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Unstructured proteins of the malaria parasite  
*Plasmodium falciparum* as vaccine candidates

Kelwalin Dhanasarnsombut

A thesis submitted for the degree of Doctor of Philosophy  
University of Edinburgh  
2013

## **Declaration**

I hereby declare that this thesis is my own work and it has not been submitted anywhere for any award. All work in this thesis was done by me, unless otherwise acknowledged. The MSP-1 hybrid was designed by Dr. David Cavanagh, while the development on large-scale production of this protein presents in this thesis was carried out in collaboration with Dr. Graeme Cowan. The mass spectrometry work in this thesis was performed by SIRCAMS (School of Chemistry, University of Edinburgh). All sources of information in the text have been acknowledged by reference.

Kelwalin Dhanasarnsombut

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## Abstract

Malaria vaccine research has been battling with persistent challenges, including polymorphisms of vaccine antigens, difficulties with production processes, and limited immune protection against the disease. Intrinsically unstructured proteins (IUPs) are a fairly newly classified group of proteins that have no stable 3D structure and are generally heat-resistant. They usually contain low complexity regions and repetitive sequences, both of which are distinct characteristics of the malaria proteome. Surprisingly, some of the vaccine candidates that have been extensively studied were later reported to have unstructured regions, some of which serve as targets of protective immunity. In keeping with their interesting immunological profiles and their unique properties, which are exceptionally beneficial for vaccine production, malarial IUP antigens may be good vaccine candidates.

This PhD project has the following aims:-

- 1) to develop a synthetic unstructured protein antigen based on the Block 2 region of MSP-1, named the MSP-1 hybrid
- 2) to characterize a novel vaccine antigen derived from the MSP-3.3 protein, namely an IUP region of PF10\_0347 gene product, for its potential as a vaccine candidate
- 3) to develop a second-generation vaccine by combining the MSP-1 hybrid, with two allelic variants of MSP-2, to overcome antigenic polymorphism and strain-specific immune responses
- 4) to validate protocols for IUP identification from proteins extracted from the malaria parasite.

This study showed that 1) MSP-1 hybrid production was scalable, yielding high protein yields with comparable immunological properties to small-scale production. MSP-1 hybrid was shown to be compatible with different adjuvants, and elicited specific antibodies covering the whole range of Block 2 allelic diversities. 2) A novel antigen, MSP-3.3C, an IUP based on the 3' region of the PF10\_0347 gene, was cloned, expressed and purified. Anti-MSP3.3C antibodies showed very strong parasite growth inhibitory effects *in vitro*. 3) The MSP-multihybrid antigen was expressed using simple techniques, but only at low levels. It contains epitopes from all three parasite antigen components, and is recognized by specific naturally acquired antibodies. 4) an unconventional 2D gel technique was tested as a method of malaria parasite IUP identification. Plans for further validation of this technique were discussed.

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## Abbreviations

ADCC	Antibody-dependent cellular cytotoxicity
ADCI	antibody-dependent cellular inhibition
AMA-1	Apical membrane antigen-1
APAD	3-acetylpyridine adenine dinucleotide
CAPS	<i>N</i> -cyclohexyl-3-aminopropanesulfonic acid
CD	Circular dichroism
CIDR	cysteine-rich interdomain region
CR	complement receptor
CSA	Chondroitin sulfate A
CSP	Circumsporozoite protein
CTL	Cytotoxic T-Lymphocyte
Da	Dalton
DAPI	4',6'-diamino-2-phenylindole
DBL	Duffy binding like
DC	Dendritic cell
DNA	Deoxyribonucleic acid
E.coli	Escherichia coli
EBA	Erythrocyte-binding antigens
ELISA	Enzyme-linked immunosorbent assay
ELISA	Enzyme-linked immuno sorbent assay
EMP	Erythrocyte membrane protein
Fc	fragment crystallizable
FITC	Fluorescein isothiocyanate
GIA	Growth inhibition assay
GLURP	Glutamate-Rich Protein
GMP	Good Manufacturing Practice.
GMP	Good manufacturing practices
GPI	Glucophosphatidylinositol domain
GST	glutathione S-transferase
His	Histidine
HRP	Horse radish peroxidase
i.p.	Intraperitoneal
IEM	Immunoelectron microscopy

