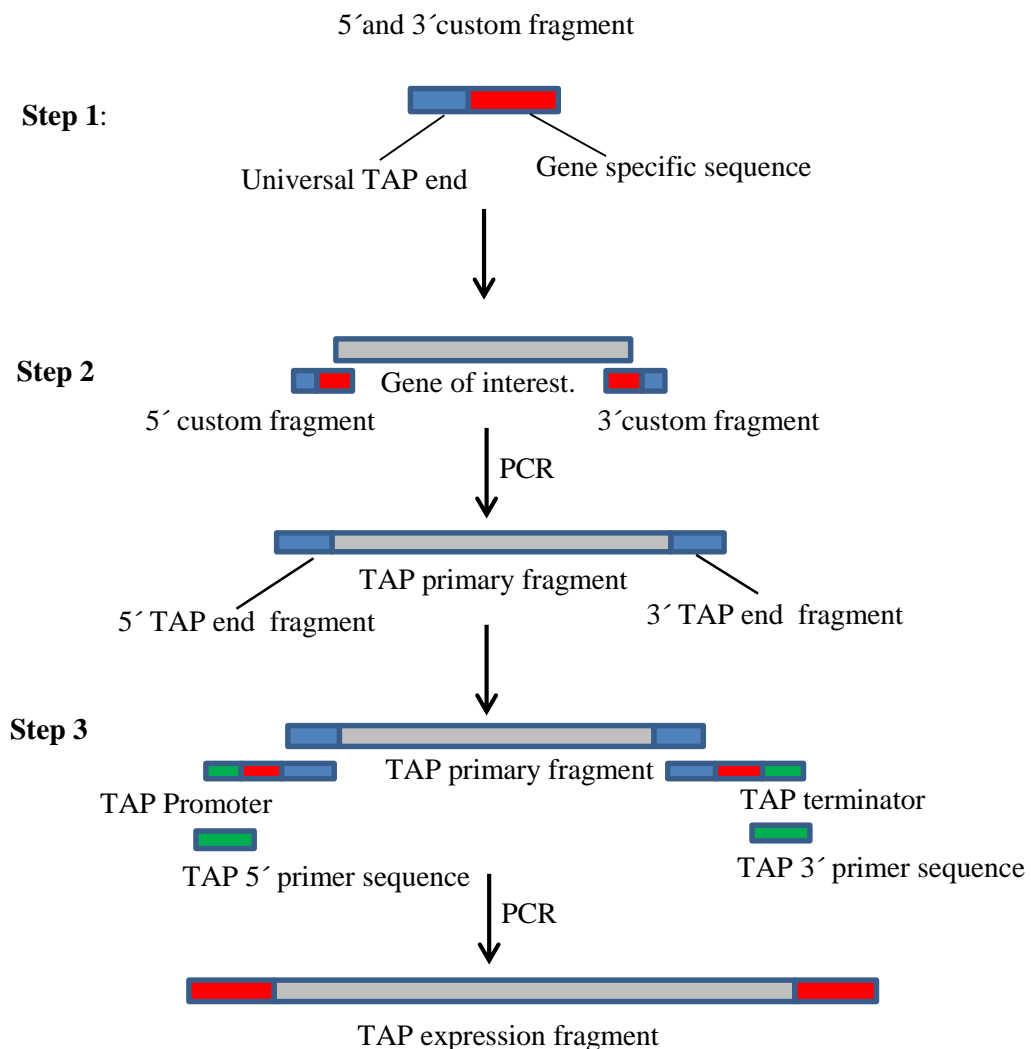


Figure 3.4: Schematic representation TAP PCR.

In the first step of TAP, gene specific primers containing universal TAP ends are synthesized. Primers are designed complementary to the 5' and 3' ends of the gene of interest. In the second step, the gene of interest with custom primers to generate TAP primary fragment. In step 3, add promoter and terminator fragments to the TAP primary fragment in a nested PCR to yield TAP expression fragment. Figure modified from (Liang *et al.*, 2002).

The 5' and the 3' TAP fragments are used in the second PCR and appended to the amplified gene of interest to generate a TAP primary fragment. In the final step, the TAP

primary fragment is then amplified to yield TAP expression fragment which is used for transfection. The 5' TAP fragment and the 3' chain specific TAP fragments for the first step of the TAP method were generated following the setup shown in **Table 3.1**.

Table 3.1 Reaction setup for 5' and 3' TAP fragments.

PCR reaction	5' fragment (CMV promoter)	3' Gamma fragment (Gamma chain Fc + 3'UTR)	3' Kappa fragment (Kappa chain Fc + 3'UTR)	3' Lambda fragment (Lambda chain Fc + 3'UTR)
Primer 1 (10uM)	J2620	J2708	J2707	J2707
Primer 2 (10uM)	iPCR Rv	Abvec iPCR G long	AbVec K iPCR	AbVec λ iPCR
Template plasmid	Mini 1:1000 B4 γ #127	Mini 1:1000 B4 γ #127	1:1000 miniprep #127 A7K	1:1000 miniprep #127 A3 λ
PCR programme	98°C – 30 secs (98°C – 10 secs 65°C – 30 secs 72°C – 80 secs) x 35 cycles 72°C – 5 mins 4°C - hold	98°C – 30 secs (98°C – 10 secs 72°C – 80 secs) x 35 cycles 72°C – 5 mins 4°C - hold	98°C – 30 secs (98°C – 10 secs 63°C – 30 secs 72°C – 1 min) x 35 cycles 72°C – 5 mins 4°C - hold	
Product size	~1.2kb	~1.3kb	~0.6kb	

The PCR reaction mix for the TAP fragments contained 1x HF Phusion mix, primer 1 and primer 2 of each chain, plasmid template and deionized water in a final volume of 50 μ l. The PCR products were run on 1.2% agarose gel and the fragments purified as described earlier.

To generate the TAP expression fragments, random V_H/V_L gene pairs that were amplified previously (PCR 2) were used as templates for the second TAP step. The PCR reaction mix contains gel extracted variable region cDNA, 5' common TAP fragment, 3' chain specific TAP fragment, 1x HF phusion mix, chain specific primer 1 and primer 2 (See appendix Table 1 for primer sequences) and deionized water in a final volume of 25 μ l. The cycling conditions for the TAP were 98°C for 30s, 35 cycles (98°C for 10s; 60°C

for 30s; 72°C for 2min), 72°C for 10min, 4°C hold. The TAP expression fragments were confirmed by running a gel and subsequently used in transfecting HEK 293 adherent cells. Supernatants from the transfectants were then tested in ELISA for specificity to PfRH5 and PfCyRPA. The V_H/V_L gene pairs that produced functional antibodies specific to our antigens of interest were then cloned by restriction cloning.

3.14 Transfection

Nearly confluent adherent HEK293 cells were re-suspended in DMEM media supplemented with ultra-low fetal bovine serum (FBS) (Thermofisher Scientific, UK), 2mM L-glutamine (Thermofisher Scientific, UK), 1mM sodium pyruvate (Thermofisher Scientific, UK), 100U/mL penicillin (Thermofisher Scientific, UK) and 0.1mg/mL streptomycin (Thermofisher Scientific, UK). The cells were seeded at 100 cells/ well in 96-well U-bottom cell culture plates and incubated at 37°C overnight. On the next day, a transfection mix was prepared by mixing 60µg/mL of 25kDa polyethylenimine (PEI) (Thermofisher Scientific, UK) and 200ng of matching heavy and light chain TAP expression fragments. The PEI/DNA mix was added to the previously plated HEK 293 cells and incubated at 37°C. DMEM media was added the next day to the cells. The transfection supernatants were harvested 3-4 days later and screened by ELISA for specificity to PfRH5 and PfCyRPA.

3.15 Restriction digest of plasmid vector and variable region cDNA

After screening supernatants from transfectants using the TAP expression fragments for specificity to PfRH5 and PfCyRPA, V_H/V_L gene pairs that produced functional antibodies with specificity to PfRH5 and PfCyRPA were cloned by restriction cloning. The plasmid vectors were obtained from Simon Draper (University of Oxford). The

plasmids were AbVec-hIgG for the V_H , AbVec-hIgKappa for V_K and AbVec-hIgLambda for V_λ . The restriction enzymes used for both the backbone and gene inserts are Fast digest BshT1 (Thermofisher Scientific, UK), Sall (Thermofisher Scientific, UK), Pfl23II (Thermofisher Scientific, UK), XhoI (Thermofisher Scientific, UK). BshTI was used for 5' end digestion of the plasmids and Sall, Pfl23II, XhoI were used for the 3' end digestion of AbVec-hIgG1, AbVec-hIg-Kappa and AbVec-hIgLambda respectively. The plasmid digestion reaction mix contained 1µg plasmid, 1µL each of the 5' and 3' digestion enzyme, 2µL of rSAP (New England Biolabs, UK), 4µL fast digest buffer (Thermo scientific, UK) and deionized H₂O in a final volume of 40µL. The digestions were carried out at 37°C for 1hr and subsequently inactivated at 80°C for 10 mins. The digested products were run on an agarose gel and the linear backbone purified using the QIAquick gel extraction kit (Qiagen, UK) following manufacturer's protocol. The reaction mix for digestion of the variable region cDNA contained, 0.5µL each of the 5' and 3' digestion enzyme, 3.4µL fast and 30µL of the gel extracted cDNA from PCR2. The digestion were carried out at 37°C for 1hr and subsequently inactivated at 80°C for 10 mins.

3.16 Ligation of plasmid vector and variable region cDNA

For ligations, an insert: plasmid molar ratio of 1:1 was used in a reaction mix containing 0.5µL quick ligase (New England biolabs, UK), 1.5µL fast digest buffer, 15µL H₂O in final volume of 20µL. Ligations were carried out at room temperature for 5-10 min.

3.17 Transformation of bacterial cells.

Stellar™ competent cells (Takara Bio, USA) were used for transformations. The cells were thawed on ice before use and 15µL of cells aliquoted into ice-cold tubes. In brief, 2µL of the ligation mix was then added to the cells and incubated on ice for 20 min. The

cells were then heat shocked at 42°C for 30 s and placed on ice again for 2 min. Subsequently, 50µL SOC media (Sigma Aldrich, UK) was added to the tubes and incubated at 37°C. After incubation, the cells were plated out on carbenicillin plates and incubated at 37°C overnight.

3.18 Thawing of parasites

Cryovials containing frozen parasites were retrieved from liquid nitrogen and allowed to rapidly thaw at 37°C. The contents of the vials were transferred into 50mL Falcon tubes. The volume of the thawed iRBC pellet was estimated and 0.1x volume of thawed iRBCs of 12% NaCl (VWR, USA) was added to the Falcon tubes whilst swirling the tubes and kept at room temperature for 5 min. After 5 min, 10x volume of thawed iRBC of 1.6% NaCl was added and centrifuged (800×g, 5min). The supernatant was discarded and the pellet washed once with incomplete parasite medium. The pellet was transferred into a T25 culture flask (Thermofisher Scientific, UK) and resuspended in parasite culture medium (RPMI with 25mM HEPES supplemented with 0.5% Albumax) (Invitrogen, USA), 0.2% normal human serum, 50 µg/mL gentamicin sulphate (Sigma Aldrich, Germany), and 4 mM Glutamine (Sigma-Aldrich, Germany). The parasites were cultured in O⁺ uninfected RBC at 2% haematocrit, gassed and incubated at 37°C.

3.19 *In vitro* parasite culture

All parasite culture work was carried out in a Class II Biosafety cabinet which was regularly cleaned with 70% ethanol. After thawing the parasites, they were maintained in parasite culture medium and cultured in a gas mixture of 2.0 % O₂, 5.5 % CO₂, and 92.5 % N₂ and incubated at 37°C. Culture media were changed every day and parasitemia estimated by preparing thin smears and staining with 10% Giemsa stain as described

earlier (3.4). Routine cultures were maintained at 2% parasitemia by diluting with washed O⁺ RBCs, obtained from RDT- and microscopy-negative adult volunteers. Venous blood was drawn into heparin vacutainers (Becton Dickinson, USA) and washed twice in incomplete parasite media to remove white blood cells and platelets by centrifuging at 500×g for 8 min. The washed RBCs were re-suspended at 50% hematocrit in incomplete parasite medium and stored at 4°C.

3.20 *In vitro* growth inhibition assay

In vitro growth inhibition assays were performed as previously described (Miura *et al.*, 2009). Culture-adapted field parasites were initially treated with 5% sorbitol to obtain a tightly synchronised culture. Sterile filtered monoclonal antibodies were mixed with trophozoite-iRBCs in a total volume of 40µL at 2% haematocrit in 96-well plates and incubated for 90-96h. The starting parasitemia was 0.8-0.9%. The final parasitemia was determined by lactate dehydrogenase (LDH) assay. The underlying principle of the LDH assay relies on the ability of *P. falciparum* LDH enzyme to catalyse the oxidation of lactate using 3-acetyl pyridine NAD (APAD) as a coenzyme. Percentage inhibition was calculated relative to the end parasitemia in uninhibited wells that served as controls.

$$\%GIA=100-100(\text{test sample } A_{650}\text{-uRBC } A_{650})/(\text{iRBC control } A_{650}\text{-uRBC } A_{650})$$

3.21 Statistical Analysis

The differences in antibody levels among different patient categories were evaluated using One-way ANOVA and Dunn's post-hoc test. Spearman's rank correlation test was used to assess associations between day 0 antibody levels and log transformed parasitemia among malaria positive donors. Correlations between merozoite antigen-specific IgG responses was assessed by Spearman's rank correlation test The 50%

inhibitory concentration (EC_{50}) values from the GIA assays obtained by interpolating values from a non-linear variable slope four-parameter curve using Prism 6.0 (Graphpad Software, Inc.). Data analysis was performed using the Sigma plot statistical software package (Systat Software, Inc.) and Prism 6.0 (Graphpad Software, Inc.). The level of statistical significance was tested at 0.05 or less for all the analysis.

CHAPTER FOUR

4 RESULTS

4.1 Clinical characteristics of study population

The clinical and demographical characteristics of the study participants are summarized in Table 4.1. In total, 210 children were recruited for the study with an age range from 1 to 12 years. Of these, 45% were females and 55% were males. One hundred and eighteen children were recruited at the hospital, of which 66, 42 and 10 suffered uncomplicated malaria, severe malaria and febrile illness respectively. The parasitemia on the day of admission ranged from 2,639 to 1,600,795 parasites/ μ L in the symptomatic group with a median parasitemia of 40,200 parasites/ μ L in the uncomplicated malaria patients and 52,510 parasites/ μ L in the severe malaria group. The asymptomatic children had parasite densities ranging from 296 to 1,924 parasites/ μ L with a median parasitemia of 1,370 parasites/ μ L. The median parasitemia among the uncomplicated and severe malaria donors were significantly higher ($P < 0.001$) than in the asymptomatic group. Within the symptomatic group, there was no significant difference in the median parasitemia between the uncomplicated and severe malaria children. Haemoglobin levels differed significantly among the different patient groups with severe malaria children having significantly lower haemoglobin levels compared to uncomplicated patients ($P < 0.001$), febrile patients ($P = 0.017$), healthy donors ($P < 0.001$), and asymptomatic donors ($P < 0.001$). The haemoglobin levels among the uncomplicated patients was significantly lower compared to the healthy donors ($P = 0.004$), but not the asymptomatic donors or febrile patients ($P > 0.05$).

Table 4.1: Clinical characteristics of study participants

Category	Uncomplicated Malaria (n=66)	Severe malaria (n=42)	Febrile Control (n=10)	Asymptomatic control (n=32)	Healthy control (n=60)
Gender					
Female	33 (50%)	14 (33.3%)	1 (10%)	16 (50%)	31 (51.7%)
Male	33 (50%)	28 (66.7 %)	9 (90%)	16 (50%)	29 (48.3%)
Age (years)¹	4 (1-12)	5 (1-8)	5 (2-12)	6 (1-10)	5 (1-10)
Haemoglobin level (g/dL)¹	10 (5.4-14.2)	7.8 (4.1-11.8)	11.05 (6.3-13)	11 (65-12.8)	11.55 (4.2-15)
Parasitemia (parasites/uL)¹	40200 (2893-649257)	52510 (2639-1600795)	0	1370 (296-1924)	0

Study participants reporting to the hospital were classified as uncomplicated *P. falciparum* malaria and severe *P. falciparum* malaria based on WHO guidelines. Febrile controls refer to children who had fever but not malaria and reported to the hospital. Asymptomatic controls were age matched community controls who were parasitemic but were not symptomatic. Healthy controls were age matched community controls who were non-parasitemic and without clinical symptoms.¹Median (range).

4.2 Seroprevalence and levels of IgG specific for PfRH5 complex proteins and other merozoite proteins.

To establish the immunogenicity of PfRH5 and its interacting partners PfCyRPA and Pf113, the seroprevalence, levels and subclass of merozoite antigen-specific IgG amongst 108 Ghanaian children who reported to the hospital sick and were confirmed to be infected with *P. falciparum* were examined. Seroprevalence to PfRH5 and PfCyRPA was 20% and 18% respectively (**Figure 4.1A**). In contrast, more than half the patients were seropositive to other well-studied merozoite antigens, GLURP R2 and EBA-175. The prevalence of IgG responses to Pf113 and GLURP R0 was intermediate with a seroprevalence of 40% and 38% respectively. PfRH5- and PfCyRPA-specific IgG levels were lower, with only one patient having PfRH5-specific IgG levels and none of the patients having PfCyRPA-specific IgG levels above 5x the negative cutoff (**Figure 4.1B**). Thus, immunogenicity to PfRH5 complex antigens appeared to be low compared to the other studied merozoite antigens among the children in this study following natural exposure to *P. falciparum* infection. The predominant IgG subclasses specific for PfRH5

complex antigens were IgG1 and IgG3 (Fig 4.1C). PfRH5- and EBA-175-specific IgG levels correlated significantly with each other ($P = 0.01$) but not with the other studied merozoite antigens (Table 2), suggesting that antibody levels to these antigens are regulated differently from the other merozoite antigens.

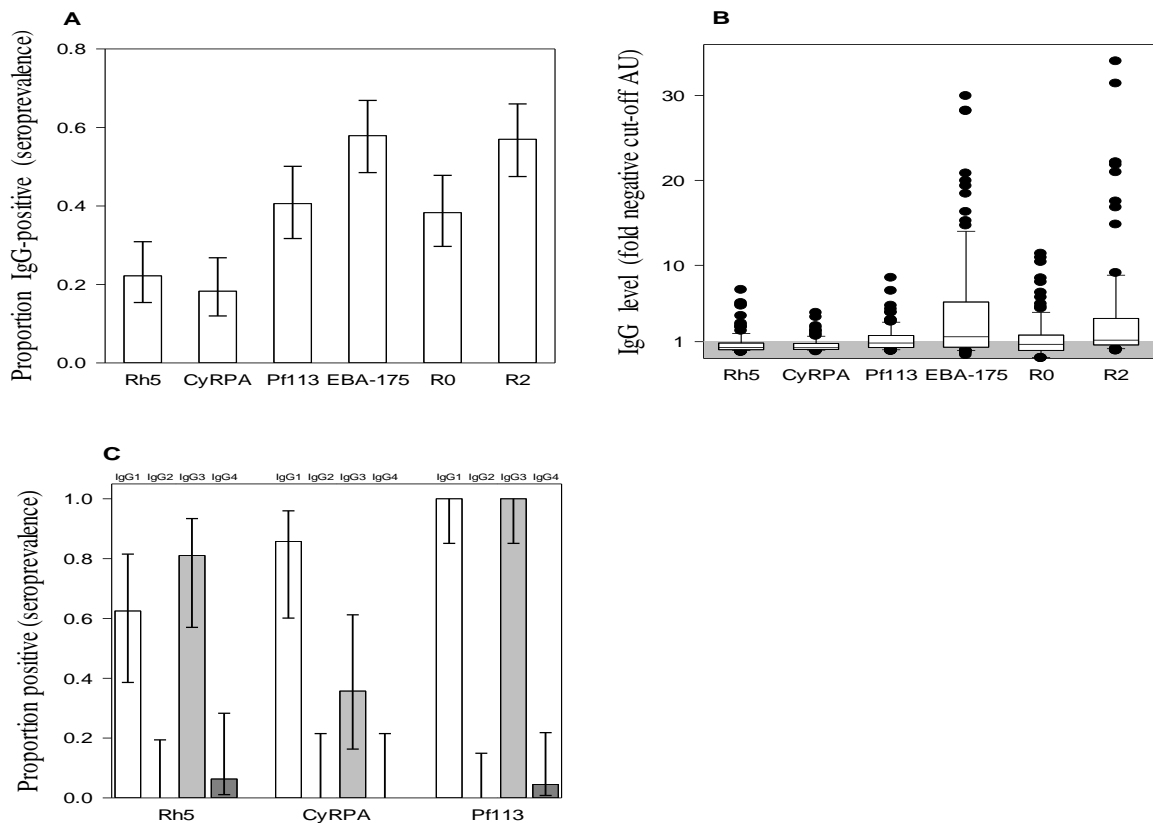


Figure 4.1: Seroprevalence of merozoite specific IgG and IgG subclass responses to merozoite antigens in acute malaria children.

A) Proportion of participants positive (antigen specific IgG levels above the negative cutoff) for merozoite antigens in children suffering acute malaria. Error bars indicate 95% confidence interval. **B)** Merozoite specific IgG levels (arbitrary units, AU) in acute malaria patients. Data represented as box plots with median (central line) dividing the interquartile range (IQR) and outliers (dots). Shaded area represents negative cutoff for each antigen. Negative cutoff was calculated as the mean AU of non-exposed controls + 3S.D. **C)** IgG subclass reactivity in plasma to PfRH5, PfCyRPA and Pf113. Bars indicate mean proportions of IgG1 (white), IgG2 (black), IgG3 (gray) and IgG4 (dark gray). Error bars indicate 95% confidence interval.

Table 4.2: Correlations between merozoite antigen-specific IgG responses

Antigens	Spearman correlation coefficient ρ [associated P-values]				
	PfCyRPA	Pf113	EBA-175	GLURP R0	GLURP R2
PfRH5	0.16 [0.1]	0.08 [0.4]	0.24 [0.01]	0.10 [0.3]	0.12 [0.2]
PfCyRPA		0.62 [<0.001]	0.03 [0.8]	0.32 [0.001]	0.36 [<0.001]
Pf113			0.18 [0.06]	0.28 [0.004]	0.39 [<0.001]
EBA-175				0.003 [1.0]	0.16 [0.1]
GLURP-R0					0.29 [0.002]

Correlation coefficient was determined by Spearman's rank correlation. IgG specific for PfCyRPA, P113, GLURP R0 and GLURP R2 correlated significantly with each other at P values < 0.05 . Significant correlation between PfRH5-specific IgG and EBA-175-specific IgG levels at P values < 0.05 but no correlation with the other antigens.

4.3 Association of merozoite-specific IgG levels with clinical presentation

Previous studies have associated IgG levels to merozoite antigens including some of the antigens under investigation here with protection from *P. falciparum* malaria in natural infections and following vaccination (Tran *et al.*, 2014, Douglas *et al.*, 2015, Weaver *et al.*, 2016). To determine if each of the antigen-specific IgG levels measured in the donors here were associated with protection from, or decreased susceptibility to *P. falciparum* malaria, the study participants were divided into different clinical categories: uncomplicated malaria (UM), severe malaria (SM), asymptomatic malaria (AC), febrile control (FC) and healthy control (HC) (**Figure 4.2**).

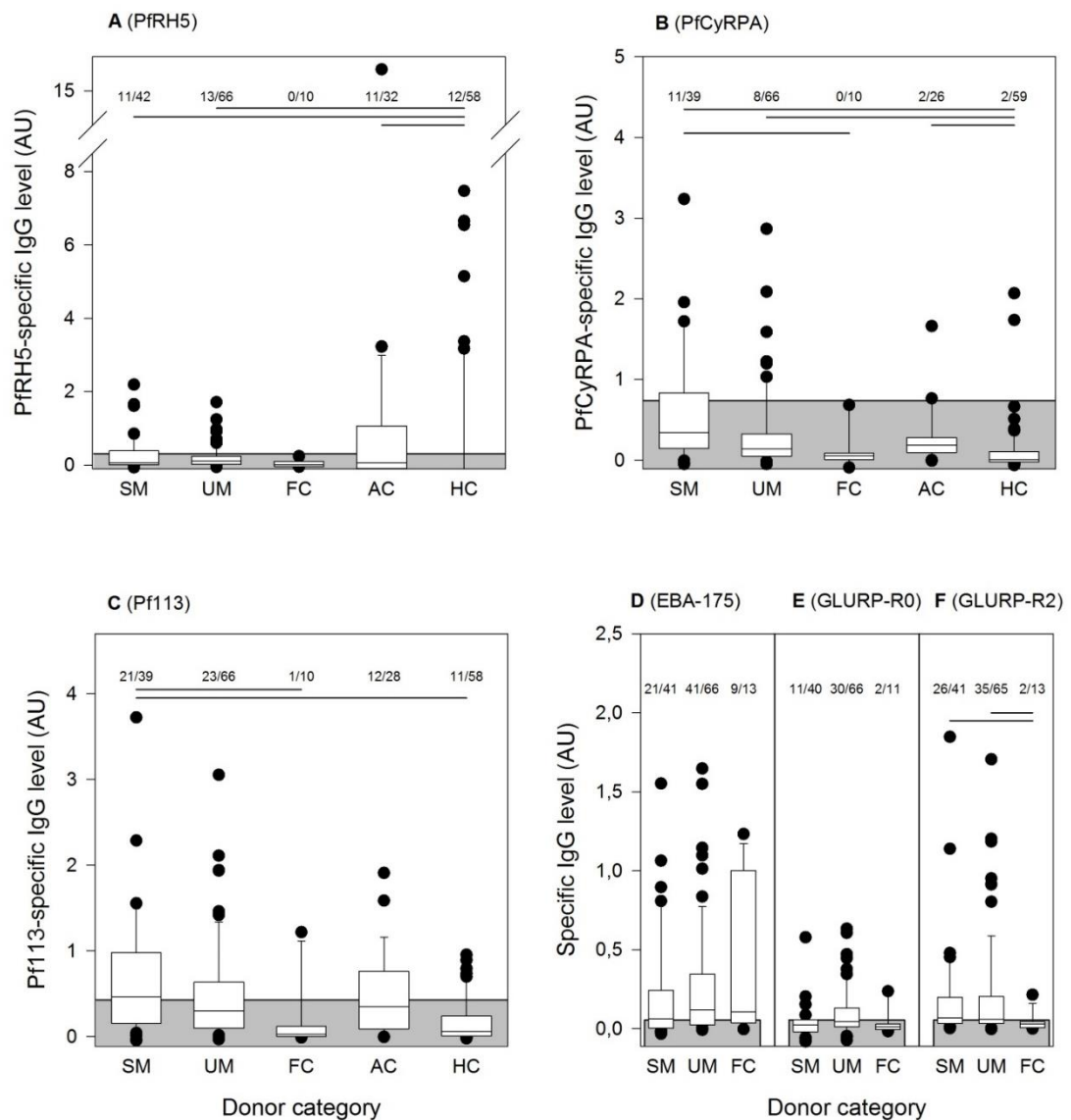


Figure 4.2: Association of IgG levels with clinical *P. falciparum* categories

A) PfrH5 specific IgG levels amongst patients suffering severe *P. falciparum* malaria (SM), uncomplicated *P.falciparum* malaria UM, febrile patients(FC), asymptomatic donors (AC) and healthy donors (HC). PfcyRPA (B) PF113 (C), EBA 175 (D), GLURP R0 (E) and GLURP R2 (F) specific IgG levels amongst the different patient categories. Shaded area represents negative cutoff for seropositivity. The number of individuals with IgG levels above the negative cutoff together with the total number of participants is indicated for each group. Horizontal lines above each panel indicate statistically significant differences between groups.

