DEVELOPMENT OF ANTIBODY DEPENDENT RESPIRATORY BURST ASSAY AS A TOOL FOR EVALUATING CELLULAR IMMUNITY TO MALARIA

BY

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University

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DEDICATION

To my loving mother, Mrs. Esther Magaka Owuor and father, the late Mr. Samuel Isaac Owuor Wauna, who laid the indispensible foundation for our family education to make us better citizens in the society.

To my daughter Abigail, sister Marceline and brothers: Philip, Ayub and Enoch.

ABSTRACT

Malaria vaccine clinical trials in exposed populations have not always been consistent in finding robust associations between the predicted immune responses and protection against disease. The selection of most promising vaccine candidates have been based on direct antibody inhibition assays that have performed dismally. This suggests that such immune correlates employed in assessing their efficacies may not be surrogate markers of protection. Immunity to asexual blood stage malaria is complex and likely involves multiple mechanisms. IgG antibodies are thought to play a critical role and a corresponding reliable *in vitro* correlate of antibody-mediated cellular immunity has long been sought to facilitate malaria vaccine development. The aim of present work was to establish an effector cell antibody-dependent respiratory burst assay as a correlate of naturally acquired immunity that may be a useful tool to evaluate vaccine-induced immunity in malaria studies. The specific objectives of the study were: to determine if polymorphonuclear neutrophils (PMNs) and monocytes could be triggered by malaria merozoites to produce reactive oxygen species (ROS); to determine the minimum number of merozoites required for the trigger; to determine the reproducibility of the assay, to determine the applicability of the assay by testing if the blood stage malaria vaccine candidates MSP–1, MSP–2 and AMA–1 elicited antibodies that trigger effector cell respiratory burst, and to associate total antibody titres to respiratory burst activity after vaccination. This study adopted an experimental design where the characteristics of the antibody mediated effector-cell-dependent respiratory burst induction were established and the key variables determining the performance of the assay evaluated. Merozoites opsonised with either malaria hyper immune IgG from Africa or IgGs raised from vaccinations with either MSP-1, MSP-2 or AMA-1 were incubated with PMNs/monocytes and production of ROS determined by isoluminol-amplified chemiluminesence in triplicate assays. ELISA assays were performed alongside to determine if total antibody titres elicited by vaccination had any association with the magnitudes of ROS measured. Results show that monocytes (fresh or cryopreserved) and PMNs are effective at respiratory burst induction of ROS. At equivalent cell numbers, PMNs exhibited higher ROS production than autologous monocytes ($p<0.001$) and for both cell subsets, burst induction was dependent on intact merozoites. The respiratory burst activity was achieved at ratios as low as one opsonised merozoite per effector cell. For the malaria vaccine candidates, the antibody mediated ROS production increased following vaccination, with the magnitude of increase depending on the vaccine antigen. However, there was no correlation between the total serum antibody titres and the respiratory burst activity induction ($r^2 = 0.2419$). These results postulate that the *ex vivo* antibody-mediated ROS assay which the study has shown to be reproducible, may be a vital tool and recommends its use in evaluating the functional relevance of anti-malaria antibodies in studies. However, given the complexity of malaria infection and the corresponding immune response, it is probable that protective immunity against the pathogen require multiple effector mechanisms and additional assays measuring other mechanisms could be needed to correlate unequivocally with protection. In conclusion, this assay offers a useful platform for elucidating malaria vaccine candidates in clinical trials and in performing malaria immuno-epidemiologic studies.

TABLE OF CONTENTS

DEFINITION OF TERMINOLOGIES

Breakthrough infection:	Infection in a vaccinated individual where the same vaccine is meant to prevent
Leaky vaccine:	A vaccine that confers only partial protection to successfully vaccinated individuals (vaccinated individuals may be prone to the same infection the vaccine is meant to prevent).
Direct assay/mechanism:	An assay/mechanism where antibodies impair malaria parasite growth and development by directly binding onto them.
Indirect assay/mechanism:	An assay/ mechanism where antibodies impair parasite growth and development through other effector mechanisms without necessarily binding onto them

LIST OF ABBREVIATIONS

Ab	Antibody
ACD	Active Case Detection
ACT	Artemisinin-based Combination Therapy
ADCI	Antibody Dependent Cellular Inhibition
AFR	African Region
AFRO,	Regional Office for Africa
AIDS	Acquired Immuno Deficiency Syndrome
AL	Artemether-Lumefantrine
ALMA	African Leaders Malaria Alliance
AMA – 1	Apical Membrane Antigen – 1
AMFm	Affordable Medicine Facility–malaria
AMP	Alliance for Malaria Prevention
AMR	Region of the Americas
AMRO	Regional Office for the Americas
ANC	Antenatal Care
ANVR	Africa Network for Vector Resistance
API	Annual Parasite Index
AQ	Amodiaquine
ARDS	Acute Respiratory Distress Syndrome
AT	Atovaquone
AU	Arbitrary Unit

AusAID	Australian Agency for International Development
BER	Annual Blood Examination Rate
CDC	US Centres for Disease Control and Prevention
CFR	Case Fatality Rate
CGD	Chronic Granulomatous Disease
CHAI	Clinton Health Access Initiative
CHMI	Controlled Human Malaria Infection
CIDA	Canadian International Development Agency
CPS	Counts Per Second
CS	Circumsporozoite
CSP	Circumsporozoite Protein
DDT	Dichloro-diphenyl-trichloroethane
DFID	The United Kingdom Department For International Development
DHS	Demographic and Health Survey
DIPI	Domestic Investment Priority Index
DTP	Diphtheria–Tetanus–Pertussis
DXFM	Desferoxide Mesylate
E8	Elimination Eight
EBA	Erythrocyte Binding Antigen
ELISA	Enzyme Linked Immunosorbent Assay
ELISpot	Enzyme-Linked Immunospot
EMR	Eastern Mediterranean Region

EMRO	Regional Office for the Eastern Mediterranean
EPI	Expanded Programme on Immunisation
ERAR	Emergency Response to artemisinin resistance in the Greater Mekong sub-region
ERG	Expert Review Group
EUR	European Region
EURO	Regional Office for Europe
FIND	Foundation for Innovative New Diagnostics
G6PD	Glucose-6-phosphate Dehydrogenase
GIA	Growth Inhibition Assay
Global Fund	The Global Fund to Fight AIDS, Tuberculosis and Malaria
GMAP	Global Malaria Action Plan
GMP	Global Malaria Programme
GNI	Gross National Income
GPARC	Global Plan for Artemisinin Resistance Containment
GPI-anchored	Glycosylphosphatidylinositol anchored
GPIRM	Global Plan for Insecticide Resistance
GSK	GlaxoSmithKline
HIV	Human Immunodeficiency Virus
HMIS	Health Management Information System
iCCM	Integrated Community Case Management
ICS	Intracellular Cytokine Staining
IEC	Information, Education and Communication

IFA	Immunofluorescent Assay
IgG	Immunoglobulin G
IHME	Institute for Health Metrics and Evaluation
IM	Intramuscular
IPT	Intermittent Preventive Treatment
IPTc	Intermittent Preventive Treatment for children
IPTi	Intermittent Preventive Treatment in infants
IPTp	Intermittent Preventive Treatment in pregnancy
IRS	Insecticide Residual Spraying
IRS	Indoor Residual Spraying
ISGlobal	Barcelona Institute for Global Health
ITNs	Insecticide Treated Nets
LLIN	Long-lasting insecticidal net
MAP	Malaria Atlas Project
MDG	Millennium Development Goal
MERG	RBM Monitoring and Evaluation Reference Group
MICS	Multiple Indicator Cluster Survey
MIS	Malaria Indicator Survey
MPAC	Malaria Policy Advisory Committee
MPO	Myloperoxidase
MSP – 1	Merozoite Surface Antigen-1
MVI	Malaria Vaccine Initiative
NIAID	National Institute of Allergies and Infectious Diseases

NMCP	National Malaria Control Programme
OD	Optical Density
OECD	Organisation for Economic Co-operation and Development
PATH	Program for Appropriate Technology in Health
PATH NGO	Non-governmental Organization
PCD	Passive Case Detection
PEMs	Parasitophorous Membrane Enclosed Merozoite structures
PMI	The United States President's Malaria Initiative
QA	Quality Assurance
RAM	Rotarians Against Malaria
RBM	Roll Back Malaria
RDT	Rapid Diagnostic Test
ROS	Reactive Oxygen Species
SAGE	WHO Strategic Advisory Group of Experts on Immunization
SEAR	South-East Asia Region
SEARO	Regional Office for South-East Asia
SMC	Seasonal Malaria Chemoprevention
SOD	Superoxide Dismutase
SP	Sulfadoxine-pyrimethamine
SPR	Slide Positivity Rate
TDR	Special Programme for Research and Training in Tropical Diseases
TEG	Technical Expert Group
UNAIDS	Joint United Nations Programme on HIV/AIDS

UNDP	United Nations Development Programme
UNICEF	United Nations Children's Fund
UNSE	Office of the United Nations Special Envoy for Malaria
USAID	United States Agency for International Development
VCAG	Vector Control Advisory Group
WER	WHO Weekly Epidemiological Record
WHA	World Health Assembly
WHO	World Health Organization
WHOPES	WHO Pesticide Evaluation Scheme
WPR	Western Pacific Region
WPRO	Regional Office for the Western Pacific

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE: INTRODUCTION

1.1 Background Information

Development of a malaria vaccine is an age-old puzzle that presents a compelling scientific challenge. Several factors may explain the historic failure to produce an effective vaccine. There is an immense challenge to identify appropriate malaria vaccine candidate(s). The stages in the life cycle of malaria that are targeted by vaccine developers are multiple (Fig. 1.1) and it is difficult to zero-in as to which one would give the much-needed candidate. The options for vaccine development include the sporozoites, liver stages, blood stages, sexual stages (gametocytes) and intra-mosquito stages (von Seidlein and Bejon, 2013). In addition, the parasite shows great complexity. The *Plasmodium* genus presents a myriad of antigens which vary throughout the different stages of the parasite's life cycle, and against which sequential consecutive immune responses are required. Many of the parasite proteins exhibit high polymorphic variants, and a single parasitic clone may have up to 50 different copies of the gene coding for an essential protein expressing a different version of such protein in each successive wave of parasitemia (Aide *et al*., 2007). This particular antigenic variability appears to be critical for the parasite's survival and, clearly is a disadvantage not only for the infected individual but also for the scientists aiming to design a vaccine against malaria (Fig 1.1)

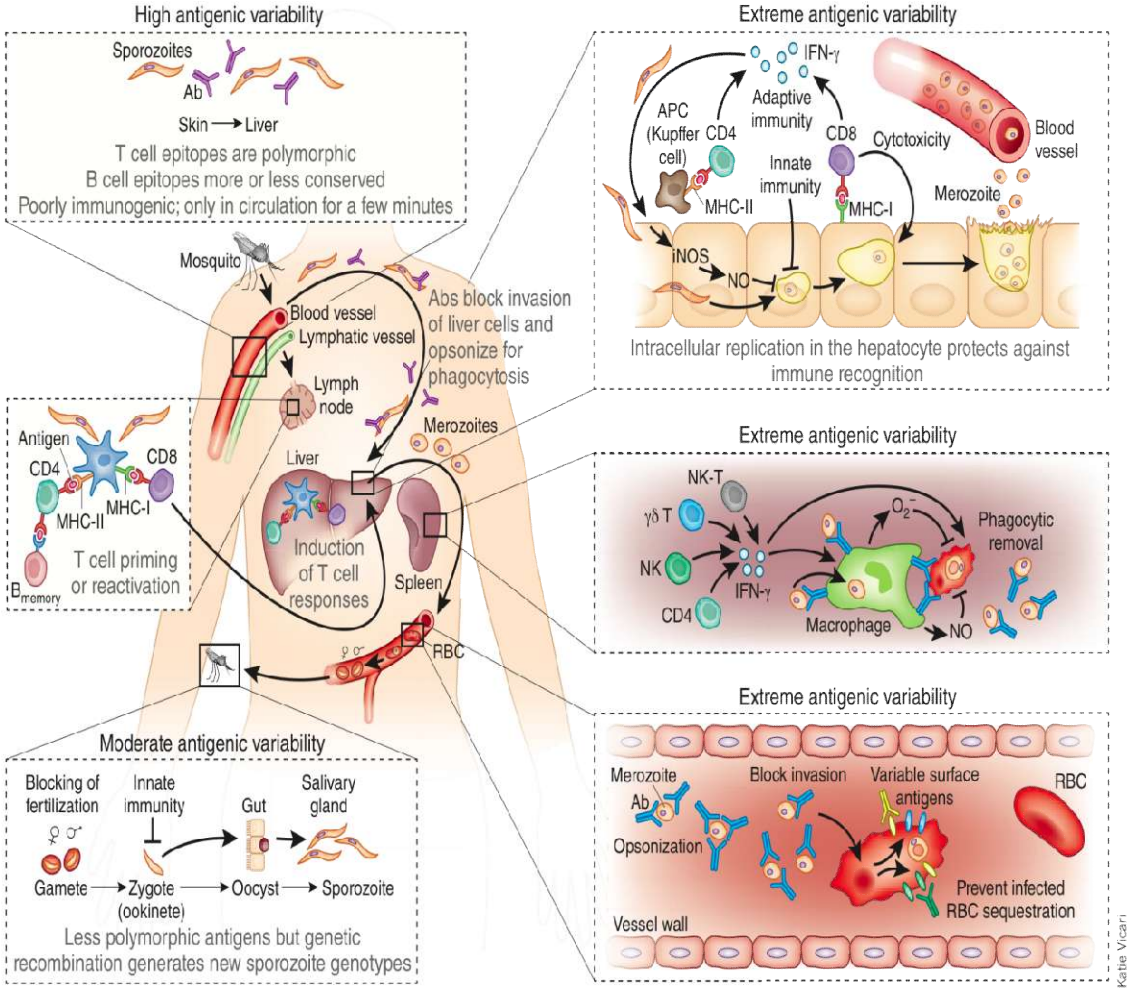


Fig 1.1 Plasmodium falciparum life cycles showing the potential challenges for vaccine developers at the different parasite developmental stages (Sauerwein *et al*., 2011)

In addition, there is no appropriate animal model to study human malaria, implying that the only way of testing the efficacy of a potential vaccine depends on logistically complex clinical trials being carried out in malaria endemic areas (Bonn,

2005). This is exacerbated by the limited and incomplete knowledge as to how acquired immunity is developed against malaria and robust correlates of immunity to evaluate such. So far, no reliable surrogates of immunity have been found (von Seidlein and Bejon, 2013).

In the blood-stage malaria vaccine development, only two functional assays have currently been characterized to measure antibody-mediated immunity to malaria: the growth inhibition assay (GIA) and the antibody-dependent cellular inhibition (ADCI) assay (Cavanagh *et al*., 2011). Whereas both assays measure the capacity of antimalarial antibodies to affect parasite replication *in vitro*, GIA is thought to measure direct parasite growth inhibition, with the ADCI measuring inhibition of parasites when the antibodies are incorporated with monocytes. Despite ADCI being the only assay postulated to measure antibody mediated cellular response to malaria, it has not been widely reproduced in other laboratories and its relevance to clinical protection in humans has not been validated (Joos *et al*., 2010).

GIA on the other hand, despite having been standardised with preliminary data in controlled human malaria infection (CHMI) studies indicating a possible association between the *in vitro* and *in vivo* parasite growth rates (Duncan *et al*., 2012), continue to demonstrate conflicting results in immunoepidemiology studies, where associations with exposure rather than protection have been observed (Duncan *et al*., 2012). In addition, GIA-interfering antibodies in vaccinated individuals from endemic regions may limit assay sensitivity in heavily malaria-exposed populations (Duncan *et al*., 2012). This spells a formidable challenge in the development of a protective malaria vaccine and more effort is needed to establish other robust and more utilisable assays, particularly

when there is no certainty about which specific antigens play a key role in the development of immunity (Aide *et al*., 2007). The current study attempted to make valuable contribution towards filling this gap of knowledge, striking an in-depth understanding on acquired immunity with the subsequent postulation of a new surrogate marker of protection against malaria.

The approach used in the current study to develop an antibody-mediated cellular response assay (given the aforementioned shortcoming in previous efforts) is based on clinical observations from malaria endemic areas where populations persistently exposed to *Plasmodium falciparum* develop, after 10–20 years, a non-sterile type of immunity against the blood stages of the parasite (Sergent, 1950) and, having reached this state of protection, individuals are able to control their parasite densities at very low levels, thereby preventing the appearance of clinical symptoms of the disease (Druilhe and Perignon, 1994; Sabchareon *et al*., 1991; Bouharoun-Tayoun *et al*., 1990; Sergent, 1950). These past observations, together with the postulation that an effective malaria vaccine would likely mimic naturally acquired immunity, prompted the interest in the present study to establish *ex vivo* and *in vitro* experiments which can be transformed into a vital tool aimed at elucidating the basis of the interaction of *P. falciparum* with the immune system and the very particular pattern of infection/ immunity that results (Druilhe and Perignon, 1994).

In particular, application of malaria sensitized merozoites to trigger monocytes and neutrophils to undergo respiratory burst (as an immune effector function) has not been fully evaluated so as to help understand the dynamics involved in such an elaborate immune mechanism. Precisely an assay set up on this platform is vital in understanding,

for example, the minimum number of merozoites necessary to trigger burst activity as this can provide understanding as to how low of the low parasitemias is potent for immunition (Jafarshad *et al*., 2007; Sergent, 1950). This platform is also an indispensible tool for testing the functional capability of vaccine-induced antibodies to induce burst activity as compared to the antibodies characterised in naturally acquired immunity. It is also paramount to assess the reproducibility of such an assay through use of mononuclear and polymorphonuclear cells from different donors and of different batches so as to specifically monitor their viability and applicability in the assay under different conditions in attempts to overcome some of the problems observed with the ADCI assay (Joos *et al*., 2010).

1.2 Statement of the Problem

New tools are required to expedite the development of an effective malaria vaccine against the blood-stage infection. To succeed in this endeavour, there is need to establish reliable immune correlates of protection and establish assays capable of evaluating potential candidates. Studies on assays that particularly elucidate the contribution of antibody mediated immune effector cell response have not been successfully evaluated despite the numerous body of knowledge pointing at the immense contribution of antibodies to malaria immunity. In particular, it is not known as to how antibodies sensitize malaria parasites, including the minimum amount of antibodies involved in the mechanism and the parasite copy numbers that are required to trigger effector cell respiratory burst induction. Establishing these dynamics is paramount and will subsequently help towards understanding whether the blood-stage malaria vaccine

candidates including apical membrane antigen – 1, merozoite surface protein – 1 (full length), and merozoite surface protein – 1 (block 2 hybrid); and indeed other present and/or future promising candidates, can trigger effector cell respiratory burst activity as a vital immune effector arm that could result into the much needed immunity to malaria parasites; with the ultimate goal of providing insight on the efficacies of potential candidate malaria vaccines.

1.3 Study Objectives

1.3.1 General objective

To develop an effector-cell antibody dependent respiratory burst induction assay as a tool to evaluate cellular immunity to malaria.

1.3.2 Specific objectives

1. To determine the activity of malaria sensitized merozoites to trigger monocyte and neutrophils to undergo respiratory burst by producing reactive oxygen species
2. To determine the minimum number of sensitized merozoites required to trigger the respiratory burst
3. To determine the reproducibility of the respiratory burst assay
4. To determine the respiratory burst activity of antibodies elicited after vaccination with MSP–1, MSP–2 and AMA–1
5. To assess the relationship between total antibody titres and the respiratory burst induction upon vaccinations with MSP–1, MSP–2 and AMA–1 malaria vaccine candidates.

1.3.3 Null Hypotheses (H_o)

1. Malaria sensitized merozoites cannot trigger monocyte and neutrophils to undergo respiratory burst
2. There is no minimum number of sensitized merozoites required to trigger respiratory burst in monocytes and neutrophils
3. The respiratory burst assay is not reproducible
4. Antibodies elicited after vaccination with MSP–1, MSP–2 and AMA–1 does not have any respiratory burst activity
5. There is no relationship between total antibody titres and the respiratory burst induction upon vaccinations with MSP–1, MSP–2 and AMA–1 malaria vaccine candidates.