IFA	Indirect Fluorescent Antibody
IFN $\gamma$	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
IPTG	Isopropyl- $\beta$ -D-thiogalactopyranoside
iRBC	Infected red blood cell
IUP	Intrinsically Unstructured Protein
Kb	Kilobase
KDa	Kilodalton
LB	Luria-Bertani
LCR	low complexity regions
LPS	Lipopolysaccharide
LSA	liver-stage antigen
M2a-H	MSP-2A and MSP-1 hybrid (M2a-H)
M2b-H	MSP-1 hybrid and MSP-2B (H-M2b)
mAb	Monoclonal antibody
MC	Maurer's clefts
MES	2-( <i>N</i> -morpholino)ethanesulfonic acid
MHC	Major histocompatibility complex
MSP	Merozoite Surface Protein
MSP-3.3C	Merozoite Surface Protein 3.3 C-terminal
MVA	modified vaccinia virus Ankara
NO	Nitric oxide
NMR	<i>Nuclear magnetic resonance</i>
OD	Optical density
OPD	o-Phenylenediamine dihydrochloride
ORF	Open reading frame
PAXEL	Plasmodium export element
PBS	Phosphate Buffered Saline
PCR	Polymerase chain reaction
pI	Isoelectric point
pLDH	Parasite lactate dehydrogenase
pNPP	<i>para</i> -Nitrophenylphosphate
R&D	Research and development
RAP	Rhoptry associated proteins

RAP	Rhoptry-associated protein 1
RAS	radiated-attenuated sporozoite
Rh	Reticulocyte-binding proteins
Rif	repetitive interspersed family
s.c.	Subcutaneously
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl-sulphate polyacrylamide gel electrophores
SNP	single nucleotide polymorphisms
SPAM	secreted polymorphic antigen associated with merozoites
stevor	Subtelomeric variable open reading frame)
TAE	Tris-acetate-EDTA
TEMED	Tetramethylethylenediamine
TLR	Toll-like receptor
TNF $\alpha$	Tumour necrosis factor-alpha
TRAP	Thrombospondin-related adhesion protein
Tris	tris(hydroxymethyl)aminomethane



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# Chapter 1: Introduction

## 1. Malaria and human immunity to malaria

### 1.1 Malaria

The malaria parasite has long been known to infect humans. Despite efforts to control this disease, it still remains a public health problem and has been named among the top most life-threatening communicable diseases (Sachs & Malaney 2002). It affects the lives of people in 100 different countries and is responsible for approximately one million deaths per year, mostly amongst young children under the age of five and pregnant women in sub-Saharan Africa (World Health Organization 2010a). Malaria is endemic in tropical and subtropical areas, including Sub-Saharan Africa, Asia and South America, occupying a broad banded area around the equator (Figure 1.1). The geographical patterns of malaria endemicity presented by Gething *et. al.* in 2011 showed that the risk of *P. falciparum* is varied, but the highest level of parasite incidence was seen in Africa (Gething et al. 2011). Although malaria has been endemic mainly in African countries, outbreaks have been reported in areas outside the tropics, such as in North America and Korea. This suggests that the parasites can also reach well into temperate climates (Doolan & Dobano 2009).

### 1.2 Challenges to the control and eradication

Although, many efforts to control and eradicate malaria have been tried by many organisations worldwide, around 48% of the global population still remain at risk (Hay et al. 2004). The World Health Organisation's Global Malaria Eradication Program (GMEP) has successfully eliminated malaria from many parts of the world, but the disease persists in some economically poor countries (Sachs & Malaney 2002a). Malaria has not totally been removed from these regions due to difficulties of access to endemic areas, socio-political issues, the costs of effective anti-malaria drugs, and the lack of an effective vaccine.