For PfrH5, the median IgG level was significantly lower in the healthy uninfected individuals (HC) in comparison to the UM patients ($P < 0.001$), SM patients ($P < 0.001$), and AC donors ($P = 0.007$) (Figure 4.2A). Within the parasitemic group the median

PfRH5-specific IgG levels did not differ between the UM, SM and AC donors. The prevalence of PfRH5 responders did not differ significantly among the different patient categories ($P(\chi^2) = 0.24$), although the proportion of responders was modestly higher (34%) within the asymptomatic group compared with the other patient groups (<27%). With respect to PfCyRPA, the median IgG level was significantly lower among the non-parasitemic HC donors compared to UM ($P < 0.001$), SM ($P < 0.001$) and AC donors ($P < 0.001$). The PfCyRPA-specific IgG levels among the FC group was significantly lower compared to the SM group ($P = 0.020$). The median PfCyRPA-specific IgG levels did not differ between the UM, SM and AC donors. Although the proportion of seropositive individuals did not differ significantly between the different donor groups ($P(\chi^2) = 0.24$), PfCyRPA responders were also higher in the SM (28%) than in the other patient categories (<13%) (**Figure 4.2B**). For Pf113, only the SM patients had a significantly higher median IgG level than the febrile ($P = 0.004$) and healthy donors ($P < 0.001$). Similar to PfRH5 and PfCyRPA, prevalence of Pf113 responders did not differ significantly among the different patient categories ($P(\chi^2) = 0.22$) (**Figure 4.2C**). Median IgG levels and seroprevalence to our control antigens, GLURP R0 and EBA175 did not differ significantly across the different patient categories (**Figure 4.2D**) (AC and HC groups were not tested in the present study). For GLURP R2, there was a significant increase in the median IgG level and proportion of responders in the UM ($P = 0.029$) and SM ($P = 0.016$) patients compared to the febrile children. Median IgG levels to all the merozoite antigens under study did not correlate significantly with parasitemia in clinical patients on day of admission (**Figure 4.3**).

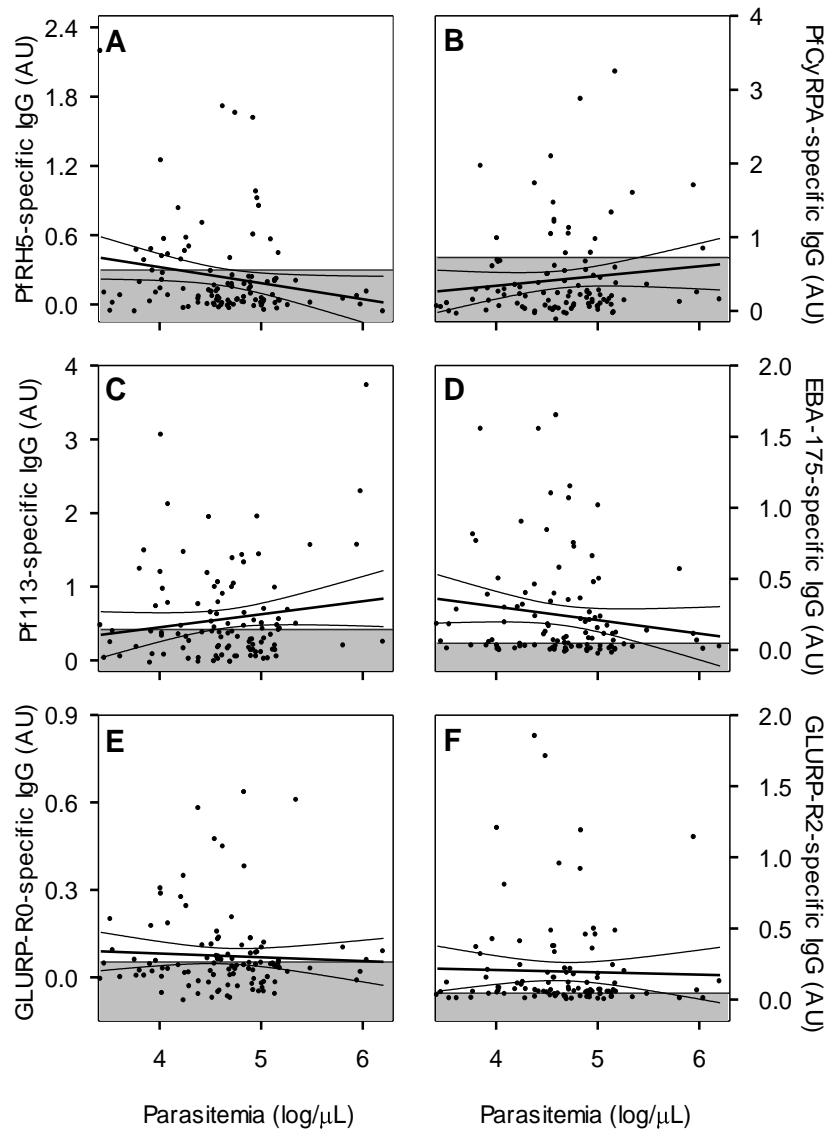


Figure 4.3: Correlation of IgG levels with parasitemia on day of admission
 Relation of median IgG levels to PFRH5 (A), PfCyRPA (B), PF113(C), EBA175 (D), GLURP R0 (E) and GLURP R2 (F) with parasitemia amongst clinical patients. Black dots represent individual parasitemia with corresponding IgG level. Lines indicate linear regression and 95% confidence interval. Shaded area represents negative cut-off for seropositivity.

4.4 Kinetics of merozoite-specific IgG levels following *P.falciparum* malaria

With the exception of EBA 175, merozoite-specific IgG levels in the different patient categories were significantly higher in donors with detectable parasitemia (UM, SM, AC) than in non-parasitemic controls (FC) (Figure 4.2). This suggests that IgG levels in this

cohort were markers of recent exposure to *P. falciparum* parasites and the induced antibody secreted may be short-lived.

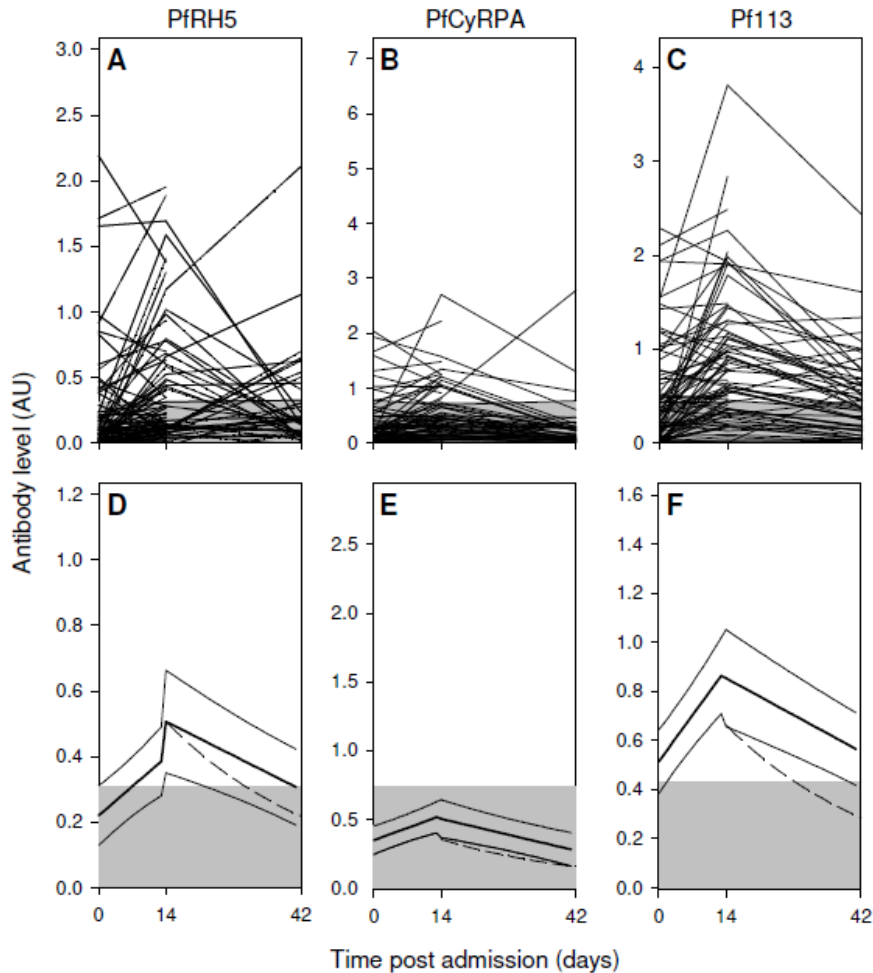


Figure 4.4: Kinetics of merozoite specific IgG levels following *P.falciparum* infection.

Antigen specific IgG levels for fFRH5 (A, D), PfCyRPA (B, E) and Pf113 (C, F) in symptomatic *P. falciparum* patients (UM and SM) measured in plasma collected on days 0, 14, and 42. Temporal changes in IgG levels in individual patients (top panel). Mean IgG levels of cohort indicated in heavy line with 95% C.I in thin lines (bottom panel). Dashed line indicates catabolic decay of IgG. Negative cutoff represented in gray in all panels.

Hence, the kinetics and longevity of IgG levels to the PfrRH5 complex proteins (PfrRH5, PfCyRPA and Pf113) in our malaria patients from the day of admission until six weeks later were examined. A large proportion of the donors did not have antibodies against the

tested antigens on the day of admission and remained so during the follow-up period (**Figure 4.4A-C**). However, a few of the donors had IgG levels above the negative cutoff, which peaked by day 14 and declined by day 42. In general, responses to the studied antigens seemed to be short lived (**Figure 4.4D**). Antibody decline rate for all three antigens appeared to follow the catabolic rate of IgG (dashed line).

4.5 PfrH5-specific IgG and its role in clinical protection

Several studies have established an association between merozoite antigen-specific IgG levels and clinical protection from *P. falciparum* malaria. More recently, clinical trials with different PfrH5 vaccine formulations have demonstrated protective efficacy of vaccination in *Aotus* monkeys (Douglas *et al.*, 2015). In the present study, levels of PfrH5-specific IgG levels were generally low (**Figure 4.1**) and did not correlate with protection from clinical disease (**Figure 4.2** and **Figure 4.3**). However, a minority of the donors had PfrH5-specific IgG levels sufficiently high to be of potential clinical significance (**Figure 4.2A**). Thus, a subset of our donors were tested in an additional standard ELISA which has been used in a clinical trial (Payne *et al.*, 2017) to estimate IgG levels predictive of merozoite invasion inhibition in *in vitro* GIA. Data from the two assays strongly correlated ($r=0.92$) ($P<0.001$, $N=76$) (**Figure 4.5**). PfrH5-specific IgG titres among our study participants ranged from 5.5ng/mL to 1.5 μ g/mL. Thus, PfrH5 specific IgG levels among the donors were not sufficiently high enough to predict GIA *in vitro*.

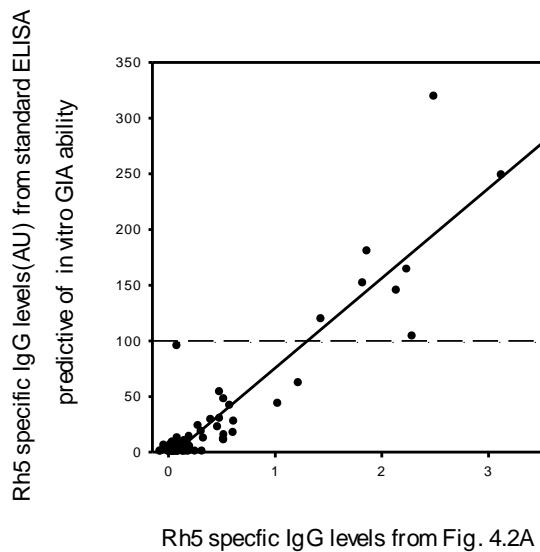


Figure 4.5: Role of Rh5 specific IgG levels in clinical protection.

Correlation of Rh5 specific IgG levels from standard ELISA assays showing proportion of donors with IgG levels above 100 A.U. Dashed line represents minimum IgG level predictive of *in vitro* growth inhibitory ability.

The data presented from the child cohort showed that PfRH5-specific antibodies were sustained in a minority of asymptomatic children (**Figure 4.2A**). Hence, the seroprevalence of PfRH5-specific IgG levels among adults living in an area of medium malaria transmission intensity was examined. During the first year of sampling (2014), only about 35% of the adults had PfRH5-specific IgG above cut-off (**Figure 4.6**). The proportion of responders in the adult cohort was similar to that in the asymptomatic children. In the subsequent year (2015), a subset of only the individuals who were seropositive in the previous year were recruited and plasma IgG tested again. About half of them [46 % (24.8-69.9%) 95% confidence interval] had maintained their PfRH5-specific plasma IgG levels a year later. This suggests some of the donors had sustained PfRH5-specific plasma IgG levels for about a year or had been recently exposed to *P. falciparum* infection. Although the PfRH5-specific plasma IgG levels in 2015 were

generally low, the IgG levels could not be directly compared to IgG levels in 2015 as the results were from two independent ELISA assays.

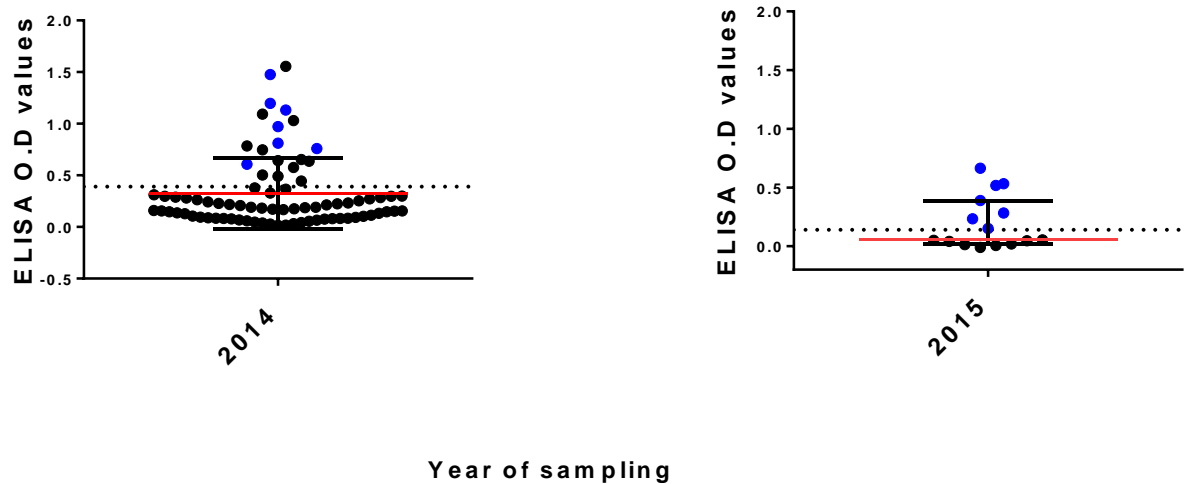


Figure 4.6: PfrH5 specific IgG levels in semi-immune adults.

Immune reactivity of semi immune adult plasma collected in 2014 (A) and 2015 (B). Individual ELISA O.D plotted with median (red horizontal line) and interquartile range (Error bars). Negative cut-off (short dashed horizontal line). Data points in blue are samples that were positive in both 2014 and 2015.

4.6 Isolation of human anti-PfrH5 and anti-PfCyRPA monoclonal antibodies

Monoclonal antibodies are important biological tools with wide applicability in research and therapy. The high specificity and complete homogeneity of monoclonal antibodies make them useful research tools. Human monoclonal antibodies have aided in the identification of protective antigens or epitopes and thus guided the design of vaccines in human Cytomegalovirus (CMV) (Wang & Shenk 2005) and *P. falciparum* infections (Tan *et al.* 2016; Barfod *et al.* 2007). Therefore, the objective of the study was to produce human monoclonal antibodies to PfrH5 and PfCyRPA from semi-immune adults. The strategy employed in the present study was to immortalize isolated memory B cells with Epstein-Barr virus and subsequently clone cells producing relevant monoclonal antibodies by a single-cell cloning method.

B cells from three donors (MC107, FC105 and MC103) who were seropositive for PfrH5 were infected with EBV to produce antibody secreting polyclonal B cell cultures. The number of 96-well cell cultures per donor ranged between 20 and 30 plates. Only a few cultures from each donor produced antibodies specific for PfrH5 with success rates ranging from 0.1 to 0.2%. Some of the wells that tested positive in the first screen (**Figure 4.7B**, plate 6) lost their specificity in the second screen (**Figure 4.7E**, plate 6). In most of those wells, a closer look at the cells under the microscope revealed cell death among the B cell cultures. A total of five B cell lines that produced antibodies specific for PfrH5 were established, designated 2B, 6.8B, 3.3G, 7.12E and 10.1B.

An initial ELISA test to determine if donors from the same adult cohort acquired PfCyRPA-specific IgG antibodies after natural exposure to *P. falciparum* malaria did not identify MC107, FC105 and FC103 as having PfCyRPA-specific plasma IgG. Nevertheless, immortalization of memory B cells from donor MC107, but not FC 103 and FC105, (**Figure 4.8**) yielded antibody-secreting B cell cultures with specificity for PfCyRPA. This is not surprising, as antibodies are secreted by plasma cells resident in the bone marrow. The absence of circulating antibodies does not imply the development of antigen-specific memory is hampered as shown by others (Ampomah *et al.*, 2014). A total of three B cell lines that produced antibodies specific for PfCyRPA were established, designated 1B4E, C3C and 1B.

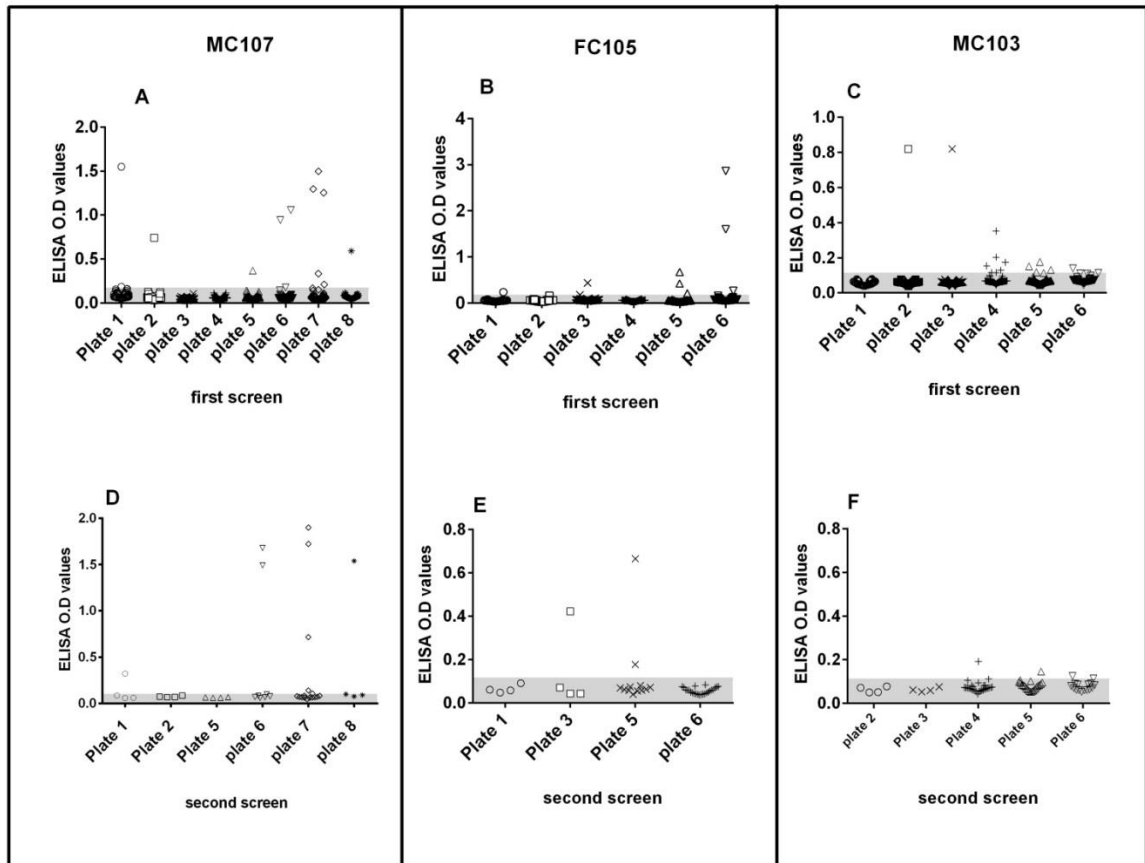


Figure 4.7: ELISA screen of EBV immortalized memory B cell culture supernatants for specificity to PfrH5

Memory B cells by binding CD22 microbeads were isolated from three donors MC107 (A), FC105(B) and MC103(C) were infected with EBV virus to generate antibody secreting B cells. Three weeks post infection, B cells were screened for reactivity to PfrH5. Each data point in top panel represents pooled supernatants collected from four adjacent wells. Positive wells from the first screen were then tested individually in a second screen (C, D, E). Cut-off for reactivity indicated in shaded area.

To clone the polyclonal B cell cultures following EBV immortalization, different strategies were attempted with no success. The polyclonal B cells were initially cloned by limiting dilution and subsequently by single-cell sorting into 384-well cell culture plates in the presence of gamma-radiated PBMC feeder layer. Both methods resulted in death of the cells. To overcome the challenges of cloning the monoclonal antibodies, the polyclonal cultures that were specific for PfrH5 or PfCyRPA were single-cell sorted,

followed by cloning of the IgG genes and expressing them as recombinant monoclonal antibodies.

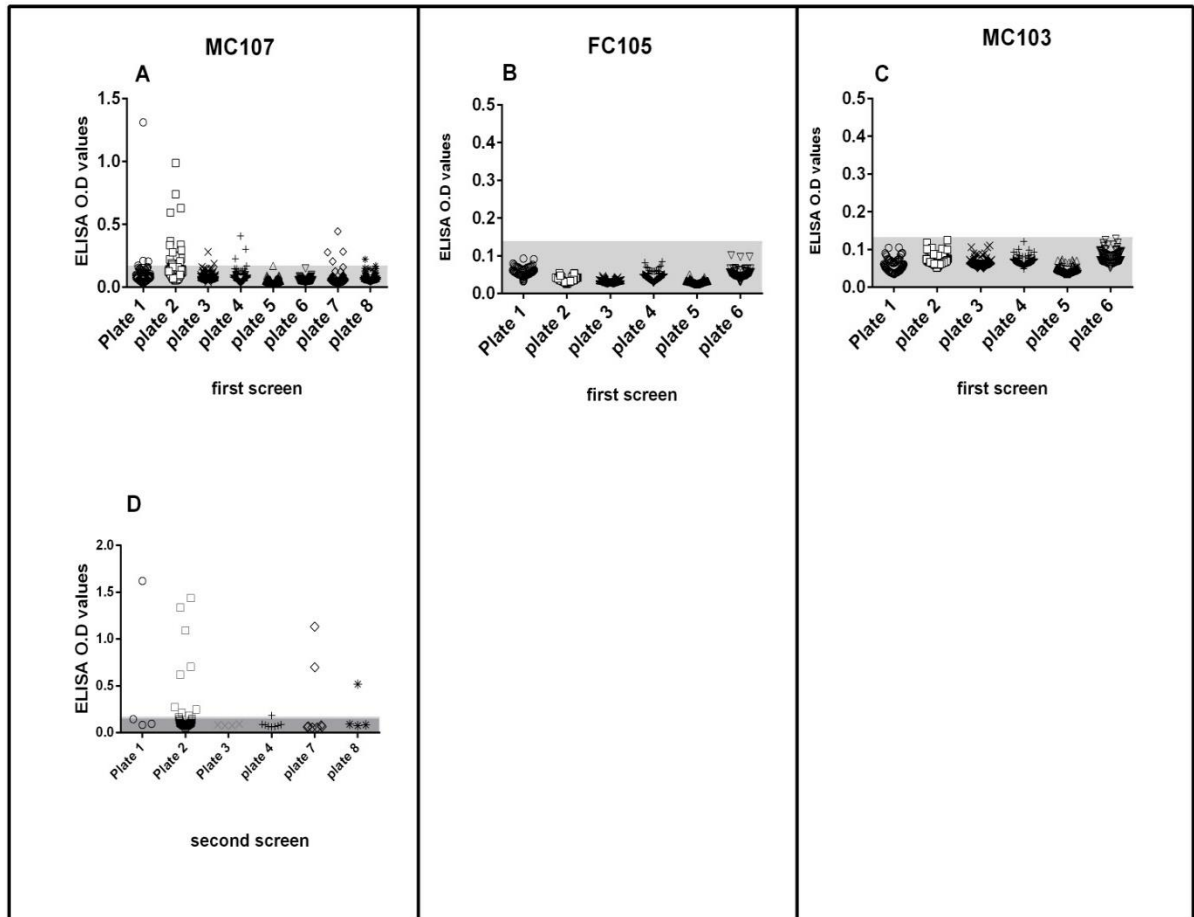


Figure 4.8: ELISA screen of EBV immortalized memory B cell culture supernatants for specificity to PfCyRPA.