1.4 Significance of the Study

Creating an effective malaria vaccine remains a major challenge. Despite the prospects of having a partially effective candidate like RTS,S being licensed in the foreseeable future (Vogel and Roberts, 2011), its protection is short-lived and does not provide sterile immunity (Vogel and Roberts, 2011). It seems probable that such a vaccine will require additional blood stage component(s) to control breakthrough infections. Efforts are underway by various groups to test other novel antigens and formulations both in humans and in non-human primates. However, absence of robust immunological measurements that reliably predict protection against malaria infection continue to be a drawback. The only two functional assays, the growth inhibition assay (GIA) and the antibody-dependent cellular inhibition (ADCI) assay have demonstrated

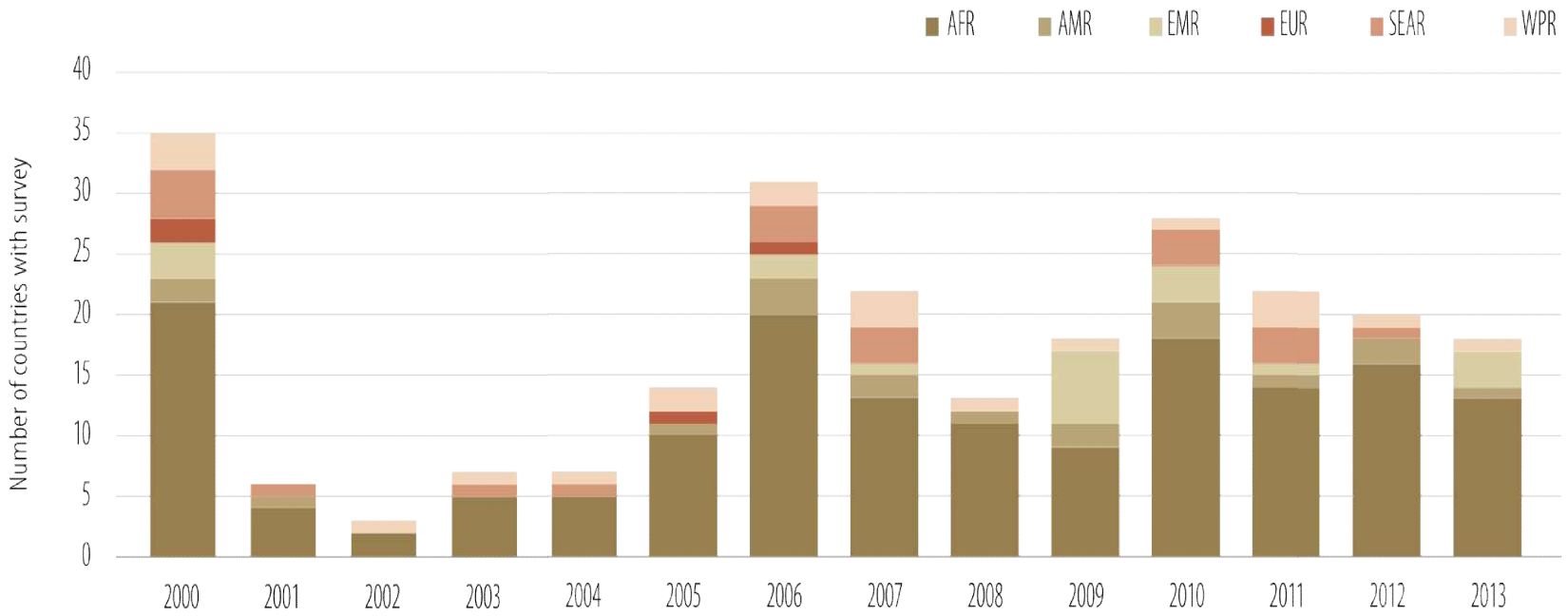
conflicting results in immunoepidemiology studies (GIA) and the relevance of ADCI to clinical protection in humans has not been validated. In addition, despite ADCI being the only antibody mediated effector cell assay, it has not been reproducible (Joos *et al*., 2010) and even then it exclusively measures the contribution of monocytes as the immune effector cells, yet the neutrophils are quite more plausible effectors for the control of *Plasmodium* blood stage infection because they are the most numerous blood leucocytes (about 50–75%) and the best circulatory phagocytes. In effort to address such drawbacks, the present study established an antibody-dependent cellular respiratory burst functional assay that employs both the neutrophils and monocytes as key effector cells. This work elucidated some of the dynamics of the established assay; including the assay reproducibility, dose dependency of the assay on key variables/factors such as antibody concentration and their opsonising ability and above all demonstrated that different test candidate antigens elicit antibodies of varied potencies; demonstrating the use of this assay in determining vaccine efficacy. The assay is less time consuming to perform and in the overall, can help expand the scope of the existing knowledge about the mechanisms involved in malaria immunity.

CHAPTER TWO: LITERATURE REVIEW

2.1. The Epidemiology of Malaria and the Life Cycle

2.1.1 Malaria Epidemiology

Malaria remains the world pre-eminent parasitic disease in the tropics (Duncan *et al.*, 2012). Currently, there are about 97 countries and territories with ongoing malaria transmission, and 7 countries in the prevention of reintroduction phase, making a total of 104 countries and territories in which malaria is presently considered endemic. Globally, an estimated 3.4 billion people are at risk of malaria (WHO, 2015). World Health Organisation estimates that 207 million cases of malaria occurred globally in 2013 and 627 000 deaths. Most cases (80%) and deaths (90%) occurred in Africa (Figure 2.1), and most deaths (77%) were in children under 5 years of age (WHO, 2013).



AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR, Western Pacific Region0

Source: *Household surveys*

Fig 2.1 Number of countries with household surveys measuring at least one malaria-specific indicator, 2000–2012

(WHO, 2013).

2.1.2 Malaria Life Cycle

Malaria in human is caused by the different species of the genus *Plasmodia* namely *P. ovale*, *P. vivax, P. malariae* and *P. falciparum*, and recently, *P. knowlesi* (Breman, 2009). Despite the fact that these species induce varied clinical syndromes, they share strong similarities in their life cycle. Their life cycle comprises of several developmental stages and require both invertebrate and vertebrate host. The female *Anopheles* mosquitoes exclusively transmit human malaria, and the infection is initiated when an infectious mosquito inoculates sporozoites during a blood meal (Meuwissen and Ponnudurai, 1988). The sporozoites travel through the blood stream and invade hepatocytes where they multiply asexually as pre-erythrocytic stage parasites. The pre-erythrocytic forms mature in 5 to 7 days leading to the release of thousands of tissue merozoites that invade erythrocytes and initiate the blood stage phase of infection. During this erythrocytic phase, free merozoites invade erythrocytes, undergo asexual maturation into trophozoites and schizonts, and ultimately rupture the erythrocyte releasing new merozoites. It is this phase of the life cycle that leads to the clinical disease known as malaria. During this stage, the parasite undergoes huge amplification increasing the probability of differentiation into gametocytes, the stage infectious to mosquitoes (Miller *et al*., 1994). After ingestion by mosquitoes, gametocytes emerge from the erythrocytes to become male and female; which then undergo fertilization to form zygotes. Within 24 hours, the zygotes transform into ookinete, an elongate and motile form which traverses the midgut wall, lodging under the basal lamina where it develops into an oocyst. In approximately two weeks after the gametocyte-infected blood meal, the oocyst breaks open causing hundreds to thousands of sporozoites to enter the

haemolymph. The sporozoites migrate to the salivary glands of the mosquito waiting to be inoculated into the next human host to continue the life cycle (See Appendix I – source: Sauerwein *et al*., 2011).

2.2. Malaria Control Strategies

Malaria is an entirely preventable disease and is also treatable with proper implementation of the recommended interventions. These interventions include: vector control through the use of insecticide treated bed nets (ITNs), indoor residual spraying (IRS) and, in some specific settings, larval control; chemoprophylaxis for the most vulnerable populations, especially pregnant women and infants; confirmation of malaria diagnosis through microscopy or rapid diagnostic tests (RDTs) for every suspected case; and timely treatment with appropriate anti-malarial medicines according to the parasite species and any documented drug resistance (WHO, 2015).

The effective intervention measures and control strategies such as the use of artemesinin – combination therapies (ACT) to treat infections and the use of insecticide – treated bed nets (ITNs) to prevent infective mosquito bites have contributed significantly to this cause, resulting in considerable and impressive reductions in malaria incidences in some countries (O'Meara *et al*., 2008). This has prompted renewed calls for disease eradication (Roberts and Enserink, 2007). However, evolution of vector resistance (Trape *et al*., 2011) to insecticides as well as parasite resistance to drugs (Dondorp, 2009) have continued to challenge such noble control efforts, making the development of an effective malaria vaccine a prominent global public health priority.

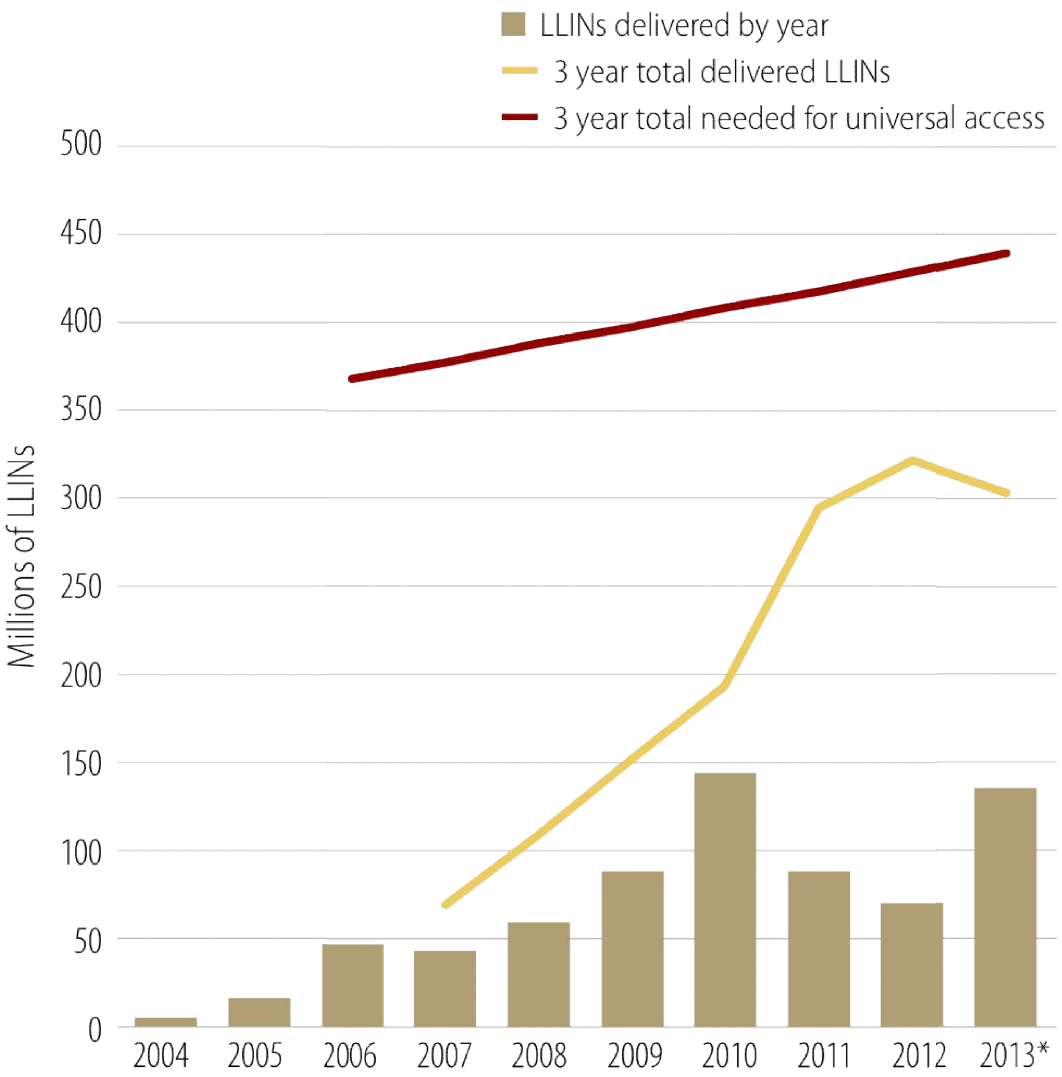
2.2.1 Vector Control for Malaria

Areas of high malaria risk are considered most in need of vector control interventions. Primarily, the use of ITNs or IRS has been adopted as a major vector control strategy. The choice of ITNs or IRS depends on a number of entomological, epidemiological and operational factors, including seasonality of transmission, housing density and distribution, and insecticide susceptibility of *Anopheline* vectors (Pluess *et al*, 2010). In sub-Saharan Africa, where the characteristics of the predominant malaria vectors and the widespread presence of malaria risk indicate that almost all of the 800 million people at risk would benefit from vector control with ITNs or IRS, at least 150 million ITNs would be required each year (assuming that they are long-lasting insecticide treated nets [LLINs], that the typical LLIN lifespan is 3 years, and that 1 LLIN is distributed per 1.8 persons). If the average LLIN lifespan is actually less than 3 years, as suggested (WHO, 2009), then true replacement needs could be greater.

A total of 88 countries distributed ITNs free of charge, including 39 of 44 countries in the African region with ongoing malaria transmission (WHO, 2015). In 83 countries, ITNs are distributed to all age groups; in 64 of those countries, the ITNs were distributed to all age groups through mass campaigns (WHO, 2015). Of 39 countries in the African region that distribute ITNs free of charge, 34 distribute them through, antenatal clinics (reflecting policies directed at reducing the burden of malaria in pregnancy) and 26 distribute ITNs through Expanded Programme on Immunization (EPI) clinics (WHO, 2015). In quantifiable figures, the number of nets delivered by the seven World Health Organization Pesticide Evaluation Scheme (WHOPES)-approved manufacturers that supply nearly all of the LLINs for public sector distribution in Africa (Lengeler, 2002)

increased dramatically, from 6 million in 2004 to 145 million in 2010 (Figure 2.2); it then decreased in 2011 (92 million) and 2012 (70 million). However, information on projected LLIN deliveries beyond 2013 suggests that the increase in deliveries in 2013 may continue and the 3-year total of available LLINs may increase (WHO, 2015).

In addition, WHO recommends that the core vector control interventions of IRS and ITNs may be complemented by other methods (e.g. mosquito larval source control, including environmental management), in a few specific settings and circumstances (WHO, 2015). WHO larval control is appropriate and advisable only in settings where mosquito-breeding sites are few, fixed and easy to identify, map and treat (WHO, 2012a). In 2012, national programmes in 31 malaria-endemic countries worldwide reported (WHO, 2015) information on the use of larval control in certain specific foci of malaria transmission, including six countries in the African region, nine in the region of the Americas, four in the Eastern Mediterranean region, four in the European region, five in the South-East Asia region and three in the Western Pacific region (WHO, 2015). Various larval control strategies were reported, and many countries engaged in more than one type of larval control activity. Among countries reporting on larval control, 15 countries reported activities involving habitat manipulation, and six reported some form of habitat modification (WHO, 2015). Larval control through chemical larviciding was reported by 18 countries, and through biological larviciding by 13 countries (WHO, 2015).



LLIN, long-lasting insecticidal net

* The total number delivered for the first three quarters of 2013 has been multiplied by 4/3 to provide an annual estimate.

Source: *Data from 7 WHOPES-approved manufacturers, collated by Milliner Global Associates.*

Fig. 2.2 Number of LLINs delivered by manufacturers to countries in sub-Saharan Africa, 2004–2013.

2.2.2 Malaria Chemoprophylaxis

World Health Organization recommends three major strategies for the use of antimalarial medicines for the prevention of morbidity and which targets groups at high risk of *Plasmodium falciparum* malaria infection and who live in or visit moderate to high malaria transmission settings (WHO, 2015). These strategies include: intermittent preventive treatment in pregnancy (IPTp) with sulfadoxine-pyrimethamine (SP) (IPTp-SP), delivered at each scheduled antenatal care (ANC) visit after the first trimester (van Eijk *et al*, 2013); intermittent preventive treatment in infants (IPTi) with SP (IPTi-SP), delivered at the time of the second and third diphtheria–tetanus–pertussis (DTP) and measles vaccination (Dellicour *et al*, 2010); and seasonal malaria chemoprevention (SMC) with amodiaquine plus SP (AQ+SP) for children aged 3–59 months in areas of highly seasonal malaria transmission across the Sahel sub region (Cairns *et al*, 2012).

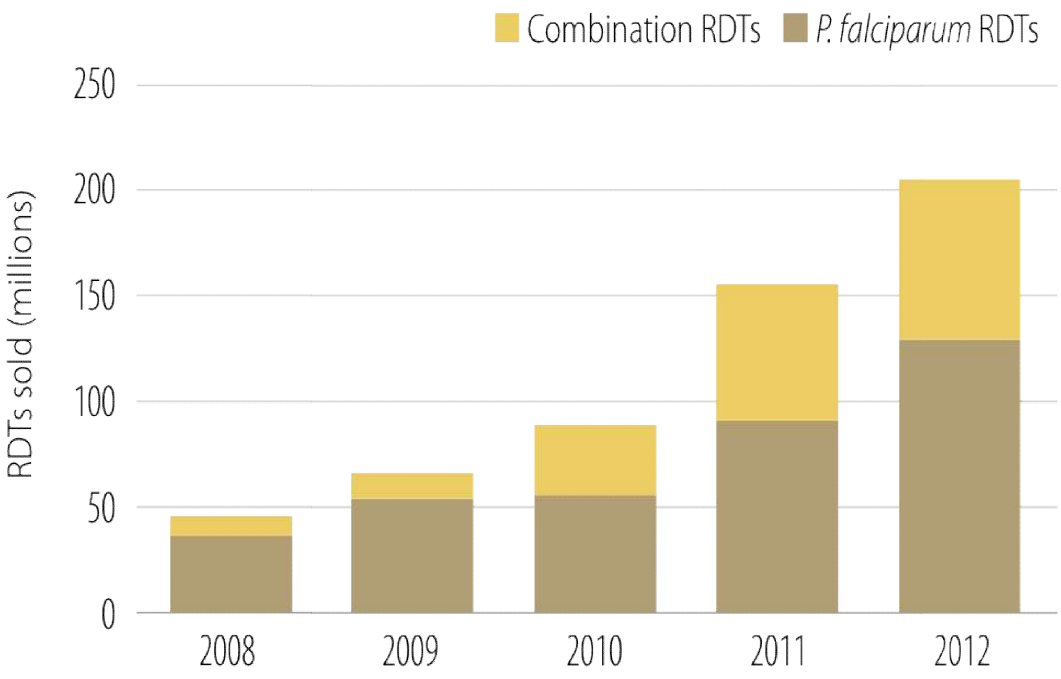
Considering the substantial burden of malaria in groups targeted for preventive treatments, important reductions in infant and childhood morbidity and mortality could be achieved through expanded implementation of IPTp, IPTi and SMC (WHO, 2015). intermittent preventive treatment in pregnancy reduces low birth weight arising from malaria in pregnancy, which is estimated to result in as many as 100 000 infant deaths each year in sub-Saharan Africa (van Eijk *et al*, 2013). intermittent preventive treatment in infants has been shown to reduce clinical malaria cases by 30% in the first year of life. Implementation of SMC could reduce approximately 108 000 deaths in children under 5 years of age with malaria estimated to occur during the first year in areas of the Sahel targeted for this intervention (Cairns *et al.*, 2012).

2.2.3 Diagnostic Testing and Treatment of Malaria

The WHO emphasizes the need for diagnostic testing and prompt treatment of malaria upon confirmed infection. World Health Organization recommends that all persons of all ages in all epidemiological settings with suspected malaria should receive a parasitological confirmation of diagnosis by either microscopy or rapid diagnostic test (RDT), and that uncomplicated *Plasmodium falciparum* malaria should be treated with an artemisinin-based combination therapy (ACT) (WHO, 2010b). Diagnostic testing for malaria is the cornerstone of WHO's initiative of the 3Ts: Test, Treat and Track (WHO, 2010a) – whereby testing of every suspected malaria case ensures appropriate anti-malarial treatment and improves malaria surveillance. WHO provides guidance for quantifying, at the national programme level, the diagnostic needs using malaria surveillance data (WHO, 2011), and treatment needs based on malaria morbidity (WHO, 2010a). These data can be used to assess the scale of global and regional diagnostic and treatment needs of a given population.

The rapid diagnostic testing of malaria have dramatically scaled up in the public and private sectors in malaria-endemic regions in the past five years preceding 2015 - according to data supplied by the WHO (WHO, 2015), Figure 2.3, with use of combination test kits that can detect more than one malaria species – reaching 205 million in 2012. WHO and other organizations i.e. Centres for Disease Control and Prevention, Foundation for Innovative New Diagnostics, Special Programme for Research and Training in Tropical Diseases, have all undertaken product-quality testing of such kits and have demonstrated an improvement in test quality of the kits over time (WHO, 2012b). They also suggest that information on test quality is being used, because

organizations funding diagnostic testing programmes are procuring proportionally more high-quality test kits over time (WHO, 2015).



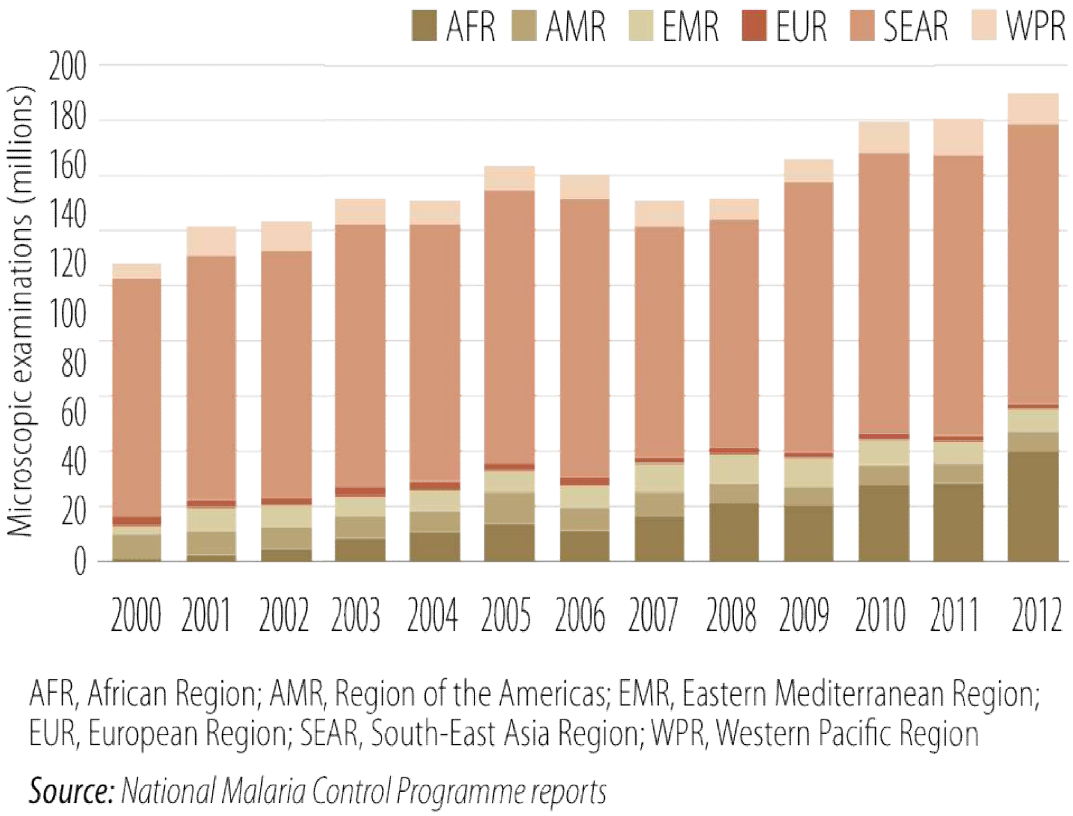
CDC, Centers for Disease Control; FIND, Foundation for Innovative New Diagnostics; RDT, rapid diagnostic test

Source: Data provided by 31 (2008-2010), 24 (2011), 24 (2012) manufacturers eligible for the WHO FIND/CDC Malaria RDT Product Testing Programme

Fig. 2.3 RDT supply to public and private sectors, 2008–2012.