One of the important reasons for persistence of malaria in Africa is the presence of mosquito vectors of the *Anopheles* genus. Insecticides and insecticide-treated bednet programmes were introduced into Africa in order to cope with this problem. DDT spraying, in parallel with the environment improvement strategies (for example, removing mosquito breeding areas) and drug treatment had been used successfully, resulting in reduction of the vector populations and decrease in malaria distribution in many areas. These interventions face new problems, including the development of insecticide resistance (Tanner & de Savigny 2008; Guerin et al. 2002). Bednet use is associated with reduced mortality and



incidence of mild malaria episodes. However, the emergence of mosquito resistance to insecticides in Africa has now diluted the efficiency of this method (Lengeler 1996).

Increasing frequency of drug-resistant parasites also causes complications in malaria control, resulting in re-emergence of malaria and increased death rates (Sachs 2002). The dynamics of resistance may be mediated by factors such as the level of drug use in the population (Hastings & Watkins 2005). Chloroquine resistance, for example, is widespread in African countries where chloroquine is continuously used. The efficacy of sulphadoxine-pyrimethamine, which is commonly used in some countries as an alternative to chloroquine, has also declined rapidly (Frosch et al. 2011). In SouthEast Asia, *P. falciparum* is resistant to most of the available antimalarial drugs. Chloroquine and sulfadoxine-pyrimethamine resistance was reported in the Thai-Cambodian border as early as 1950 (Wernsdorfer & Payne 1991) and then again in the mid-1960s (Björkman & Phillips-Howard 1990). Recently, a decline in efficacy of Artemisinin was reported (Dondorp et al. 2009; Phyo et al. 2012). Since artemisinin is recommended by the WHO as a first-line treatment of uncomplicated malaria in endemic areas, the emergence of resistance adds more complications to global malaria control (World Health Organization 2010b).

With the completed genome sequences of several *P. falciparum* parasite isolates (Gardner et al. 2002; Jeffares et al. 2007), advances in biomedical research and financial support from charitable foundations, the possibility of malaria eradication has been recently reconsidered (Tanner & de Savigny 2008). Although research on drug target identification and new drug discovery are important, these efforts must be complemented by malaria vaccine development, which will hopefully provide effective protection in the long-term against clinical disease and support the goal of malaria control and eradication globally (Holder & Holder 2009; Genton 2008; Moorthy & Hill 2002).