ELISA screen of supernatants from EBV immortalized memory B cells isolated from three donors MC107 (A), FC105 (B) and MC103 (C). Three weeks post immortalisation, only MC107 produced B cell cultures secreting antibodies specific to PfCyRPA. Each data point in top panel (A-C) represents supernatants collected from four adjacent wells. Positive wells from the first screen were then tested individually in a second screen (D). Cut-off for reactivity indicated in shaded area.

4.7 Single-cell RT-PCR and IgG gene amplification of antigen specific polyclonal cell lines

To increase the efficiency of cloning the EBV-transformed polyclonal B cells specific for PfRH5 or PfCyRPA, a strategy was adapted that enabled the generation of monoclonal antibodies by amplifying the IgG genes and subsequently cloning them into

an expression vector. The method used allowed the preservation of related pairs of heavy chain (HC) and light chains (LC). In all, an attempt was made to clone three CyRPA-specific (1B, 1B4E and C3C) and five RH5-specific (2B, 6.8B, 10.1B, 3.3G, and 7.12E) polyclonal B cell lines into monoclonal antibodies. Results of one PfCyRPA (1B4E) and two PfrH5 (2B and 6.8B) specific mAbs are shown in this section (see appendix for rest of the results).

Figure 4.9 shows amplification of the V_γ and V_λ genes of single B cells sorted from a PfCyRPA-specific polyclonal cell line, 1B4E. For this cell line, cDNA was amplified from twenty-four single B cells. All the 24 cells produced amplicons for the V_γ (**Figure 4.9**, top panel) that correspond to ~420 bp and only 19 cells produced amplicons for V_λ (**Figure 4.9**, bottom panel) of ~430 bp (Lanes A2, A5, B2, B7 and B9 did not yield products for V_λ).

For PfrH5, cDNA was amplified from twenty-four single B cells sorted from PfrH5-specific polyclonal cell line 2B (**Figure 4.10**) and forty-eight single B cells from polyclonal cell line 6.8B (**Figure 4.11** & **Figure 4.12**). The IgG light chain for both 2B and 6.8B was kappa light chain. All the 24 cells from cell line 2B produced products for V_γ (**Figure 4.10**, top panel) and V_κ of ~395 bp (**Figure 4.10**, bottom panel).

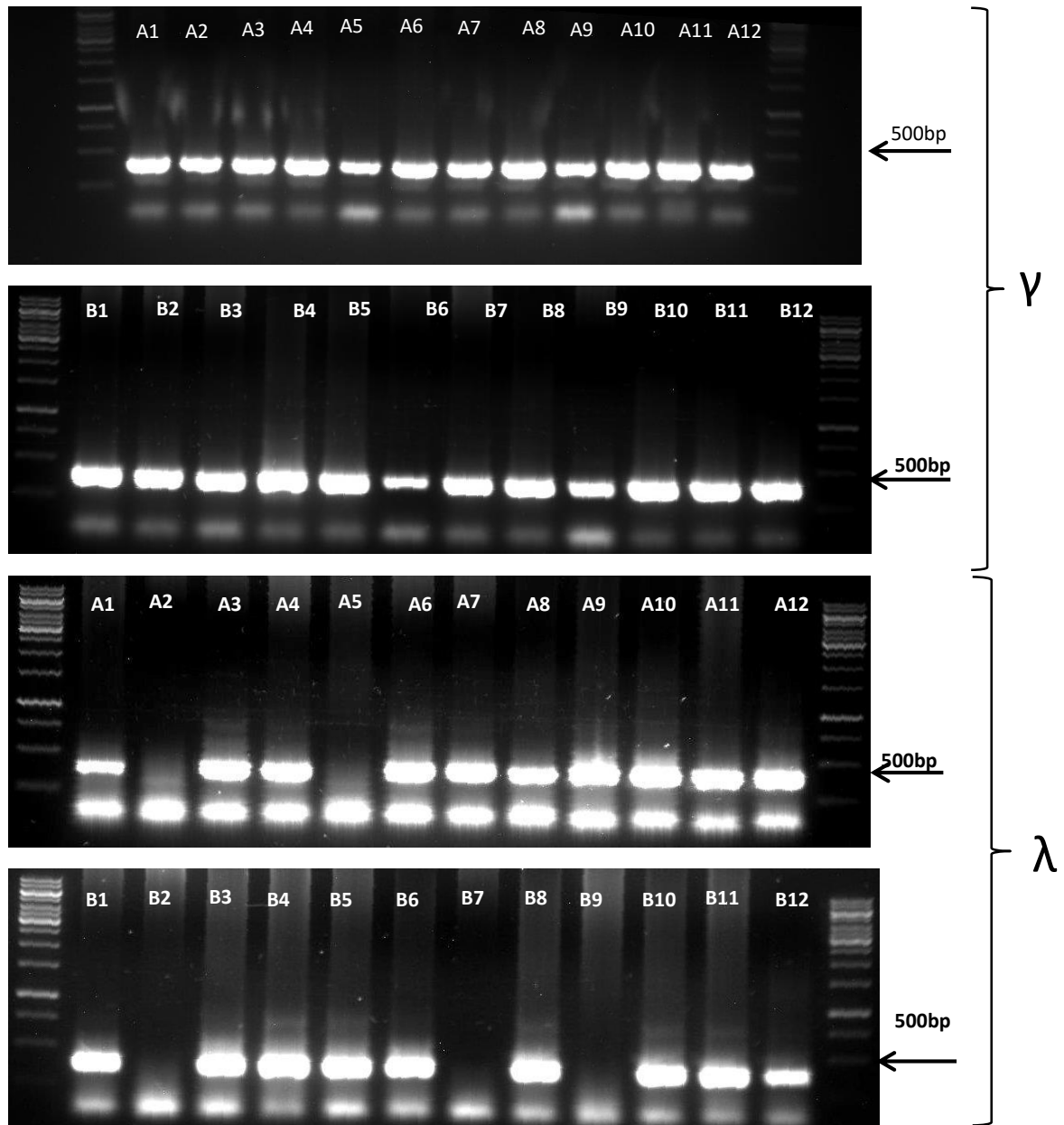


Figure 4.9: Amplification of V_γ and V_λ genes from CyRPA specific polyclonal cell line 1b4E which was single cell sorted into 24 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is 420 bp and 430 bp for V_γ and V_λ . PCR products were run on 1% agarose gel.

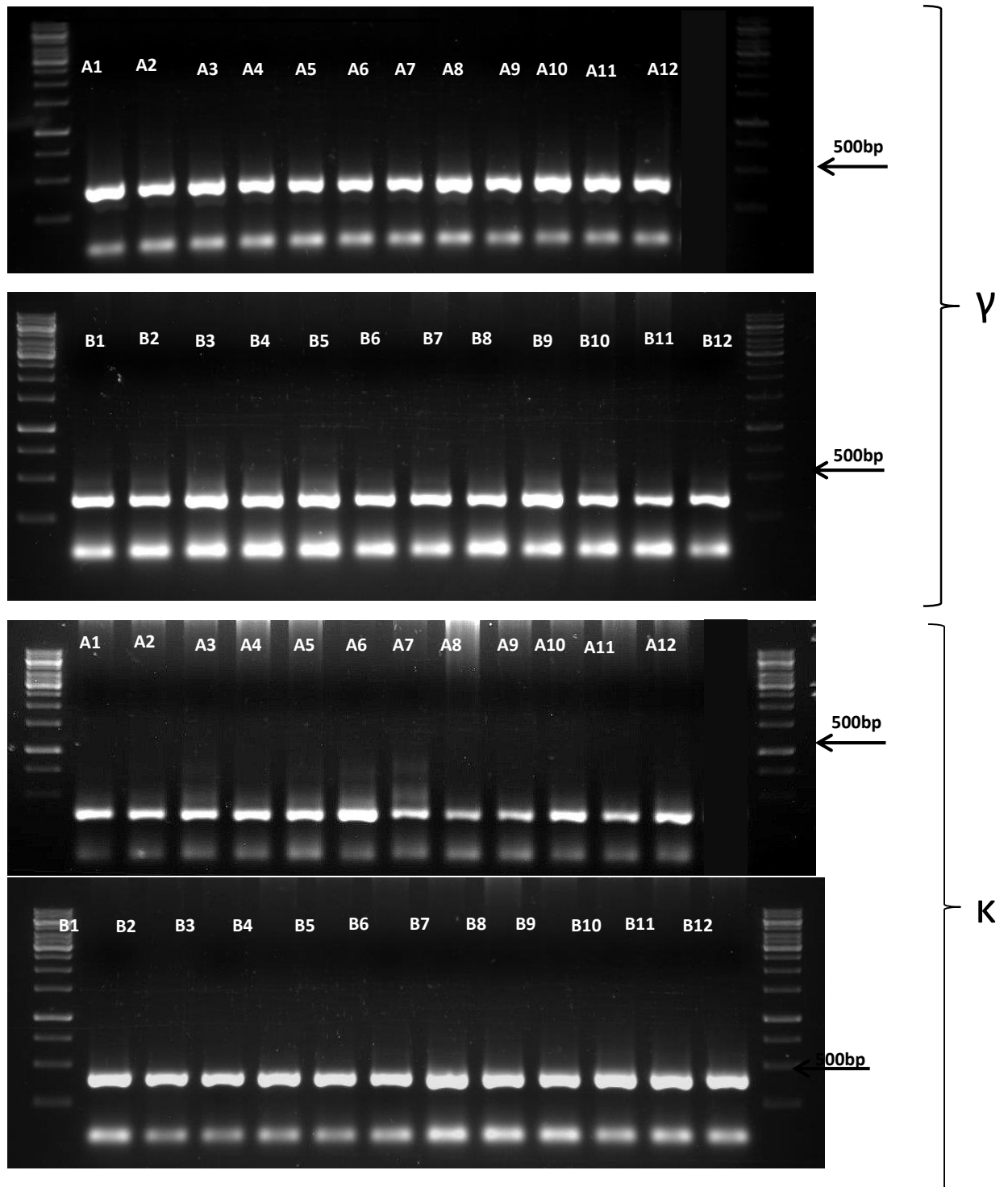


Figure 4.10 Amplification of V_{γ} and V_{κ} genes from RH5 specific polyclonal cell line 2B which was single cell sorted into 24 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is 420 bp and 395 bp for V_{γ} and V_{κ} . PCR products were run on 1% agarose gel.

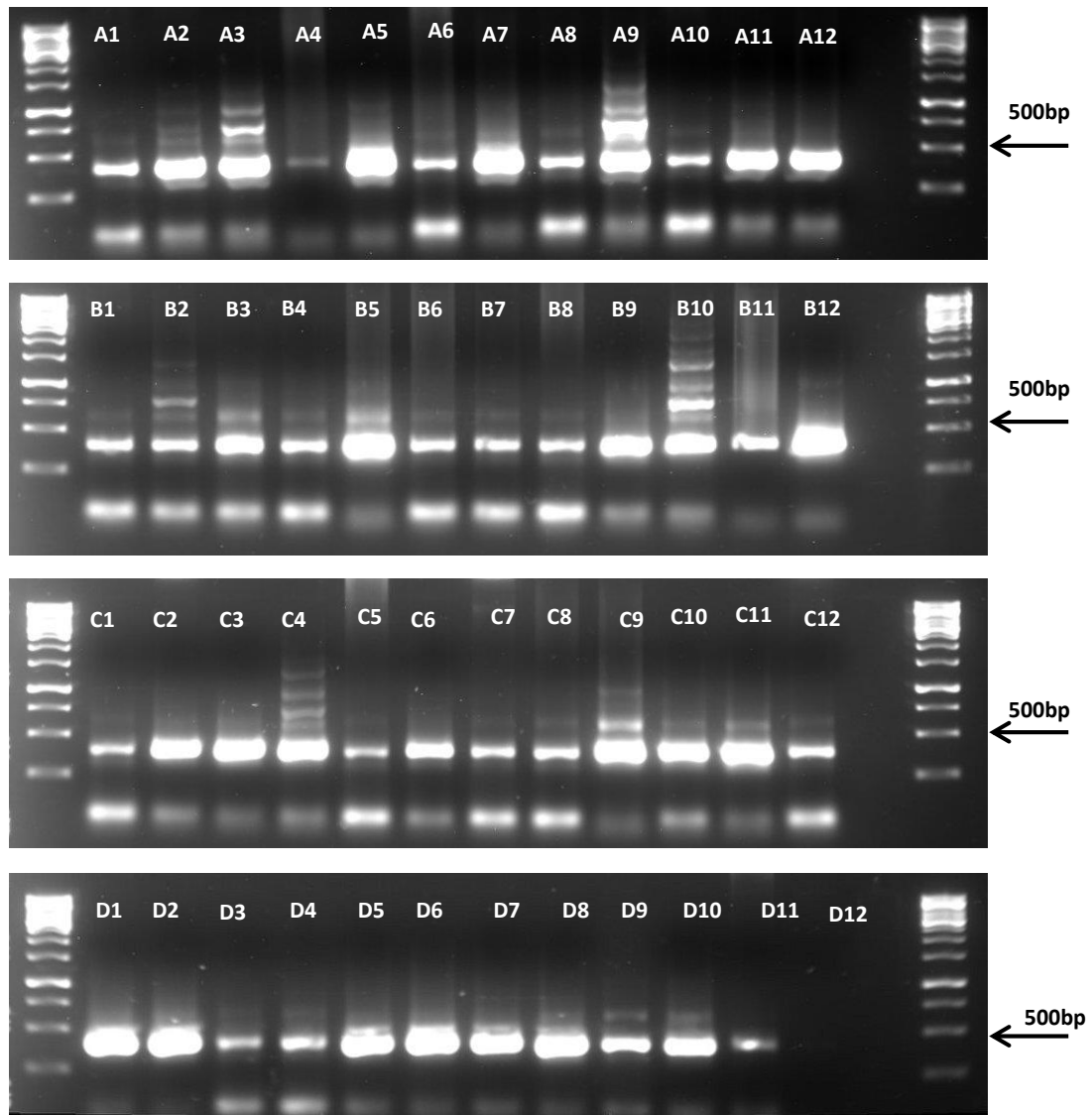


Figure 4.11: Amplification of V γ genes from RH5 specific polyclonal cell line 6.8B which was single cell sorted into 48 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is 420bp for V γ . PCR products were run on 1% agarose gel.

For PfrH5-specific polyclonal cell line 6.8B, out of the forty-eight single B cells sorted, forty-seven produced amplified products for V γ (Figure 4.11) (lane D12 did not yield product) and forty-three produced amplified products for V κ (Figure 4.12) (lane A1, A9, A12, B1 and B12 did not yield product)

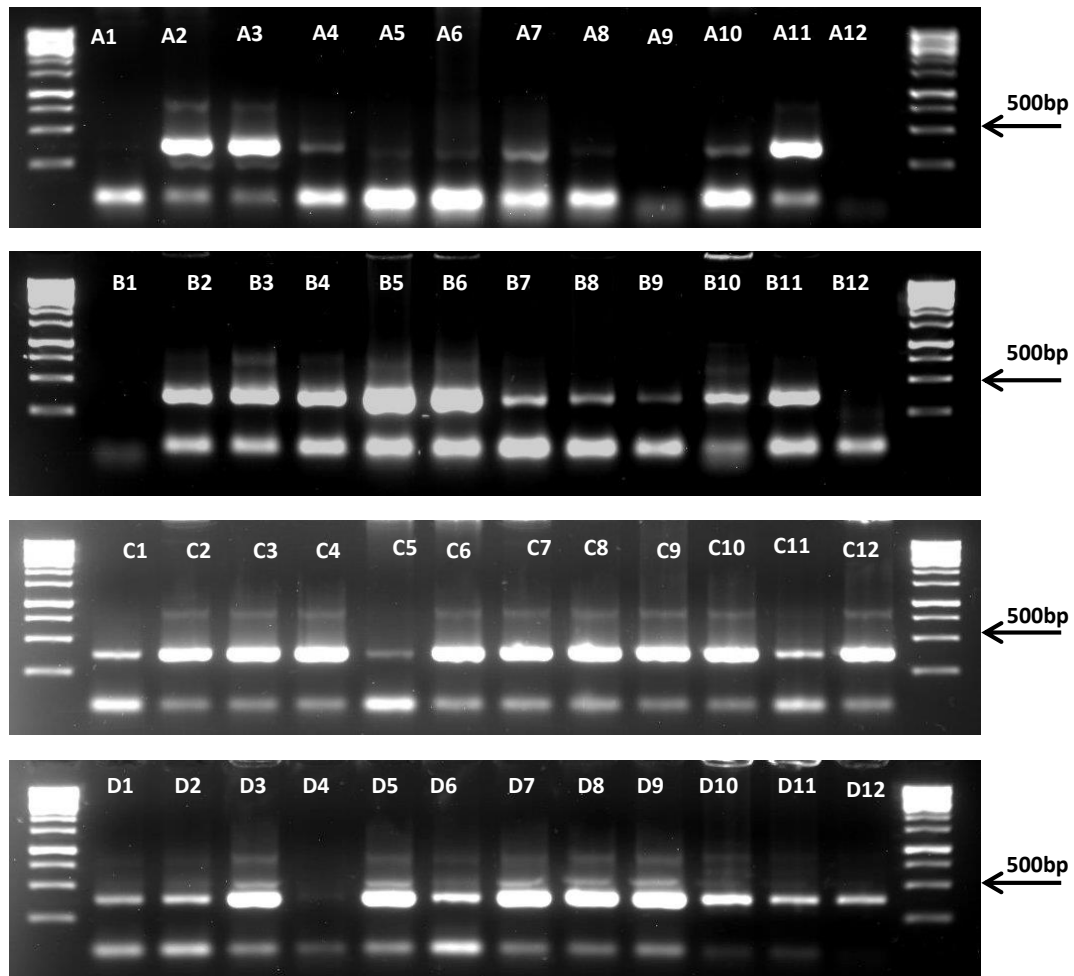


Figure 4.12: Amplification of V_{κ} genes from RH5 specific polyclonal cell line 6.8B which was single cell sorted into 48 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is 395bp for V_{κ} . PCR products were run on 1% agarose gel

4.8 Cloning of V_H/V_L gene pairs into expression vectors

After amplifying cDNA from the variable regions of the IgG heavy and light chains of the single B cells, random V_H/V_L gene pairs from each antigen-specific cell line were used in the TAP process to generate linear DNA expression fragments. In this experiment, eight, twelve and thirteen random V_H/V_L gene pairs from cell lines 1B4E, 2B and 6.8B respectively were used. **Figure 4.13** shows the TAP products for PfCyRPA-specific cell line 1B4E. **Figure 4.14** and **Figure 4.15** shows the TAP products for PfRH5-specific cell line 2B and 6.8B respectively. The TAP method was efficient as all the gene

pairs from the three different cell lines produced TAP fragments with the V_H TAP fragment of ~3 kb and V_L TAP fragment ~2.3 Kb.

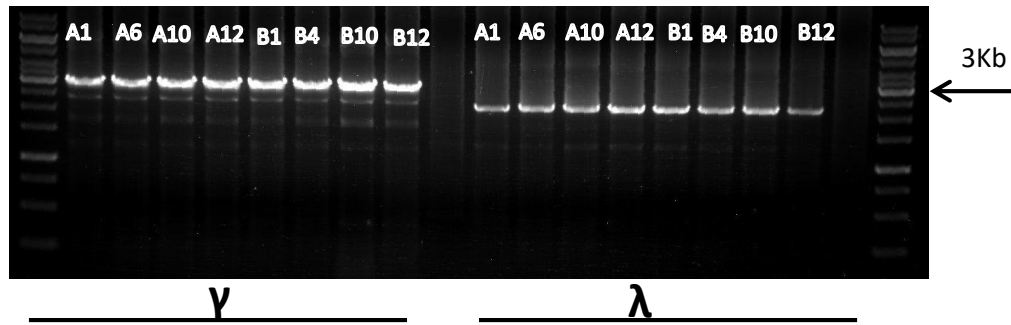


Figure 4.13: TAP expression fragments generated from amplified V_H/V_λ gene pairs from cell line 1B4E

Random V_H/V_L gene pairs previously amplified in PCR 2(Fig 4.4) were used in producing TAP expression fragments for transfecting HEK cells. Expected band size for V_H TAP is 3kb and V_λ is 2.3 kb. PCR product were run on 0.8% agarose gel

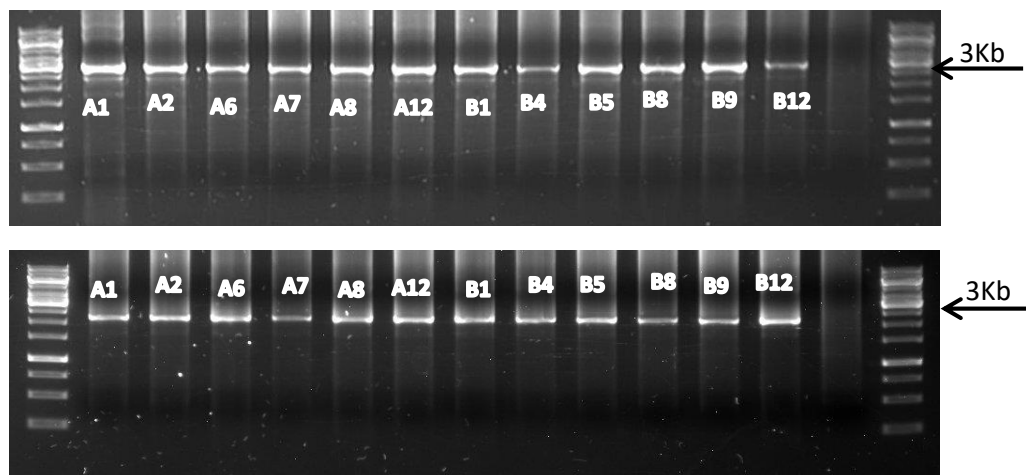


Figure 4.14: TAP expression fragments generated from amplified V_H/V_κ gene pairs from cell line 2B

Random V_H/V_κ gene pairs previously amplified in PCR 2(Fig 4.5) were used in producing TAP expression fragments for transfecting HEK cells. Expected band size for V_H TAP (top panel) is 3kb and V_κ (bottom panel) is 2.3 kb. PCR product were run on 0.8% agarose gel

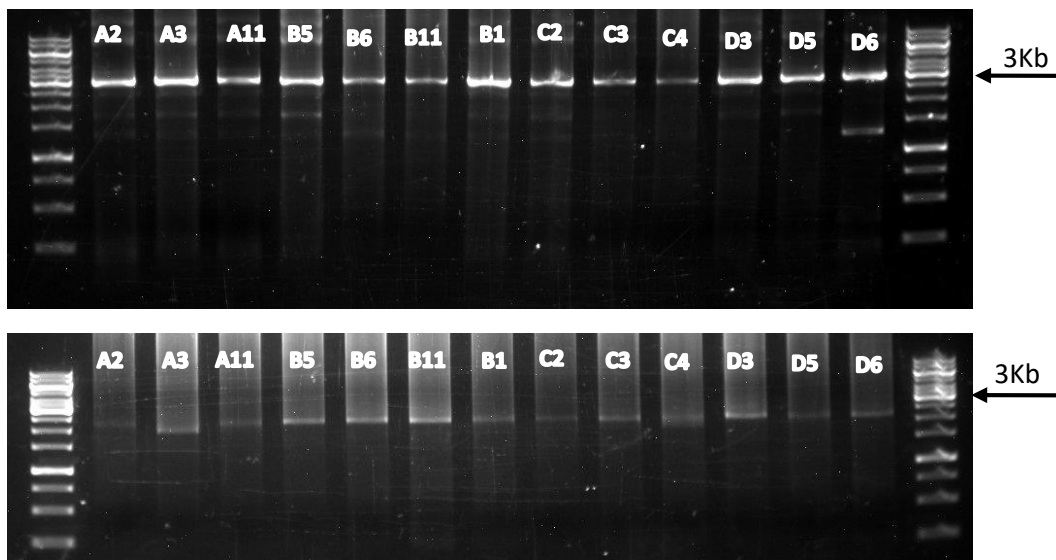


Figure 4.15: TAP expression fragments generated from amplified VH/VL gene pairs from cell line 2B

Random V_H/V_L gene pairs previously amplified in PCR 2 (Fig 4.6-7) were used in producing TAP expression fragments for transfecting HEK cells. Expected band size for V_H TAP (top panel) is 3kb and V_κ (bottom panel) is 2.3 kb. PCR products were run on 0.8% agarose gel.