In terms of diagnostic testing by microscopy, the number of microscopic examinations for malaria as reported by national malaria control programmes by different countries and territories increased to a peak of 188 million globally in 2012 (Figure 2.4, WHO, 2015). The global total is dominated by India, which accounted for over 120 million slide examinations in 2012. The global increase in microscopy from 2011 to 2012 is accounted for by the nearly 52 million examinations undertaken (a 42% rise) from

Africa. Several countries in Africa reported increased microscopy in 2012, with seven countries accounting for 85% of the increase (WHO, 2015).



AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; SEAR, South-East Asia Region; WPR, Western Pacific Region

Source: *National Malaria Control Programme reports*

Fig. 2.4 Number of microscopic examinations performed for malaria, by WHO region.

Upon confirmation of diagnosis, the WHO has recommended the use of ACTs as first line of treatment, with 79 of the 88 countries where *P. falciparum* is endemic adopting this as a national policy (WHO, 2015). Chloroquine is still used in some countries in the Region of the Americas where it remains efficacious. Pre-referral treatment of severe malaria cases with quinine or artemether intramuscularly (IM), or with artesunate suppositories, has been adopted by 33 countries in the African region and by 52 countries globally (WHO, 2010b). Of the 58 countries with ongoing *P. vivax* transmission, 52 countries adopted a policy of using primaquine for radical treatment of *P. vivax* cases (WHO, 2015); in 26 of these 52 countries directly observed primaquine treatment is recommended and 13 of these 52 countries recommend testing for glucose-6-phosphate dehydrogenase (G6PD) deficiency before treatment with primaquine (Table 2.1, WHO, 2013)

Table 2.1 Adopted regimens for malaria treatment by WHO region, 2012

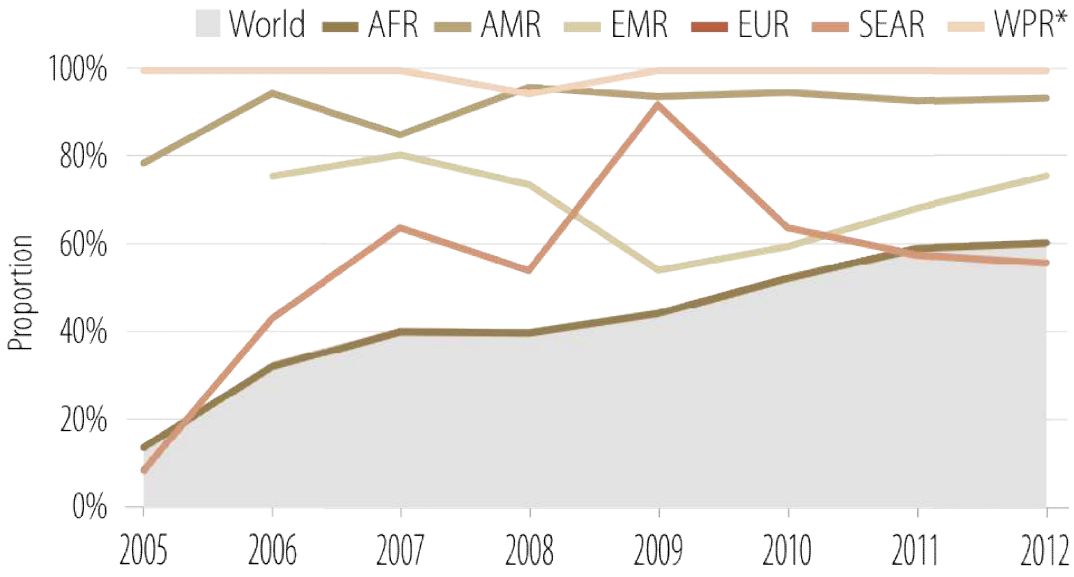
Policy	AFR	AMR	EMR	EUR	SEAR	WPR	Total
ACT for treatment of of *P. falciparum*	42	9	9	1	9	9	79
Pre-referral treatment with quinine/artemether IM/artesunate suppositories	33	4	6		6	3	52
Single dose primaquine (0.25mg base/kg) as gametocidal for *P. falciparum*	4	15	3	2	6	2	32
Primaquine for radical treatment of *P. vivax* cases	7	21	5	3	9	7	52
Directly observed treatment with primaquine	3	12	1	3	3	4	26
G6PD test is recommended before treatment with primaquine	3		3		1	6	13
Number of countries/areas with ongoing malaria transmission	**44**	**21**	**9**	**5**	**10**	**10**	**99**
Number of *P. falciparum* endemic countries/areas	**43**	**18**	**9**	**0**	**9**	**9**	**88**
Number of *P. vivax* endemic countries/areas	**7**	**20**	**6**	**5**	**10**	**10**	**58**
Number of countries/areas endemic for both *P. falciparum* and *P. vivax*	**6**	**17**	**6**	**0**	**9**	**9**	**47**

ACT, artemisinin-based combination therapy; AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; G6PD, Glucose-6-phosphate dehydrogenase; RDT, rapid diagnostic test; SEAR, South-East Asia Region; WPR, Western Pacific Region

Source: *National malaria control programme reports*

On the basis of the available data from national programmes on the number of ACT treatments distributed and the number of estimated presumed (cases treated without being tested) and confirmed *P. falciparum* cases in the public sector, it is possible to calculate the proportion of malaria cases from public facilities that could potentially be treated with ACTs (WHO, 2013). The proportion of presumed and confirmed *P. falciparum* cases potentially treated by distributed ACTs has varied over time (Figure 2.5, WHO, 2013). The trend in the African Region, which accounts for the over 90% of the estimated ACT treatment need (WHO, 2015), has risen steadily since 2005, in line with the increasing ACT deliveries by manufacturers and distributions by NMCPs (WHO, 2015), whereby in 2012 it reached 60%.

Inconsistent reporting by certain countries may heavily influence the observed trends in the regions from which the countries are located. However, increasing number of countries continue to provide updated information on ACT distributions over time and the proportions of presumed and confirmed *P. falciparum* cases potentially treated with ACTs have been less subject to reporting bias in more recent years (WHO, 2015). Such data are therefore more likely to reflect true access to ACTs.



*WPR does not include Papua New Guinea due to incomplete data

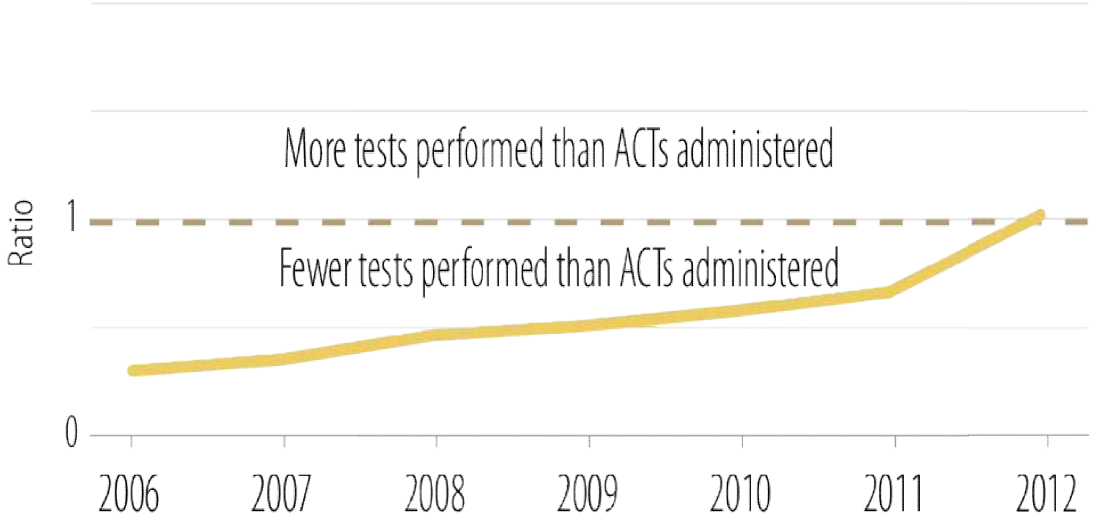
ACT, artemisinin-based combination therapy; AFR, African Region; AMR, Region of the Americas; EMR, Eastern Mediterranean Region; EUR, European Region; RDT, rapid diagnostic test; SEAR, South-East Asia Region; WPR, Western Pacific Region

Source: *National Malaria Control Programme reports*

Fig 2.5 Proportion of estimated presumed and confirmed *P. falciparum* cases at public facilities potentially treated with distributed ACTs, by WHO region, 2005–2012

Despite many patients with suspected malaria still not receiving a parasitological test, the recent expansion of malaria diagnostic testing – as evidenced by the increase in RDTs distributions by country programmes combined with microscopic tests performed – has resulted in an increase in the proportion of suspected malaria cases tested at public facilities (WHO, 2013). In the African Region during 2006–2012, the total number of tests conducted in the public sector increased compared with the number of ACTs distributed by NMCPs during the same period (Figure 2.6, WHO, 2015). In 2012, nearly

as many patients were tested as received an ACT. This is an encouraging trend; however, considering that test positivity rates in most areas in Africa are less than 50%, if diagnostic testing is fully implemented, the ratio of diagnostic tests to ACTs should be ≥2 (WHO, 2013). The data indicate that, although substantial progress has been made, the scale-up of diagnostic testing through RDTs and microscopy remains incomplete in the public sector, and to an even greater extent in the private sector. Expanding diagnostic testing, particularly through the scale-up of RDTs, can significantly reduce the need for ACTs and can thus reduce expenditures on anti-malarial drugs (Thiam *et al*, 2011).



ACT, artemisinin-based combination therapy; RDT, rapid diagnostic test

Source: *National malaria control programme reports*

Fig 2.6 Ratio of RDT and microscopy performed to ACTs distributed, African Region, 2006-2012

In the context of malaria treatment, recent spread of resistance to anti-malarial drugs has led to intensified efforts to prohibit the use of oral artemisinin-based monotherapies and to expand anti-malarial drug efficacy monitoring. To implement this, the WHO has long recommended the withdrawal of oral artemisinin-based monotherapies from the market, and their replacement by ACTs, as endorsed by all WHO Member States at by the World Health Assembly in 2007 (WHO, 2010b). WHO also calls upon manufacturers to cease the marketing of oral artemisinin-based monotherapies. In 2012, eight countries withdrew marketing authorization of oral artemisinin-based monotherapies, and by October 2013, another seven countries had taken regulatory steps to remove these products from their markets (WHO, 2015). In contrast, a total of nine countries still allow oral artemisinin-based mono- therapies; six of the countries are in the Africa region and one each from the American, Eastern Mediterranean, and South-East Asian Regions (WHO, 2015). The number of countries conducting therapeutic efficacy studies for antimalarial drugs has also increased, particularly in the African Region, where the reliance on ACTs is high (WHO, 2015). Despite the observed changes in parasite sensitivity to artemisinins, ACTs remain efficacious in curing patients, provided that the partner drug is still efficacious (WHO, 2015).

2.3 Malaria Vaccine Development

The rationale for accelerated development of a malaria vaccine is majorly anchored on the observation that all effective vaccines as a group represent the single most cost-effective public health intervention. In addition, and as has been mentioned previously, the current methods of malaria control have limited effectiveness, for example, drug resistance is on the increase (WHO, 2015). Recent scientific advances in malaria

immunology and advances in vaccinology have made the development of malaria vaccines an achievable goal (WHO, 2015). To say the least, vaccines in conjunction with other measures could greatly improve the effectiveness of malaria control if not eradication. However, scientific hurdles facing malaria vaccine developers are still imposing, including the fact that there remains no known *in vitro* correlate of protection and part of which this study addressed.

2.3.1 Promising Malaria Vaccine Candidates in Perspective

Until 1987, no malaria vaccine candidate had attracted more attention than SPf66, a synthetic peptide polymer containing four different peptides; one from the circumsporozoite protein, which is a protein from the pre-erythrocytic stage of the parasite life cycle and three asexual-stage antigens, one from the merozoite surface protein and two others which at some stage were thought to derive from *Plasmodium falciparum* blood-stage antigens (Graves *et al*., 2006). SPf66 was initially said to be promising in early trials but later clinical trials indicated lack of efficacy (Graves *et al*., 2006). Numerous other stage specific vaccine candidates have failed at earlier stages of their development.

The leading vaccine candidate so far that could be licensed in the near future is RTS,S (von Seidlein and Bejon, 2013). The core of the vaccine consists of a region of the circumsporozoite fused to the surface antigen of the hepatitis B virus and targets the sporozoite stage in the *Plasmodium* life cycle (Vekemans and Ballou, 2008). Early challenge studies suggested that the RTS,S antigen construct on its own has limited immunogenicity and it has since been combined with adjuvants such as AS01 or AS02

(Stoute *et al*., 1997). Although it has progressed to phase III trials, findings suggest that RTS,S/AS01 only confers 50% protection against *P. falciparum* malaria in children vaccinated at over 5 months of age (Agnandji *et al*., 2011). But the developers aimed to license the vaccine for infants at ages 2, 3 and 4 months based on the need to incorporate RTS,S/AS01 in the Expanded Programme on Immunisation (EPI) schedule, thus simplifying the logistics of deployment and administration (von Seidlein and Bejon, 2013). However, the component of the phase III trial assessing vaccine efficacy in infants with co-administration of other EPI vaccinations showed only 30% protection in younger infants (Agnandji *et al*., 2012). The reason for the lower protection in infants is not clearly understood. It is hypothesised that protection varied significantly by study sites, a variation of which was partly predicted by transmission intensity (von Seidlein and Bejon, 2013); and in which case, children with frequent exposure were less well protected than children with low malaria exposure. In this background, RTS,S appears to be a 'leaky' vaccine, providing protection against a limited number of infectious bites but does not provide protection against the onslaught of infectious bites experienced in the malaria hyper endemic regions (von Seidlein and Bejon, 2013). In addition, the protection conferred by RTS,S wanes over time and disappears by 3–4years after vaccination (Bejon *et al*., 2013; Olotu *et al*., 2013).

Taken together, these factors diminish enthusiasm for RTS,S, and there's need to either add additional components to boost its efficacy or to find a better alternative; of which various blood stage antigens have shown promise (Vannice *et al*., 2012; Boyle *et al.*, 2013; Heppner *et al*., 2013; Olotu *et al*., 2013; de Souza, 2014). A number of parasite proteins located on the surface of the merozoite and largely involved in the erythrocyte

invasion process have been shown to be transiently accessible to circulating antibodies (Ballou *et al*., 2004). The most well studied antigens include the merozoite surface protein-1 and the apical membrane antigen-1 (Ballou *et al*., 2004).

2.3.1.1 Merozoite Surface Protein – 1

One important vaccination strategy is the production of antibodies directed against the carboxyl-terminal portion of merozoite surface protein–1 (MSP–1). The MSP-1 is an abundant protein on the surface component of the merozoite, accounting for upto 40% of the GPI-anchored protein coat on the surface of the merozoite (Sanders *et al*., 2005). Upon synthesis from a high molecular weight (195kDa) protein precursor, MSP–1 undergoes proteolytic processing to yield fragments of varying sizes: 83 (Block-2), 42, 36, 28 – 30 and 19kDa respectively (Holder *et al*., 1994; Holder and Blackman, 1994; Wipasa *et al*., 2002).

Studies have shown that MSP–1 plays a vital role in the binding and subsequent invasion of red blood cells by the merozoites (Cowman *et al*., 2000), with a secondary proteolytic processing of the 42 kDa fragment resulting in a final C-terminal membrane-anchored 19kDa moiety (Cowman *et al.*, 2000). Immune responses to both the 83 (Block-2) kDa and 42 kDa fragments have been associated with protection against natural infection to malaria in West African children (Riley *et al*., 1992; Tolle *et al*., 1993). In addition, antibodies to the 19-kDa fragment have been found abundantly in naturally exposed people from endemic areas (Cavanagh *et al*., 2004). Initial clinical trials showed that *Aotus* monkeys vaccinated with MSP-1_{42} were protected from *P. falciparum* challenge (Stowers *et al*., 2002, Darko *et al*., 2005) and antibodies derived from such

animals exhibited *in vitro* growth inhibition activity (Chang *et al*., 1996). In malaria-naïve adults in the United States, an MSP1 component vaccine induced high titre, functional antibodies that recognized *P. falciparum* merozoites by indirect fluorescent antibody (IFA) and exhibited *in vitro* growth inhibitory activity (Ockenhouse *et al*., 2005) although this did not confer protection to malaria when tested in Africa (Ogutu *et al*., 2009).

2.3.1.2 Apical Membrane Antigen-1 (AMA-1)

Apical membrane antigen – 1 (AMA – 1) is an 83kDa membrane protein located in the micronemes and rhoptry organelles of the merozoites (Crewther *et al*., 1990). AMA – 1 is thought to be involved in the reorientation and formation of a tight-junction that facilitates merozoite invasion of the red blood cells, making it a potential vaccine candidate. Studies done both in mice and in rhesus macaques have shown that the animals are protected from clinical malaria upon vaccination with recombinant AMA – 1 (Anders *et al*., 1998; Stowers *et al*., 2002). In human, antibodies to the full length AMA-1 have also been associated with protection (Polley *et al*., 2004)

However, while AMA-1 is an attractive candidate antigen, there is considerable antigenic polymorphism that could limit its impact in mass clinical trials (Ballou *et al*, 2004). Data from animal studies indicate that antisera raised against one form of the molecule that efficiently block the growth of the homologous parasite are less efficient at blocking the growth of heterologous parasites (Ballou *et al*, 2004).

In summary, a protective vaccine able to clear the pre-erythrocytic stages could prevent the establishment of a blood-stage infection whereas a vaccine to prevent the

sexual and intra-mosquito stages would have an impact on malaria transmission but would provide no direct benefit to the vaccinated person as the sexual stages do not provoke clinical manifestations. However, the blood-stage infection is the critical stage at which disease and a risk of mortality arises with potentially exponential parasite growth. Vaccines against blood stages may therefore, in theory either prevent blood-stage infection from becoming established or may allow infection in the absence of disease. This has informed the motivation to focus on the blood-stage antigens: merozoite surface protein – 1 and the apical membrane antigen–1, despite many other potential candidates, for the purpose of the current study that is geared towards developing an antibody-dependent respiratory burst assay as a surrogate marker of acquired immunity.

2.4 Antibody-Mediated Cellular Immunity to Malaria

Polymorphonuclear neutrophils (PMNs) and monocytes are key players of the innate immune defence against invading pathogens and may be more involved in controlling blood-stage malaria infection than have been generally appreciated. The PMNs are much more numerous than monocytes and consist up to 75% of blood leucocytes (Joos *et al*., 2010). They are known for microbicidal mechanisms that include phagocytosis, the release of proteolytic enzymes and antimicrobial peptides, and the rapid production of reactive oxygen species (ROS), which are toxic to the intraerythrocytic malaria parasites (Clark and Hunt, 1983; Allison and Eugui, 1983; Cox, 1983; Wozencraft *et al*., 1984; Ockenhouse and Shear, 1984; Kharzami *et al*., 1987, Clark *et al*., 1989).

Several immunoepidemiological studies in different malaria endemic regions in Africa have pointed at possible involvement of reactive oxygen species (ROS) in clinical protection to disease. In Gabon, high ROS production by polymorphonuclear cells (PMNs) was reported to correlate with fast clearance of parasites in children (Greve *et al*., 1999); whereas in Senegal, acquired protection from clinical disease correlated with *ex vivo* PMNs respiratory burst induction using serum antibodies from exposed individuals (Joos *et al*., 2010).

Cytophilic antibodies, particularly IgG1 and IgG3 (Lazarou *et al*., 2009) are thought to trigger these effects when they bind to parasite antigens encountered in circulation and interact with the effector cells by mechanisms that include Fc-receptor cross linking. Establishing functional assays that gauge antibody contribution to protection is plausible since antibodies are regarded as the main arbiters in acquired immunity to malaria (Joos *et al*., 2010). Early studies that involved passive transfer of purified IgGs clearly demonstrated the important role that antibodies perform in clinical protection against the disease (Cohen and McGregor, 1961).

Since then, numerous reports (Perraut *et al.*, 2004; Marsh and Kinyanjui, 2006; Beeson *et al.*, 2008; Duncan *et al.,* 2012; de Souza, 2014; Osier *et al.*, 2014) have postulated several possible mechanisms through which antibodies offer such protective immunity. These include: directly inhibiting parasite invasion of erythrocytes, neutralizing parasite toxins, agglutinating the variable surface antigens on erythrocytes, facilitating erythrophagocytosis, blocking the adhesion of infected erythrocytes to the tissues (Perlmann and Troye-Blomberg, 2002; Druilhe and Perignon, 1994; Sabchareon *et al*., 1991; Bouharoun-Tayoun *et al*., 1990) and; fixing the complement and up

regulating the innate immune responses that include the cross linking of the Fc-receptors leading to the antibody dependent cellular cytotoxicity, a major mechanism involved in the microbicidal activity of the effector cells (Joos *et al*., 2010). This body of knowledge points to the need for the establishment of novel assays that involve antibody-mediated immune effector cell responses to act as an immune correlate of protection.

2.5 Functional Assays in Malaria Vaccine Development

Malaria vaccine clinical trials in exposed populations have not always been consistent in finding associations between immune responses and protection against disease (Dobano and Campo, 2009; Ogutu *et al*., 2009). Several malaria vaccine candidates have failed the protection test in phase II trials (See Appendix I, Ballou *et al*., 2004), suggesting that the immune correlates employed to select these vaccine candidates may not have been reliable surrogate markers of protection (Dobano and Campo, 2009; Ogutu *et al*., 2009; Spring *et al*., 2009; Ellis *et al*., 2010; Greenwood, 2011).