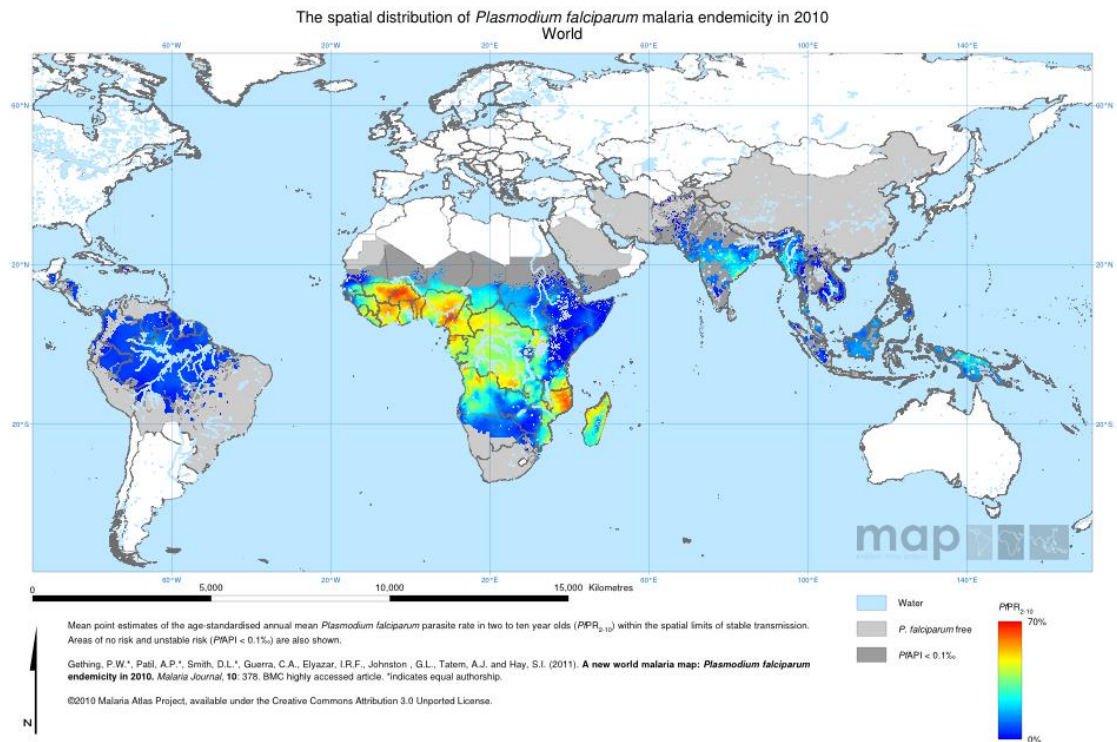


Figure 1.1: The world map shows the extent and intensity of *Plasmodium falciparum* malaria endemicity in 2010. The *P. falciparum* parasite rate surveys used to produce this map are age-standardized to the 2 to 10 year age-range (PfPR2-10). The continuum of blue to red represent PfPR2-10 from 1%-100%. The areas which were defined as stable risk (*P. falciparum* annual parasite incidence (PfAPI)  $\geq 0.1$ ), unstable (PfAPI < 0.1) and no risk (PfAPI = 0) are represented by dark grey, light grey and white area respectively (Gething et al. 2011).

### 1.3 Parasite life cycle

There are five primate malaria parasites that are regularly transmitted to humans, including *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. Among the 5 species of human malaria, *P. falciparum* is the most virulent, often resulting in severe symptoms and causing 90% of the mortality from the disease. Malaria parasites have complex life cycles involving female mosquitoes of the Anopheles genus, the primary host where parasites reproduce sexually, and in the intermediate mammalian host (Figure 1.2).

Once parasites enter the host by injection from the salivary glands of the Anopheles mosquito, malaria parasites in sporozoite form travel via the bloodstream to the liver where

they replicate asexually and asymptotically to yield thousands of merozoites, which then burst from infected hepatocytes and are re-introduced into the bloodstream, marking the initiation of the asexual blood-stage of the parasite lifecycle. Merozoites invade erythrocytes, replicate and develop through a series of life cycle stages known as rings, trophozoites and then schizonts. Approximately 16-32 new merozoites within a schizont are released during the subsequent erythrocyte rupture resulting in rising densities of parasitaemia. As a consequence, this process causes a rapid loss of red blood cells and leads to a spectrum of mild to severe symptoms (Bledsoe 2005). Released merozoites in the blood stream continue the blood-stage cycle by re-infecting new red blood cells. Erythrocyte invasion by the parasite is a complicated process, which involves many surface proteins as well as secreted proteins from the apical end of the parasite (Rodriguez et al. 2008; Farrow et al. 2011; Cowman & Crabb 2006). Soon after bursting from mature schizonts, merozoites firstly form low affinity interactions with uninfected red blood cells, a step which is suggested to be reversible (Blackman 2000). Reorientation of the parasite then follows, resulting in interaction between the apical end of the parasite and the red blood cell (Bannister & Dluzewski 1990). Stronger binding between the parasite and the red blood cell takes place when a tight junction is formed and moves along the surface of the merozoite from the apex (Blackman 2000; Farrow et al. 2011). Upon the entrance of the merozoite, the majority of the protein complexes which form the surface coat of the merozoite are discarded by the activities of proteases (Harris et al. 2005). Although the protease-mediated processing is known to be critical for parasite survival (Blackman 1994; Blackman 2000), there still no clear explanation why parasites invest a considerable amount of their time and energy in creating surface molecules that are removed later in the asexual life cycle (Kauth et al. 2003; Holder & Blackman 1994).