4.9 Antibody production and screening for specificity

The HC/LC TAP fragment pairs generated were used in co-transfecting HEK 293a cells by using 25kDa polyethylenimine (PEI). Transfections were carried out in single wells of 96-well culture plates and incubated for 3 days. The supernatants containing the secreted antibodies were collected and tested by ELISA. In the original work by (Smith *et al.* 2009), they sequenced the plasmids to check that the genes had been cloned in the correct reading frame before transfection. To cut down cost and save time, the transfectants were first screened for reactivity to PfRH5 and PfCyRPA and for human IgG production (**Figure 4.16**). All the transfectants tested produced human IgG (**Figure 4.16 A-C**). However, the antibodies produced were not specific for PfRH5 (**Figure 4.16 E-F**). A few transfectants from PfCyRPA cell line 1B4E were weakly positive (**Figure 4.16D**). The gene pairs showing reactivity were then cloned, sequenced and the monoclonal antibodies expressed in HEK 293 cells. To functionally characterize the

monoclonal antibodies produced, the abilities of the antibodies to inhibit merozoite invasion *in vitro* was tested in GIA against the *P. falciparum* 3D7 strain, but no inhibitory effect was observed with the monoclonal antibodies. A panel of human anti-PfRH5 monoclonal antibodies isolated from naïve adults vaccinated with viral vectored PfRH5 was obtained from a collaborating laboratory and tested for their growth inhibition activity

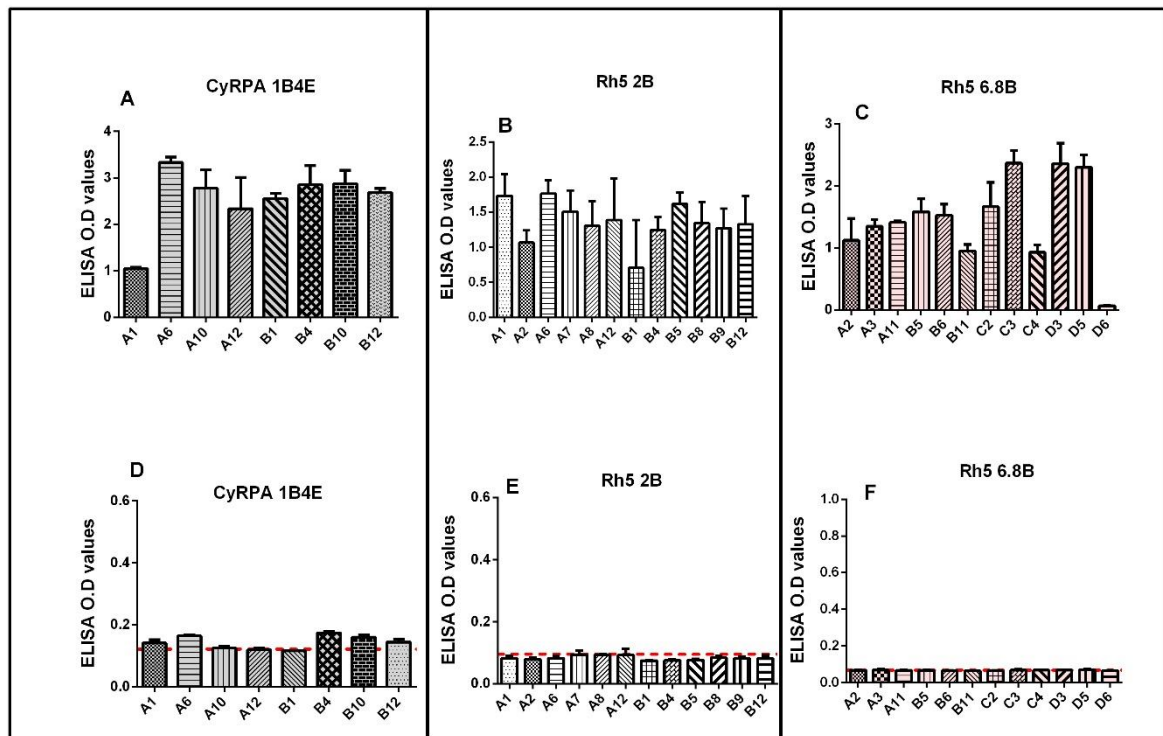


Figure 4.16: Antibody secretion and reactivity.

Supernatants from transfectants were screened for anti human IgG production (A-C top panel) and specificity of the secreted antibodies to PfCyRPA(D) and PfRH5 (E&F). Red dashed line in bottom panel represents cut-off for reactivity.

4.10 *In vitro* growth inhibition with human anti-PfRH5 monoclonal antibodies

To test the growth inhibitory effect of the anti-PfRH5 monoclonal antibodies on merozoite invasion *in vitro*, clinical isolates were cultured in the presence of the antibodies for two cycles of merozoite invasion. The panel of anti-PfRH5 monoclonal

antibodies tested showed a strain transcending inhibitory effect albeit at varying neutralizing potencies (**Figure 4.17**), with *in vitro* %inhibition ranging from 98% (R5.004) to 50 % (R5.007) at 500 μ g/mL.

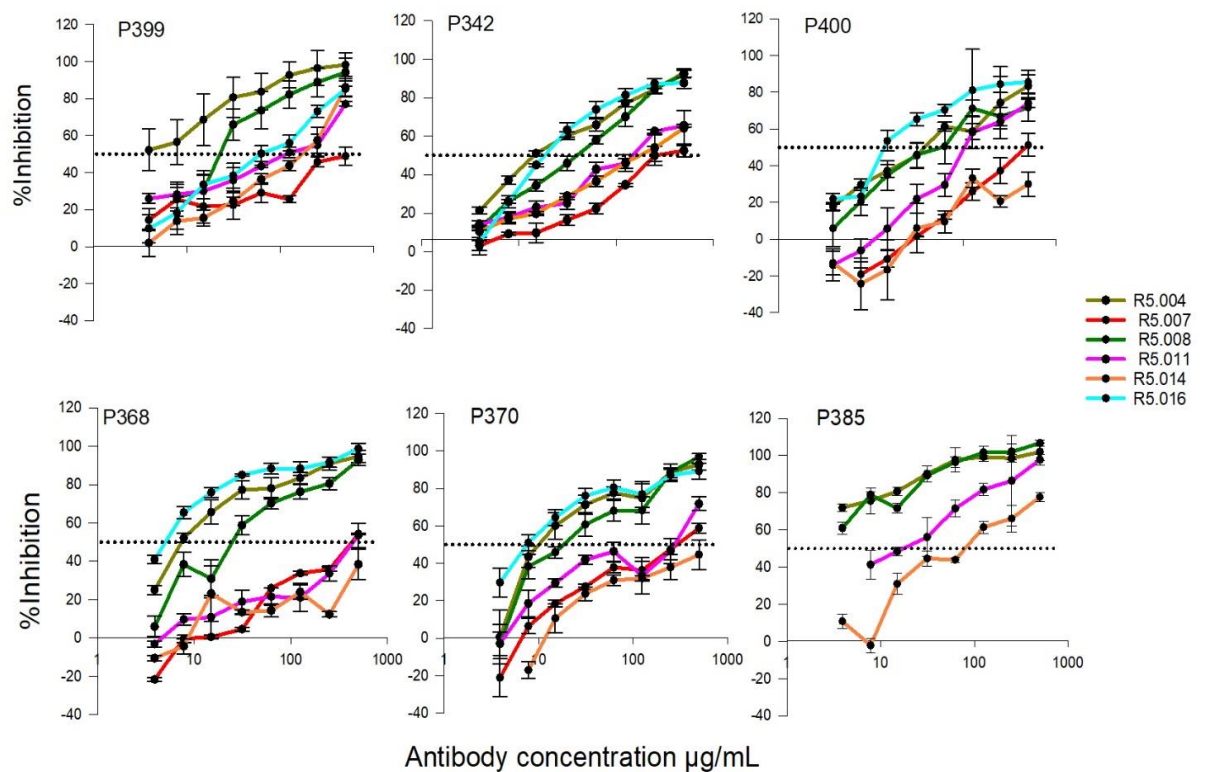


Figure 4.17: Growth inhibition effects of panel of human monoclonal antibodies

A two fold dilution series of a panel of human anti-PfRH5 monoclonal antibodies (R5004, R5007, R5008, R5011, R5014 and R5016) was tested in GIA with six different clinical isolates (P342, P368, P370, P385, P399 and P400). Data represents mean of replicates from two independent experiments. Error bars represent standard error of the mean. The EC_{50} values for each monoclonal antibodies were obtained by interpolating values from a non-linear variable slope four-parameter curve using Prism 6.0.

The antibodies tested inhibited the clinical isolates in a concentration dependent manner.

The strain transcending effect observed in this study is consistent with earlier reports using mouse anti-PfRH5 antibodies (Douglas *et al.*, 2014) and purified human anti-PfRH5 specific IgG (Patel *et al.*, 2013). Furthermore, the range of inhibitory efficiency

recorded here is comparable with anti-PfRH5 monoclonal antibodies (Douglas *et al.*, 2014) or other merozoite antigens (Aniweh *et al.*, 2016, Favuzza *et al.*, 2016, Maskus *et al.*, 2016). Based on the calculated EC₅₀ values (**Table 4.3**), the monoclonal antibodies can be grouped into three categories; highly neutralizing mAbs (R5.004, R5.008 and R5.016), medium neutralizing mAbs (R5.011 and R5.014) and low neutralizing mAb (R5.007).

Table 4.3: EC₅₀ values of anti PfRH5 monoclonal antibodies against different clinical isolates

Antibodies	EC ₅₀ values(µg/mL) of Clinical Isolates					
	P342	P400	P399	P368	P370	P385
R5.004	16.74	39.70	3.42	7.82	10.52	ND
R5.007	324.58	472.11	ND	448.72	298.41	ND
R5.008	37.63	39.60	23.47	22.12	18.26	ND
R5.011	126.40	112.13	134.68	492.56	131.10	6.56E-05
R5.014	176.43	ND	153.37	ND	ND	1.86E-06
R5.016	18.00	16.74	64.83	4.86	7.42	ND

CHAPTER FIVE

5 DISCUSSION

Developing an effective malaria vaccine remains a top priority in malaria control efforts particularly at a time when an increase in reported malaria cases is becoming evident. Much of vaccine development studies have focused on merozoite antigens of *P. falciparum*, the most virulent form of malaria. The goal of targeting merozoite antigens is to induce high titre antibodies that disrupt merozoite invasion to prevent asexual blood stage replication. However, identifying the antigen targets of protective immunity have been difficult and antigenic diversity in parasite antigens have made vaccine development challenging. The PfRH5 complex proteins have emerged as leading blood stage vaccine candidates due to the essential role of PfRH5 in merozoite invasion (Baum *et al.*, 2009), limited polymorphisms (Manske *et al.*, 2012) and the strain *transcending in vitro* inhibition of anti-PfRH5 antibodies (Douglas *et al.*, 2011, Patel *et al.*, 2013, Reddy *et al.*, 2014). Although a few studies have examined the immunogenicity of PfRH5 in natural infections (Douglas *et al.*, 2011, Reddy *et al.*, 2012, Villasis *et al.*, 2012, Patel *et al.*, 2013, Tran *et al.*, 2014), not much has been studied about the other complex proteins Pf113 and PfCyRPA. Hence, this study sought to first examine the immunogenicity of PfRH5, PfCyRPA and Pf113 following natural infections.

In the present study, children suffering from severe malaria had significantly higher parasite densities than the uncomplicated or asymptomatic donors. The high parasite densities within the severe malaria donors may contribute to but does not solely account for the severe pathogenesis. Other known factors such as host immune status, host inflammatory responses and parasite virulence all contribute to severe malaria pathogenesis. Although the median age of children within each patient category did not

differ significantly, the haemoglobin levels among the severe malaria group was significantly lower. In malaria endemic regions, severe anemia is a common complication of malaria, which is further worsened by co-infection with other infectious diseases such as HIV and helminthic infections. Indeed, previous studies have associated severe anaemia and parasitemia in 85% of hospital admissions in Kenya (Obonyo *et al.*, 2007). In Ghana, severe anaemia have been shown to be the most common manifestation of severe malaria in children under five years (Oduro *et al.*, 2007). Low haemoglobin may be caused by haemolysis of iRBC, clearance of iRBC by the spleen and dyserythropoiesis (White *et al.*, 2014).

Here, we show that PfRH5 seroprevalence among the symptomatic children was similar to that reported in previous studies (Douglas *et al.*, 2011, Tran *et al.*, 2014). Proportion of responders to Pf113 in the symptomatic donors was 40%, which is slightly lower but comparable to the observed Pf113 seroprevalence reported by others in Kenya (50%) (Osier *et al.*, 2014b) and Gabon (51%) (Imboumy-Limoukou *et al.*, 2016a). The difference in Pf113 seroprevalence could be due to differences in the malaria endemicity between the various study sites or the age range of study participants. The study reveals for the first time that PfCyRPA was immunogenic as anti-PfCyRPA-specific IgG was induced following natural infections. The proportion of donors responding to PfCyRPA was much lower than Pf113 responders. Thus, immunogenicity of PfRH5 complex proteins is relatively low in comparison to other well-studied merozoite antigens. The poor immunogenicity of PfRH5 and its binding proteins could be due to the transient exposure of these antigens during the rapid process of merozoite invasion. It is also possible the antigens are presented in a way that masks the immunogenic epitopes.

The major IgG subclass response to the PfRH5 complex proteins was IgG1 and IgG3, which are both cytophilic antibodies. Earlier reports have identified IgG1 and IgG3 as

the dominant IgG subclass response to PfRH5 (Tran *et al.*, 2014, Weaver *et al.*, 2016). A longitudinal study with a child cohort in Papua New Guinea found a strong association between IgG3 and protection from malaria (Weaver *et al.*, 2016). For PfCyRPA, we show IgG1 is the major IgG subclass response. Similarly, IgG1 and IgG3 were the dominant IgG subclass response to Pf113. However, a recent study in Gabon has reported IgG3 and IgG4 as the major IgG subclass response to Pf113 (Imboumy-Limoukou *et al.*, 2016a). The nature of IgG subclass responses induced in malaria infections have been reported to be influenced by the intrinsic nature of the antigen (Stanisic *et al.*, 2009), host factors and the levels of parasite exposure among individuals (Tongren *et al.*, 2006). It has been shown that antibodies generated by vaccinations with the PfrH5 complex proteins can inhibit merozoite invasion *in vitro* by disrupting protein-protein interactions during the invasion process (Dreyer *et al.*, 2012, Williams *et al.*, 2012a, Payne *et al.*, 2017). The cytophilic nature of the antibody responses to the PfrH5 complex proteins observed in the present study suggest antibody function may involve other immune actors such as macrophages, complement system and natural killer cells.

The data show that plasma levels of IgG to PfrH5 are boosted following natural infections and do not protect from clinical symptoms or severe disease within the study population. This was similar for Pf113 and PfCyRPA antibodies. Previous studies have reported on naturally acquired PfrH5-specific antibodies in malaria endemic populations (Douglas *et al.*, 2011, Patel *et al.*, 2013, Weaver *et al.*, 2016), although there are conflicting reports on the association of PfrH5 plasma IgG levels and protection from clinical malaria. A longitudinal study which followed Malian children from the beginning of the malaria transmission season have reported naturally acquired PfrH5-specific antibodies were associated with protection against clinical malaria (Tran *et al.*, 2014). It is clear from the present study that plasma IgG levels to the studied antigens

were a reflection of recent parasite exposure and reflect transmission intensity as children with detectable *P. falciparum* had significantly higher antigen-specific antibody levels. This observation supports findings from earlier works examining anti-PfRH5 and anti-Pf113 antibodies in Gabonese children living in areas of different transmission intensities (Imboumy-Limoukou *et al.*, 2016b). Anti-PfRH5 plasma IgG levels were found to reflect transmission intensity and could be a marker for malaria surveillance.

The strength of this study is the longitudinal design of the study which allowed for examination of the longevity and kinetics of antigen-specific antibodies induced to PfRH5 complex proteins in natural infections. In all, antibodies induced to PfRH5, PfCyRPA and Pf113 waned off rapidly following treatment suggesting antibodies to the PfRH5 complex proteins are secreted by short-lived plasma cells. Continuous antigen exposure seems to be required, at least in children to maintain antibodies to PfRH5, PfCyRPA and Pf113, a phenomenon observed with other merozoite antigens (Akpogheneta *et al.*, 2008). Data from the phase Ia clinical trial with PfRH5 have shown an antibody concentration of 2µg/ml shows a 20% inhibition against 3D7 parasite strains (Payne *et al.*, 2017). The plasma PfRH5-specific IgG levels detected in this study were too low to induce any functional activity similar to that reported by others (Tran *et al.*, 2014). This might be the case for PfCyRPA and Pf113 in natural infections given the low plasma antigen specific-IgG levels observed in this study. The present study further confirms PfRH5 complex proteins as poor immunogens in natural infections and plasma anti-PfRH5 IgG levels in natural infections may be too low to play a role in NAI to malaria. In contrast, vaccinations with viral-vectored PfRH5 in *Aotus* monkeys (Douglas *et al.*, 2015) and humans (Payne *et al.*, 2017) induced appreciable levels of PfRH5-specific antibodies than found in naturally immune adults from Ghana and Kenya, that elicited growth inhibitory activity *in vitro*. Thus, highlighting PfRH5 as highly

immunogenic and favouring the development of PfRH5 based vaccines. The differences in immunogenicity of PfRH5 between natural infections and experimental vaccinations may be due to competition between antigens in natural infections or the reduced abundance of PfRH5 on merozoites.

Although PfRH5 is already featured in Phase I clinical trials, there are still some basic questions to be answered. We still do not fully understand the fine specificities of antibodies against the PfRH5 complex proteins in natural infections. In natural infections, antibodies are induced to both the protective and non-protective epitopes of an antigen. With other merozoite antigens like MSP1 and AMA-1 (Uthaipibull *et al.*, 2001), there have been reports of 'blocking or interfering' antibodies that block neutralising antibodies. Identifying the protective or functionally relevant epitopes would greatly enhance the development of blood stage vaccines targeting the PfRH5 complex proteins. This highlights the relevance of the current study, as isolated human anti-PfRH5 and anti-PfCyRPA monoclonal antibodies from naturally immune individuals would enable characterization of such functional epitopes.

To generate the human monoclonal antibodies, B cells from semi immune adults were immortalized with EBV to produce antibody-secreting B cell lines. At the beginning of the study, a small volume (20mL) of venous blood was drawn and used for PBMC isolation. The isolated PBMCs were frozen at -80°C overnight before transferring to a liquid nitrogen tank. Our attempts to immortalize the isolated PBMCs from the seropositive individuals in 2014 did not yield any B cell cultures. It became evident that not only does the amount of isolated PBMCs affect the efficiency of the immortalization, but the quality of the cells after thawing was also critical. To increase the efficiency of the EBV immortalization, in the second year of sampling, we collected a higher volume (60mL) of venous blood and cryopreserved the isolated PBMC using a controlled-

gradient freezer (Hviid *et al.*, 1993). Freezing the cells with the gradient freezer seems to preserve the functional quality of the thawed PBMCs as EBV-infection of thawed PBMCs from four donors all generated B cell clones with different specificities.

In screening for individuals who were seropositive for PfrH5 in 2014, 35% of the donors tested had detectable PfrH5-specific plasma IgG. This result was comparable to the proportions of responders in our asymptomatic children cohort. A subset of the seropositive individuals maintained their PfrH5-specific plasma IgG levels a year later. This is in contrast to observation in the children cohort where by six weeks following natural infection, antibodies wane off. The ability of the adults to maintain their PfrH5-specific plasma IgG levels a year later could be attributed to an age dependent acquisition of long-lived plasma cells (Hviid *et al.*, 2015) or reboosting of antibodies in recent infections. Three weeks after immortalization of the memory B cells, B cell cultures were growing in almost every well in the 96-well culture plates. However, only few polyclonal B cell lines from each donor were specific for PfrH5 or PfCyRPA. The frequency of PfrH5- and PfCyRPA-specific polyclonal B cell lines produced thus was relatively low, in contrast to our observations with FV6 (a PFEMP1 protein) and with a merozoite lysate (unpublished data). The low frequencies of PfrH5- and PfCyRPA-specific polyclonal B cell lines probably reflect the low frequencies of PfrH5- and PfCyRPA-specific memory B cells in natural infections. Given the low immunogenicity of PfrH5 and PfCyRPA in natural infections, it is not surprising that frequencies of PfrH5- and PfCyRPA-specific memory B cells in natural infections were indeed low. The PBMCs used were from donors who were PfrH5-responders, which justified their use, but none of them was seropositive for PfCyRPA. Nonetheless, one of the donors had PfCyRPA-specific memory B cells, which produced antibodies reactive to PfCyRPA. Our data are consistent with an earlier study, which did not find PfCyRPA-responders among immune

adults from the Kasena-Nankana district in the Northern region of Ghana (Dreyer *et al.*, 2012). Previous studies have also shown the development of memory B cell without detectable plasma IgG levels (Dorfman *et al.*, 2005).

Testing of the polyclonal B cell culture supernatants for specificity to the studied antigens yielded a higher number of reactive B cell cultures in the first screen than in the subsequent screens. Some of the B cell cultures lost their specificity as they were kept in culture for long. It is possible that the antibody-secreting cells were not fully transformed and hence died out over time. Another explanation is that the antibody-secreting cells might only be a small minority of the polyclonal B cell cultures, which gets crowded out by other cells in the culture which are not specific to the antigens of interest. To avoid loss of the antigen-specific polyclonal B cell cultures, we decided to clone the polyclonal B cells into monoclonal B cells right after the second screen, which was about four weeks after immortalization.

To clone the polyclonal B cells, we first sorted single cells from the B cell cultures into 384-well microtitre culture plates containing γ -irradiated PBMCs. There have been several studies reporting that the addition of irradiated feeder cells enhances EBV transformation and cloning (Hwang *et al.*, 2012, Sadreddini *et al.*, 2016). To our surprise, there was not a single B cell clone in the wells 2 weeks post sorting. The feeder layer cells were also, as expected dying, out after the 2 weeks. Our next approach was to clone the cells by limiting dilution and plate the cells out such that there were 10 cells/well or 0.5 cell/well. The 10 cells/well thrived and formed oligoclones whereas the 0.5 cell/well did not produce any B cell clone. It appears the transformed B cells do not thrive when cultured as single cells. Previous studies have successfully cloned EBV-immortalized polyclonal B cells into monoclonal B cell lines by limiting dilution (Traggiari *et al.*, 2004, Barfod *et al.*, 2007a).

Due to the difficulty in cloning the EBV-transformed polyclonal B cells by either FACS or limiting dilution, we decided to clone using the single-cell RT-PCR method. A similar approach was used by Liao *et al.* (2009) to clone EBV-transformed cell lines that produced antibodies neutralizing the HIV-1 viral protein gp41. In amplifying the V_H and V_L genes, most of the wells had amplicons, with a few negative wells without any PCR product. The negative wells without amplicons could be due to mRNA degradation or no cell placed in the wells at all. The sorting step did not include an antigen-staining step, which could be a limitation in the cell sorting process. The TAP system used offered a high throughput method to screen the V_H and V_L genes on large scale thereby avoiding the tedious and time-consuming steps of restriction cloning, transformation and screening for bacterial colonies with gene insert of interest. The process between generating TAP products for transfection and screening for the secreted antibodies was approximately 4 days.

In screening for the monoclonal antibodies, conscious efforts were made to reduce the detection of false-positive antigen-specific monoclonal antibodies. The media used for the transfection of HEK 293 cells was supplemented with Ultra low IgG fetal calf serum (FCS) to prevent cross-reactivity of bovine IgG in the ELISA. Following incubation of the transfection supernatant with the antigens, the plates were washed at least 6 times to reduce the binding of low-affinity monoclonal antibodies to the studied antigens. In addition, the secondary antibody used for detection in the ELISA was goat anti-human γ -chain-specific alkaline phosphatase. This was to select for IgG antibodies.

The secreted monoclonal antibodies from the tested V_H/V_L pairs isolated from the antigen-specific polyclonal B cells had low OD values in ELISAs to both PfRH5 and PfCyRPA. Nonetheless, plasmids were made for the positive monoclonal antibodies and were expressed in HEK 293 suspension cells. The purified antibodies were tested *in vitro*

GIA for their ability to inhibit merozoite invasion. None of the PfrH5-specific or PfCyRPA-specific monoclonal antibodies showed any inhibition *in vitro* (data not shown).

The next logical step was to go back to the original single-cell sorted plate and start the process over with new single cells. However, considering the time constraints for the study, PfrH5-specific human monoclonal antibodies were obtained from a collaborating laboratory. The human monoclonal antibodies were isolated from naïve adults immunized with PfrH5 in a Phase Ia clinical trial (Payne *et al.*, 2017). The monoclonal antibodies have been thoroughly characterized with respect to their interaction with PfrH5 complex proteins (unpublished data) and tested in *in vitro* GIA to several laboratory adapted *P. falciparum* strains. However not much is known about their ability to inhibit field stains *in vitro*. Hence, the PfrH5-specific monoclonal antibodies were tested in GIA with different field strains.

Antibodies have been shown to be the major immune effectors against the blood stages of *P. falciparum* infection (Cohen *et al.*, 1961b, McGregor, 1964, Sabchareon *et al.*, 1991). Mechanism of action of the antibodies involves opsonization targeting iRBC for phagocytosis, inhibiting merozoite invasion, inhibiting intraerythrocytic parasite development, or steric hindrance of essential protein-protein interactions. The neutralizing abilities of the human anti-PfrH5 monoclonal antibodies on clinical isolates from Hohoe were tested. Examining the inhibitory effects of the monoclonal antibodies does not only allow for characterization of each monoclonal antibody, but also identifying the binding sites of neutralizing monoclonal antibodies could lead to identification of protective epitopes on PfrH5. Although PfrH5 is believed to be relatively conserved with only 5 non-synonymous SNPs, testing the monoclonal antibodies on clinical isolates allows for the examination of effects of genetic diversity

of malaria parasites in natural infections. Genetic diversity of parasite antigens is one of the major challenges in developing blood stage vaccines, resulting in strain-specific vaccine induced immune responses and evolution of vaccine resistant strains.

To this end, all the monoclonal antibodies tested showed *in vitro* inhibition against all the tested clinical isolates. The strain transcending inhibitory ability of anti-PfRH5 monoclonal antibodies uniquely distinguishes PfRH5 and its complex proteins from other well studied merozoite antigens, favouring PfRH5 as potential next generation blood stage vaccine target. The neutralizing potencies of the panel of human anti-PfRH5 monoclonal antibodies were in the order of R5.004>R5.016>R5.008>R5.011>R5.014>R5.007. A similar observation was made in GIA with the homologous 3D7 strain and other heterologous laboratory-adapted strains (Draper laboratory, unpublished data). The inhibitory effect of the highly potent mAbs (R5004, R5008 and R5016) is comparable to previously published works with mouse anti-PfRH5 monoclonal antibodies (Douglas *et al.*, 2014) and rabbit anti-PfRH5 polyclonal IgG (Douglas *et al.*, 2011).