For the pre-erythrocytic malaria vaccine development, the immunological down-selection for the most promising candidates to progress to clinical trials has been based on non-harmonized pre-clinical IgG and T-cell based assays and there are no well developed functional assays (Cavanagh *et al*., 2011) to support this venture even to clinical trials, implying that assay development remains one of the key priorities to effectively test the most promising candidates. The immune mechanisms in the pre-erythrocytic stage of the parasite have demonstrated evidence for protective immunity through cellular responses to disease, where both enzyme-linked immunospot (ELISpot)

and intracellular cytokine staining (ICS) assays have been used as identifiers to these mechanisms.

In the blood-stage malaria vaccine development, the only two notable functional assays are the Growth Inhibition Assay (GIA) and Antibody-Dependent Cellular Inhibition (ADCI) assay (Cavanagh *et al*., 2011). The GIA measures the capability of antibodies to directly impair parasite invasion and/or their intraerythrocytic development (Cavanagh *et al*., 2011) whereas ADCI measures antibody-dependent cell-mediated effects on parasite growth (Jafarshad *et al*., 2007).

Several malaria vaccine investigators proficient in GIA have participated in harmonization efforts resulting in conformity in some aspects of the assay procedure and a satellite laboratory has been selected and supported as an intramural NIAID laboratory of reference centre to this extent (Reed *et al*., 2009; Arnot *et al*., 2008). However, GIA only measures the direct contribution of antibodies in impairing parasite growth/development and does not envisage the contribution of effector cells such as monocytes and neutrophils.

On the other hand, the ADCI despite being the only assay measuring antibody mediated cellular response and which is thought to have the advantage of requiring far lower IgG concentrations for activity compared to the GIA (Galamo *et al.*, 2009), has not been widely reproduced in other laboratories. The relevance of the assay itself to clinical protection in humans has not been validated (Joos *et al*., 2010). Besides, ADCI exclusively measures the contribution of monocytes as the immune effector cells, whereas the neutrophils are quite more plausible effectors for the control of *Plasmodium* blood stage infection because they are the most numerous blood leucocytes (about 50–

75%) and the best circulatory phagocytes (Joos *et al*., 2010). Opsonized merozoites are known to be phagocytosed by neutrophils *in vitro* (Khusmith *et al*., 1982; Celada *et al*., 1983) and *in vivo* (Sun *et al*., 1985). In addition, neutrophils are particularly much more effective generators of reactive oxygen species (Joos *et al*., 2010). This underscores the need to identify additional (and probably more inclusive) assays that can measure antibody-dependent cell mediated immunity.

The postulated antibody dependent respiratory burst assay if established and comprehensively developed would be a vital tool to circumvent some of these shortcomings. Because it has been previously used in the study of respiratory burst activity in various clinical conditions involving bacterial infections (Lundqvist and Dahlggren, 1996), the same chemiluminescent technique can be used with malaria antigens in place of bacteria. In addition to using neutrophils, the elaborate assay could also potentially be set-up using monocytes, the immune effector cells that have been demonstrated to be effective in ADCI (Jafarshad *et al*., 2007). Using this approach would allow the assessment of the contribution of each effector cell subset in the immunological outcome of this mechanism. No studies have previously undertaken to evaluate this.

It has been observed time and again that in the state of protection, individuals are able to control their parasite densities at very low levels, thereby preventing the appearance of clinical symptoms of the disease (Sergent, 1950). However, there have never been any *ex vivo* assays to evaluate this in the context of vaccine-induced immunity. An assay that would determine the minimum number of sensitised merozoites that can potently induce monocytes and/or neutrophils to undergo respiratory burst could provide insight into the mechanism of naturally acquired immunity. Subsequently such an

undertaking could be a good platform to compare the dynamics of natural immunity and immunity elicited by vaccination. A study dwelling on this has not been reliably achievable with the current functional assays.

The hallmark of a reliable assay is its ability to be simple to perform and to be reproducible; some of the factors which have made the ADCI assay difficult to implement (Joos *et al*., 2010). Consistency in the outcome of the assay irrespective of the batch variations of the key assay materials used/parameters is important in sustaining such reliability. Because this has been a drawback with ADCI, it is paramount that newer assays in the pipeline be designed, evaluated and appropriately tested. The respiratory burst induction isoluminol chemiluminescence is a technique in which the amplifying molecule reacts with the oxygen species generated by immune cells to produce an excited state intermediate that emits light. It is a sensitive and simple method, which has been demonstrated, in clinical investigations of the chronic granulomatous disease (CGD) (Lundqvist and Dahlggren, 1996).

In the ROS production model of CGD, the enzyme system responsible for the generation of these oxygen radicals is the neutrophil NADPH oxidase, which is located in the plasma membrane and in the specific granules (Jesaitis *et al.*, 1990; Bjerrum and Borregaard, 1989). The NADPH oxidase reduces molecular oxygen to superoxide and hydrogen peroxide, that are further metabolized to other toxic substances, such as hypochlorite, choloramines, and hydroxyl radicals (Lundqvist and Dahlggren, 1996). NADPH serves as the electron donor, and the electrons are ferried either to a phagosome or to the extracellular milieu (Dahlgren and Stendahl, 1983). The three main components of the luminol amplified chemiluminescence reaction in neutrophils involve a peroxidase

(usually MPO originating from azurophil granules), oxygen metabolites produced by the NADPH oxidase, and capture by extracellular reactions that measure it. The amplifying molecule luminol (5-amino-2,3,dihydro-l,4-phthalazinedione) reacts with the oxygen species generated by the neutrophils to produce an excited state intermediate that emits light (chemiluminescence) upon relaxation to the ground state (Lundqvist and Dahlggren, 1996). A scheme showing this is demonstrated in figure 2.7

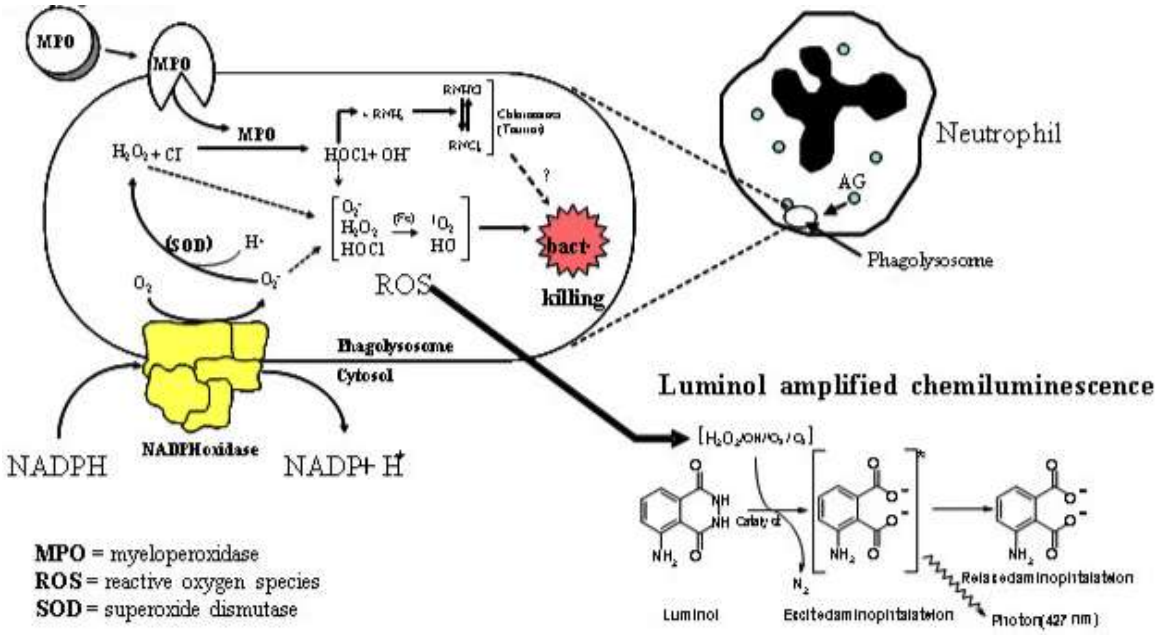


Fig 2.7 Schematic diagram showing neutrophil respiratory burst induction as can be captured by luminol amplified chemiluminescence.

However, key parameters/dynamics of the reactions that led to the founding of the assay need to be studied further, especially in the scope of malaria vaccine studies. Starting with the illuminometer that captures the output parameter (fluorescence due to oxygen radical), it is paramount to determine an optimum measurement condition that

supports the readout as the assay develops. Whereas some illuminometers are calibrated to work at 37^0C (Joos *et al*., 2010), others work at room temperature. It is likely that measuring in the latter conditions would require more time to reach the peak value as compared to illuminometers at 37^0C. One way to optimise the instrument measurement conditions is to try different measurements over different time intervals and plotting dose response curves under the different conditions and temperatures to establish the most convenient option.

Another outstanding issue is to ascertain whether the chemiluminescent readouts are indeed the result of oxygen radicals and not just excited states of other reactions. One way of doing this would be by use of radical quenchers or biological catalysts that break down radicals into non-excitable compounds that do not fluoresce (Lundqvist and Dahlggren, 1996). The other assay component that is vital is to establish that such observable stimulation is the direct result of sensitized merozoites that come into the vicinity of effector cells. To rule out burst induction due to non-specific stimulation, other parameters that come into play, either during the *in vitro* culture of malaria parasites or during preparation of merozoite structures need to be tested to affirm that only merozites are central to the mechanism observed (Kumaratilake *et al.*, 1992; Malhotra *et al.*, 1988). In addition, and equally of importance, is to analyse whether antibody sensitisation of merozoites that eventually lead to burst induction with effector cells is dependent on intact merozoites. This calls into question the quality and specificity of the antibodies involved in the process. A mechanism that is able to interfere with merozoite antigenic determinants before sensitization would be a necessary tool to achieve this (Joos *et al.*, 2010). Until such antibody specificity involvement in the

process would there be confidence that the assay can reliably measure the functional capability of elicited antibodies equivalent to those characterised in immunity.

It is not known if different malaria vaccine candidates can elicit antibodies with capabilities to sensitise parasites so as to induce immune effector cells such as neutrophils to undergo respiratory burst. It would be important to develop a tool that can evaluate this since it has been previously reported that clinical protection to malaria correlated with the reactive oxygen species generated by neutrophils in individuals with naturally acquired immunity (Joos *et al*., 2010). Such knowledge would allow the gauging of the quality of the antibodies raised by vaccination and if this can be satisfactory for protection. This can help malaria vaccine investigators to identify more promising antigens needed in formulating efficacious vaccine candidates.

2.6 Conceptual Framework

A new assay was developed to measure antibody-mediated cellular immune response to malaria; the output parameter (which in this case was chemiluminescence) was deciphered and its reliability evaluated. Key assay variables/parameters involved were then determined in the effort to optimise the set-up after which its applicability was tested using representative material from vaccine studies. At every stage in all the work done, knowledge was gained that is instrumental in understanding malaria immune response mechanisms and that may aid in overall vaccine design and development as shown in Figure 2.8

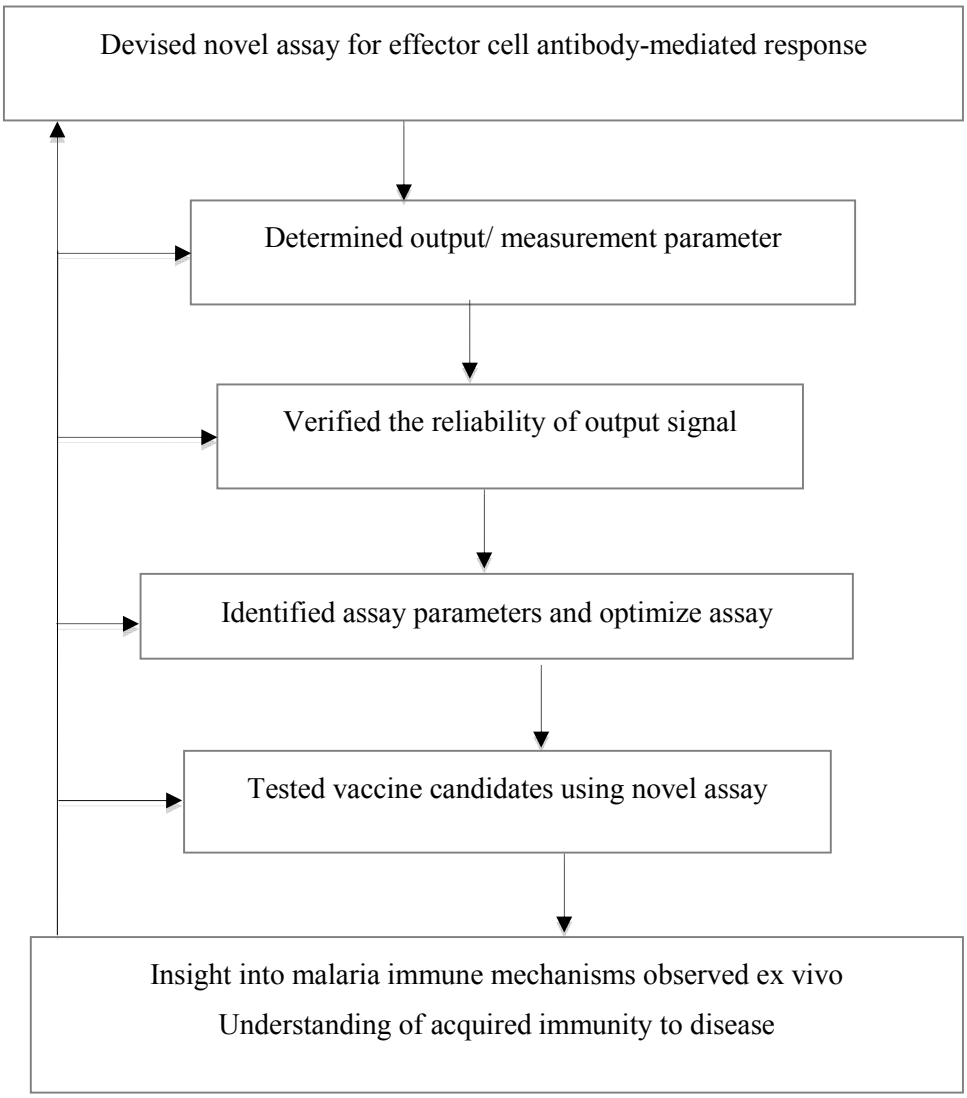


Fig 2.8 Antibody-mediated effector cell respiratory burst assay development

CHAPTER THREE: STUDY DESIGN AND METHODS

3.1 Study Design

This was an experimental-based study exclusively performed in the laboratory and that did not involve any direct recruitment of study participants on stratified epidemiologic statistical criteria. Because the study proposed to develop an antibody-mediated respiratory burst assay as a tool; it was performed in the laboratory and consisted of assays that formed three major segments; with each assay component performed to yield the feeder materials that were eventually collated in the sum total of respiratory burst process analysis.

The first component involved the continuous *in vitro* culture of malaria parasites from which the parasitophorous enclosed merozoite structures (PEMs) were isolated for subsequent sensitization with antibodies. The second component of the study involved assays that led to the isolation of IgG antibodies from serum samples; antibodies of which were used in the merozoite sensitization procedures. The third component of the assay involved purification of monocytic and polymorphonuclear immune effector cells that were needed for testing the sensitized merozoites for their capability to trigger burst activity induction, a procedure that involved the measurement of chemiluminescent activity of the effector cell-antibody-merozoite complex mixtures. Schematic outlay of the study design is shown in Figure 3.1, and the detailed procedures of how each arm of the design was executed is explained in the methods.

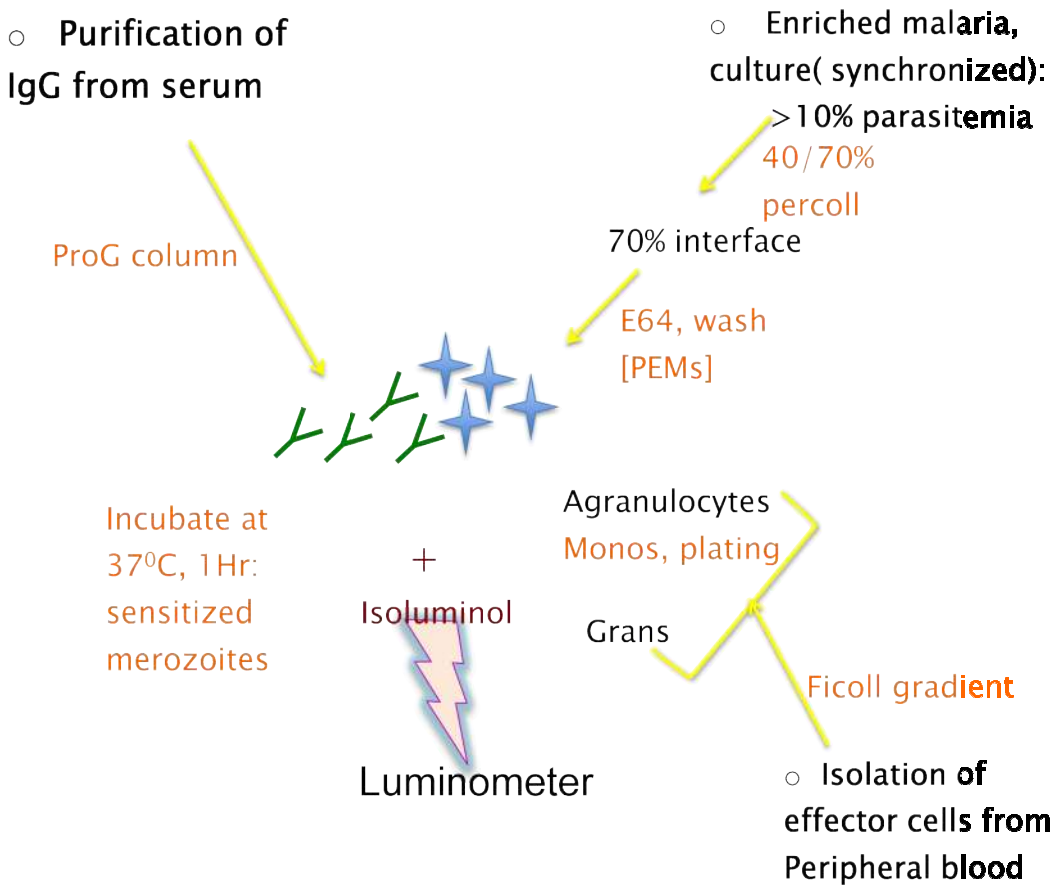


Fig 3.1 Schematic presentation of the experimental design

This study was done at the Biomedical Primate Research Centre (BPRC), Netherlands. The samples used in executing this study included human blood, rhesus blood, rhesus sera, human sera and the vaccine candidate construct proteins: apical membrane antigen – 1, merozoite surface protein – 1 full length, and merozoite surface protein – block 2 hybrid.

Human blood was obtained from the local blood bank in the Netherlands after informed consent as per the blood bank procedures. Rhesus blood was obtained from rhesus macaques in the colony at BPRC, Netherlands. An existing African test sera pool

(malaria hyper immune sera) in the BPRC lab freezers was used as positive control in the study experiments. This had been obtained from Mali, Africa and had been utilised in previous studies (Mahdi-Abdel-Hamid *et al*., 2011). The vaccine candidates were synthesised as recombinant proteins expressed in *Pitchia pastoris* at the BPRC. According to the institute schedule routine, a healthy *Rhesus macaque* was euthanized every Monday morning as source of vital materials required for the sum total of research samples needed for respective studies at BPRC. In all experimental tests, the malaria hyper immune sera pool extract was used as positive control whereas malaria naïve sera pool extract was used as negative control.

3.2 Blood Processing

For this study, human (RBCs used for parasite culture; PBMCs for sensitization of merozoites) and rhesus blood (PBMCs for sensitization of merozoites) was collected into lithium heparin tubes (Greiner Bio-One GmbH, KremsmÜnster, Austria) and centrifuged at 500Xg for 10 minutes at room temperature. The buffy coat (opaque layer on top of the erythrocyte pellet) was then removed into clean 50 ml conical flasks for subsequent procedures and the RBC pellet pooled together. Any storage of cells was done at 4^0C prior to use.

3.2.1 Purification of Erythrocytes

Pooled RBC pellet was washed twice in RPMI 1640 (Gibco) and resuspended in the same medium at 50% concentration by volume (haematocrit). This was kept at 4^0C

for no more than two weeks for use in parasite culture and other subsequent functional assays that required autologus erythrocytes.

3.2.2 Effector Cells Isolation

The buffy coat obtained from spun whole blood was removed and layered onto lymphocyte separation medium, Lymphoprep (Axis-Shield, Oslo, Norway) and centrifuged at 500Xg for 30 minutes at room temperature. Mononuclear cells was obtained from the gradient interface and monocytes were subsequently isolated by adherence to plastic culture flasks for one hour at 37^0C and harvested using a cell scraper. The pellet consisting of erythrocytes and granulocytes was used to harvest PMNs. Residual erythrocytes were lysed using erythrocyte lysis solution (Miltenyi Biotech, Bergisch Gladbach, Germany) for ten minutes at room temperature according to the manufacturer's instructions. The purified cells (PMNs or monocytes) were then washed twice in complete medium (RPMI with 10% FCS, 2 μM L-glutamin and 100 U/ml penicillin/streptomycin). Cells were subsequently resuspended in Hank's balanced salt solution, HBSS (Gibco) prior to use. Monocytes or PMNs were enumerated by differential counts following MayGrunwald staining (VWR International) and purity established at over 80% for either of the desired cell-populations. Viabilities in all preparations were assessed to be above 98% by trypan blue staining prior to use. The PMNs were then used within 4 - 5 hours following blood sample collection and monocytes within 48 hours. For longer periods before use, monocytes were stored at 4^0C in complete medium (RPMI with 10% FCS, 2 μM L-glutamin and 100 U/ml penicillin/streptomycin)

3.3 *In Vitro* Parasite Culture and Isolation of Merozoites

Plasmodium falciparum (NF54 line) was maintained in continuous culture at 5% haematocrit in purified human erythrocytes with RPMI 1640 (Gibco) containing 10% A+ human non-immune serum and 15µg/ml gentamycin in gassed (5% CO_2, 5% O_2 and 90% N_2) culture flasks and placed on a shaker at 37^0C, in a modification of the Trager and Jensen method (Trager and Jensen, 1976). Before isolation of parasitophorous membrane enclosed merozoite structures (PEMS), cultures were synchronized 2 – 3 cycles by treatment with alanine for 30 minutes at 37^0C and saturated up to 10% parasitemia at 2% haematocrit. Schizonts were harvested at the 70% interface following centrifugation at 700Xg for 16 minutes at 4^0C on a 40% / 70% percoll gradient (Invitrogen Life Technologies). The isolated schizonts were incubated with the reversible cysteine protease inhibitor E64 (Sigma-Aldrich) as described previously (Jafarshad *et al*., 2007) to obtain the parasitophorous membrane enclosed merozoite structures (PEMs). Following two washes in RPMI 1640 to remove E64, the PEMs were freeze-thawed at -20^0C to kill the merozoites and rupture the erythrocyte membranes to release them. Thawed PEMs were washed in RPMI 1640 and stored frozen in aliquots at a protein concentration of 300µg/ml until use. Protein concentrations of the purified merozoite lysates were determined spectroscopically by the bicinchoninic acid (BCA) protein assay reagent kit using BSA as standard. Merozoite numbers in the preparations were estimated by counting the number of schizonts per volume using a haemocytometer counter and multiplying with the average number of merozoites per schizont (i.e. 300µg/mL corresponding to approximately 10^8 merozoites per ml).