During this stage, by mechanisms that are still unclear, some parasites develop into sexual stages called male and female gametocytes, which circulate freely in the blood stream. These forms are taken up by mosquitoes during a blood meal. The two types of gametocytes then fuse, producing a zygote, beginning the infection in the mosquito. This diploid form undergoes meiosis and differentiates to form ookinetes. This form of the parasite can penetrate the mosquito gut, and differentiate into oocysts, which contain thousands of haploid sporozoites. These sporozoites are then released from the bursting oocyst, migrating to the mosquito salivary glands, ready to be released into host skin during the salivation which occurs during mosquito blood meal feeding.

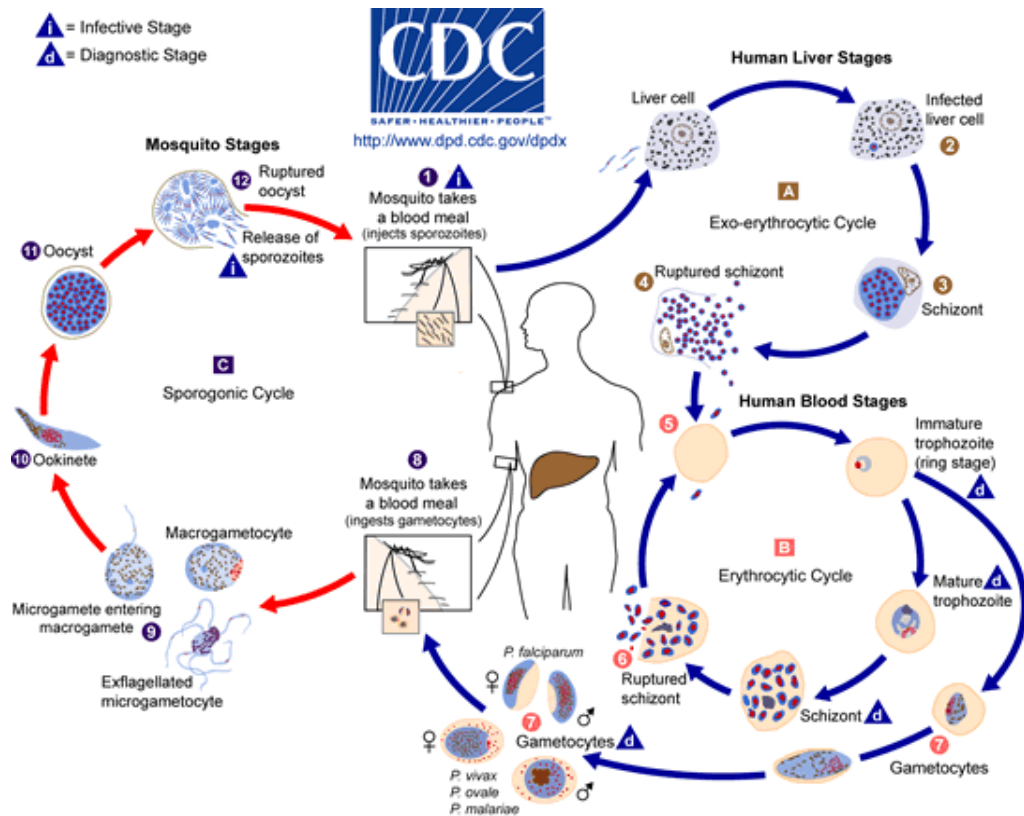


Figure 1. 2: The malaria parasite life cycle represents (A) Exo-erythrocytic cycle, (B) Erythrocytic cycle and (C) Sporogonic cycle. Following the bite of a Plasmodium-infected mosquito, sporozoites migrate to hepatocytes, where multiple rounds of cell division occur. Many thousands of merozoites then released, and invade erythrocytes. Some invading merozoites develop into male or female gametocytes, are taken up by the mosquito during a blood meal. In the mosquito gut, these sexual forms of the parasite develop into gametes, fuse to form zygotes and then develop into an oocyst, from which sporozoites are released. These sporozoites travel to the insect salivary glands and are re-introduced into a human host during the mosquito's next blood meal. Diagram from Centers for Disease Control and Prevention at [http://www.cdc.gov/malaria/biology/life\\_cycle.htm](http://www.cdc.gov/malaria/biology/life_cycle.htm)

## 1.4 Antigenic polymorphism and antigenic variation

The effectiveness of protective immunity can be diluted by the parasite's polymorphisms in the surface proteins encoded from single copy genes, such as merozoite surface protein 1 (MSP-1) (Bledsoe 2005; Galamo et al. 2009; Lyon et al., 2008) and merozoite surface protein 2 (MSP-2) (Genton et al. 2002; McCarthy et al. 2011) and due to antigenic variation in the gene products of multicopy gene families, notably the *var* (Kyes, Kraemer, et al. 2007b), *stevor* (subtelomeric variable open reading frame) (Blythe et al. 2004; Niang et al. 2009), and *rifin* (repetitive