GIA is one of the functional assays, which is generally used in the area of blood stage vaccine development. Notwithstanding its broad usage, it is still unclear if GIA is an appropriate assay to predict protection in clinical trials in endemic populations. It was observed that vaccine induced growth inhibitory activity could vary depending on the antigen used (Miura *et al.*, 2011b) and the study population vaccinated (Malkin *et al.*, 2005, Dicko *et al.*, 2007, Miura *et al.*, 2009). A classic example was vaccination with AMA-C1/Alhydrogel resulting in increased antibody titers in Malian adults and children compared to naïve US adult controls. Malian children without pre-immunization inhibitory activity and the US adults elicited an increased growth inhibitory activity compared to the Malian adults although antibody levels did not differ between Malian

adults and children (Miura *et al.*, 2011b). Other studies have reported the presence of malaria-specific interfering antibodies that disrupt vaccine induced growth inhibitory activity of affinity purified antigen-specific IgG in Malian children (Miura *et al.*, 2011a) and adults (Miura *et al.*, 2008). It is unclear if the malaria-specific interfering IgGs actually reduce vaccine efficacy in endemic settings or if the interfering IgGs is just associated with *in vitro* GIA.

Developing the next generation of blood-stage malaria vaccines that overcome the challenges of antigenic variation and polymorphism would require identifying parasite antigens that are essential but are not under immune pressure. Going forward, studies must continue to improve the immunogenicity of PfRH5 vaccines and enhance the quality of the vaccine-induced antibody responses. It is anticipated Phase Ia studies with PfRH5 vaccines in different adjuvant systems would commence in the not so distant future to assess the safety and immunogenicity of PfRH5-based vaccines. This should then be followed by a controlled human malaria infection (CHMI) study to assess the efficacy of PfRH5-based vaccines in blood stage challenge.

To achieve sterile immunity against blood stage parasites by vaccinations would be quite challenging if not impossible due to the antigenic variation, high parasite multiplication and mutation. However, not achieving sterile immunity but sufficient protection against disease and death may not necessarily be bad as vaccinated individuals may still benefit from repeated exposure that may boost vaccine-induced memory B cells. In the case of PfRH5, boosting might not happen given the poor immunogenicity of the antigen. Rather, boosting of antibodies to other parasite proteins such as PfEMP1 and *P. falciparum*-encoded repetitive interspersed family of polypeptides (PfRIFINS) that mediate cytoadhesion and sequestration of parasites would consequently prevent

sequestration and allow sufficient time for vaccine-induced immune responses to effectively neutralize parasites and clearance of iRBC by the spleen.

Targeting PfrH5 as a stand-alone blood stage vaccine may not be effective. Incorporating PfrH5 into a multi-antigen or multistage vaccine could be the way forward. Preliminary data have shown other merozoite antigens such as PfrIPR, PfrEBA175, PfrRH2, PfrRH4 (Williams *et al.*, 2012b), *P. falciparum* apical asparagine-rich protein (PfrAARP) (Pandey *et al.*, 2013) and PfrCyRPA act synergistically with PfrH5, inducing cross strain inhibition *in vitro*. Nonetheless, it is unknown if such synergistic effects exist *in vivo*. Thus, studies must continue to identify other antigens that have high synergistic effects in combination with PfrH5 vaccines.

5.1 Conclusions

In the present study, we sought to examine the immunogenicity of potential vaccine candidates PfrH5, PfrCyRPA and Pfr113 in natural infections. The longevity and kinetics of the antigen-specific plasma IgG were also studied. The study shows in malaria-infected children that PfrH5 complex proteins are immunogenic in natural infections although seroprevalence to PfrH5 complex proteins is lower compared to other well studied merozoite antigens. Levels of antibodies to PfrH5, PfrCyRPA and Pfr113 among our cohort of children did not correlate with protection from malaria or severe malaria disease but were rather markers of recent parasite exposure. Plasma IgG induced to the studied antigens following natural infections increased and peaked by day 14 and declined by day 42, suggesting that antibodies to PfrH5 complex proteins are secreted by short-lived plasma cells.

Finally, we attempted to isolate human anti-PfRH5 and anti-PfCyRPA monoclonal antibodies from semi-immune adults by immortalizing memory B cells with EBV. We have successfully produced polyclonal B cell lines that are specific for PfRH5 and PfCyRPA through EBV immortalization of memory B cells isolated from semi-immune Ghanaian adults. The study demonstrates an efficient method for cloning fully human monoclonal antibodies from memory B cells by isolating human antibody-coding genes from single cells sorted from EBV-transformed polyclonal B cell lines. The use of TAP system provides a rapid pipeline to screen and down-select for monoclonal antibodies with desired specificity before cloning. Although the TAP protocol has been widely applied, this is the first time the method has been applied in isolating fully human mAbs from memory B cells. The isolated human IgG genes from the polyclonal B cell lines in this study remain a useful resource, that can be further exploited to obtain anti-PfRH5 and anti-PfCyRPA mAbs of high specificity and functional quality.

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APPENDICES

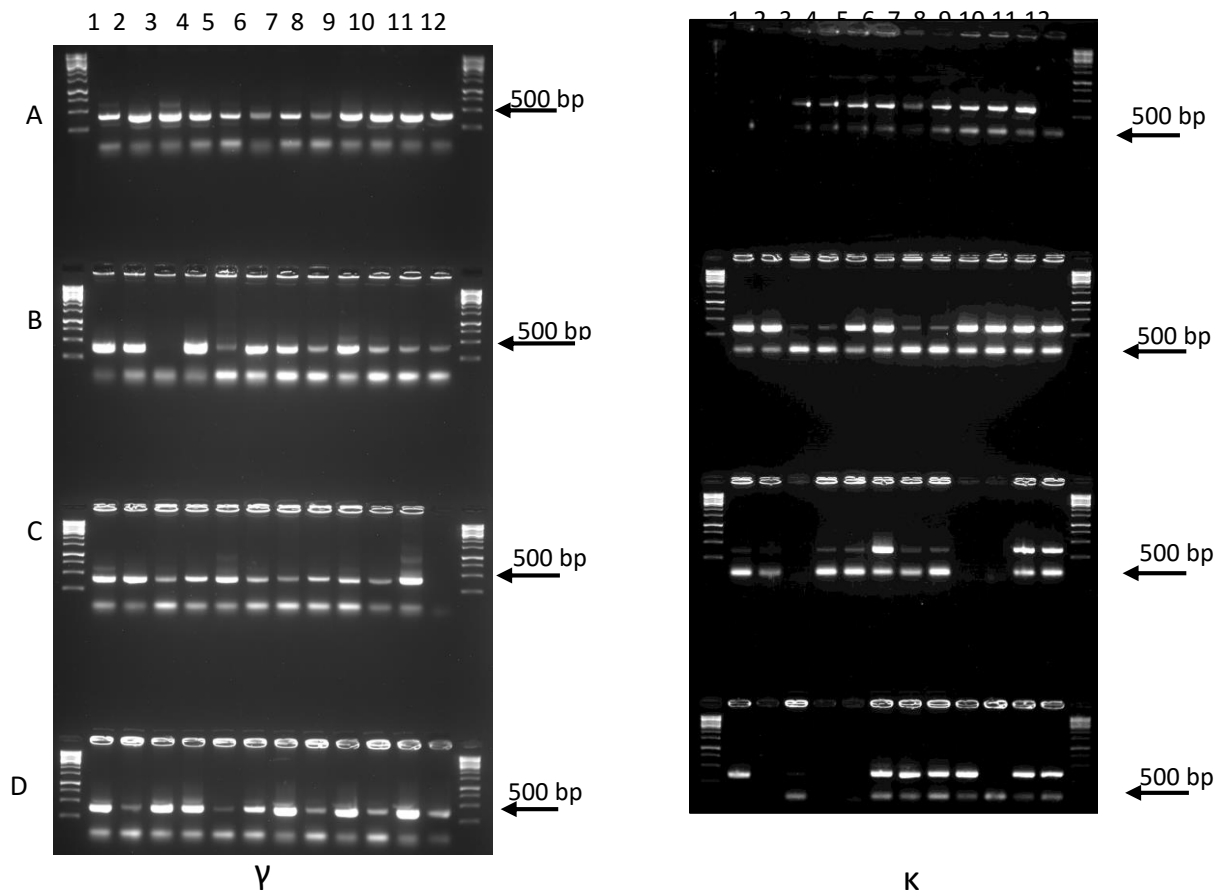
Table 1: List of primers for amplification of human IgG variable genes from single cell sorted B cells

	Primer name	5' - 3' Sequence	Melting Temperature for HF Phusion polymerase
RT PCR	5' L VH1	ACAGGTGCCCACTCCCAGGTGCAG	78
	5' L VH3	AAGGTGTCCAGTGTGARGTGCAG	70
	5' L VH4/6	CCCAGATGGGTCTGTCCCAGGTGCAG	81
	5' L VH5	CAAGGAGTCTGTTCCGAGGTGCAG	72
	3' C γ CH1	GGAAGGTGTGCACGCCGCTGGTC	79
	5' L VK1/2	ATGAGGSTCCCYGCTCAGCTGCTGG	80
	5' L VK3	CTCTTCCTCCTGCTACTCTGGCTCCCAG	75
	5' L VK4	ATTTCTCTGTTGCTCTGGATCTCTG	66
	3' CK 543	GTTTCTCGTAGTCTGCTTTGCTCA	66
	5' L V λ 1	GGTCCTGGGCCCAGTCTGTGCTG	77
	5' L V λ 2	GGTCCTGGGCCCAGTCTGCCCTG	79
	5' L V λ 3	GCTCTGTGACCTCCTATGAGCTG	66
	5' L V λ 4/5	GGTCTCTCTCSCAGCYTGTGCTG	72
	5' L V λ 6	GTTCTTGGGCCAATTTTATGCTG	67
	5' L V λ 7	GGTCCAATTCYCAGGCTGTGGTG	71
	5' L V λ 8	GAGTGGATTCTCAGACTGTGGTG	65
		3' C λ	CACCAGTGTGGCCTTGTTGGCTTG
IgH (PCR2)	CPEC VH1/5/7	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCG AGGTGCAGCTGGTGCAG	87,8
	CPEC VH3	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AGGTGCAGCTGGTGGAG	86

	CPEC VH4	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTGCAGCTGCAGGAG	87,3
	CPEC VH3- 23	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AGGTGCAGCTGTTGGAG	85,2
	CPEC VH4- 34	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTGCAGCTACAGCAGTG	86,4
	CPEC VH1- 18	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTTTCAGCTGGTGCAG	86,5
	CPEC VH1- 24	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTCCAGCTGGTACAG	85,2
	CPEC VH3- 9/30/3 3	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AAGTGCAGCTGGTGGAG	85,2
	CPEC VH6- 1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGGTACAGCTGCAGCAG	85,7
	CPEC VH4- 39	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCCC AGCTGCAGCTGCAGGAG	87,3
	CPEC VH3- 33	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTC AGGTGCAGCTGGTGGAG	86
	CPEC JH1/2 /4/5	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACC AG	89,4
	CPEC JH3	GATGGGCCCTTGGTCGACGCTGAAGAGACGGTGACC ATTG	88,8
	CPEC JH6	GATGGGCCCTTGGTCGACGCTGAGGAGACGGTGACC GTG	91
IgK(P CR2)	CPEC VK1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG ACATCCAGATGACCCAGTC	84,8
	CPEC VK1- 9/1- 13	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG ACATCCAGTTGACCCAGTCT	84,7
	CPEC VK1 D- 43/1- 8	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTGTG CCATCCGGATGACCCAGTC	87,8
	CPEC VK2	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGG ATATTGTGATGACCCAGAC	87,8
	CPEC VK2- 28/2- 30	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATGGGG ATATTGTGATGACTCAGTC	83,7
	CPEC VK3-	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCTG AAATTGTGTTGACACAGTC	82,7

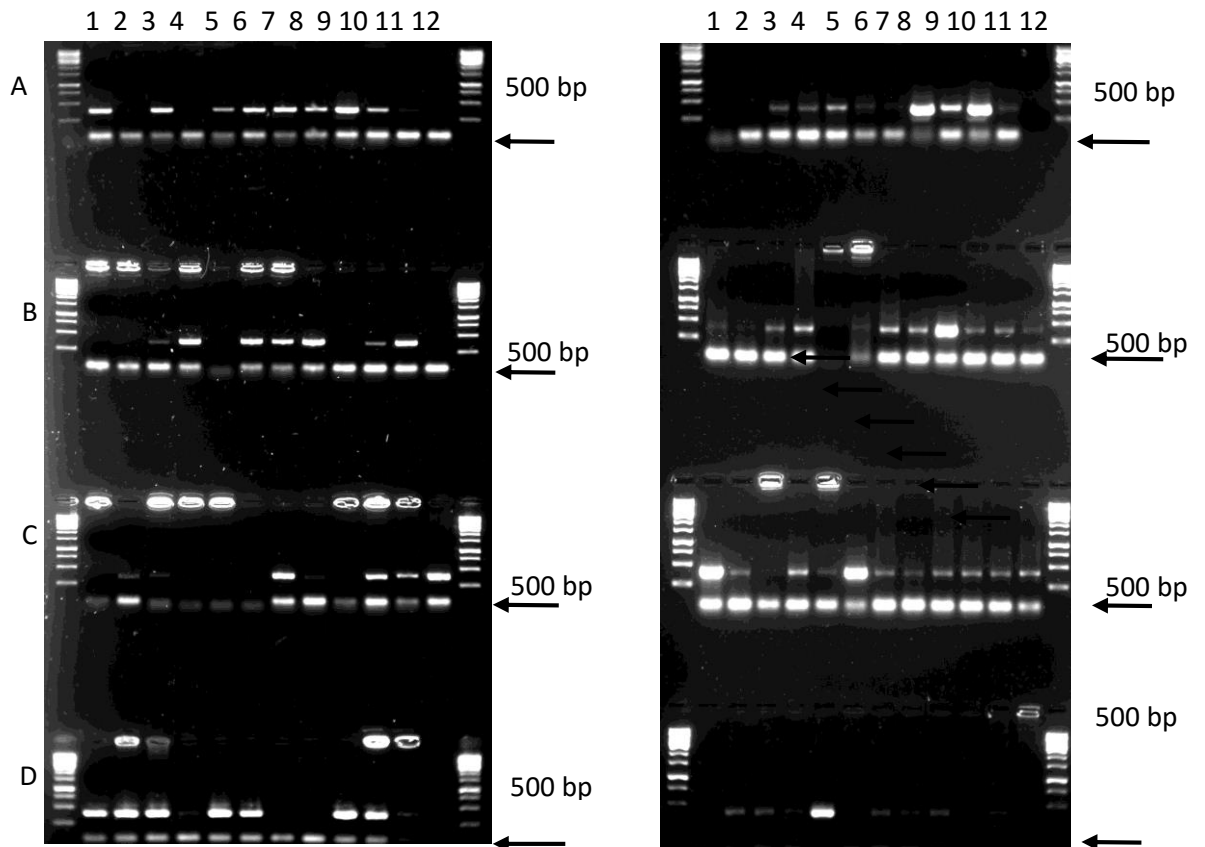
	11/3D -11		
	CPEC VK3- 15/3D -15	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAG AAATAGTGATGACGCAGTC	82,8
	CPEC VK3- 20/3D -20	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCAG AAATTGTGTTGACGCAGTCT	84
	CPEC VK4- 1	CTTTTTCTAGTAGCAACTGCAACCGGTGTACATTCGG ACATCGTGATGACCCAGTC	86,5
	CPEC JK1/2 /4	ATGGTGCAGCCACCGTACGTTTGATYTCCACCTTGGT C	84,6
	CPEC JK3	ATGGTGCAGCCACCGTACGTTTGATATCCACTTTGGT C	82,1
	CPEC JK5	ATGGTGCAGCCACCGTACGTTTAATCTCCAGTCGTGT C	82,5
	CPEC JK15 0/3	ATGGTGCAGCCACCGTACGTCTGATTCCACCTTGGT C	84,9
Igλ(PC R2)	CPEC VL1	CTTTTTCTAGTAGCAACTGCAACCGGT <u>TCCTGGGCC</u> <u>AGTCTGTGCTGACKCAG</u>	88,9
	CPEC VL2	CTTTTTCTAGTAGCAACTGCAACCGGT <u>TCCTGGGCC</u> <u>AGTCTGCCCTGACTCAG</u>	88,8
	CPEC VL3	CTTTTTCTAGTAGCAACTGCAACCGGT <u>TCTGTGACCTC</u> <u>CTATGAGCTGACWCAG</u>	84,1
	CPEC VL4/ 5	CTTTTTCTAGTAGCAACTGCAACCGGT <u>TCTCTCTCSA</u> <u>GCYTGTGCTGACTCA</u>	86,3
	CPEC VL6	CTTTTTCTAGTAGCAACTGCAACCGGT <u>TCTTGGGCCA</u> <u>ATTTTATGCTGACTCAG</u>	84,2
	CPEC VL7/ 8	CTTTTTCTAGTAGCAACTGCAACCGGT <u>CCAATTCYC</u> <u>AGRCTGTGGTGACYCAG</u>	86,2
	CPEC CL revers e	GGCTTGAAGCTCCTCACTCGAGGGYGGGAACAGAGT G	85
	TAP PCR	J2620	GAGTCAGTGAGCGAGGAAG
J2707		CTAACTGACACACATTCCACAGC	
J2708		CTAACTGACACACATTCCACAGCTGG	
iPCR Rv		ACCGGTTGCAGTTGCTACTA	62.7
Abve c iPCR G		GTCGACCAAGGGCCCA	66.7

AbVc c K iPCR	CGTACGGTGGCTGCAC	63.4
AbVc c λ iPCR	CTCGAGTGAGGAGCTTCAA	62.4



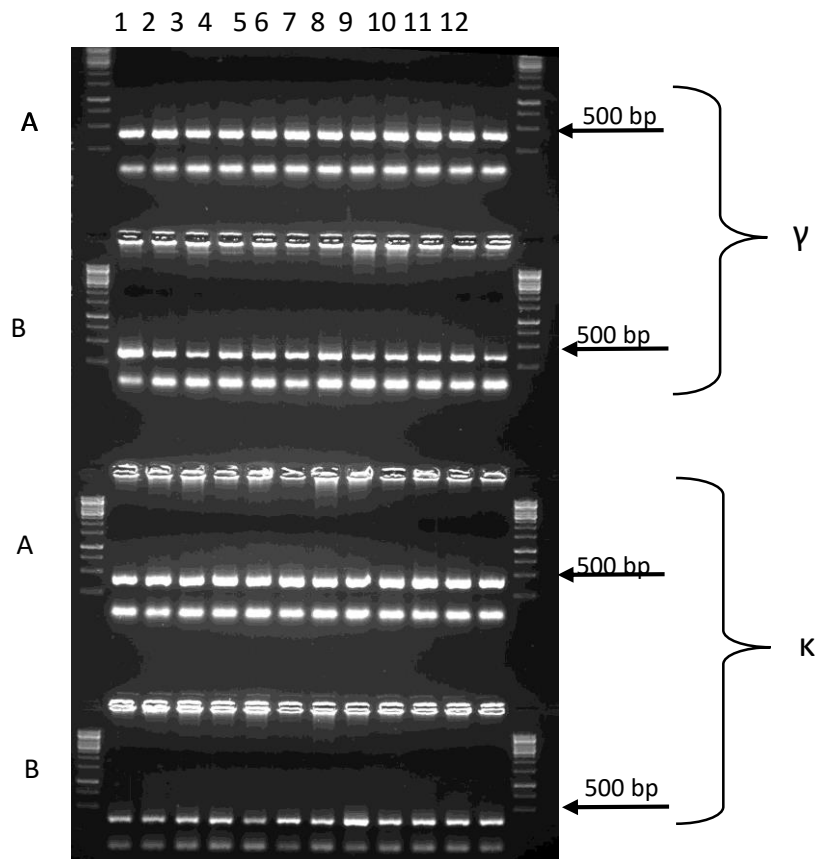
Appendix A: Amplification of V_γ and V_κ genes from RH5 specific polyclonal cell line 3.3G which was single cell sorted into 48 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is V_γ is 420 bp and 395 bp for V_κ . PCR products were run on 1% agarose gel



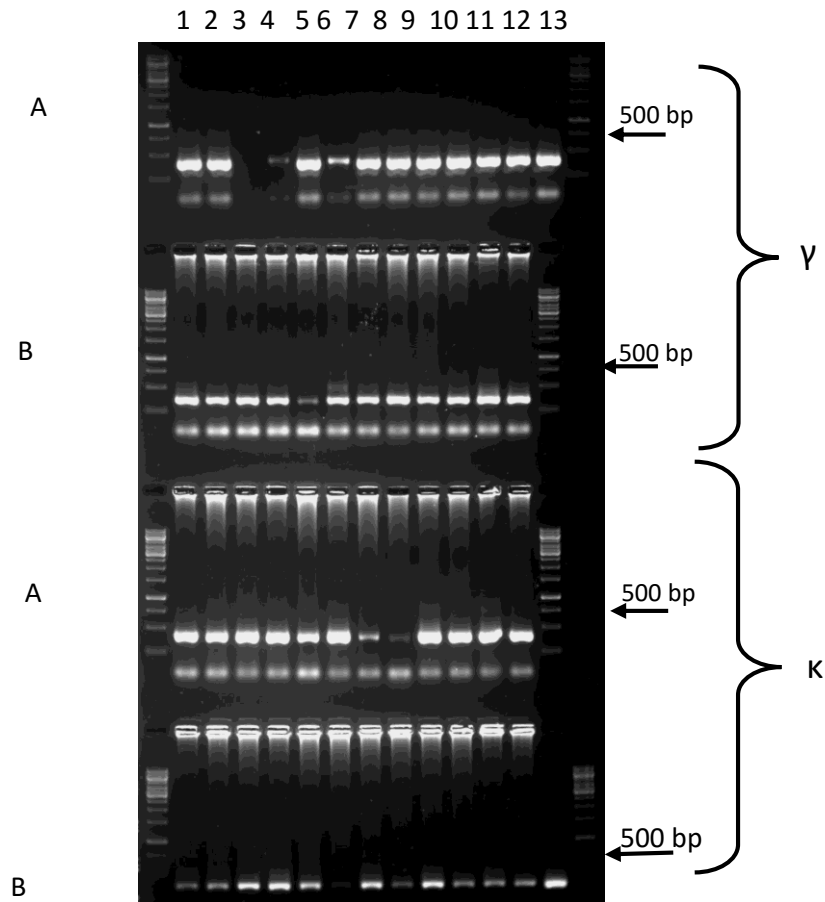
Appendix B. Amplification of V_γ and V_λ genes from RH5 specific polyclonal cell line 7.12E which was single cell sorted into 48 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is V_γ is 420 bp and 430 bp for V_λ. PCR products were run on 1% agarose gel



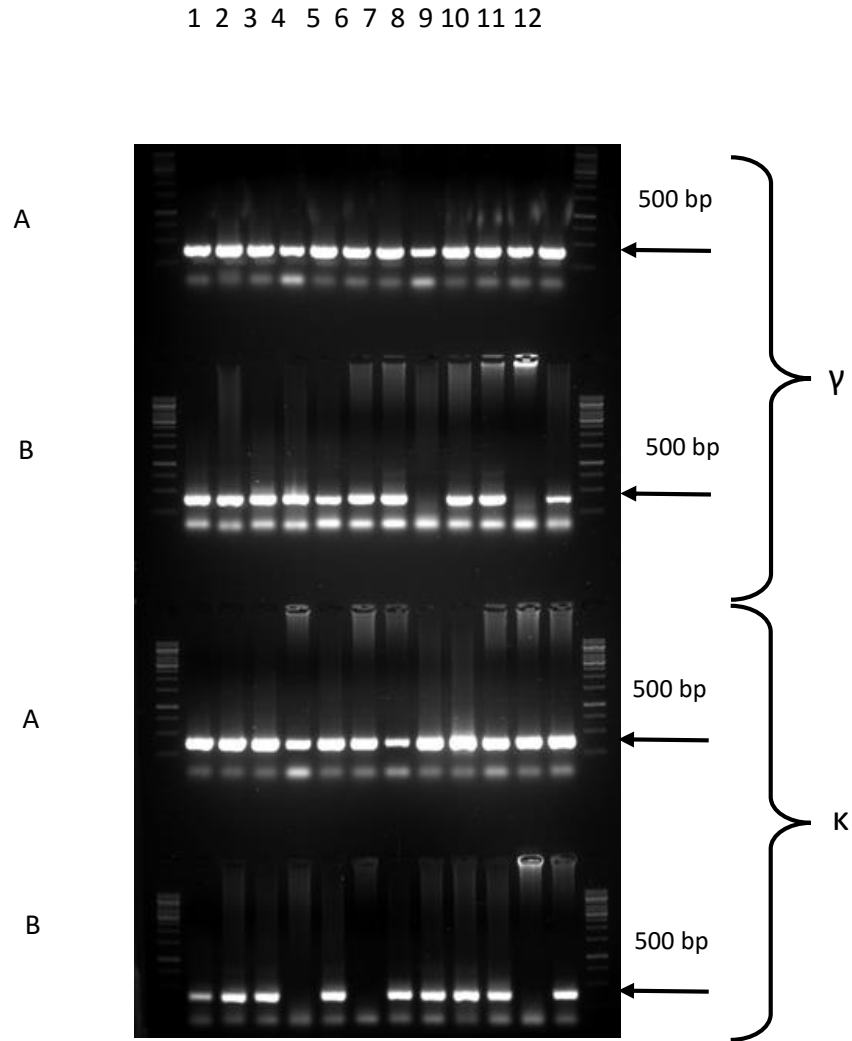
Appendix C: Amplification of V_{γ} and V_{κ} genes from PfrH5-specific polyclonal cell line 10.1B which was single cell sorted into 24 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is V_{γ} is 420 bp and 395 bp for V_{κ} . PCR products were run on 1% agarose gel



Appendix D: Amplification of V_{γ} and V_{κ} genes from CyRPA specific polyclonal cell line C3C which was single cell sorted into 24 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is V_{γ} is 420 bp and 395 bp for V_{κ} . PCR products were run on 1% agarose gel



Appendix E: Amplification of V_{γ} and V_{κ} genes from CyRPA specific polyclonal cell line 1B which was single cell sorted into 24 well plates.