3.4 IgG Purification

Total IgG (for both test and control samples) were isolated from sera using protein G columns (Pierce, Thermo Scientific) and corresponding buffers. IgG's were then purified by exchanging into RPMI 1640 by repeated concentration/dilution using Amicon Ultra-15 concentrators (30-kDa cut-off; Millipore BV, Amsterdam, The Netherlands) as described previously (Mahdi Abdel Hamid *et al.*, 2011). Purified IgG's were filter-sterilized and stored at -20^{0}C, at a concentration of 20 mg/ml until use. The IgG concentrations in the purified IgG fractions were determined using a Nanodrop-ND 1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE)/the bicinchoninic acid (BCA) protein assay reagent kit using BSA as standard. Due to high cost of purchasing new protein G columns, a novel technique to recycle used columns was developed. This method involved a simple cleaning method and employed the use of polyethylene glycol (PEG8000) that renewed the column matrix-binding capacity comparable to a new resin while retaining high selectivity for the IgG purified.

3.5 Antibody ELISA

For antibody ELISA, 96-well micro titre ELISA plates were coated with 50 ng vaccine protein antigens per well and incubated overnight at 4^{0}C for subsequent steps as described previously (Mahdi Abdel Hamid *et al.*, 2011). Briefly, the coated plates were washed and blocked. Test antibody samples were diluted in serial two fold dilutions, starting at 1/5,000 and were in duplicate added to antigen-coated wells and incubated for 1 h at 37^{0}C. A duplicate control dilution series of standard malaria hyper immune serum

pool was prepared from immune serum and included on every plate. After extensive washing, the plates were incubated with secondary antibodies goat anti-human immunoglobulin G conjugated to alkaline phosphatase (Pierce, Rockford, IL). Bound antibodies were visualized by adding the substrate solution pNPP (para nitro phenyl phosphate, Sigma, Zwijndrecht, The Netherlands) and incubated for 30minutes at room temperature. Absorbance at 405 nm was measured with a Bio-Rad Microplate Reader 3550 (BioRad, Veenendaal, The Netherlands). A standard curve was constructed by four-parameter curve fitting for every plate and titres of unknowns were calculated from the four-parameter fit. Antibody titres were expressed as arbitrary units (AU), where 1 AU corresponds to an amount of IgG theoretically yielding an OD of 1 over background. Thus the titre in AU indicates the dilution at which an OD of 1 over background is achieved.

3.6 Immunofluorescent Staining of Opsonised Merozoites

For immunofluorescent microscopy, smears of purified merozoite preparations were made on glass slides, fixed with chilled methanol for 1 minute and allowed to dry at room temperature. Antibodies (a two-fold dilution series over 10 wells starting at 4 mg/ml) or sera was then applied onto slides and incubated for 1 hour at room temperature in a moist chamber. The slides were then washed 4 times in PBS, 5 minutes each round. After gently tapping the slides dry, the secondary goat anti-human IgG conjugated to FITC diluted 1:100 in PBS/ 1% FCS (GIBCO) was added. Slides were incubated for another 1 hour in a moist chamber in the dark and washed 4 times in PBS, 5 minutes each round. A drop of DAPI stain diluted 1:5000 in Slow-Fade anti-fade (Molecular Probes)

was then added onto the staining area and a cover slip added. Fluorescent stains were then visualized using an Olympus X100 oil-immersion lens. Composite images showing FITC fluorescence onto DAPI were scored as sensitised merozoites.

3.7 Respiratory Burst Monitoring by Chemiluminescence

Chemiluminescence assay was done as described previously (Lundqvist and Dahlgren, 1995; Joos *et al*., 2010) with some modifications; 40 µL of the isolated merozoites or alternatively infected/non-infected erythrocytes were pre-incubated for 1 hour at 37^0C with or without 120 µL of purified IgG or serum at different concentrations to establish any possible dose-dependent curve. These were then added respectively at a volume of 53.3 µL each to triplicate wells containing either PMNs or monocytes at cell suspensions of 100 µL at $2X10^6$ cells mL-1. In some experiments, the oxygen radical scavengers desferoxamine mesylate (Sigma), or superoxide dismutase (Sigma) were added. 20 µL of isoluminol (4-aminophthalhydrazide; Sigma) prepared from a stock concentration of 4mg.mL-1 in DMSO and kept in the dark was then pre-diluted ten times in RPMI and added to all wells to a final concentration of 0.04mg.mL-1, and well volumes adjusted using RPMI 1640 (Gibco) to a total of 200 µL each. Plate measurement was initiated immediately using the TopCount NXT™ Scintillation and Luminescence Counter (PerkinElmer), that recorded luminescence as counts per second (CPS). Each well was monitored automatically over 142 minutes at 2 minutes per cycle, with the plate reading chamber at about 20^0C and attaining peak value within the last two minutes. Alternatively, measurements were also performed for 25 minutes in the TopCount, either after leaving the plate at room temperature for 120 minutes, or after incubating the plate

at 37^0C for 40 minutes to achieve peak measurements within the first 2 minutes and to establish the optimum measurement condition for burst activity.

3.8 Ethical Considerations

This study was approved by the ethical committee of the Biomedical Primate Research Centre (BPRC), which follows the Guide for the Care and Use of Laboratory Animals (1996). BPRC has acquired accreditation from the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Data confidentiality was highly maintained and all donated samples were coded and stored in monitored computers with password-protected computers with restricted access.

All laboratory procedures and sample handling were done by personnel who had undergone Good laboratory/Clinical laboratory QA/QC practises and that conform to the European Molecular Biology (EMBL) requirements.

3.9 Study Limitations

This study was laboratory-based and did not enjoy the benefit of population-based recruitment/and enrolling volunteers under stratified epidemiologic groupings. This limitation however did not have a negative impact on the outcome of an established novel assay because all the parameters that impact on assay reliability were properly evaluated in the laboratory setting.

3.10 Data Analysis and Presentation

Data collected from this study included observational analyses and fluorescent microscopy images. Data analyses, including plotting of graphs were done using either Microsoft Excel or the Graph Pad Prism software (San Diego, California). Burst activity measurements were presented either as absolute value, as relative burst or as an index.

The relative burst of the test sample was expressed as the absolute burst activity of the unknown divided by the absolute burst activity of the positive control at equivalent concentrations of the opsonising serum antibody.

Relative burst = Absolute burst, test/Absolute burst, hyper immune IgG positive control.

This fraction was also represented as the antibody dependent respiratory burst index (ADRB index).

Alternatively, unknown burst activity was expressed as a derivative equivalent of a known concentration of the hyper immune control IgG titrated out and plotted on a 4-parameter curve fit.

In accordance with the respective specific objectives, the following analyses were performed:

Determination of whether malaria sensitized merozoites trigger immune effector cells to undergo respiratory burst was analysed by visual observation of stained fluorescent microscopic images. End point hyper immune dilution was then determined by titration. In addition, analysis of the difference between the levels of ROS produced by autologous PMN and monocyte effector cell subsets were evaluated using the F-test.

Determination of the minimum number of sensitized merozoites required to trigger the respiratory burst process was done by titration of antibody and merozoite copy numbers. These data were plotted to elucidate dose dependent changes, with each plot as a representative mean of triplicate measurement with a standard error of mean.

Determination as to whether the different malaria vaccine candidates MSP-1, MSP-2 and AMA-1 elicit antibodies that trigger effector cell respiratory burst after vaccination was evaluated by plotting graphs of burst activity before and after vaccination with each of the candidates, where the magnitudes of increases in burst activity was depicted. The plots represented means of triplicate measurements at every assay point.

Analysis of the relationship between total antibody titres and the effector cell respiratory burst induction upon vaccinations with the MSP-1, MSP-2 and AMA-1 malaria vaccine candidates was done by assessing the degree of association between antibody titres and burst activity induction using Pearson's correlations.

CHAPTER FOUR: RESULTS

4.1 Malaria Sensitized Merozoites Trigger Neutrophils/Monocytes to Undergo Respiratory Burst by Producing Reactive Oxygen Species

4.1.1 Opsonisation of Merozoites

It was established that the isolated merozoites could be bonded with antibodies purified from a pooled hyper immune serum and not with antibodies purified from a pooled malaria naïve serum. Figure 4.1 confirms that the hyper immune antibodies bound to the merozoite preparations and the naïve antibodies did not, as detected by immunofluorescent assay (IFA) staining. In addition, it was found out that isolated merozoites reacted to similar endpoint titre as merozoites smeared directly from cultures, both reaching an equivalent serum dilution of 1:2000 (Figure 4.1: [1] A, B and [2] A, B respectively).

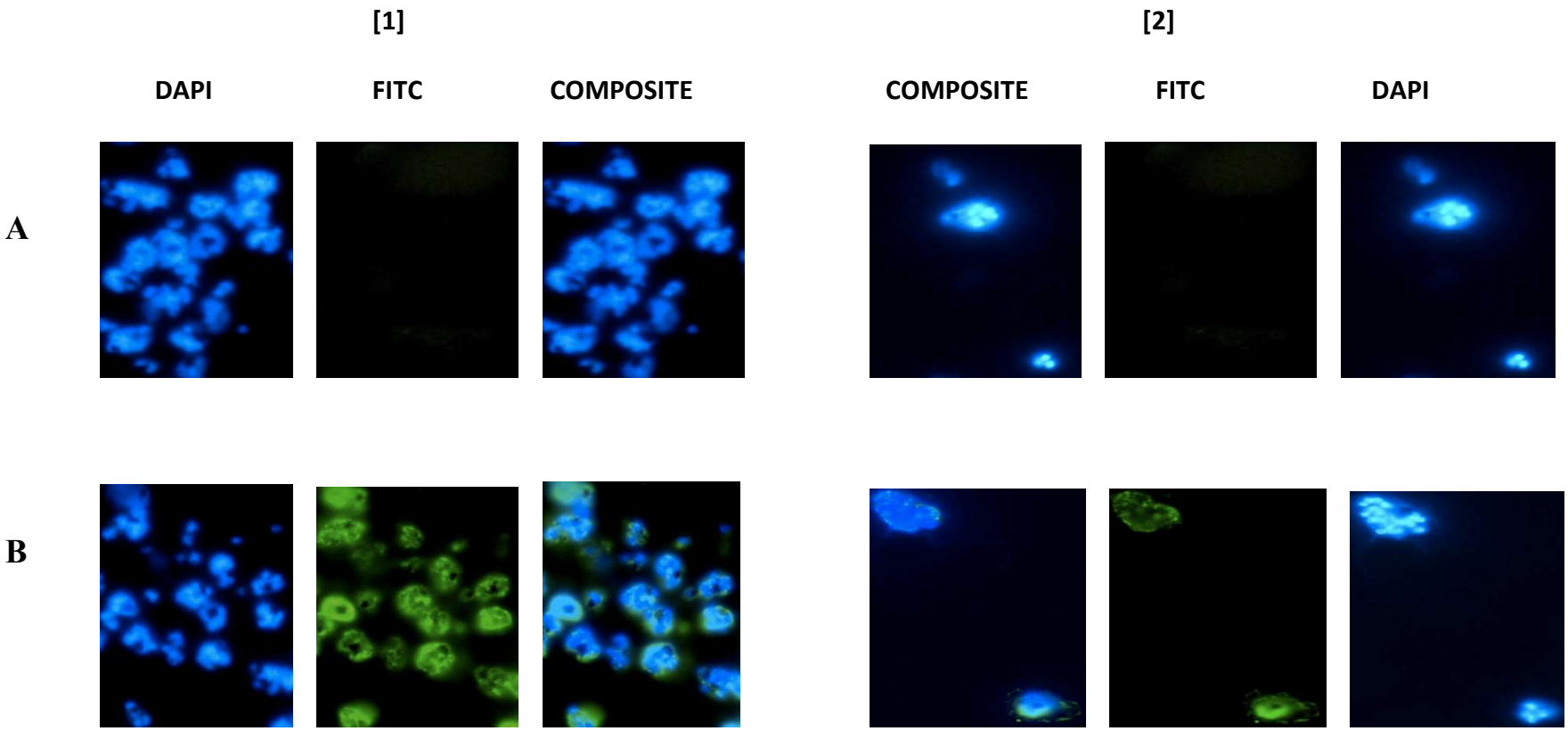


Fig 4.1 Immunofluorescent staining (IFA) of merozoites. Presence of anti-malarial antibodies on merozoite surfaces as visualized by FITC staining. Presence of antibodies detected on staining with malaria hyper immune IgGs (B) and not with the malaria naïve IgGs that do not bind to merozoites (A), with an end-point titre equivalent to 1:2000 hyper immune serum dilution. Panel [1], isolated merozoites; Panel [2] merozoites smeared directly from malaria culture.

4.1.2 Opsonised Merozoites Induce Respiratory Burst Activity with Neutrophils/Monocytes with Optimised Measurement Conditions

The ability of the isolated merozoites to trigger ROS production was tested, either on their own or when opsonised with antibodies. When incubated with either PMNs or monocytes, malaria hyper immune IgG-opsonised merozoites significantly induced more respiratory burst activity than either merozoites alone or merozoites with malaria naïve IgG, both of which remained at baseline (Figure 4.2, A, B). The PMNs were able to generate over two-fold higher burst activity (arbitrary units) as compared to autologous monocytes at equivalent cell numbers (Figure 4.2 A, C and E versus B, D and F). Because this was a functional assay, the time taken in the measuring instrument (TopCount) and the temperatures involved were determined to be able to adopt the most efficient option. In Figure 4A and B, chemiluminescence measurements were undertaken in the TopCount at room temperature immediately after bringing opsonised merozoites in presence of effector cells and it was observed that it took 142 minutes to attain burst activity peak value. However, when effector cells were pre-incubated at 37^0C in presence of opsonised merozoites and then transferred directly to the TopCount, peak burst activity measurement values were realised instantly, decaying out within the next fifteen minutes of measurement (Figure 4.2 C, D, E and F). In addition PMNs were observed to lose their ability to produce ROS within 4-5 hours, whereas monocytes remained able to produce ROS up to 48 hours after isolation.

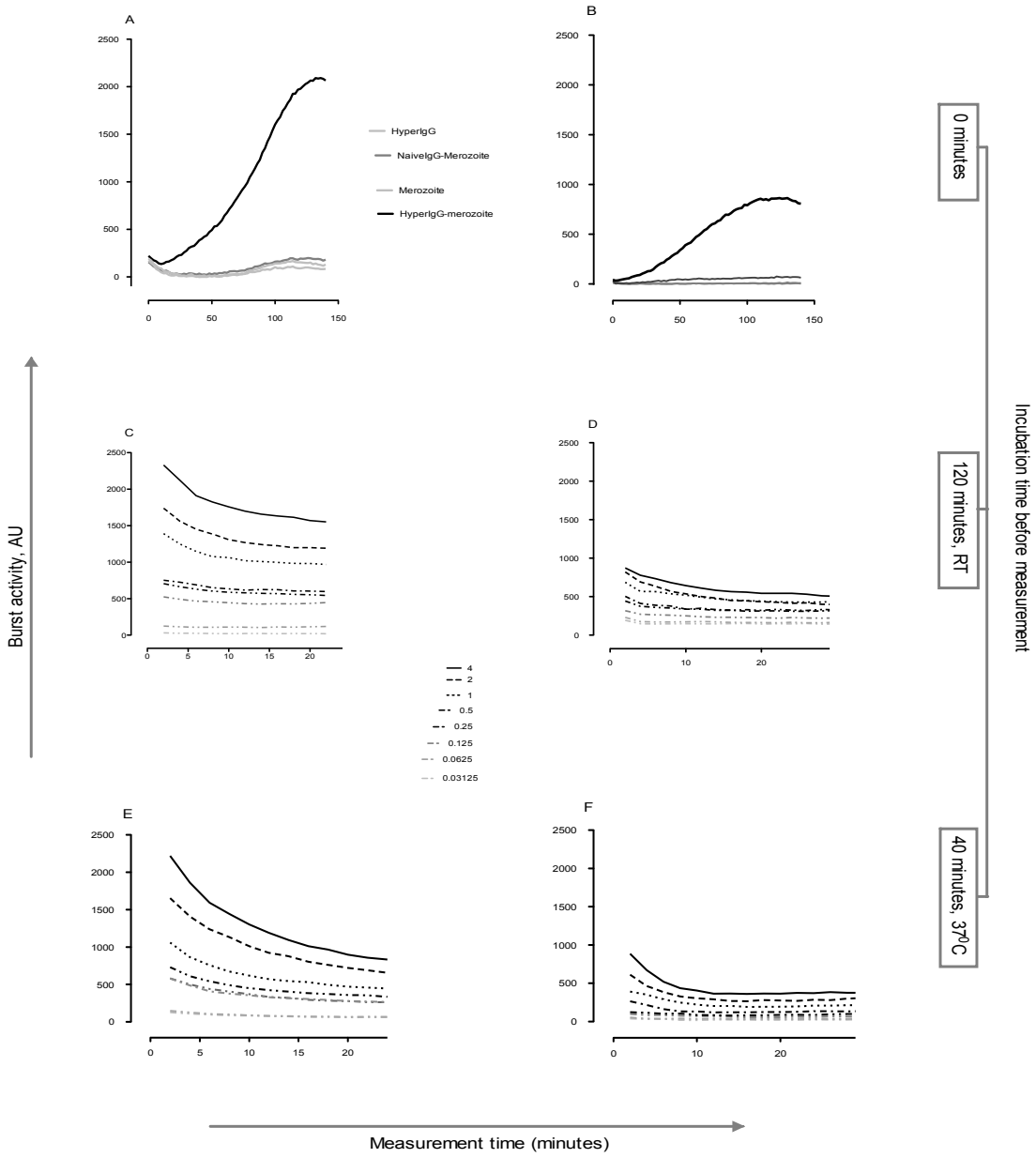


Fig 4.2 Isoluminol-amplified respiratory burst (ROS) profiles by chemiluminescence, for PMNs and autologous monocytes. Malaria hyper immune IgG-opsonised merozoites robustly induce burst activity, resulting detectable levels. Merozoites similarly treated with malaria naive IgGs, merozoites on their own or malaria antibodies on their own remain at baseline (top panel), cont. next page

Measurement in Topcount after incubating assay plate at room temperature for 120 minutes (C, D) or at 37^0C for 40 (E, F) minutes result in similar burst activity peak values within the first two minutes of reading as that obtained after direct measurement for 142 minutes (A, B). The different curves (E to F) depict hyper immune IgG-opsonised merozoites at doubling antibody dilutions from 4mg.mL^{-1}. Plots A, C and E show granulocytes while B, D and F show autologous monocytes. C – F are plots of cells from same donor. All plots in each graph represent the means of triplicate measurement at every interval. The differences between autologus effector cell PMN/Monocyte subsets ROS production levels were found to be statistically significant ($p<0.001$)

4.1.3 Respiratory Burst Activity Characteristics

To study the characteristics of burst activity induction and to rule out possible involvement of contaminants such as hemozoin and erythrocyte membrane debris present in isolated merozoite preparations that could trigger ROS production, chemiluminescence was measured in presence of effector cells with or without antibodies and merozoites on their own (no opsonisation), presence of effector cells with normal/non-infected erythrocytes and in presence of infected erythrocytes (at 5% parasitemia) without antibodies, either freshly prepared or freeze-thawed. In a parallel set-up, merozoites were sonicated before opsonising them with hyper immune antibodies for subsequent burst activity induction challenge. Figure 4 A and B show that sonication of merozoites before incubation with hyper immune IgGs abrogated most of the burst activity (Figure 4.3 B). Chemiluminescent measurement using opsonised intact merozoites in the presence of the oxygen radical scavengers desferoxamine mesylate (DFXM) and/or superoxide dismutase (SOD) significantly decreased the ROS signal, with the combination of DFXM and SOD demonstrating an additive effect (Figure 4.3B).