interspersed family) (Kyes et al. 1999). Immunity to heterologous parasite strains is not as effective as with previously encountered homologous strains (Pombo et al. 2002; Lyon et al., 2008) and cross-reactions between antibodies to different serotypes of some antigens are rare (Felger et al. 2003). If high titre strain- or variant- specific antibodies are required to provide efficient protection, multiple exposures to a mix of malaria strains may be required to induce acquired immune responses to the wide repertoire of antigenic variations prevalent in parasite populations. Thus, this may partly explain why protective immunity develops slowly (Malaguarnera & Musumeci 2002).

### 1.4.1 Antigenic polymorphism

Antigenic polymorphism or allelic diversity in the malaria parasite can be found in many of its antigens, especially the surface antigens. Accordingly it is widely assumed that antigenic polymorphism is associated with immune interference and immune evasion. Diversity of surface proteins may arise from point mutations, complementary-strand slippage (Brown et al. 2002; Tanabe et al. 1987), intragenic recombination, or miss-alignment (Brown et al. 2002; Rich et al. 1997). Genes that encode surface proteins of *P.falciparum*, such as *msh-1* and circumsporozoite protein (*csp*), are thought to be very old evolutionarily (Rich et al. 1998; Rich et al. 1997). Many of these genes contain repeating nucleotide sequences that code for repetitive amino acid sequences which are highly mutable (Mwingira et al. 2011). The variations in these genes are mostly in the form of duplication and/or deletion of the repeated segments, caused by intragenic recombination (IGR). The repetitive DNA sequences within these genes are subject to higher rates of mutation than non-repeated sequences (Rich et al. 2000). Unlike the repetitive central region sequences of *csp*, which is very conserved and has been maintained within each allele, the *msh-2* of *P.falciparum* shows much greater diversity in length, number of repeats and amino acid content, which is thought to result from proliferation of repeats within the *msh-2* gene (Rich et al. 2000). For *msh-1*, polymorphisms are rare in most regions, except notably the Block 2 region, which contains a set of repetitive tripeptides in two of three allelic types (Miller et al. 1993).