Each lane represents PCR amplified product of single cells sorted into each well. The expected amplicon size is V_{γ} is 420 bp and 395 bp for V_{κ} . PCR products were run on 1% agarose gel

Human vaccination against RH5 induces neutralizing antimalarial antibodies that inhibit RH5 invasion complex interactions

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Conflict of interest: S.J. Draper is a named inventor on patent applications relating to RH5 and/or other malaria vaccines and immunization regimens; is a cofounder of, shareholder in, and consultant for SpyBiotech, and declares research funding support from Pfizer and GSK BioPharm. A.D. Douglas, G.J. Wright, and A.V.S. Hill are named inventors on patent applications relating to RH5 and/or other malaria vaccines and immunization regimens. L. Siani and S. Di Marco are employees of RaiThera (formerly Okaikos), which is currently developing vectored vaccines for a number of diseases. J. Vekemans was an employee of GSK, which has acquired the ChAd63 vector from Okaikos. R.Ashfield is a director of Ducatis and holds shares in the company, which is developing a therapy for autoimmune disease. A.M. Minassian has an immediate family member who is an inventor on patents relating to RH5 and/or other malaria vaccines and immunization regimens and who is a cofounder of, shareholder in, and consultant for SpyBiotech. S. Biswas is a cofounder and CEO of, and shareholder in, SpyBiotech and is a contributor in a patent application relating to multimerisation technology. J. Jin is a cofounder of and shareholder in SpyBiotech.

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The development of a highly effective vaccine remains a key strategic goal to aid the control and eventual eradication of *Plasmodium falciparum* malaria. In recent years, the reticulocyte-binding protein homolog 5 (RH5) has emerged as the most promising blood-stage *P. falciparum* candidate antigen to date, capable of conferring protection against stringent challenge in *Aotus* monkeys. We report on the first clinical trial to our knowledge to assess the RH5 antigen – a dose-escalation phase Ia study in 24 healthy, malaria-naïve adult volunteers. We utilized established viral vectors, the replication-deficient chimpanzee adenovirus serotype 63 (ChAd63), and the attenuated orthopoxvirus modified vaccinia virus Ankara (MVA), encoding RH5 from the 3D7 clone of *P. falciparum*. Vaccines were administered i.m. in a heterologous prime-boost regimen using an 8-week interval and were well tolerated. Vaccine-induced anti-RH5 serum antibodies exhibited cross-strain functional growth inhibition activity (GIA) in vitro, targeted linear and conformational epitopes within RH5, and inhibited key interactions within the RH5 invasion complex. This is the first time to our knowledge that substantial RH5-specific responses have been induced by immunization in humans, with levels greatly exceeding the serum antibody responses observed in African adults following years of natural malaria exposure. These data support the progression of RH5-based vaccines to human efficacy testing.

Introduction

The most severe form of human malaria is caused by the *Plasmodium falciparum* parasite. Despite recent and encouraging advances in malaria control measures, current estimates suggest that, in 2015, there were still over 200 million clinical cases leading to 438,000 deaths (1). Consequently, the development of an effective and durable vaccine remains a key strategic goal to aid the control, local elimination, and eventual eradication of this disease (2). The mainstay approach to vaccination against the blood-stage of malaria infection is to induce antibodies against the merozoite form of the parasite that invades erythrocytes (3). Such a vac-

cine would protect against disease severity and could reduce transmission by accelerating the control and clearance of blood-stage parasitemia, either in a standalone manner or by complementing preerythrocytic immunity induced by partially effective subunit (4) or whole sporozoite vaccine strategies (5).

However, despite the fact that naturally acquired immunity to malaria is largely directed against the blood-stage parasite (6), the development of an effective vaccine has proved exceptionally challenging. The most widely studied merozoite candidate vaccine antigens have been hindered by substantial levels of polymorphism (7) alongside redundant erythrocyte invasion pathways (8). Extremely high concentrations of functional antibody against these antigens have also been required to neutralize the parasite (9), likely linked to the kinetic constraints imposed by very rapid erythrocyte invasion (10). Nevertheless, substantial progress has been made in recent years, with the identification of a new generation of merozoite antigen targets that are essential and that exhibit relatively low levels of polymorphism, leading to induction of strain-transcending antibodies by vaccination in preclinical models (11). Three of these targets form a complex, including the *P. falciparum* reticulocyte-binding protein homolog 5 (RH5) (12), the cysteine-rich protective antigen (CyRPA) (13), and the RH5-interacting protein (Ripr) (14). Of these, vaccine development efforts are currently most advanced for RH5 (15).

The first vaccination studies with RH5 used regions of the antigen made in *E. coli* that failed to induce functional antibodies (16, 17). However, functional neutralizing antibodies were subsequently generated by using immunogens based on the full-length RH5 (RH5_FL) sequence. These antibodies raised by vaccination of animals can cross-inhibit all *P. falciparum* lines and field isolates tested to date (12, 18–20), notably with higher efficiency than other historical target antigens (18). RH5 is also essential (16, 21) and forms a critical nonredundant interaction with its receptor basigin (CD147) on the RBC surface (22). Somewhat surprisingly, RH5 appears to be under relatively low-level immune pressure following natural infection (12, 23–26), with functional constraints also linked to basigin binding and host RBC tropism (21, 27, 28) — both of these factors potentially explain its relatively high degree of sequence conservation. Recently, the N-terminal region of RH5 (RH5Nt) has been shown to bind the essential glycosylphosphatidylinositol-anchored (GPI-anchored) merozoite protein P113, providing a mechanism for anchoring RH5 to the merozoite surface (29). When bound by P113, RH5_FL is able to bind basigin and CyRPA, but not Ripr. CyRPA, however, can bind Ripr and likely recruits this molecule to the complex leading to its one-way release from P113, either by proteolytic cleavage of RH5Nt or mutually exclusive binding of P113 and Ripr. The function of the RH5-CyRPA-Ripr complex has been linked to pore formation between the merozoite and the RBC, allowing for the movement of Ca^{2+} into the host cell and alterations in RBC cytoskeleton architecture, prior to establishment of the tight junction (30, 31).

The traditional approach to antibody induction by vaccination has been the delivery of recombinant protein, or particle-in-adjuvant formulations. An alternative strategy, developed over the last decade, has seen the use of recombinant viral vectored vaccines to deliver protein antigens of interest, whereby the design and administration of these vectors has been optimized to induce antibodies in conjunction with the T cell responses for which they were historically favored (32, 33). Such a strategy is particularly attractive when the antigen of interest is refractory to production in recombinant form using heterologous expression platforms. Indeed, the RH5_FL protein proved particularly difficult to express for many years, with the first highly promising vaccine data generated by use of the viral vector platform (12). In the case of this approach, a recombinant replication-deficient adenovirus (of human or simian serotype) is used to prime the immune response, followed by a booster vaccination (typically 8 weeks later) with an attenuated poxvirus recombinant for the same antigen (33). This heterologous prime-boost approach has shown antibody induction against difficult-to-express malaria antigens in numerous animal models, including nonhuman primates (32, 34, 35). These vectors, delivering antigens from *P. falciparum*, have now been shown to be safe and immunogenic for T cell and antibodies in healthy European and American adult volunteers (36–40), as well as African adults, children, and infants (41, 42). More recently, similar adenovirus-poxvirus vectored vaccine technologies have been used to immunize humans against numerous other pathogens including *P. vivax* malaria (43), Ebola virus (44), hepatitis C virus (HCV) (45), respiratory syncytial virus (RSV) (46) and HIV-1 (47).

Here, we report the safety and immunogenicity of a replication-deficient chimpanzee adenovirus serotype 63 (ChAd63) and an attenuated orthopoxvirus modified vaccinia virus Ankara (MVA) encoding monovalent RH5_FL based on the sequence from the 3D7 clone of *P. falciparum*. These vaccines were tested in an open-label dose-escalation phase Ia study in healthy United Kingdom (UK) adults. These vaccines have been previously shown to be immunogenic in mice and rabbits (12) and exhibited significant *in vivo* protec-

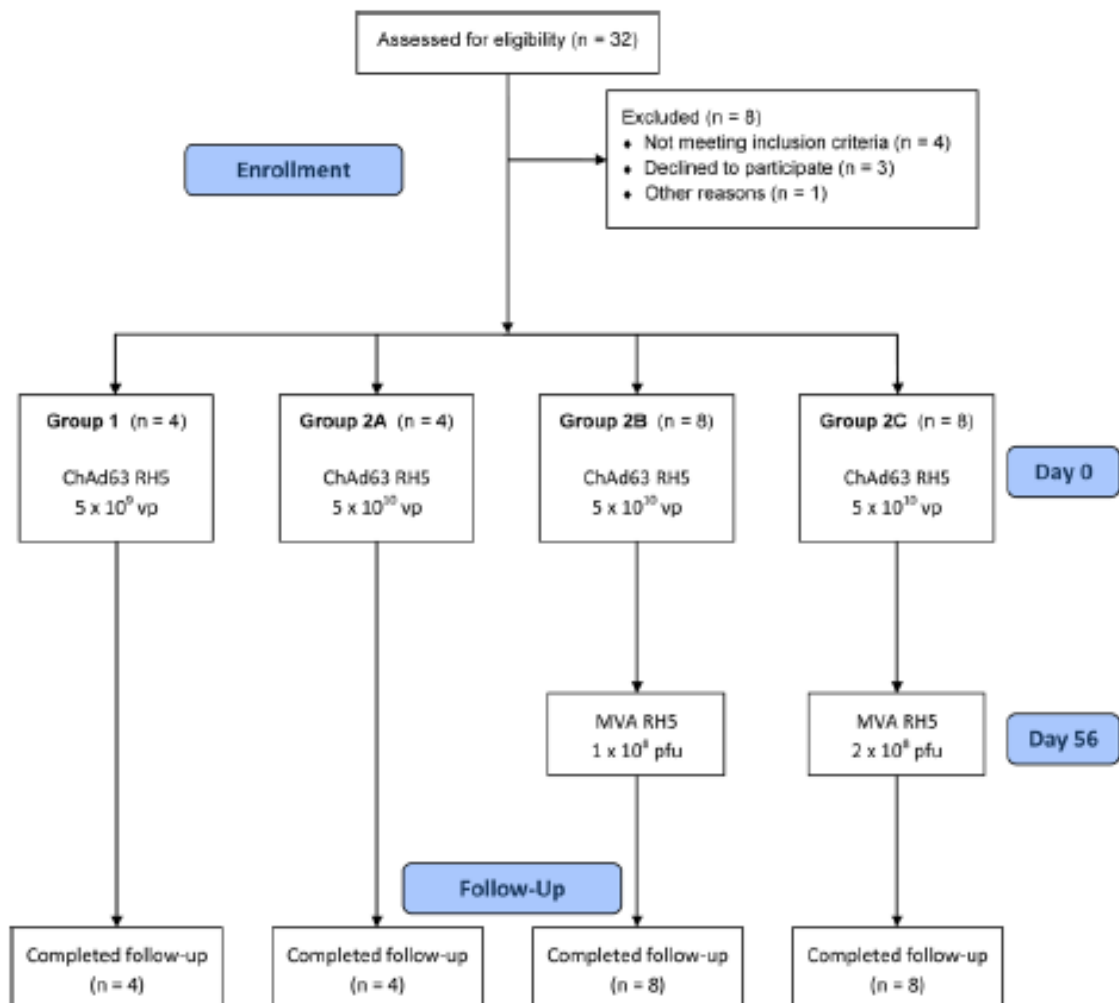


Figure 1. VAC057 flow chart of study design and volunteer recruitment. Enrolment into the VAC057 study began in August 2014, and all follow-up visits were completed by October 28, 2015. All immunizations were administered i.m. into the deltoid of the nondominant arm preferentially.

tion against a stringent heterologous strain blood-stage *P. falciparum* challenge in *Aotus* monkeys (34). Protection was strongly associated with anti-RH5_FL serum IgG antibody concentration and in vitro growth inhibition activity (GIA) measured using purified IgG (34). Now, we report that these vaccines demonstrate a favorable safety profile in malaria-naïve adults and confirm for the first time to our knowledge that substantial RH5-specific antibodies, B cell, and T cell responses can be induced by immunization in humans. Vaccine-induced serum antibodies exhibited cross-strain in vitro GIA, targeted linear and conformational epitopes within RH5, and inhibited key interactions within the RH5 invasion complex, including those between RH5 and P113, CyRPA and busigin.

Results

Twenty-four healthy adult volunteers were enrolled into the VAC057 trial to assess the ChAd63-MVA RH5 vaccine in an open-label, dose-escalation study design. Thirty-two volunteers were screened in total, across 2 sites, and 24 of these were enrolled (Figure 1). Vaccinations began on August 18, 2014, and all follow-up visits were completed by October 28, 2015. All vaccinees received their immunizations as scheduled, and there were no withdrawals from the study. Similar numbers of males and females were enrolled (13 females, 11 males).

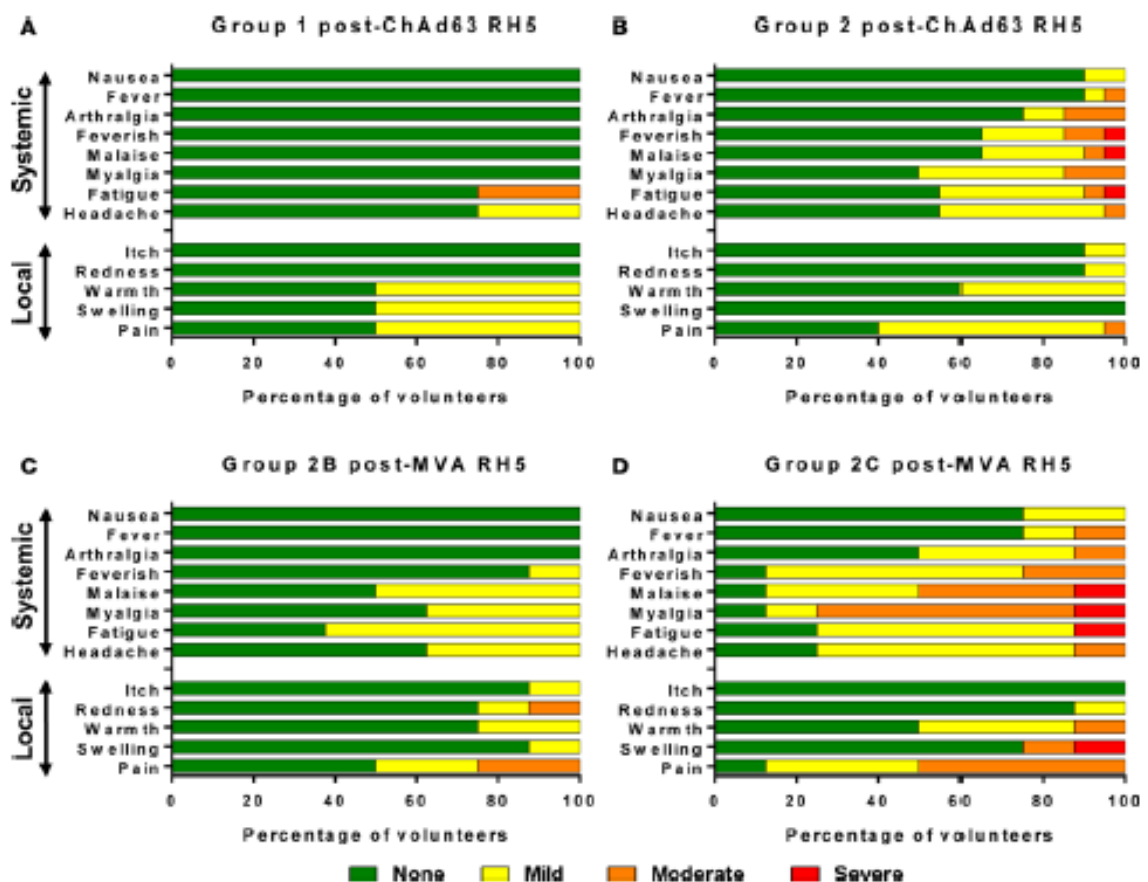


Figure 2. Solicited AEs following vaccination with ChAd63 and MVA RH5. The solicited local and systemic adverse events (AEs) recorded for 7 days following ChAd63 RH5 and MVA RH5 are shown at the maximum severity reported by all volunteers. (A) Four volunteers received 5×10^9 viral particles (vp) ChAd63 RH5 (Group 1), and (B) 20 received 5×10^{10} vp (Group 2). (C) Eight of the Group 2 volunteers went on to receive MVA RH5 1×10^8 plaque-forming units (pfu) (Group 2B), and (D) 8 received 2×10^8 pfu (Group 2C).

The mean age of volunteers was 28 years (range 19–48 years). The 4 Group 1 volunteers received 5×10^9 viral particles (vp) of the ChAd63 RH5 vaccine. Following a safety review, the dose of ChAd63 RH5 was increased for Group 2 and volunteers received 4.26×10^{10} to 4.77×10^{10} vp (a nominal figure of 5×10^{10} vp is otherwise used throughout this report). Four volunteers in Group 2A received ChAd63 RH5 alone. Eight volunteers were enrolled into Group 2B, and a further 8 were enrolled into Group 2C. These volunteers received ChAd63 RH5, followed 8 weeks later with a boost vaccination of MVA RH5 at a dose of 1×10^8 plaque-forming units (pfu) (Group 2B) or 2×10^8 pfu (Group 2C). There was a 2-week interval between the final vaccination in Group 2B with MVA RH5 at the lower dose of 1×10^8 pfu and the first vaccination with MVA RH5 at the full dose of 2×10^8 pfu in Group 2C, with a safety review prior to dose escalation.

ChAd63 and MVA RH5 show a favorable safety profile in healthy UK adult volunteers. There were no serious adverse events (SAEs) or unexpected reactions and no safety concerns during the course of the trial. The reactogenicity of the vaccines was similar to that seen in previous malaria vaccine trials using the same viral vectors at similar doses in healthy adults (37, 43, 48), with the higher doses of both vaccines associated with an increased number and higher severity of reported AEs (Figure 2). The majority of AEs following ChAd63 RH5 were mild, but moderate AEs were reported by some volunteers in both groups, and 2 volunteers who received the full dose reported severe AEs on the day of vaccination that resolved within 24 hours. All moderate or severe solicited systemic AEs following MVA RH5 occurred in volunteers who had received the higher dose of vaccine. The

majority of solicited AEs occurred within the first 2 days after vaccination, and the median duration of each systemic AE was between 1 and 2 days following either vaccine. The unsolicited AEs considered possibly, probably, or definitely related to either vaccine are shown in Supplemental Table 1 (supplemental material available online with this article; <https://doi.org/10.1172/jci.insight.96381DS1>). The majority were mild in nature, and all resolved spontaneously. There were no severe laboratory AEs following ChAd63/MVA RH5 vaccination. One volunteer had a moderately raised ALT (123 IU/l) at day 7 (d7) following ChAd63 RH5 that had resolved fully by d28. One volunteer had moderate thrombocytopenia ($123 \times 10^9/l$) and mild leukopenia ($3.3 \times 10^9/l$) at d28 following ChAd63 RH5 but had commenced postexposure prophylaxis for a possible HIV exposure the day before these bloods were taken; therefore, causality is unclear. All other laboratory AEs were mild and had resolved fully by d84, except for 1 volunteer who had a persistent mild anemia. This had been present at screening and had not worsened over the course of the study, so it was not considered significant.

ChAd63 and MVA RH5 expand IFN- γ T cell responses in healthy UK adult volunteers. The kinetics and magnitude of the RH5-specific T cell response were assessed over time by ex vivo IFN- γ ELISPOT following restimulation of PBMC with 20-mer peptides overlapping by 10 amino acids (aa) spanning the entire RH5 insert present in the vaccines (Figure 3 and Supplemental Table 2). Vaccination with ChAd63-MVA RH5 induced antigen-specific T cell responses in all volunteers, with individual responses shown in Supplemental Figure 1 and median responses to the total vaccine insert shown for each group in Figure 3A. Following ChAd63 RH5 prime, there was no significant difference between median responses in the lower-dose Group 1 in comparison with Group 2 at the peak of the response on d14 (median 499 [range 96–993] vs. 797 [range 3–3,269] spot forming units [SFU]/million PBMC in Groups 1 vs. 2 respectively [$n = 4$ vs. 20, $P = 0.27$ by Mann-Whitney test]) (Figure 3B). Responses subsequently followed classical T cell kinetics and contracted by d56 (Figure 3A). Administration of MVA RH5 significantly boosted these responses in all volunteers as measured 1 week later on d63 (Groups 2B and 2C vs. 2A, Kruskal-Wallis test with Dunn's multiple comparison test) (Figure 3C), reaching medians of 2092 [range 628–4,102] and 2,281 [range 935–4,257] SFU/million PBMC in Groups 2B and 2C, respectively, vs. 213 [range 15–363] SFU/million PBMC in Group 2A. However, there was no significant difference between the 2 groups who received the different doses of MVA RH5 ($P = 0.33$, Mann-Whitney test). T cell responses were detected in all 6 of the peptide pools used in the ELISPOT assay (Supplemental Figure 2), confirming these were spread across the whole RH5_FL antigen, and thus including both structured and disordered regions of the molecule (49, 50). Following the peak at d63, responses contracted and were maintained above baseline at the end of the study period, again with no significant difference between Groups 2B and 2C ($P = 0.27$, Mann-Whitney test) (Figure 3D).

ChAd63 and MVA RH5 induce serum antibody responses and memory B cells (mBCs) in healthy UK adult volunteers. The kinetics and magnitude of the anti-RH5 serum IgG antibody response were assessed over time by ELISA against RH5_FL recombinant protein (Figure 4). Priming vaccination with 5×10^{10} vp ChAd63 RH5 followed by MVA RH5 boost induced antigen-specific IgG responses in all volunteers (Groups 2B and 2C), with individual responses shown in Supplemental Figure 3 and median responses shown for each group in Figure 4A. Responses are reported in $\mu\text{g/ml}$ following conversion of ELISA arbitrary units (AU) by calibration-free concentration analysis (CFCA) (Supplemental Figure 4). Following ChAd63 RH5 prime with 5×10^6 vp, 2 of 4 volunteers showed a detectable response on d28, in contrast to 16 of 20 volunteers (median: 0.2, range: 0–2.3 $\mu\text{g/ml}$, $n = 20$) following priming with 5×10^{10} vp ($P = 0.13$, Mann-Whitney test) (Figure 4B). Responses were subsequently maintained in Group 2 volunteers prior to administration of MVA RH5, which led to a boost as measured 4 weeks later on d84 (Figure 4A); this reached significance for Group 2C vs. 2A ($P = 0.008$, Kruskal-Wallis test with Dunn's multiple comparison test) (Figure 4B). Responses in Group 2C (median: 9.3, range: 0.5–14.5 $\mu\text{g/ml}$, $n = 8$) tended to be higher than in Group 2B, but this did not reach significance (median: 4.0, range: 2.1–17.5 $\mu\text{g/ml}$, $n = 8$) at this peak time point ($P = 0.33$, Mann-Whitney test). Serum antibody responses decreased by d140 but were well maintained above preboost levels, with significance of Group 2C over 2A ($P = 0.005$, Kruskal-Wallis test with Dunn's multiple comparison test) (Figure 4B). We also compared postboost responses induced by the vaccine (Groups 2B and 2C combined) with those induced by natural malaria exposure in 79 Ghanaian adults and 96 Kenyan adults (Figure 4C). Anti-RH5_FL IgG responses were weak in the malaria-endemic populations, with median responses less than 0.1 $\mu\text{g/ml}$ in both and the highest 4 $\mu\text{g/ml}$ in a single Kenyan adult. The vaccine-induced responses were significantly higher ($P < 0.0001$, Kruskal-Wallis test with Dunn's multiple comparison test).

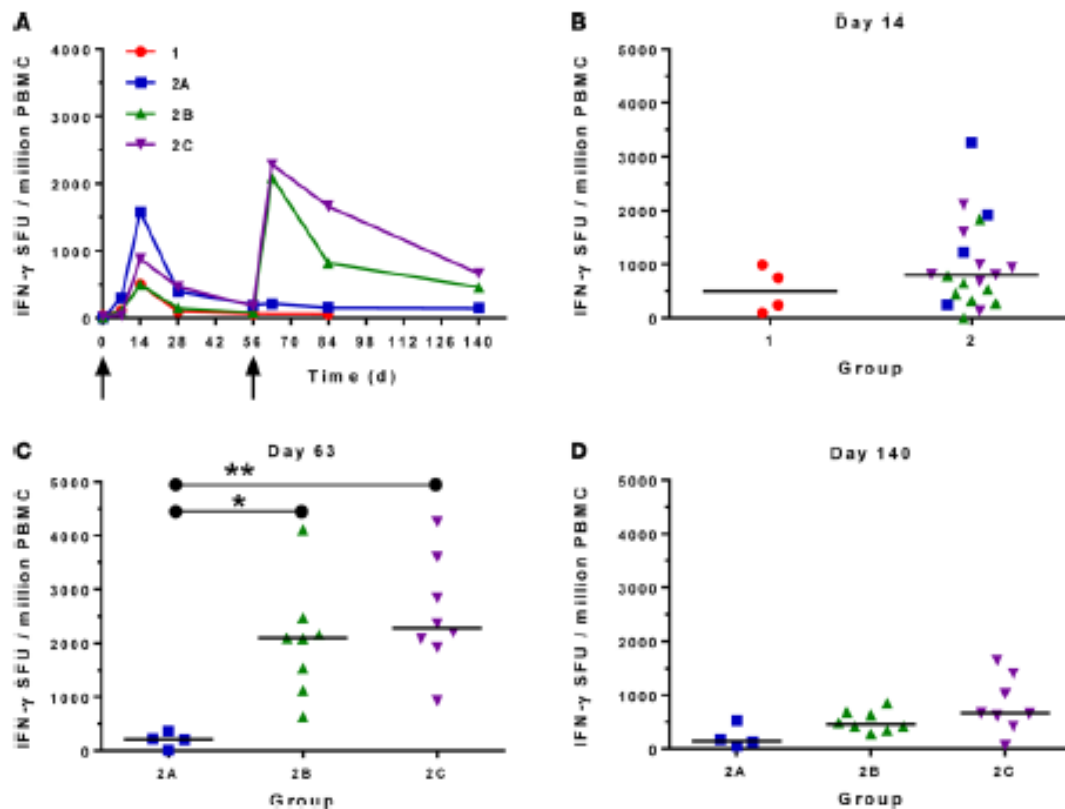


Figure 3. Ex vivo IFN- γ T cell response to vaccination. (A) Median ex vivo IFN- γ enzyme-linked immunospot (ELISPOT) responses in peripheral blood mononuclear cells (PBMC) to the RH5 insert (summed response across all the individual peptide pools) shown for all groups. Individual responses are shown in Supplemental Figure 1. Median and individual responses are shown at (B) d14 ($n = 4$ vs. 20); (C) d63 (G2A, $n = 4$; G2B, $n = 8$; G2C, $n = 8$) assessed by Kruskal-Wallis test with Dunn's multiple comparison test; and (D) d140 (G2A $n = 4$, G2B $n = 8$, G2C $n = 8$). Symbols are coded according to group. * $P < 0.05$, ** $P < 0.01$.