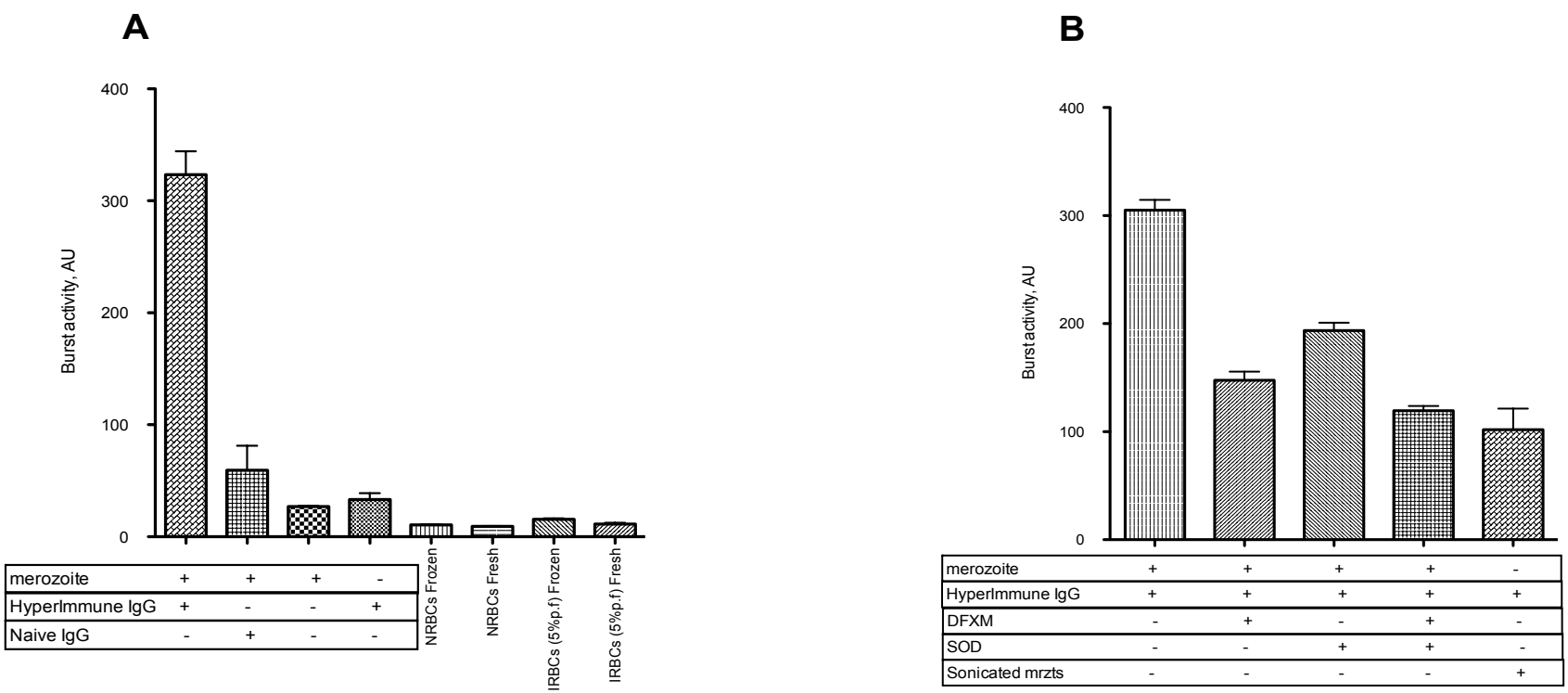


Fig 4.3 Respiratory burst characteristics in two independent experiments.

Burst activity induction (in arbitrary units) levels dependent on opsonised intact merozoites. Merozoites or malaria hyper immune IgGs on their own or with normal red cells (NRBCs) or infected red cells (IRBCs, 5% parasitemia) -fresh or freeze-thawed, do not induce burst activities above background (A, B). Levels of ROS measured after respiratory burst induction is reduced in the presence of superoxide scavengers: superoxide dismutase (SOD) or desferoxamine mesylate (DFXM). SOD and DFXM together have additive effect. Sonication of merozoites prior to opsonisation with antibodies compromise respiratory burst induction (B). Each plot is mean of triplicate measurement at every probe with standard error of mean.

4.2 Low Numbers of Parasites and Antibodies Are Sufficient to Trigger ROS Production by Neutrophils/Monocytes

The minimum number of merozoites required to trigger ROS production was studied using a range of merozoite to neutrophil/monocyte ratios in the assay. The maximal ROS production was reached at ratios as low as one merozoite per effector cell, with substantial effects seen from the ratios of two merozoites (Fig 4.4A); approaching plateau ROS values at between two to four merozoite per effector cell.

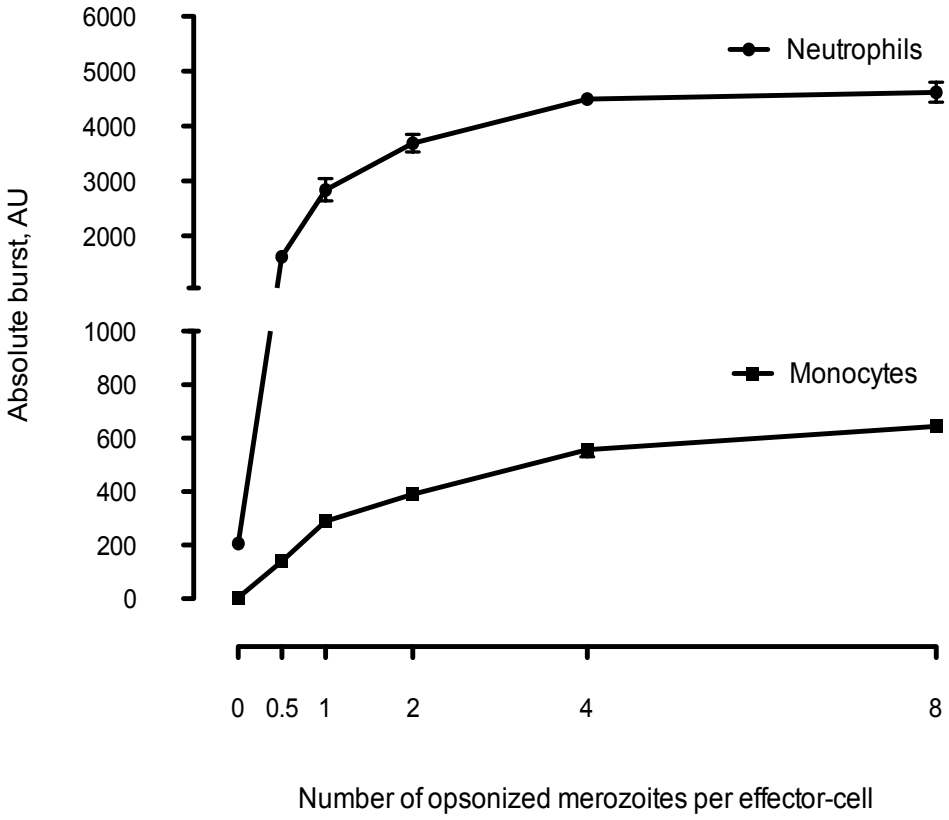


Fig 4.4A Antibody mediated respiratory burst induction is titratable with merozoite copy numbers. Titrating merozoite numbers at constant antibody concentration showed that maximal burst activity could be achieved at very low opsonised merozoite to effector cell ratios with substantial effects seen from 1-4 merozoites per effector cell (B). Plots represent means of triplicate measurements at every point.

At constant merozoite numbers, effector cell ROS production increased with increasing concentrations of the opsonising anti-malaria IgGs (Figure 4.4B). Substantial ROS production is achieved from as low IgG concentrations as from 0.25 mg/ml (equivalent 1/dilution ratio of 0.0625). Doubling IgG concentrations demonstrate titratable increases of effector cell ROS production.

In addition, pooled malaria hyper immune serum showed similar titration profile as purified IgGs (Fig 4.4B); and at all doses tested, ROS production was absent in IgG-depleted pooled hyper immune serum (Fig 4.4B, Depl. Serum curve).

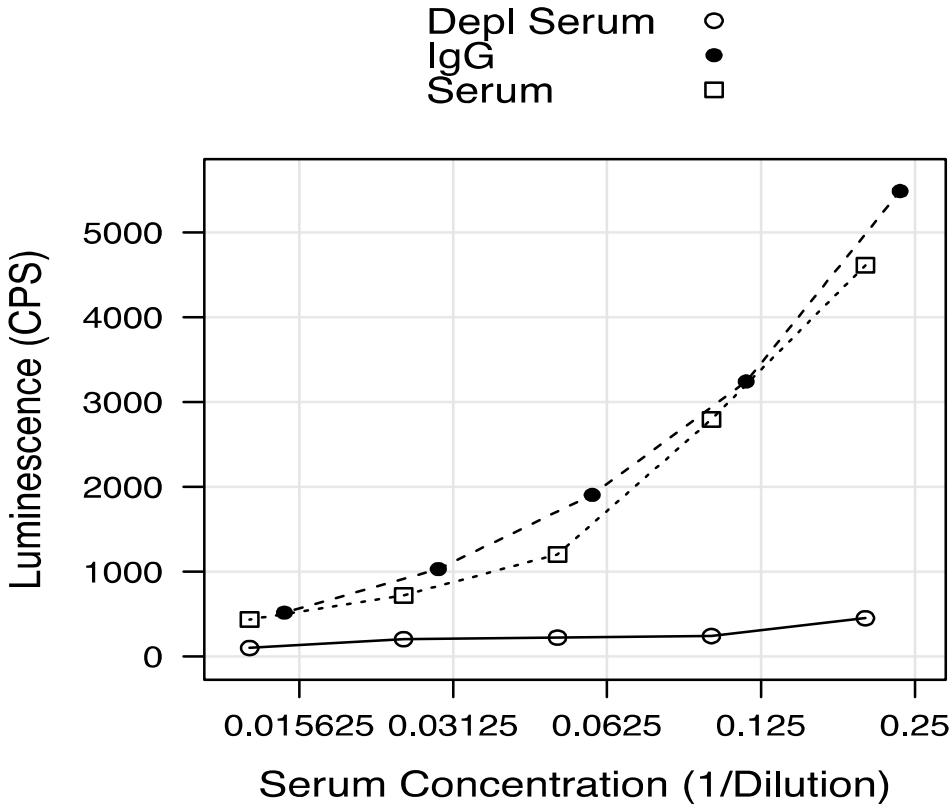


Fig 4.4B Antibody mediated respiratory burst induction is titratable with opsonising antibody. Increasing opsonising antibody concentrations led to increased burst activity induction for purified malaria immune (IgG) and for malaria immune serum pool (serum). Burst activity induction with the same serum pool is abrogated when depleted of the IgGs [(Depl serum.); (Depl serum = depleted serum)]. Plots represent means of triplicate measurements at every point.

4.3 Respiratory Burst Activity Induction is Reproducible

Additional experiments were performed to evaluate how some of the key variables might impact on the reproducibility of this assay. These included different effector cell- and merozoite batches. The pooled hyper immune Africa serum antibody positive control was assayed along as an internal control that was used to normalize the burst activity of unknown test samples as described in methods. Figure 4.5A shows that the relative burst score rankings for test samples are retained despite different effector-cell batches. Different batches of merozoites prepared over different weeks also demonstrated similar relative burst rankings for other two samples tested (Figure 4.5B). Figure 4.5C shows that burst activity induction was similar for fresh monocytes as for monocytes that were thawed after freezing (-20^0C), i.e. monocytes isolated from cryopreserved PBMCs. There were no differences observed between rhesus and human cells, with rankings retained either for equivalent monocytes or granulocytes.

The two methods of expressing burst activity; either as relative value or as equivalent to a known a concentration of the hyper immune control IgG as derived from a 4-parameter curve fit strongly correlated as shown in Figure 4.5D, with correlation coefficient (r^2) of 0.999.

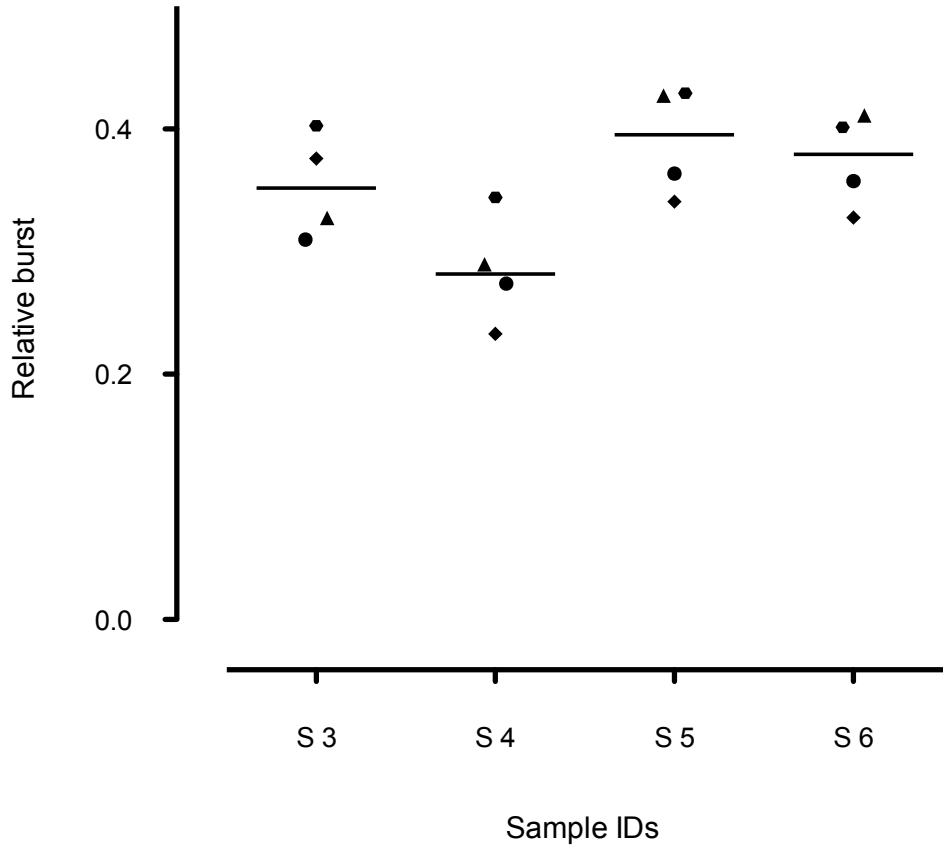


Fig 4.5A. Antibody mediated respiratory burst assay reproducibility with effector-cells from different donors. Four samples tested in four different experiments, each time using different donor monocytic effector cells. Mean of the four experiments for each sample, demonstrating that rankings are retained irrespective of donor cells used.

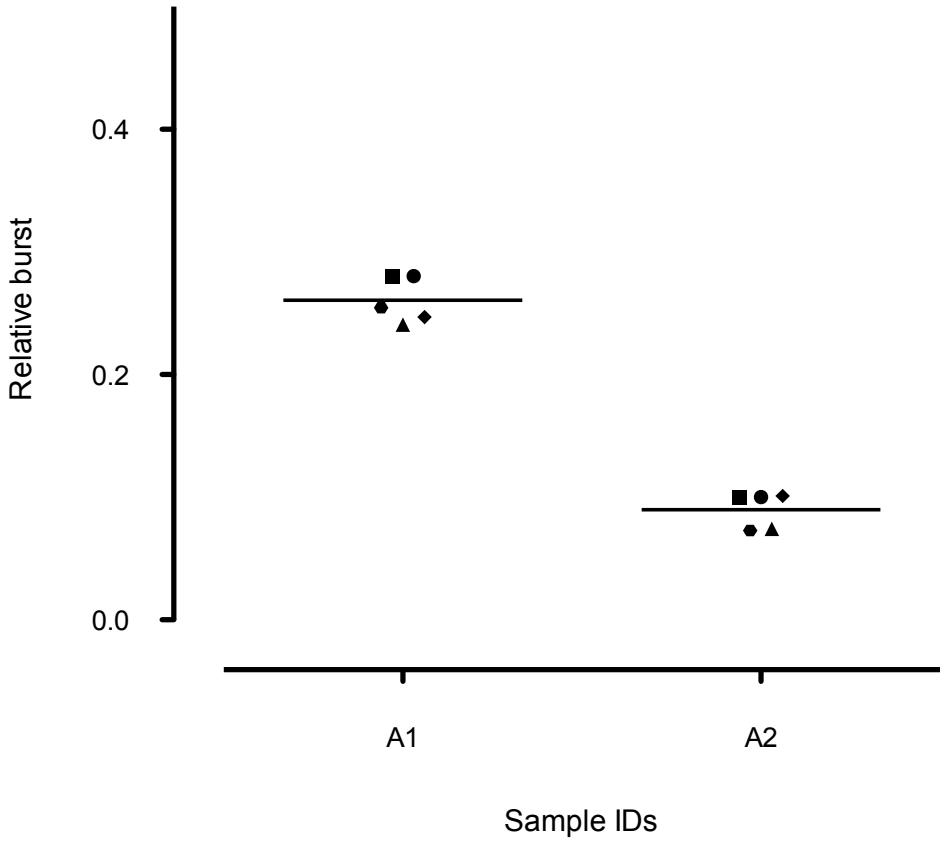


Fig 4.5B. Antibody mediated respiratory burst assay reproducibility with different merozoite batch preparations. Testing two samples with five different batches of merozoites prepared in different weeks show retention of sample rankings. Each plot is the mean of triplicate readings.

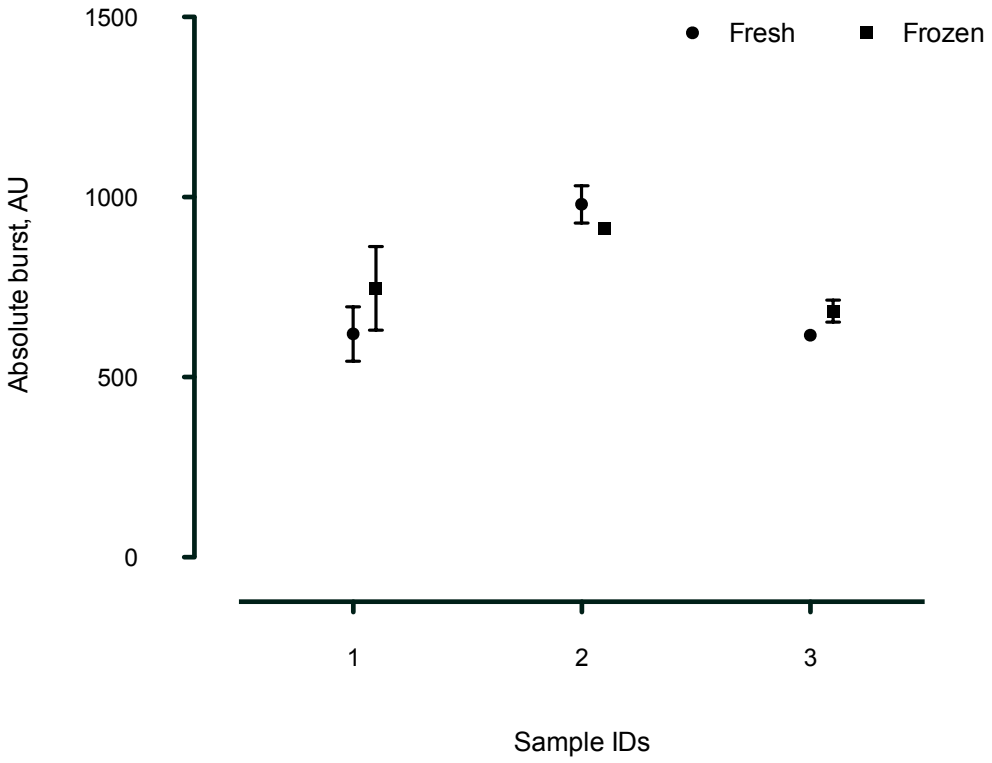


Fig 4.5C. Antibody mediated respiratory burst assay reproducibility when performed with fresh and with thawed autologous monocytes. Thawed monocytes (after freezing) demonstrate similar burst activity rankings for three samples tested (using the same batch of cells) when fresh and when thawed after storage. Samples were coded with the numerals 1, 2, and 3. Each plot is mean of triplicate measurement with standard error of mean.

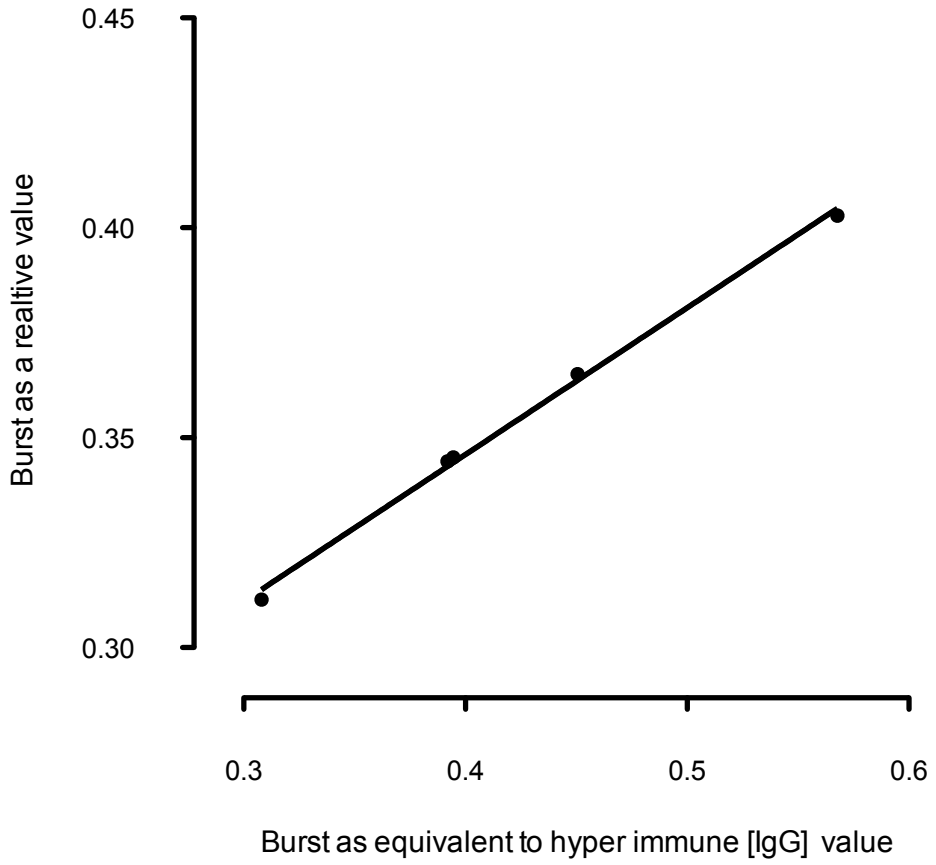


Fig 4.5D Comparison of two methods of expressing burst activity. Burst activity expressed as relative value (index) or as equivalent to a known a concentration of the hyper immune control IgG derived from a 4-parameter curve fit show strong correlation, $r^2 = 0.99$. Plots are means of triplicate measurements.