The vaccine-induced serum antibody response against RH5_FL as measured by ELISA at d84 was composed of IgG1 and moderate levels of IgG3 (Figure 4D) with little to no IgG2 or IgG4 (Supplemental Figure 5A). Low levels of IgA and IgM were detectable above baseline (d0) levels in only a few volunteers (Supplemental Figure 5A). This profile was maintained at the end of the study period (d140); however, responses were measured at lower levels, consistent with the anti-RH5_FL total IgG ELISA kinetic (Supplemental Figure 5B). The avidity of the anti-RH5_FL IgG, as measured by a sodium thiocyanate displacement (NaSCN displacement) ELISA, was similar at d84 for all volunteers in Groups 2A, 2B, and 2C, with the IC_{50} (concentration of NaSCN required to reduce the starting OD in the ELISA by 50%) ranging from 0.8 to 1.7 M. Avidity showed a significant increase in Groups 2B and 2C from d28 to d84 ($n = 16$, $P = 0.001$, Wilcoxon matched-pairs signed rank test); however, the same trend was observed in Group 2A ($n = 2$), suggesting that this may relate to IgG affinity maturation over time, as opposed to a direct consequence of MVA RH5 boost (Figure 4E).

Previous studies have shown that antibody-secreting cells (ASC) can be detected in peripheral blood for a short time (around d7) after MVA boost when using the ChAd63-MVA regimen (51, 52). RH5_FL-specific ASC responses were assessed by ex vivo ELISPOT using fresh PBMC collected at the d63 visit for volunteers in Groups 2B and 2C. Median responses of 52 vs. 180 RH5_FL-specific ASC per million PBMC were observed, respectively, but there was no significant difference between the 2 groups ($P = 0.15$, Mann-Whitney test) (Figure 5A). ASC responses across both groups showed a significant correlation, with peak serum antibody responses at d84 (Figure 5B).

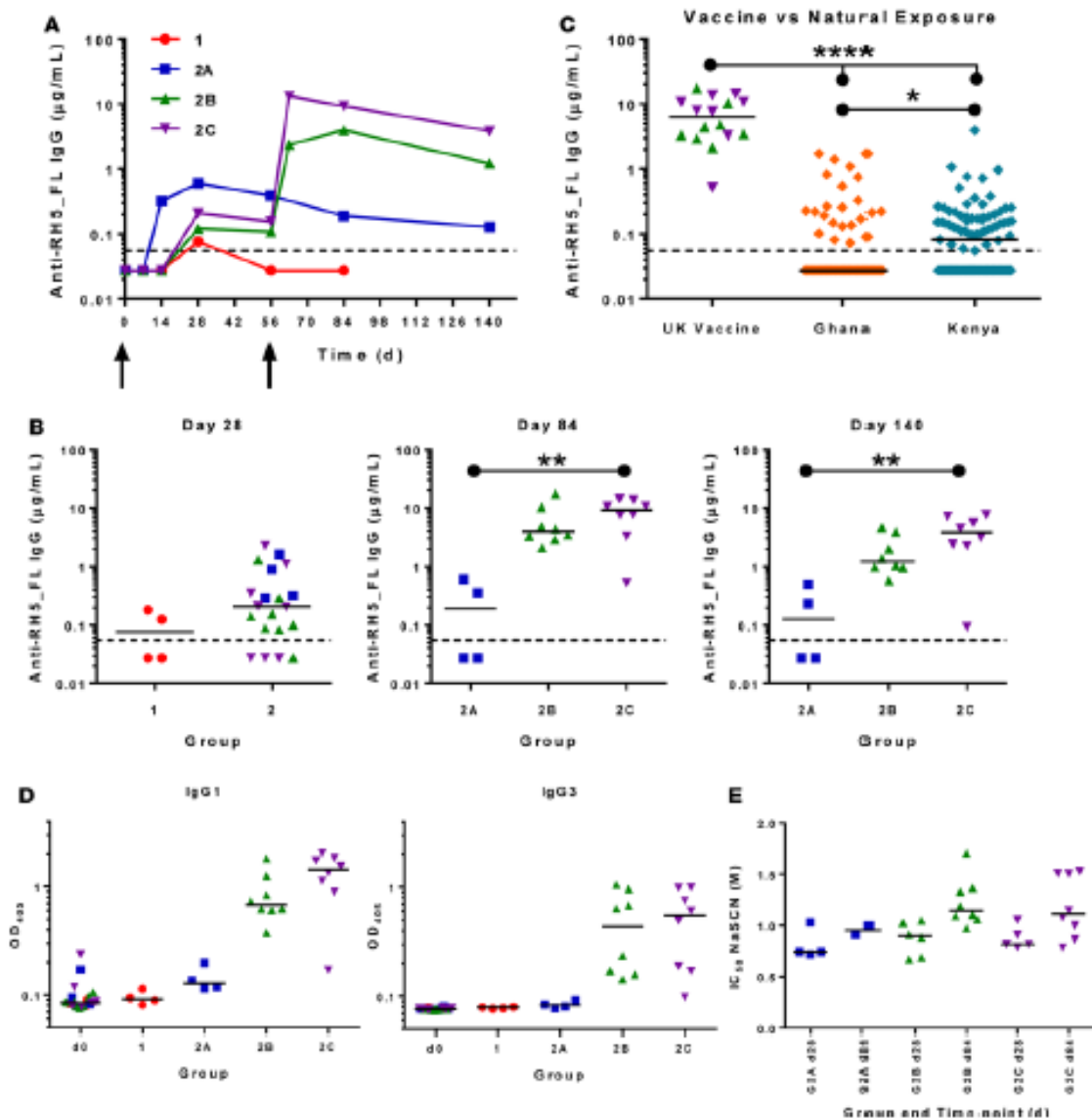


Figure 4. Serum antibody response to vaccination. Group 1 ($n = 4$), Group 2A ($n = 4$), Group 2B ($n = 8$), Group 2C ($n = 8$). (A) Median anti-RHS_FL serum total IgG responses shown for all groups over time. Individual responses are shown in Supplemental Figure 3. Median and individual responses are shown at (B) d28, d84, and d140. The horizontal dotted line indicates the limit of detection of the assay. Statistical analysis using Kruskal-Wallis test with Dunn's multiple comparison test. (C) Vaccine-induced responses shown for Groups 2B and 2C combined ($n = 16$) vs. responses following natural exposure in Ghanaian adults ($n = 79$) and Kenyan adults ($n = 96$); analysis by Kruskal-Wallis test with Dunn's multiple comparison test. (D) Isotype profiles of serum antibody responses against RHS_FL were assessed by ELISA. Responses are shown at baseline (d0) and for all groups at d84. Individual and median responses are shown for IgG1 and IgG3; results for IgG2, IgG4, IgA, and IgM are shown in Supplemental Figure 5. (E) Avidity of serum IgG responses at d28 and d84 was assessed by sodium thiocyanate (NaSCN) displacement RHS_FL ELISA and is reported as the molar (M) concentration of NaSCN required to reduce the starting OD in the ELISA by 50% (IC_{50}). Only samples with a positive response by anti-RHS_FL total IgG ELISA could be assayed for avidity. Symbols are coded according to group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.0001$.

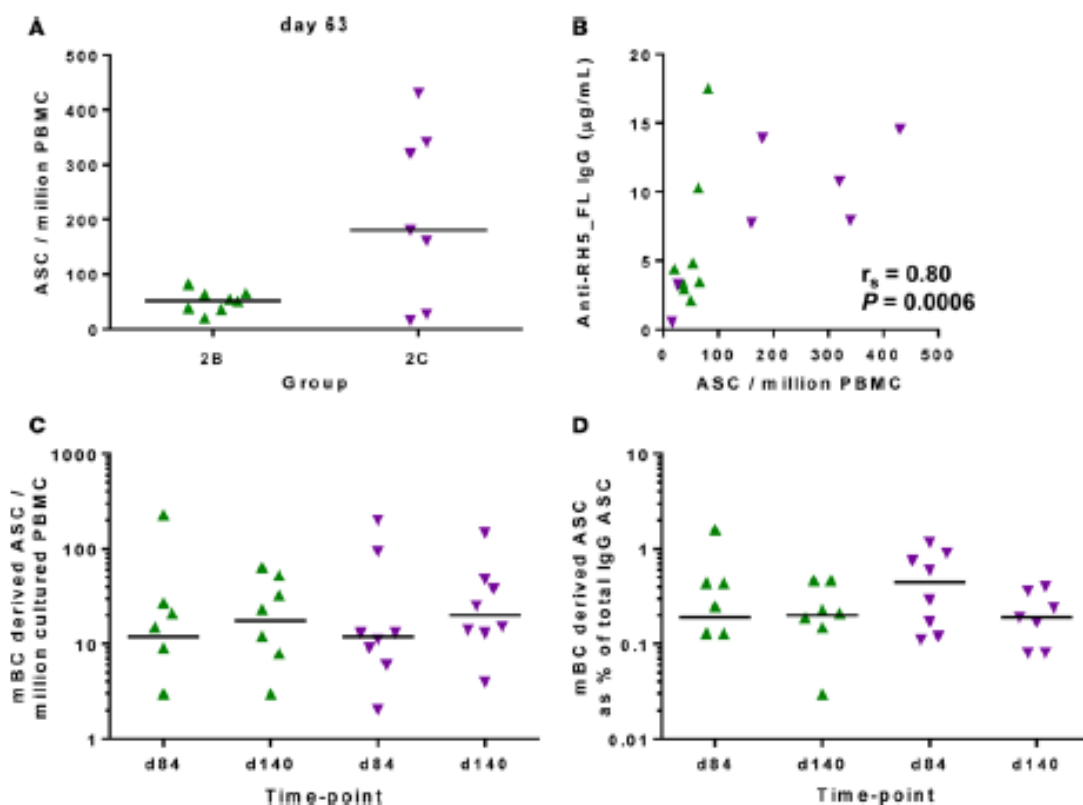


Figure 5. B cell response to vaccination. (A) R2H5-specific antibody-secreting cell (ASC) responses were assessed by ex vivo enzyme-linked immunospot (ELISPOT) using R2H5_FL protein and fresh peripheral blood mononuclear cells (PBMC) from the d63 time point. Individual and median responses are shown for each group and reported as R2H5-specific ASC per million PBMC used in the assay ($n = 8$ for Group 2B and $n = 7$ for Group 2C because 1 sample was not tested in this group). (B) Correlation of the ASC response vs. the concentrations of serum anti-R2H5_FL IgG measured at d84. Spearman's rank correlation coefficient (r_s) and P value are shown by Spearman's rank correlation. (C) R2H5-specific memory B cell (mBC) responses were assessed by ELISPOT assay using R2H5_FL protein ($n = 8$ for Groups 2B and 2C). Frozen PBMC were thawed and underwent a 6-day polyclonal restimulation, during which ASC are derived from mBC, before testing in the assay. Individual and median responses are shown from the d84 and d140 time points and are reported as mBC-derived R2H5-specific ASC per million cultured PBMC or as (D) % of total number IgG-secreting ASC ($n = 7$ for Group 2C at the d140 time point in D, otherwise $n = 8$). Groups 2B and 2C are coded by color and symbol.

mBC responses were also measured using an established cultured ELISPOT protocol, whereby mBC within PBMC undergo a 6-day polyclonal stimulation to form ASC, which are then measured using the same protocol as for the ex vivo assay. These were measured for volunteers in Groups 2B and 2C at the d84 and d140 time points (4 and 12 weeks after MVA boost). D84 was most consistently identified as the peak of the mBC response in other trials of ChAd63-MVA *P. falciparum* blood-stage malaria vaccines (51, 52). Responses are reported as number of mBC-derived R2H5-specific ASC per million cultured PBMC (Figure 5C), and as a percentage of total IgG-secreting ASC (Figure 5D). The d84 mBC responses across both groups did not correlate with peak serum antibody responses at d84 (data not shown). Overall, there were no significant differences between the groups or time points, indicating the mBC response was well maintained to the end of the study period.

Vaccine-induced antibodies show cross strain in vitro GIA. Serum was analyzed at the GIA Reference Center at NIH, with IgG purified from each sample prior to initial testing against vaccine homologous 3D7 clone parasites. Baseline samples prior to vaccination (d0) using pooled sera, as well as individual samples from Groups 1 and 2A from d84 after vaccination, did not demonstrate any GIA above baseline. Samples from Group 2B and 2C volunteers taken at d84 showed in vitro GIA of median 36.0% (range 19.7%–61.6%) and 50.6% (range 7.2%–68.1%) using 10 mg/ml purified IgG (Figure 6A). We confirmed

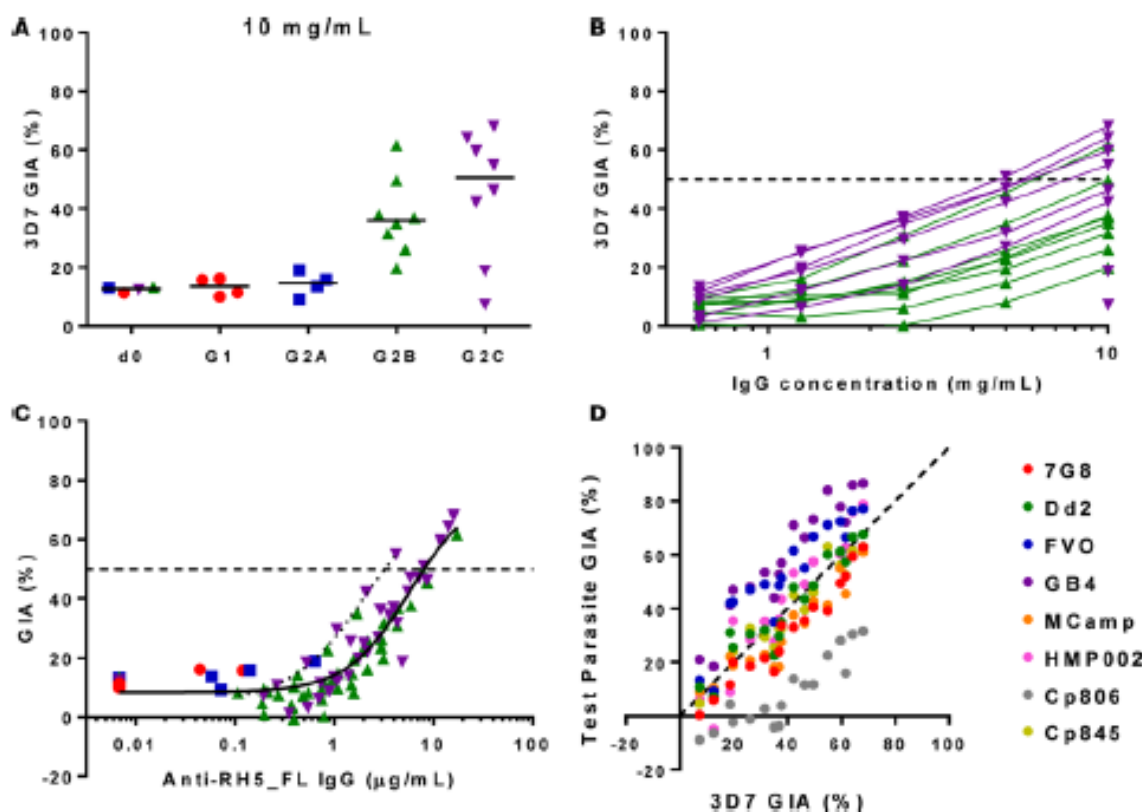


Figure 6. Functional GIA induced by ChAd63-MVA RH5 vaccination. (A) In vitro growth inhibition activity (GIA) of purified IgG was assessed at 10 mg/ml against 3D7 clone *P. falciparum* parasites. Individual data and medians are shown for each group at d84 (G1, $n = 4$; G2A, $n = 4$; G2B, $n = 8$; G2C, $n = 8$); pooled sera were used for each group ($n = 4$) at baseline (d0). (B) Dilution series of purified IgG from Group 2B and 2C d84 samples. (C) Relationship between GIA data from the dilution series shown in B and concentration of anti-RH5_FL purified IgG used in the assay as measured by ELISA. The EC_{50} (concentration of anti-RH5_FL polyclonal IgG that gives 50% GIA, dashed line) was 8.2 $\mu\text{g/ml}$ (95% CI, 7.2–9.5 $\mu\text{g/ml}$); nonlinear regression curve is shown (solid line, $r^2 = 0.90$, $n = 74$). Two volunteers (1 in Group 2B and 1 in 2C) showed a reproducibly higher EC_{50} of 3.3 $\mu\text{g/ml}$ (95% CI, 2.8–3.9 $\mu\text{g/ml}$); nonlinear regression curve is shown (dotted line, $r^2 = 0.99$, $n = 10$). (D) Purified IgG from Group 2B and 2C d84 samples, plus 1 pooled d0 preimmunization sample, were tested at 10 mg/ml against a panel of 8 other laboratory-adapted parasite lines and short-term culture-adapted parasite isolates. GIA for each parasite and test sample is plotted against corresponding GIA against 3D7 clone parasites on the x axis.

that GIA decreased as purified IgG was diluted in the assay (Figure 6B) and also that GIA was not enhanced by inclusion of complement (Supplemental Figure 6). The GIA (as routinely assessed, without complement) was related to RH5_FL-specific IgG concentration (Figure 6C), as seen for the merozoite surface protein 1 (MSP1) and apical membrane antigen 1 (AMA1) antigens following human immunization (9, 52, 53). Notably, the concentration of anti-RH5_FL polyclonal IgG that gives 50% GIA (EC_{50}) was only 8.2 $\mu\text{g/ml}$. Two volunteers also consistently showed a better quality of response, with a 2.5-fold improvement in the EC_{50} (3.3 $\mu\text{g/ml}$ RH5_FL-specific IgG) against the 3D7 clone parasite, although no other obvious differences were apparent in their antibody profile, as measured by the various ELISA assays. This EC_{50} concentration is substantially lower than previous results with vaccines against the AMA1 and MSP1 antigens (9, 52, 53). In line with this result, the overall levels of GIA using 10 mg/ml purified IgG from these RH5 vaccinees against 3D7 clone parasites were higher than those achieved with the same ChAd63-MVA platform used previously to deliver AMA1 or MSP1 (54), reaching significance for MSP1 ($P = 0.02$, Kruskal-Wallis test with Dunn's multiple comparison test comparing RH5 with AMA1 and MSP1) (Supplemental Figure 7A). However, the levels of GIA using 10 and 2.5 mg/ml purified IgG against 3D7 clone parasites were comparable with those reported previously in healthy UK adults immunized with an AMA1 recombinant protein vaccine delivered in the proprietary adjuvant sys-

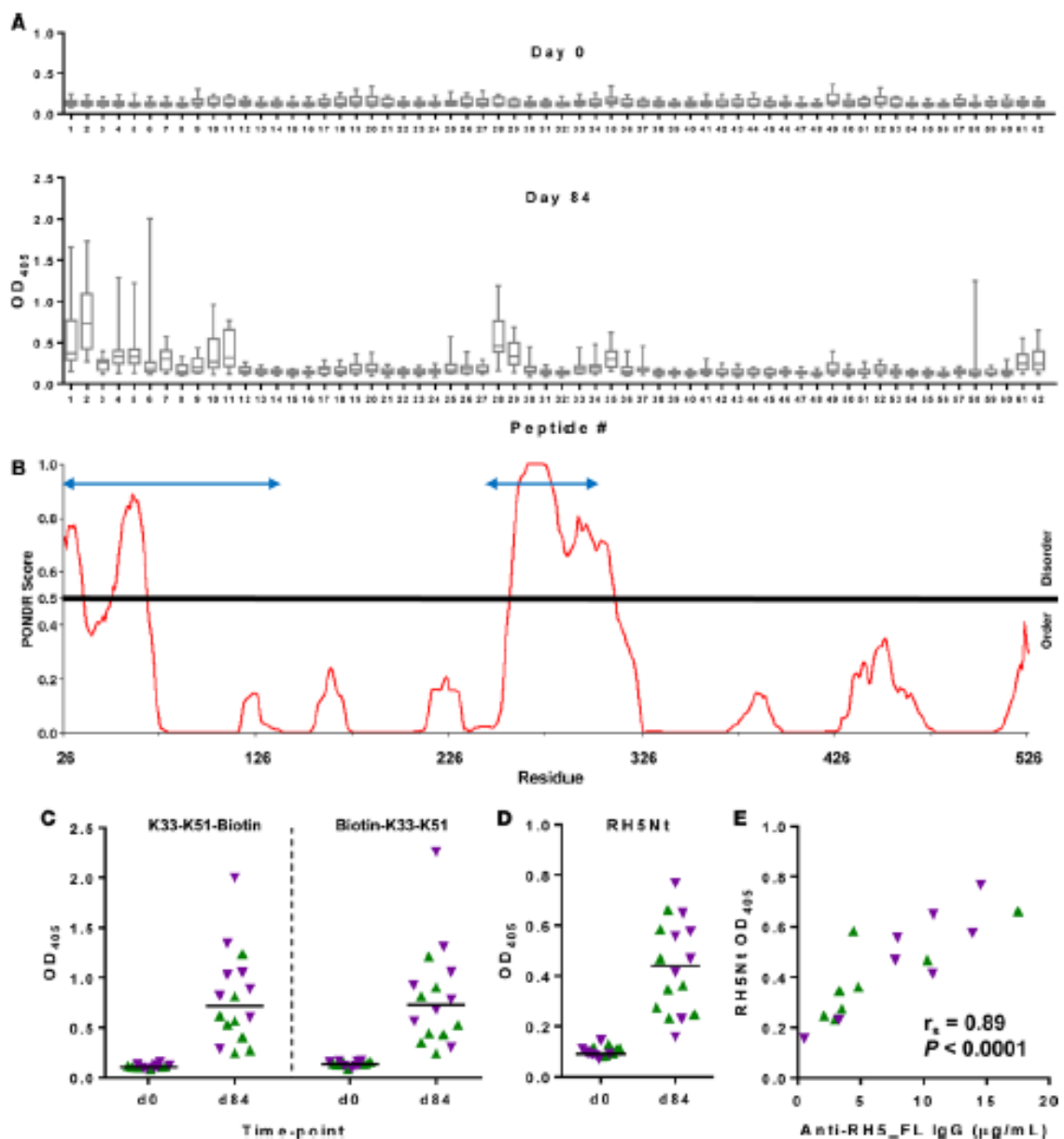


Figure 7. Vaccine-induced anti-RHS antibodies recognize linear epitopes and RHSNt. (A) D0 and d84 sera for volunteers in Groups 2B and 2C ($n = 16$) were diluted 1:100 and tested against linear overlapping peptides spanning the RHS vaccine insert. Median, interquartile range (IQR), and range are shown for each peptide. (B) Plot of disorder within the RHS vaccine construct predicted by POND R. Blue arrows indicate the regions removed in the RHS Δ NL protein (E26-Y139 and N248-M296). (C) D0 and d84 sera for volunteers in Groups 2B (green triangles) and 2C (purple triangles) ($n = 16$) were diluted 1:100 and tested against 19-mer peptides that represent the minimal P113 binding region within RHSNt (K33-K51). Peptides with N- and C-terminal biotinylation were tested to allow for binding to streptavidin-coated plates in both orientations. Individual and median results are shown. (D) D0 and d84 sera for volunteers in Groups 2B and 2C were diluted 1:100 and tested against RHSNt protein. Individual and median results are shown ($n = 16$). (E) Correlation of d84 serum IgG responses in Groups 2B and 2C ($n = 16$) against RHS_FL and RHSNt. Spearman's rank correlation coefficient (r_s) and P value are shown by Spearman's rank correlation.

tem AS01B from GSK ($P = 0.16$ and $P = 0.78$, respectively, Mann-Whitney test) (ref. 53 and Supplemental Figure 7, A and B). This relates to the fact that the AMA1/AS01B formulation was quantitatively 10 times more immunogenic, achieving approximately 100 $\mu\text{g/ml}$ AMA1-specific IgG on average in these vaccinees (53), unlike the 9.3 $\mu\text{g/ml}$ RH5_FL-specific IgG measured on average in Group 2C.

We next assessed the purified IgG from Groups 2B and 2C against a panel of 8 other laboratory-adapted parasite lines (7G8, Dd2, FVO, GB4, MComp) and short-term culture-adapted parasite isolates (from Cambodian patients with malaria [Cp845, Cp806; ref. 18] or from an Australian resident who contracted malaria in Ghana [HMP002; ref. 55]), that between them include RH5 sequences that encompass the 5 most common RH5 single nucleotide polymorphisms (SNPs) (18, 19). All parasites were neutralized by the IgG, with results for each parasite significantly correlating with 3D7 ($n = 16$, $P < 0.0001$ and Spearman's rank correlation coefficient [r_s] > 0.88 for all parasites) (Figure 6D). Notably, %GIA against 1 parasite was significantly lower: Cp806 ($P = 0.0001$); while 2 strains showed significantly higher %GIA: FVO ($P = 0.029$) and GB4 ($P = 0.0009$) by Friedman test with Dunn's multiple comparison test.