4.4.1 Antibody Mediated Respiratory Burst Induction Increases Following Vaccination

To investigate how vaccination impacts on burst activity as measured by ROS production and whether different malaria vaccine candidates elicited different levels of antibody-mediated burst activity, serum antibodies from rhesus macaques that had been vaccinated with the three candidate vaccine antigens: merozoite surface protein, block 2 hybrid, apical membrane antigen 1 and the merozoite surface protein 1, full length were tested.

Serum antibodies from the animals in each of the vaccine antigen study groups were purified from samples obtained on day 0 (-pre) and on day 70 (two weeks after the 3rd vaccination). Figure 4.6 shows that there are differences in respiratory burst induction following vaccination (day 70 compared to day 0) and that differences in the ability to induce burst activity following vaccination exist between different candidate antigens used in vaccine formulations; with the AMA-1 showing the least increase, block 2 hybrid intermediate and MSP1 full length the most.

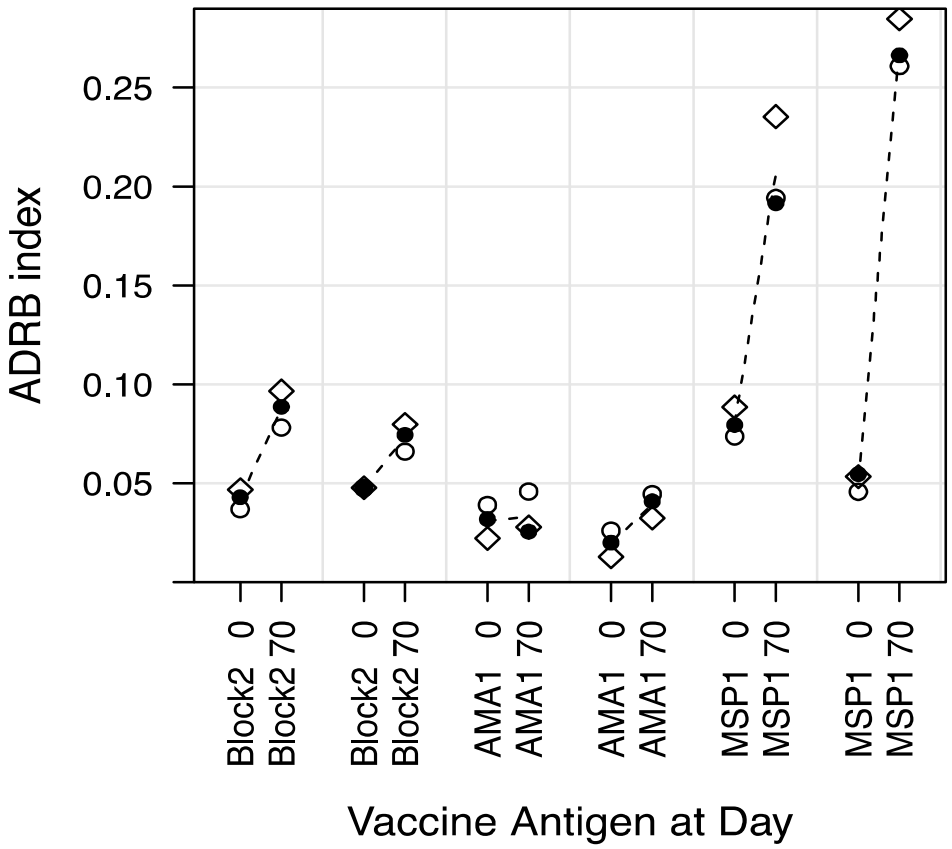


Fig 4.6. Antibody mediated burst activity induction increases upon vaccination. Serum antibodies from animals in three vaccination groups representing MSP1 full length, AMA-1 and MSP1 block 2 were tested for burst activity induction at day 0 (pre-) and day 70 (post-) vaccinations. Differences in burst activity induction occurred after vaccination, with the magnitudes of increase reflecting the respective vaccination antigen used formulation. Plots represent means of triplicate measurements at every assay point.

4.5 Respiratory Burst Induction Does Not Depend On the Quantity of Antibodies Elicited After Vaccination

To assess the relationship between burst activity induction and the quantity of antibodies raised after vaccination, the quantities of serum antibodies from animals in three vaccination groups representing MSP1 full length, AMA-1 and MSP1 block 2 at day 0 (pre-) and day 70 (post-) vaccinations were measured by ELISA and the increases in antibody titres plotted as shown in Figure 4.7. Compared to the trend in burst activity profiles characterised there was negative correlation between the quantities of antibodies raised and the burst activity inductions observed ($r^2 = 0.2419$, Figure 4.8); in which case, of the three antigens, AMA-1 vaccinated animals had the most increase in antibody titres following vaccination (Figure 4.7) but the least burst activity induction profile (Figure 4.6).

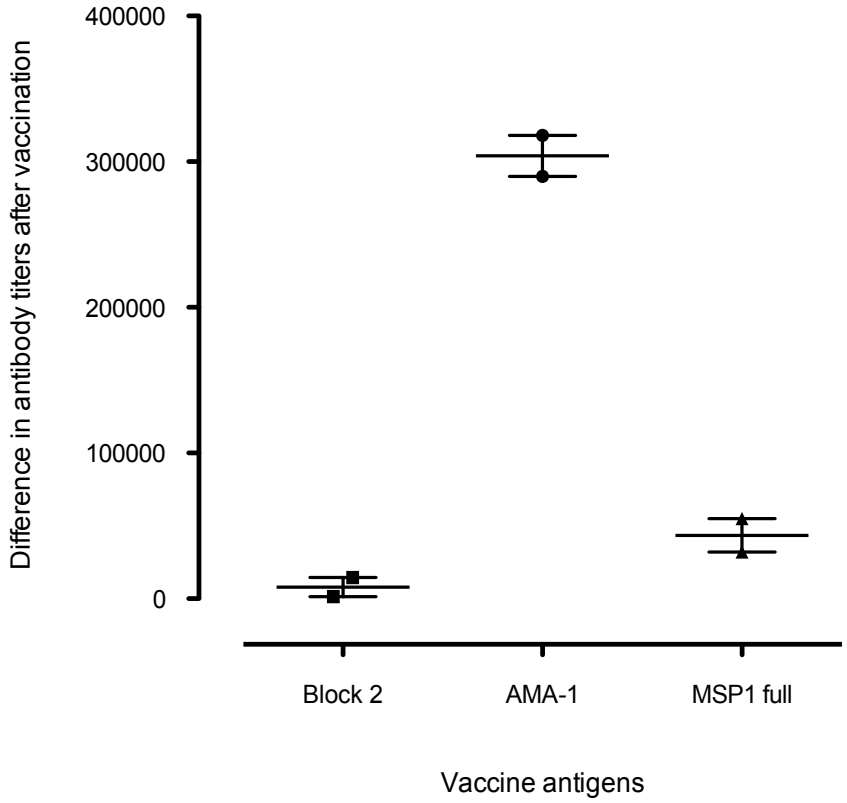


Fig 4.7 Increases in antigen-specific antibody levels after vaccination with test antigens. Serum antibodies from animals in three vaccination groups representing MSP – 1 full length, AMA – 1 and MSP – 1 block 2 were measured to determine vaccine induced increments post vaccination. Differences have been plotted for each antigen with the magnitudes of increase reflecting the respective vaccination antigen used formulation.

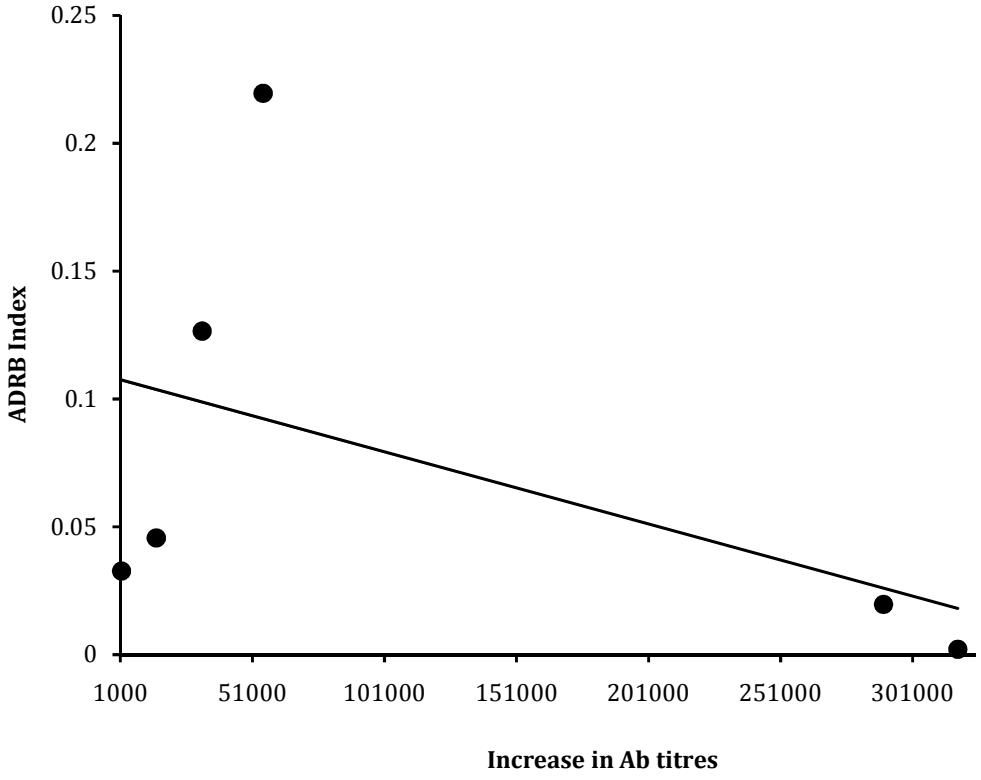


Figure 4.8 Correlation plot between antibody titres and ADRB Indices after vaccination with the test antigens in Fig 4.7. The graph depicts a negative association between antibody titres elicited and their ability to induce respiratory burst, $r^2 = 0.2419$. Plots represent the means of triplicate measurements at each assay point

CHAPTER FIVE: DISCUSSIONS

5.1 Malaria Sensitized Merozoites Trigger Neutrophils/Monocytes to Undergo Respiratory Burst By Producing Reactive Oxygen Species

Results from this study have further highlighted that extracellular ROS release is induced in PMNs by sensitized merozoites as had been suggested in previous reports (Joos *et al.*, 2010). Observations from this study have also demonstrated for the first time that monocytes may also perform key immune effector function via this mechanism and their postulated role in protection against blood stage malaria infection is not misplaced. Monocytes may as well be good candidates for use in the establishment of an antibody-mediated respiratory burst assay. In addition, these results have shown that the PMNs ROS production is much more when compared to autologous monocytes, corroborating the postulation that the PMNs could be more plausible effectors against malaria infection than has previously been studied (Joos *et al.*, 2010).

5.1.1 Opsonisation of Merozoites

From the observed dynamics of antibody opsonisation of merozoites, the findings that isolated merozoites were able to titrate to the same endpoint titres as the merozoites still in culture demonstrated that the procedure and processes used in merozoite isolation does not impact on the relative purity of the isolates. This would allow direct head to head comparison of the burst activity induction assay with other functional assays like the GIA (also ADCI), which employ the use of merozoites in culture rather than isolates. Subsequently, this gives room for direct head-to-head comparisons, i.e. the antibody concentrations expended in respective assay set-ups.

5.1.2 Opsonised Merozoites Induce Respiratory Burst Activity with Neutrophils/Monocytes with Optimised Measurement Conditions

The effort to develop the antibody-mediated respiratory burst assay was scrutinised further by evaluating the optimal measurement conditions under which the ROS produced by neutrophil and monocytic ROS can be gauged. Because the antibody-mediated respiratory burst is a functional assay, the measurement parameters, including the time and the measuring-instrument temperatures are critical aspects that can immensely determine the output parameters. According to the study by Joos *et al*., chemiluminescence measurements were performed at 37^0C over a measurement period of 1 hour with ROS peak values resolving within the first five minutes (Joos *et al*., 2010). In other studies by Helen Lundqvist and Claes Lundqvist, the measurement periods lasted only 25 minutes; again with ROS peak values resolving within the first 10 minutes (Helen Lundqvist and Claes Lundqvist, 1996); whereas for Adame-Gallegos *et al*., measurements took over two hours with ROS peak values resolving after about 40 minutes (Adame-Gallegos *et al*., 2012).

In comparison to the previous studies, the current study established that undertaking the measurement at 37^0C would realise the ROS peak values within a shorter period compared to measuring at room temperature. The measuring durations (when burst activity is decayed completely) were 15 minutes and 142 minutes respectively; suggesting the use of the 37^0C option for convenience, especially in testing large sample batches as those that may be encountered in clinical trials. If the measuring instrument cannot calibrate to this temperature, and as our results have shown, the assay mix can be pre-incubated at 37^0C for a period and transported to the measuring instrument at this temperature before actual measurement is initiated. In addition a shorter measurement time is also paramount when using PMNs in the assay

since they've been demonstrated to have shorter viability period and can lose their ability to produce ROS within 4-5 hours after isolation (Joos *et al*., 2010).

5.1.3 Respiratory Burst Activity Characteristics

To evaluate the specificity and fidelity of the new assay, it was necessary to measure some of the assay characteristics so as to establish that the quantifiable chemiluminescence signals detected were actually by reactive oxygen species and not due to other excitable molecules and that the PMN/monocyte respiratory bursts causing their production were triggered by only merozoites and no other factors in the assay mix.

To understand if opsonised merozoites are the sole inducers of burst activity, chemiluminescence was measured in the presence of effector cells with (i) opsonised merozoites (both fresh batch and thawed batch [-20^{0}C], (ii) merozoites on their own, (iii) normal RBC and (iv) infected RBC, 5% parasitemia, fresh and thawed [-20^{0}C]. Results from this study show that burst activity depends exclusively on opsonised merozoites (fresh or thawed [-20^{0}C] and not on possible contaminants present in merozoite preparations, including normal or parasitized RBC, lysed parasitized RBC (containing hemozoin, food vacuoles, membrane debris etc.), or on immune antibodies in the absence of merozoites. These observations corroborated the findings by Joos *et al*. (Joos *et al.*, 2010), where neither any contaminants during merozoite preparations nor the immune sera on their own, could elicit chemiluminescence readout above background levels.

In another set-up to establish if the burst activity induction depended on the abilities of the opsonising antibodies to recognise specific epitopes on the merozoite surface that would lead to recognition before subsequent binding, the observation that

merozoites that were sonicated before opsonisation massively lost their ability to induce effector cells, leading extreme abrogation of the ROS measured. This demonstrated that the burst activity induction is dependent on intact merozoites and is likely determined by the antigenic determinants on the same. It also implores the postulation that this assay can reliably measure the functional capacity of the antibodies involved in the opsonisation process and is in keeping with other previous findings (Osier *et al.*, 2014; Joos *et al.*, 2010).

In efforts to test the veracity of the chemiluminescence readout signals, the observations from this study that the presence of oxygen radical chelator desferoxamine mesylate (DFXM) or the enzyme, superoxide dismutase (SOD), led to reduction in the levels of measured ROS confirmed that the applied isoluminol chemiluminescence technique reliably detects ROS which may constitute different species of radicals, some of which, for example, are substrates of the enzyme used in the test. This is especially confirmed when addition of both DFXM and SOD demonstrate additive effect on ROS signal abrogation. However, further elaborate studies would be needed to characterise the respective individual radical species that may be involved in this vital immune mechanism.

5.2 Low Numbers of Parasites and Antibodies are Sufficient to Trigger ROS Production by Neutrophils and Monocytes

Results from this study show that as low as one merozoite can substantially induce burst activity, with maximal ROS production seen with as low as two to four merozoites. These findings are consistent with the very low parasitemias harboured by immune subjects (Bouharoun-Tayoun *et al*., 1990; Sergent, 1950). Indeed, it has been previously argued that an indirect mechanism such as ADCI (and now as

demonstrated this study) is consistent with the phenomenon of chronicity which is characteristic of malaria in hyper-endemic regions (Druilhe and Perignon, 1997). The resulting low-grade chronic infection makes sense in evolutionary terms as it satisfies the need to ensure parasite survival, though at a low density to also ensure host survival (Jafarshad *et al.*, 2007).

Similarly, IgG molecules tested in this study showed titratable effects; with very low levels of Abs sufficient to trigger burst activity. Specific effector cell activation was observed at concentrations as low as 175 ng/ml and became substantial at 250 ng/ml. It can be postulated from these results that low levels of Abs maybe sufficient to trigger anti-parasitic effects; suggesting further that optimal antibody-mediated cellular effect may not require effector-cell stimulation via contribution of multiple combination of antibodies. In contrast, the concentrations observed in this study are far much lower compared, for example, to those required to achieve even 50% inhibition of merozoites invasion by Abs in a direct inhibition assays such as GIA (Woehlbier *et al.*, 2006; Ockenhouse *et al.*, 2005). The number of Ab molecules required to neutralize all antigenic targets in such an invasion-inhibition mechanism is understandably far much larger than that required to trigger burst activity induction, which is postulated to take place through the Fcγ Rs (Jafarshad *et al.*, 2007). The results from this study also corroborate *in vivo* findings that relatively low concentrations of cytophilic antibodies in malaria exposed individuals (for example anti-MSP-3 IgG3) are very strongly associated with protection (Singh *et al*., 2004).

From the practical point of view, the observation that low concentrations of Abs are sufficient to trigger burst activity implies economic use of clinical samples to run this assay. This confers an advantage in the execution of this assay given the scarcity in sample volumes such as in Phase 2b clinical trials involving young

children. In addition, the observation that the immune serum that was tested together with the purified IgGs showed similarity in titration profile indicate that test serum samples maybe utilisable in this assay without having to purify IgGs, an extra step which is both expensive and time consuming. Indeed the observation that the IgG depleted serum does not indicate any burst activity induction is a confirmation that the burst activity observed in the un-depleted serum is due to the presence and contribution of the IgGs.

5.3 Respiratory Burst Activity Induction Is Reproducible

One of the major difficulties encountered in the development of ADCI is its reproducibility (Cavanagh *et al*., 2011; Joos *et al.*, 2010; Jafarshad *et al.*, 2007). This study analysed some of the aspects that may impact on assay robustness and reproducibility. One of the issues is the effector-cell variability from one donor to another. In ADCI development, some groups have reported difficulties in reproducing results due to the varied sourcing of monocytes (Jafarshad *et al.*, 2007). The antibody dependent respiratory burst assay on the contrary have demonstrated that the burst score rankings for test samples are retained irrespective of the different sources of the effector-cells used in independent assay runs. The observation that both PMNs and monocytes can be used to run the assay makes it more versatile and robust to perform. Our observations have however shown that PMNs had to be used immediately after isolation, with up to 75% loss of activity within 24 hours whereas fresh monocytes retained burst activity up to 48 hours after isolation. In addition, our study demonstrated that the assay can be performed using cryopreserved monocytes, which show similar burst activity as fresh monocytes. It is tempting to postulate that aliquots

from a single cytapheresis can be cryopreserved to support a desired number of assay-runs such as in a clinical trial.

The other key parameter in this assay that has shown not to impact on assay reproducibility is the merozoites. Because the procedures of culturing and isolating the merozoites is elaborate, it was thought that any differences in handling the same over periods of time would impact on the outcome of PMN/monocyte stimulation. However, the retention of burst activity rankings with different merozoite batches prepared over different weeks affirmed that these factors do not impact on assay reproducibility and corroborated the previous findings by Joos et al. (Jafarshad *et al.*, 2007). In addition, and like the monocytic effector cells, batches of merozoites can be cryopreserved. This can limit time expenditure on frequent preparations.

In conclusion, the interpretation of burst activity as a relative value to a known positive control at equivalent concentration as adopted in this assay can allow tests to be done without having to use a fixed amount of the test sample, making it easier to utilize this assay in situations where there are only limited amounts of sample to run as mentioned previously. The notion that either of the two methods of expressing burst activity (as relative value or as equivalent to a known a concentration of the hyper immune control IgG that is derived from a 4-parameter curve fit) makes it possible to choose the most suitable option that can be analysable alongside other results from concurrent assays such as ELISA where the 4-parameter curve fit is a utilisable analysis tool.

5.4 Antibody Mediated Respiratory Burst Induction Increases Following Vaccination

This study has corroborated other findings that PMN and monocytes produce a respiratory burst in response to opsonised merozoites, and its findings support the observation that the merozoite-triggered burst activity of sera from individuals living in endemic areas correlated with naturally acquired clinical protection from malaria (Joos *et al.*, 2010). However, it has never been known as to how burst activity would be impacted upon by antibodies elicited after vaccination. The clinical implications of deploying this assay to measure such outcome are two fold: first it would gauge the candidate vaccine's ability to elicit functionally relevant antibodies as compared to those observed in naturally acquired immunity; but importantly it would provide a platform to compare and evaluate candidates so that only the most promising ones are prioritized. This would cut down on the high cost of clinical testing (Joos *et al.*, 2010). In assessing the applicability of this assay in vaccine development, serum antibodies from rhesus macaques that had been vaccinated with the three candidate vaccine antigens: merozoite surface protein 1, block 2 hybrid, apical membrane antigen 1 and the merozoite surface protein 1, full length were tested. The increases in burst activities observed showed that vaccination can up-regulate burst activity and that respective vaccine candidates elicit functionally relevant antibodies; the magnitudes of which depend on the antigen formulation of the candidate. Even though it remains to be seen if these observations translate into how vaccine efficacy is gauged in a clinical trial, it is tempting to postulate that the approach demonstrated here is a vital assay platform to prioritize vaccine candidates for selection.

In the context of malaria vaccine development, these findings lend more emphasis to and fulfil the calls to develop reliable *in vitro* functional tests that correlate with immune protection in humans (Joos *et al.*, 2010). This would facilitate the down-selection of the promising candidates and formulations as early as possible within the development pathway in accordance with the malaria vaccine technology roadmap (WHO, 2006) of a highly effective second generation construct. The assay evaluated in this work has demonstrated that it can reliably be utilised as a dependable tool in achieving this goal. However, given the complexity of malaria infection and the corresponding immune response, it is probable that protective immunity against the pathogen requires multiple effector mechanisms. It seems less likely that any single assay measuring a subset of such mechanism would correlate unequivocally with protection. Instead, a panel of assays may be required to correctly capture the signatures of acquired immunity to malaria in order to propel integrated success in clinical vaccine trials. The malaria community needs to assess and agree upon additional robust set of evaluation criteria to qualify promising vaccine candidates.

5.5 Respiratory Burst Induction Does Not Depend On the Quantity of Antibodies Elicited After Vaccination

This study assessed if there was any relationship between the increases in total antibody titres after vaccination with the observed increases in burst activity. If the three vaccinating antigens were considered, there was negative correlation, implying that with some candidates, only a fraction of the Ab titres elicited may be functionally relevant in burst activity induction. These findings contrast with most previous studies that have variously correlated (positively) the specific antibody levels with clinical protection (Cavanagh *et al.*, 2004: Perraut *et al.*, 2004; Polley *et al.*, 2006; Soe *et al.*,

2004), without looking at their functional attributes. This could be an explanation as to why the predicted outcomes in vaccine studies have not always been consistent with data from field trials (Ogutu *et al.*, 2009).

Observations in this study show that the AMA–1 formulated vaccines does not elicit antibodies that induce burst activity, with MSP–1 full length performing better than MSP-2 (block 2). Whereas it is understandable that MSP-2 (block 2) is an 83-kD fragment of the MSP1 full length that is a fluffy protein spanning over the merozoite surface, it is tempting to think that the poor performance of AMA–1 molecule may be because it is a less abundant apical antigen. Despite being highly immunogenic compared to the other two antigens, it remains to be investigated if its orientation at the apex, which make the AMA–1-specific antibodies bind in a punctuated apical fashion on the merozoite surface, may lead to a poor spatial orientation of such antibodies, making them less relevant for effector cell mediation as observed in this study.

CHAPTER SIX: SUMMARY OF FINDINGS, CONCUSIONS, RECOMMENDATIONS AND SUGGESTION FOR FUTURE WORK

6.1 Summary of Findings

Results from this study show that malaria sensitized merozoites trigger immune effector cells to undergo respiratory burst. For the respiratory burst induction to occur, effector cells need the functional involvement of antibodies. Relatively low concentration of functional antibodies with as low as one sensitized merozoite per effector cell may be enough to trigger substantial burst activity induction. The antibody-dependent respiratory burst assay is a reproducible tool that can be used to interrogate the functional capacity of anti-malarial antibodies. The different malaria vaccine candidates MSP–1, MSP–2 and AMA–1 elicit antibodies that trigger effector cell respiratory burst after vaccination and the magnitudes of burst activity induced vary depending on the candidate vaccine antigen tested. Respiratory burst induction does not depend on the quantity of antibodies elicited after vaccination. The AMA – 1 vaccine candidate elicited the highest antibody titres after vaccination but these antibodies were the poorest in inducing effector cell respiratory burst activity. MSP – 1 was not as immunogenic as AMA – 1 when antibody titre measurements were compared but the antibodies elicited due to vaccination with MSP – 1 formulation had bigger magnitudes of burst activity induction.

6.2 Conclusions

From this study, we can conclude that:

1. Malaria sensitized merozoites trigger neutrophils/monocytes to undergo respiratory burst by producing reactive oxygen species
2. Low numbers of parasites and antibodies are sufficient to trigger ROS production by neutrophils and monocytes
3. Respiratory burst activity induction is reproducible
4. Antibody mediated respiratory burst induction increases following vaccination
5. Respiratory burst induction does not depend on the quantity of antibodies elicited after vaccination

6.3 Recommendations

1. Use the developed tool (antibody-dependent respiratory burst) to test sera from clinical trials for acquired immunity after vaccinations and thereby evaluate candidate vaccine efficacy
2. Use the developed tool to perform immunoepidemiological surveys in different malaria endemic regions to understand the pattern(s) of naturally acquired immunity to disease
3. It is recommended that immunologists and vaccine developers put more emphasis on the possible contributions of PMNs, alongside the monocytes, to malaria immunity.
4. Chemiluminescent ROS measurements of biologically active mechanisms in malaria studies may achieve optimised measurement condition at the ambient temperatures of 37^0C.

5. Malaria vaccine designers need to focus more on antigens that are of functional relevance in burst activity induction

6.4 Suggestions for Future Work

Based on the findings from this study, the following are suggested for subsequent studies:

1. Test and analyse the newly developed assay in different satellite malaria laboratories in effort to harmonise it further and subsequently standardise its use by setting up agreed laboratory assay conditions, sample volumes and the standard operating procedure that can be published in the Malaria Research and Reference Reagent Resource Centre, (MR4).
2. Test the new developed tool as a comparator in malaria functional antibody studies alongside other established tools like the growth inhibition assay (GIA) and evaluate the similarities and differences in terms of immunological outcomes
3. Establish and understand possible correlation between respiratory burst and other effector cell immune mechanisms i.e. phagocytosis (e.g. fate of opsonised merozoites)
4. Evaluate and analyse possible modulatory effect of immune mediators such as cytokines on the onset of respiratory burst related acquired immunity to malaria

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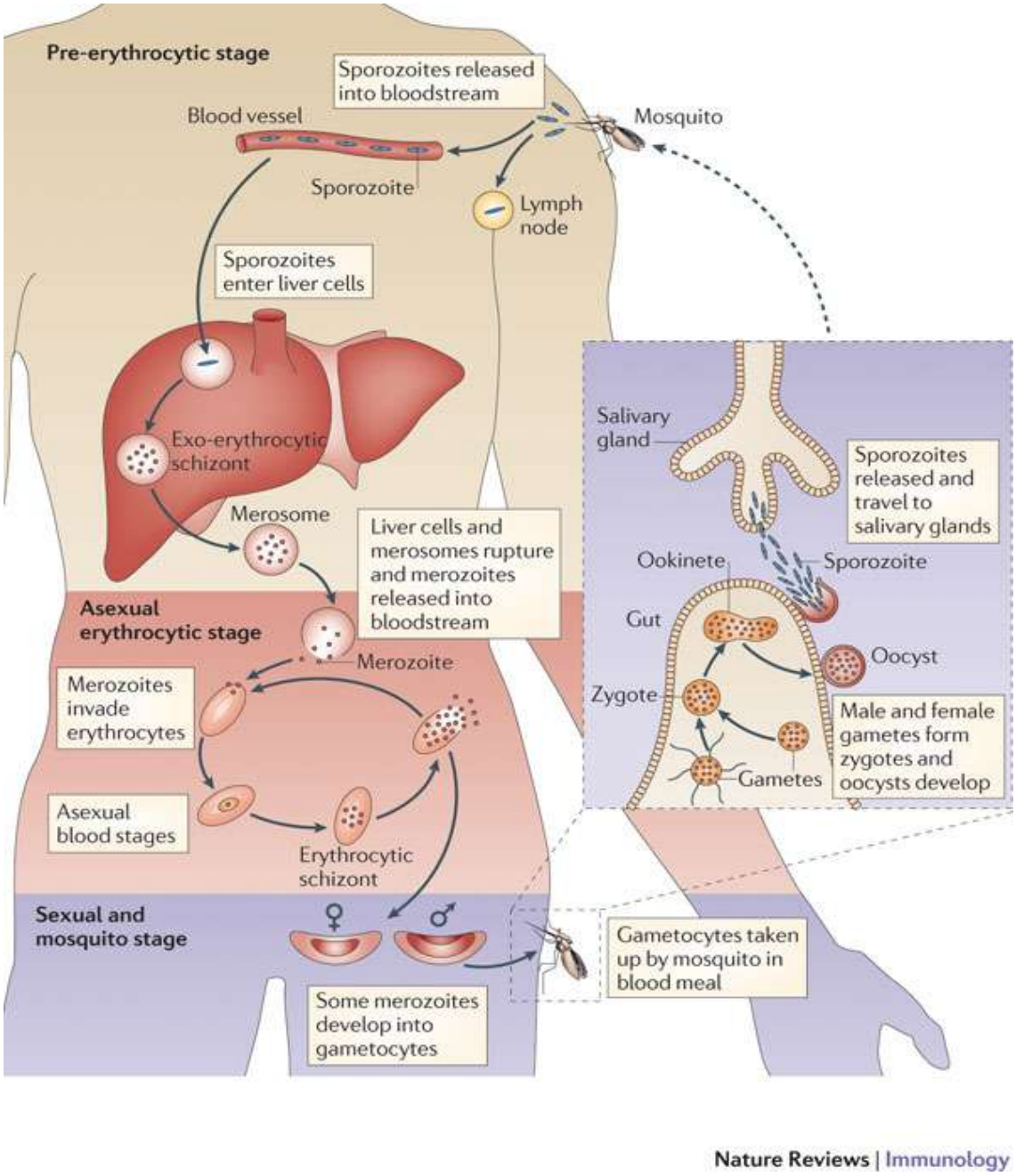
APPENDICES

APPENDIX I: Malaria Life Cycle Showing Parasite Developmental Stages as Possible Vaccine Targets



(Sauerwein *et al*., 2011).

APPENDIX II: Malaria Candidate Vaccines and their Stages in Clinical Development

Candidate malaria vaccines currently in clinical development*

	Pre-erythrocytic vaccines						
	Research	Preclinical development	Phase 1a	Phase 2a	Phase 1b	Phase 2b	Pivotal
CSP							
RTS,S/AS02A (CSP/HBsAg particle in AS02A adjuvant) (GSK)	X	X	X	X	X	X	
HBcAg-CSP VLP (Malarivax Apovia)	X	X	X	X			
Modified Vaccinia Ankara (MVA) CSP tested in combination with RTS,S, SA02 (Oxford-GSK)	X	X	X	X			
Long synthetic CSP peptide (Lausanne)	X	X	X				
CSP DNA immunization (Vical/NMRC)	X	X	X	X			
Long Vivax CSP C-terminus 72 amino acid synthetic peptide (MVDC)	X	X	X				
Long Vivax CSP N-terminus 77 amino acid synthetic peptide (MVDC)	X	X	X				
Long Vivax CSP repeat 48 amino acid synthetic peptide (MVDC)	X	X	X				
Other antigens							
Long synthetic LSA-3 peptide (Pasteur Institute)	X	X	X				
Lactococcus lactis recombinant LSA3 (Full length) in AS02 adjuvant (Pasteur Institute)	X	X	X	X			
Lipopeptide LSA3 without adjuvant (Pasteur Institute)	X	X	X	X			
Modified Vaccinia Ankara (MVA) CSP + LSA-1 epitope (Oxford) only a phase 1 with MVA. Combination noted above)	X	X	X				
Fowl Pox 9 CSP + LSA-1 epitope (Oxford)	X	X	X				
Escherichia coli LSA-1 recombinant protein (WRAIR)	X	X					
MVA prime-boost DNA Multi-epitope string + TRAP (Oxford)	X	X	X	X	X	X	
MVA prime-boost Fowl Pox 9 Multi-epitope string + TRAP (Oxford)	X	X	X	X	X		
Asexual stage vaccines							
MSP-1							
Recombinant full length MSP-1 3D7 + FCB1 (Heidelberg/WRAIR)	X	X					
E. coli expressed recombinant protein MSP-1 42-kd 3D7 (FMP-1 WRAIR)	X	X	X	X	X		
E. coli expressed recombinant protein MSP-1 42-kd FVO (WRAIR)	X	X					
Pichia pastoris expressed recombinant protein MSP-1 42-kd 3D7 + FVO (MVDU)	X	X					
Baculovirus recombinant protein MSP-1 19-kD (Pasteur Institute)	X	X					
Baculovirus recombinant protein MSP-1 42-kD FUP (U of Hawaii/Antigenics)	X	X					
P. pastoris recombinant AMA-1 and MSP-1 chimera (SMMU)	X	X	X				
Other MSPs							
Long synthetic peptide MSP-2 (Lausanne)	X	X					
Long synthetic peptide MSP-3 (Pasteur Institute)	X	X	X		X		
L. lactis MSP-3 recombinant (Pasteur Institute)	X	X	X				
MSP-3-GLURP hybrid vaccine (SSI)	X	X					
AMA-1							
E. coli expressed recombinant protein AMA-1 3D7+FVO (MVDU)	X	X	X				
P. pastoris expressed recombinant AMA-1 protein (BPRC) Doubt	X	X					
E. coli recombinant protein AMA-1 3D7 (FMP-2.1 WRAIR)	X	X					
Other proteins							
Long synthetic GLURP peptide (SSI)	X	X	X				
Transmission-blocking vaccines							
Saccharomyces recombinant protein PvS25 (MVDU)	X	X	X				
P. pastoris recombinant protein PfS25 (MVDU)	X	X					
Combination (multistage) vaccines							
Multi-stage pre-erythrocytic DNA vaccination (Vical/NMRC)	X	X	X				
Recombinant FMP-1 plus RTS,S MSP-1 3D7 + CSP (WRAIR)	X	X	X	X			

* CSP = circumsporozoite protein; HBsAg = hepatitis B surface antigen; GSK = GlaxoSmithKline; HBcAg = hepatitis B core antigen; VLP = virus-like particle; NMRC = Naval Medical Research Center; MVDC = Malaria Vaccine Development Center; LSA = liver stage antigen; WRAIR = Walter Reed Army Institute of Research; TRAP = thrombospondin-related adhesive protein; MSP = merozoite surface protein; FMP = Falciparum merozoite protein; MVDU = Malaria Vaccine Development Unit; AMA = apical membrane antigen; SMMU = Second Military Medical University; GLURP = glutamate-rich protein; SSI = Statens Serum Institute; BPRC = Biomedical Primate Research Center; Pv = *Plasmodium vivax*; Pf = *P. falciparum*.

(Ballou *et al*., 2004)

APPENDIX III: Protocols

A. Isolation of Neutrophils from Peripheral Blood

MATERIALS

- Hank's balanced salt solution (HBSS)
- Trypan blue solution

4% trypan blue powder in 1x PBS rapped in aluminium foil, store at 4^0C

- RBC lysis buffer (Miltenyi Biotech)

Dilute 10 times in endotoxin free water

- Ficoll-Paque

PROCEDURE

1. Carefully layer 35 ml of peripheral blood suspension over 15 ml Ficoll-Paque in a 50 ml conical tube
2. Centrifuge at 400 g for 35 minutes at 20^0C in a swinging bucket rotor without brakes
3. Remove the upper plasma layer and the interface
4. Collect the granulocyte thin white layer above the red blood cell pellet with a Pasteur pipette and transfer the cells to a new conical tube
5. Lyse remaining red blood cells with excess volume of lysis red blood cell lysis buffer for 5-10 minutes at room temperature
6. Centrifuge the granulocytes at 300 g for 10 minutes at 20^0C and carefully aspirate the supernatant

7. Re-suspend the cell pellet in 50 ml of HBSS and centrifuge at 200 g for 10-15 minutes at 20^0C and carefully remove the supernatant completely (to remove platelets)
8. Repeat step 7
9. Enumerate the cells by trypan blue staining
10. Re-suspend cell pellet in an appropriate volume of HBSS to give cell concentration of 70,000 for every 200ul well plate.

NB: Stain a sample of the cells using May-Grunewald protocol to assess purity

B. Isolation of Peripheral Mononuclear Cells (PBMs) from Blood

MATERIALS

- Hank's balanced salt solution (HBSS)
- Trypan blue solution

4% trypan blue powder in 1x PBS rapped in aluminium foil, store at 4^0C

- RBC lysis buffer (Miltenyi Biotech)

(Dilute 10 times in endotoxin free water)

- Ficoll-Paque

PROCEDURE

1. Carefully layer 35 ml of peripheral blood suspension over 15 ml Ficoll-Paque in a 50 ml conical tube
2. Centrifuge at 400 g for 35 minutes at 20^0C in a swinging bucket rotor without brakes
3. Carefully remove the interface layer and transfer the cells to a new conical tube
4. Wash the cells twice in HBSS
5. Enumerate the cells using trypan blue and re-suspend in HBSS so as to give every well plate a concentration of 70, 000 cells.
6. Stain a sample of the cells using May-Grunewald protocol to assess purity

C. Isolation of Monocytes from PBMCs

MATERIALS

- 1X RPMI
- 1X RPMI 1640 with 10% FCS, 2 μM L-glutamin and 100 U/ml penicillin/streptomycin (complete culture medium)
- Trypan blue solution

4% trypan blue powder in 1x PBS rapped in aluminium foil, store at 4 0C

- Cell scrapper

PROCEDURE

1. Carefully layer PBMCs suspension in complete cell culture medium in culture flask
2. Incubate at 37^0C for one hour
3. Carefully aspirate the non adherent cells, washing them with RPMI at 37^0C
4. Add 10ml ice-cold RPMI to the adherent cells, swirl the culture flask and use cell scrapper to detach any cell remaining on the culture flask
5. Rinse the culture flask with ice-cold RPMI and re-suspend the detached cells in 50 ml conical.

Wash the re-suspended cells once; perform viability test using trypan blue and use accordingly

D. Isolation of PEMs from Saturated Malaria Culture

MATERIALS

- RPMI, 10X PBS, 1X PBS, E64
- 40% percoll: 2 ml percoll, 250 ul 10X PBS, 2.75 ml 1X PBS
- 70% percoll: 3.5 ml percoll, 350 ul 10X PBS, 1.65 ml 1X PBS
- Dilute E64, dilute 1:1000

PROCEDURE

1. Synchronize P. falciparum culture for atleast two subsequent cycles with alanine
2. Enrich the synchronized culture to parasitemia of 10% by lowering the hct to about 2.5% and changing culture medium as frequently as necessary
   (i.e. once, twice or thrice a day, depending on growth phase)
3. Transfer schizont stage cultures into 50 ml conical, wash twice in RPMI and re-suspend in 9X pellet volume with RPMI
4. Carefully layer 5 ml of resuspended culture onto 2.5 ml of 40-70% percoll cushion
5. Centrifuge at 2000 rpm on swing out rotor centrifuge for 16 minutes without brakes
6. Using a Pasteur pipette, careful aspirate the layer interface at the 70% gradient into a clean tube, and wash three times with ice-cold RPMI at 2000 rpm for 5 minutes each time to remove traces of percoll

7. Re-suspend the harvested in 10X volume of culture medium reconstituted with E64 and put back in culture for 2-6 hours in the incubator for all the schizonts to maximally ripen into merozoites
8. Wash out E64 and isolate merozoites by passing the culture through 3 μm filter, followed by 2 μm filter
9. Determine the concentration and freeze at below -20 ^{0}C until use
10. Wash the bottom layer of the percoll gradient (where majority of the red cells remain in 6 above) three time with ice cold RPMI and put back into culture.

E. May-Grunwald's Staining of Neutrophils Isolated from Peripheral Blood

[To assess the purity of neutrophils isolated from peripheral blood]

MATERIALS

- May-Grunwalds methylene blue solution
- Giemsa azur eosin solution
- PBS, pH 6.88 (particular PBS for this procedure)

PROCEDURE

1. Make thin smears of resuspended cell isolates
2. Fix for two minutes in 100% methanol
3. Air dry
4. Stain for 3 minutes in May-Grunwald's solution
5. Stain for 2 minutes in May-Grunwald's solution reconstituted in PBS, pH 6.88 (1:1)

NB: At every stage: tilt the smear to remove stain, do not wash in water

6. Stain for 15 minutes in GIEMSA reconstituted in PBS, pH 6.88 (1:25)
7. Rinse smear on water and air dry
8. Enumerate the different white blood subsets and make scores of each subset seen.

F. Protein Concentration Determination by BCA

1. Prepare the BSA standards in 1.5 or 2.0 ml tubes. Prepare the following concentrations of BSA in mg/ml: 2, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125 and 0.0 (blank) by diluting from the BSA standard ampules from the kit (2 mg/ml). For these dilutions, use solvent closest to the solvent of the unknown to be tested samples (PBS or water). Store the diluted BSA standards at –20°C.

BSA Standard

	mg/ml	Sample	From #	PBS	Volume stock
A	2,000	300	Ampule	0	300
B	1,500	375	Ampule	125	325
C	1,000	325	Ampule	325	350
D	0,750	175	#B	175	350
E	0,500	300	#C	300	350
F	0,250	250	#E	250	325
G	0,125	175	#F	175	350
H	0,000	-	-	500	500

2. If needed, dilute unknown sample in PBS or water. Pipette 10 µl (for a working range of 125-2000 µg/ml) or 25 µl (for a working range of 20-2000 µg/ml) of each standard or unknown sample into a microplate well. Perform assay for each standard or unknown sample at least in duplicate.

3. Prepare the working reagent in a 50 ml (centrifuge) tube by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B and add 200 µl of the working reagent to each well.
4. Cover plate with aluminium foil and incubate at 37°C for 30 minutes.
5. Measure the absorbance at 550 nm using the BIO-RAD plate reader.
6. Subtract the average 550 nm absorbance measurements of the blank replicates from the 550 nm measurements of all other individual standards and unknown replicates.
7. Prepare a standard curve by plotting the average blank-corrected 550 nm measurement for each BSA standard versus its concentration in mg/ml. Use the standard curve to determine the protein concentration of each unknown sample.

BCA reagent A: Bicinchonic acid

BSA reagent B: Copper (II) Sulphate solution

G. Cleaning Used Protein G Agarose for re-use in Purifying Immunoglobulin G (IgG)

MATERIALS

- Protein G agarose that has been used and recycled 10 times
- Polyethylene glycol, PEG8000 (Sigma)
- Sigma water (Sigma)
- Column loading buffer (Pierce)
- Phosphate buffered saline, PBS

PROCEDURE

1. Make PEG8000 solution at 10mg/mL using sigma water
2. Obtain the used ten-time recycled Protein G agarose resins from respective columns and pool them together
3. To the pooled resins add equal volume of reconstituted PEG8000 and incubate at room temperature on a slow shaker
4. After treatment, wash the resins in phosphate buffered saline twice
5. Re-suspend the washed resins in equal volume of the column loading buffer
6. Store at 4^0C for five days to allow for the resin matrix recovery