Vaccine-induced antibodies recognize linear and conformational epitopes and inhibit interactions within the RH5 complex. We next sought to better understand the fine specificity of the vaccine-induced anti-RH5 antibody response. We initially tested the d84 sera from all volunteers in Groups 2B and 2C by ELISA against a linear peptide array spanning the RH5_FL vaccine sequence (Supplemental Table 3 and Figure 7A). Responses were detected to the N-terminal region, as well as a central region and a small part of the C-terminus, suggesting the vaccine-induced anti-RH5_FL IgG do recognize linear epitopes within the RH5 molecule. Interestingly, these responses to linear epitopes mainly correspond to predicted regions of disorder within the RH5 molecule (Figure 7B). Further analysis of the peptide data highlighted peptide 28 — against which the sera from all 16 volunteers reacted — which is also recognized by the nonneutralizing mouse mAb 4BA7 (56), as well as peptide 5 in RH5Nt, which is recognized by the nonneutralizing mouse mAb RB3 (56). Other peptides of interest include peptides 1–3 within RH5Nt, given that the P113-binding region was previously mapped to a 19-mer sequence running from aa K33–K51 (29). These peptide reagents included the N38Q as substitution in the vaccine construct to remove a site of potential N-linked glycosylation. We therefore synthesized the native 19-mer sequence, biotinylated at either the N- or the C-terminus (Supplemental Table 3), and assessed responses by ELISA (Figure 7C). Similar to the results with the original peptide 2, all volunteers showed detectable responses irrespective of bound peptide orientation. We also confirmed these results using recombinant RH5Nt protein in ELISA (Figure 7D), and these data would suggest the sera have the potential to inhibit the RH5Nt-P113 interaction. Notably, these responses to RH5Nt significantly correlated with those against RH5_FL (Figure 7E), suggesting that in each vaccinee, the response to RH5Nt is a consistent proportion of the total anti-RH5_FL response irrespective of overall magnitude.

To assess whether responses are also directed against conformational epitopes, we next performed ELISA against heat-denatured RH5_FL protein (Figure 8A). These data showed significant loss of sero-reactivity in all vaccinees ($P = 0.008$ for both groups by Wilcoxon matched-pairs signed rank test) — similar to the conformation-sensitive mAb 2AC7 (49, 56), but not mAb 4BA7, which binds a linear peptide (56). To investigate further, we performed ELISA using RH5 Δ NL protein, which lacks the disordered N-terminal and central loop regions of RH5 (49), and quantified these responses by CFCA. Responses to this protein were concordant with those against RH5_FL (Figure 8B), suggesting that most of the vaccine-induced IgG recognize the RH5 Δ NL structured protein and that the linear peptide array and RH5Nt ELISA are measuring only a minor proportion of the total anti-RH5_FL response.

We next assayed the ability of these sera to inhibit interactions within the RH5 complex by avidity-based extracellular protein interaction screen (AVEXIS). These data confirmed that the d84 sera from the Groups 2B and 2C volunteers could inhibit the interaction between RH5 and P113, as well as RH5 and basigin and RH5 and CyRPA (Figure 9A). One volunteer in Group 2C showed no inhibition, consistent with their very low postboost anti-RH5_FL IgG concentration (Supplemental Figure 3D). Notably, the blocking activity observed by AVEXIS for each of the 3 interactions significantly correlated with the anti-RH5_FL IgG response (Figure 9B).

Discussion

This phase Ia dose escalation and safety clinical trial reports the first data to our knowledge in humans for a vaccine targeting the RH5 antigen from the blood-stage *P. falciparum* merozoite. We show in healthy malaria-naive UK adult volunteers that a recombinant ChAd63-MVA heterologous prime-boost immu-

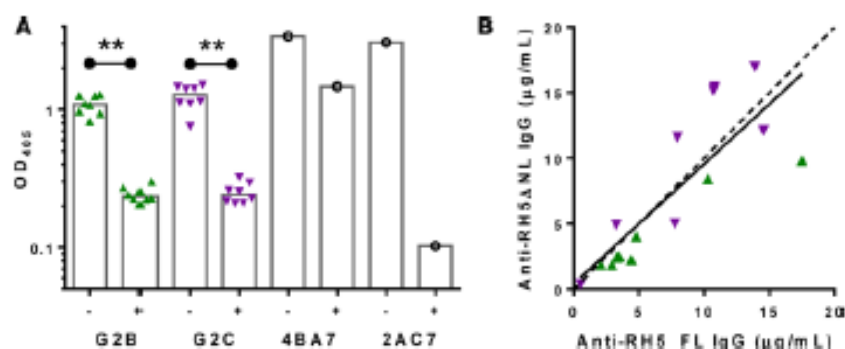


Figure 8. Vaccine-induced antibodies recognize conformational epitopes and inhibit interactions within the RHF5 invasion complex. (A) D0 and d84 sera for volunteers in Groups 2B and 2C ($n = 16$) were tested by ELISA against non-denatured RHF5_FL protein (-) and the same protein following heat denaturation (+). Individual and median responses are shown. The 4BA7 and 2AC7 mAbs were included as controls that bind a linear vs. conformational epitope, respectively. $**P < 0.01$ according to Wilcoxon matched-pairs signed rank test. (B) D84 serum ELISA responses to RHF5_FL and RHF5ΔNL for volunteers in Groups 2B and 2C ($n = 16$) were analyzed for concordance by linear regression (solid line). $r^2 = 0.69$; slope = 0.91 (95% CI, 0.56–1.27); Y intercept when X = 0.0 is 0.4 (95% CI, -2.7–3.6); X intercept when Y = 0.0 is -0.5 (95% CI, -6.0–2.3). Line of identity (X=Y) is also shown (dashed line).

nization regimen has a favorable safety profile and can induce functional RHF5-specific serum antibody responses, in addition to B and T cell responses. Reactogenicity of the ChAd63 RHF5 vector was similar to that seen with the same doses of ChAd63 vectored vaccines encoding the *P. falciparum* antigen multi-epitope string-thrombospondin-related adhesion protein (ME-TRAP), circumsporozoite protein (CSP), MSP1, or AMA1 (36–39, 52, 54) or the *P. vivax* antigen Duffy-binding protein region II (PvDBP_RII) (43). The same vectors encoding ME-TRAP have similarly been safe following immunization of adults, children, and infants residing in malaria-endemic areas (41, 42). Our data with ChAd63 RHF5 add to the growing body of evidence that this simian adenovirus vector is safe for clinical use. Likewise, the clinical safety of MVA as a recombinant vaccine vector for many infectious diseases and cancer is now well documented (57). MVA RHF5 appeared to be more reactogenic than the ChAd63 vector at the higher dose, consistent with previous vaccine trials using this orthopoxvirus vector (38, 39, 43, 44, 46, 58).

The ChAd63 and MVA vectors used here were designed to maximize induction of antibody responses against blood-stage malaria antigens, in conjunction with the T cell responses for which they are more routinely used (32, 33). In this trial, RHF5-specific IFN- γ T cell responses were induced and peaked following the MVA boost at median levels of $> 2,000$ SFU/million PBMC. No data on cellular responses to this antigen have been reported following natural *P. falciparum* infection; however, given that vaccination elicited peptide-restimulated responses spanning the entire RHF5 sequence, the RHF5_FL molecule does not appear to lack T cell epitopes. The kinetics and magnitude of the response were also similar to those previously reported following human vaccination with the same vectors encoding *P. falciparum* or *P. vivax* antigens (36–39, 43). Similarly, studies using chimpanzee adenovirus vectors followed by MVA boost (44, 46, 58) have routinely shown that a mixed antigen-specific CD4⁺/CD8⁺ T cell response is induced in humans. The possible contribution of T cells to blood-stage malaria protection remains unclear, given that previous clinical trials using whole parasite immunization (59) or ChAd63-MVA vectors encoding MSP1 or AMA1 (54) failed to show an impact on blood-stage parasite growth following controlled human malaria infection (CHMI) with *P. falciparum* despite strong T cell induction by vaccination. However, CD8⁺ T cells against blood-stage antigens can act against late liver-stage forms of the parasite once merozoite antigens are expressed (54, 60), while viral vector vaccine-induced CD4⁺ T cells will provide key help to B cell responses (61, 62).

In agreement with preclinical data in mice, rabbits, and *Aotus* monkeys (12, 34), the ChAd63-MVA RHF5 vaccines induced RHF5_FL-specific serum IgG antibody responses, peaking at a median of 0.2 $\mu\text{g}/\text{ml}$ after ChAd63 prime and 9.3 $\mu\text{g}/\text{ml}$ after MVA boost in the full-dose vaccination Group 2C. The overall kinetic and magnitude of the RHF5_FL-specific IgG, ASC, and mBC responses induced here in malaria-naïve humans are broadly comparable with those reported for the same vectors encoding PvDBP_RII (43) or *P. falciparum* MSP1 and AMA1 (38, 39, 51, 52). These vaccine-induced RHF5_FL-specific IgG concentra-

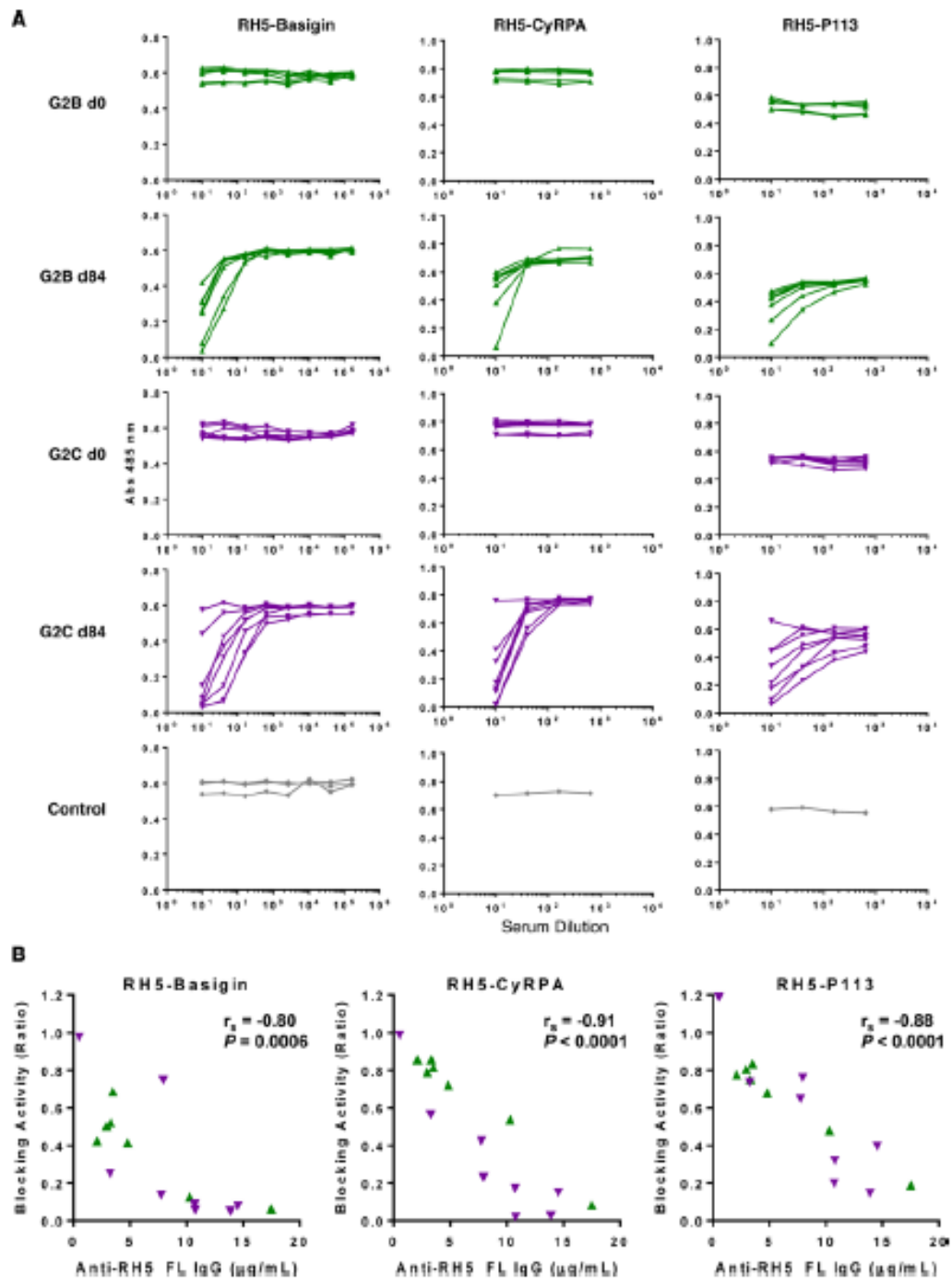


Figure 9. Vaccine-induced antibodies inhibit interactions within the RH5 invasion complex. (A) D0 and d84 Group 2B sera ($n = 7$) and Group 2C sera ($n = 8$) were tested for their ability to inhibit the interaction between proteins from the RH5 invasion complex by AVEXIS. Dilution of each test serum sample is shown starting at 1:10. Results with various assay controls also shown (no serum for RH5-Basigin, and anti-AMA1 for RH5-CyRPA and RH5-P113). Each point represents the mean of duplicate or triplicate wells. (B) Correlation of blocking activity for each interaction using d84

sera from Groups 2B and 2C ($n = 15$) against anti-RH5_FL serum IgG responses measured by ELISA. Blocking activity was calculated for each individual sample from the data in panel A as the ratio of the Abs 485 nm at 1:10 serum dilution divided by the Abs 485 nm at the highest serum dilution tested. Spearman's rank correlation coefficient (r) and P value (Spearman's rank correlation) are shown.

tions are similar to those observed following ChAd63-MVA immunization with PvDBP_R11 (43) but are lower than *P. falciparum* AMA1 (38, 52) and MSP1 (38) and higher than CSP (37). Notably, in agreement with immunoepidemiological datasets (12, 23–26), anti-RH5_FL IgG responses were weak in the Kenyan and Ghanaian adult populations assessed here for comparison, with median responses < 100 ng/ml. The vaccine data, thus, confirm that RH5 is not in itself a weakly immunogenic antigen in humans but instead suffers modest immunogenicity in the context of natural *P. falciparum* infection.

Consistent with other ChAd63-MVA malaria vaccines (43, 52, 63), the anti-RH5_FL serum IgG response was largely composed of IgG1 and IgG3, with moderate avidity as measured by NaSCN displacement ELISA. A study of naturally exposed individuals in Mali reported that the predominant IgG isotype detected against RH5 was IgG3 (23). A similar result was observed in children from Papua New Guinea (64), suggesting qualitative aspects of the vaccine-induced response may differ to those induced by natural exposure. Overall, the relevance of antibody isotype, affinity, and avidity to in vivo protection in humans against the *P. falciparum* merozoite remain debated, although the study in Papua New Guinea associated the IgG3 response against RH5 with reduced risk of malaria (64).

We subsequently assessed the functional anti-parasitic antibody activity using the standardized in vitro assay of GIA. In agreement with preclinical studies, these data confirmed the vaccine-induced antibodies in humans were strain transcending, showing activity against all tested parasites. Notably, addition of human complement did not increase levels of GIA, as reported for some merozoite surface proteins (65). This is perhaps not surprising, given that antibodies only have a very short window of opportunity to bind RH5 following its release from the rhoptries, likely leading to time constraints on complement recruitment. Notably, some individual parasite laboratory lines or short-term culture-adapted parasite isolates significantly differed in their sensitivity to neutralization in comparison with the 3D7 reference clone — with 2 being easier to neutralize and 1 less so. The reasons for these differences remain to be investigated. The panel of parasites used here covered a significant breadth of the known RH5 sequence diversity (18, 19, 21), encompassing at least 8 catalogued nonsynonymous SNPs, including the top fifth and seventh most common SNPs by global minor allele frequency (MAF) at positions 148, 147, 197, 203, 410, and 407 (MalariaGEN v4.0, ref. 66). A previous rabbit study using the same parasite isolates from Cambodian patients showed a similar range of susceptibility to vaccine-induced anti-RH5_FL IgG (18), which was not associated with the presence of small numbers of specific polymorphisms. It remains likely that other aspects of the RH5 invasion complex biology can affect the susceptibility of individual parasite isolates to anti-RH5 antibodies.

Most encouragingly, the GIA EC_{50} concentration of RH5_FL-specific polyclonal human IgG against 3D7 clone parasites was only 8 μ g/ml. A previous study affinity-purified RH5-specific human IgG from plasma of naturally exposed individuals in Mali and tested for GIA, reporting an EC_{50} concentration of 55 μ g/ml (23), which suggests the vaccine-induced IgG here may be qualitatively superior to the responses induced against RH5 following natural parasite exposure. Moreover, this level is substantially lower than previous results with vaccines tested in humans against the AMA1 and MSP1 antigens, where GIA EC_{50} concentrations were reported from 70–100 μ g/ml (9, 52, 53) and > 600 μ g/ml (9) for AMA1- and MSP1-specific human IgG, respectively. Consequently, the levels of GIA observed here with ChAd63-MVA RH5 outperformed those previously observed in humans with the same vectors encoding MSP1 or AMA1 (54), given that these vaccines all induce quantitatively similar levels of IgG. Our data, thus, identify a clear hierarchy, where by the RH5 antigen is inherently more susceptible to vaccine-induced human IgG than the historically favored AMA1 and MSP1 target antigens — consistent with preclinical data in rabbits (18) and in vivo protection data in *Aotus* monkeys (34), boding well for future efficacy testing of RH5-based vaccines.

Finally, we further sought to better understand the breadth of epitopes recognized by anti-RH5_FL IgG. We initially identified regions of linear antibody epitopes within the RH5_FL molecule by peptide array, including the disordered N-terminus and internal loop between helices 2 and 3 (49). Mouse mAbs that bind linear peptides within both of these regions have been shown to be nonneutralizing (56); however, immunization data with RH5Nt (29) and another mAb study (67) suggest the N-terminal region of the molecule can elicit functional antibodies that may block P13 binding and/or interfere with proteolytic pro-

cessing of RH5_FL. Further analysis confirmed the vaccine-induced IgG also recognized conformational epitopes. Quantitative assessment by ELISA and CFCA using the structured RH5 Δ NL protein (which lacks the disordered N-terminus and internal loop) suggested that most of vaccine-induced IgG recognize this region of RH5 and that antibodies against RH5Nt constitute only a minor, but consistent, proportion of the total anti-RH5_FL response. Subsequent testing by AVEXIS confirmed the sera from vaccinees could block the interaction of RH5_FL with P113, CyRPA, and basigin, consistent with known information relating to the binding of these molecules (29, 49, 56, 68) and the measurable antibody responses against both RH5Nt and RH5 Δ NL. Interestingly, blocking activity for all 3 interactions correlated with the anti-RH5_FL IgG response, suggesting qualitatively similar responses were induced in all vaccinees. Our ongoing work will focus on elucidating epitopes recognized by vaccine-induced human B cells in order to guide future immunomonitoring and better understand mechanisms of *P. falciparum* merozoite neutralization and inhibition of these interactions within the complex.

Overall, substantial progress has been made since RH5_FL was first reported as a promising new vaccine candidate in late 2011. Here, we demonstrate for the first time to our knowledge, using a rapidly translated viral vectored vaccine platform, that substantial RH5_FL-specific antibodies and B cell and T cell responses can be induced safely by immunization in humans, in contrast to the levels of responses observed following years of natural malaria exposure. Our recent demonstration of a blood-stage CHMI model for vaccine testing using *P. falciparum* (53) will allow for rapid phase IIa proof-of-concept efficacy testing of RH5-based vaccines in the near future.

Methods

Detailed methods are provided in Supplemental Methods.

ChAd63 and MVA RH5 vaccines. The design, production, and preclinical testing of the viral vector vaccines have been reported previously in detail (12, 34). Briefly, both recombinant viruses express the same 1,503 bp coding sequence of RH5 from the 3D7 clone of *P. falciparum*, aa E26-Q526 (NCBI accession number XM_001351508.1). ChAd63 RH5 was manufactured by Advent, which is a daughter company of ReThera. This production facility meets current Good Manufacturing Practice (cGMP) requirements of the US Food and Drug Administration (FDA) and the European Medicines Agency (EMA) to produce investigational vaccines to be used in human clinical studies. MVA RH5 was manufactured under cGMP conditions by IDT Biologika GmbH, Germany, as previously described (38).

Study design. VAC057 was a first-in-human, open-label, nonrandomized, dose-escalation phase Ia clinical trial evaluating the safety and immunogenicity of the viral vectored vaccines ChAd63 RH5 and MVA RH5 in a heterologous prime-boost regime with an 8-week interval. The study was conducted in the UK at the Centre for Clinical Vaccinology and Tropical Medicine (CCVTM; University of Oxford, Oxford, United Kingdom) and the NIHR Wellcome Trust Clinical Research Facility (WTCRF) in Southampton, UK. The trial was registered on Clinicaltrials.gov (NCT02181088). The primary endpoint of the study was to assess the safety of ChAd63 RH5 and MVA RH5, with a secondary endpoint to assess immunogenicity.

Participants. Healthy, malaria-naive males and nonpregnant females aged 18–50 were invited to participate in the study. Volunteers were recruited and vaccinated at the CCVTM, part of the Oxford Vaccine Centre (OVC), at the University of Oxford and the NIHR WTCRF in Southampton. Twenty-four volunteers were enrolled in total. A full list of inclusion and exclusion criteria is reported in Supplemental Methods.

Safety analysis. Following each vaccination, volunteers completed an electronic diary card for 28 days with any AE data. AE data were also collected at follow-up visits. Observations (heart rate, temperature, and blood measures) were taken at the clinic visits from the day of vaccination until the d28 follow-up visit. Blood tests for exploratory immunology were taken at all visits except those occurring 2 days after each vaccination (i.e., d2 and d58). Blood samples for safety (full blood count, liver function, urea, and electrolytes) were carried out at screening, d0, d7, and d28 for all groups, as well as on d56, d63, and d84 for Groups 2B and 2C. Any solicited AEs occurring during the diary card period were defined as being at least possibly related to vaccination. The likely causality of all other AEs was assessed as described in the protocol, and all AEs considered possibly, probably, or definitely related to vaccination are reported (Supplemental Table 1). Further details on grading are provided in the Supplemental Material.

Peptides. Peptides for ex vivo IFN- γ ELISPOT and the K33-K51 ELISA peptides were purchased from NEO Scientific (Supplemental Table 2), while the biotinylated overlapping 20-mer peptides for ELISA were synthesized by Mimotopes (Supplemental Table 3).

Recombinant RH5 proteins for ELISAs and B cell assays. Recombinant RH5_FL and RH5 Δ NL proteins were generated using *Drosophila melanogaster* Schneider 2 (S2) polyclonal stable cell lines (ExpreS² platform, ExpreS²ion Biotechnologies) (69), while RH5Nt protein was produced using HEK293-6E cells as previously described (29).

Ex vivo IFN- γ ELISPOT. Ex vivo IFN- γ ELISPOT was used to assess the kinetics and magnitude of the vaccine-induced T cell responses over time. Fresh PBMC were used in all assays using a previously described protocol (39). Results are expressed as IFN- γ SFU per million PBMC.

Total IgG ELISAs. ELISAs were performed using standardized methodology as previously described (38, 39), except that plates were coated with recombinant RH5_FL or RH5 Δ NL protein produced from the *Drosophila* S2 cells. Responses measured in AU are reported in μ g/ml following generation of a conversion factor by calibration-free concentration analysis (CPCA).

Avidity and isotype ELISAs. IgG antibody avidity was assessed by NaSCN displacement ELISA using previously described methodology (63), except that plates were coated with recombinant RH5_FL produced from the *Drosophila* S2 cells at 2 μ g/ml. The concentration of NaSCN required to reduce the OD₄₀₅ (optical density measured at 405 nm) to 50% of that without NaSCN was used as a measure of avidity (IC₅₀). Antibody isotype ELISAs were also performed using methodology described in detail elsewhere (63) with the same exception as for the avidity ELISA.

mBC and ASC ELISPOT. mBC ELISPOT assays using RH5_FL protein were performed as described in detail elsewhere (51). Ex vivo ASC ELISPOT assays were performed using fresh PBMC directly prepared and added to the ELISPOT plate with no preceding 6-day culture.

Assay of GIA. The ability of antibodies to inhibit growth of *P. falciparum* parasites in vitro was assessed at the GIA Reference Center (NIAID, NIH) as previously described (70). All samples were tested at 10 mg/ml in a final test well, followed by a dilution series for positive samples to determine the concentration that gave 50% GIA (EC₅₀). Serum IgG concentrations were measured using HPLC.

AVEXIS. AVEXIS was performed essentially as described (71) using d0 and d84 serum. Controls included addition of no serum or addition of polyclonal rabbit IgG raised against AMA1 (19).

Statistic. Data were analyzed using GraphPad Prism version 6.07 for Windows (GraphPad Software Inc.). All tests used were 2-tailed and are described in the text. A value of $P < 0.05$ was considered significant.

Study approval. The VAC057 study received ethical approval from the Oxfordshire Research Ethics Committee A in the UK (REC reference 14/SC/0120). The study was also reviewed and approved by the UK Medicines and Healthcare products Regulatory Agency (MHRA, reference 21584/0331/001-0001). Volunteers signed written consent forms, and consent was verified before each vaccination. The trial was conducted according to the principles of the current revision of the Declaration of Helsinki 2008 and in full conformity with the ICH guidelines for Good Clinical Practice (GCP).

Author contributions

ROP, SES, SCE, KM, AD, FG, HDG, NJB, IDP, OJG, NJE, JJ, GML, DGWA, LS, SDM, NG, SB, SNF, AVSH, and SJD conceived and performed the experiments. ROP, SES, SCE, KM, FG, CAL, and SJD analyzed the data. JJ, KM, EB, ASI, CMN, MB, FDP, MFO, LB, JW, LMM, FHO, JSM, AMM, ADD, GJW, and CAL contributed reagents, materials, and analysis tools. RR, RA, NKV, FLN, JV, and AML managed the project. ROP and SJD wrote the paper.

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