### 1.4.2 Antigenic variation

Infectious organisms such as parasitic protozoa (Kyes, Kraemer, et al. 2007b; Stijlemans et al. 2007), bacteria (Barbour et al. 2006), as well as viruses (Lindesmith et al. 2012) have a mechanism called antigenic variation, which allows them to switch their surface proteins and to evade host immune recognition and allow reinfection of host cells. In *P.falciparum* malaria, this mechanism is used in gene transcription of the *var* genes, the diverse family of genes encoding for PfEMP1. *Var* gene switching was shown to happen early after parasites invade the erythrocyte (Scherf et al. 1998; Kyes, Christodoulou, et al. 2007a) and during subsequent invasion cycles this results in a non-homogenous parasite population. Similar to *var* genes, the *rif* (repetitive interspersed family) and *stevor* are the known gene families, which encode for two further families of variant surface antigens (Khattab et al. 2008; Dzikowski et al. 2006).

## 2. Naturally acquired immunity to malaria parasites

In response to malaria infection host immune responses, which are regulated by both innate and adaptive immune system, are triggered. Passive maternal immunity was reported to provide protection against disease to babies (Doolan & Dobano 2009). Malaria symptoms are always detected in naïve individuals, while in high transmission areas, “anti-disease immunity” can be established in young children. This protection is acquired rapidly, resulting in reduction of severe clinical symptoms and children in endemic areas quickly develop immunity to death. However, this kind of immunity was reported to be short-lived in the absence of continual exposure (Doolan & Dobano 2009). The “anti-parasite immunity” which allows individuals to be protected against high-density parasitaemia is only developed slowly, whilst “sterilizing immunity” against infection is hardly ever fully achieved, as observed in asymptomatic adults, who carry gametocytes and therefore serve as reservoir for continued transmission (Good & Doolan 1999; Marsh & Snow 1997) and usually dies out quickly in the absence of parasitaemia (Crompton et al. 2010; Doolan & Dobano 2009). Immunity to malaria can be unstable due to other factors such as host genetics and polymorphism of parasites, and sterile immunity may not be achieved (Richie & Saul 2002; Langhorne et al. 2008).

Life-threatening forms of disease from malaria infection are normally found in children and become less frequent in adults (Carvalho et al. 2002; Marsh & Snow 1997). The most deadly form of severe disease is cerebral malaria, of which the clinical symptoms are such as sequestration of infected red blood cells in cerebral microvascular and presence of coma (Marsh & Snow 1997). Cytoadherence phenotype of parasites such as binding to

ICAM1 (Craig & Scherf 2001; Ochola et al. 2011; Cserti-Gazdewich et al. 2010), and rosetting (Hill 2011; Doumbo et al. 2009; Rowe et al. 1995) are showed to be correlated with this pathology.

Mathematical models using data from north-eastern Tanzania and Gambia classify clinical immunity that develops with age and exposure as relatively short lived, while immunity that reduces parasitaemia and is acquired later in life is considered to be long lived immunity, with a half-life of more than 20 years (Filipe et al. 2007). In young children and in adults in areas of low endemicity, immunity against malaria develops quite slowly, and rarely, if ever, becomes sterile immunity and usually dies out quickly in the absence of parasitaemia (Kinyanjui et al. 2007; Cavanagh et al. 1998). This suggests that routine exposure to the malaria parasite is necessary for both production and persistence of memory and effector cells. Some of the antibody responses to defined antigens are often very short-lived (Kinyanjui et al. 2007; Früh et al. 1991; Cavanagh et al. 1998) and may be unable to be boosted on re-exposure (McCallum et al. 2008). In contrast, adults may attain very long-lived immunity. This was illustrated by the re-emergence of malaria in Madagascar which showed that adults had maintained immunity to malaria disease at least for 30 years, after they had first experienced a number of infections before the eradication of malaria in that area (Migot et al. 1995).

## **2.1 Factors influencing development of immunity against malaria**

The immune response of each individual to malaria parasites is shaped by cumulative host factors. Development of immunity does not therefore correlate with any single factor but is affected by multiple factors (R. Wang et al. 2009; Doolan & Dobano 2009).

### **2.1.1 Age**

It is generally accepted that immunity to malaria is age-dependent. Children under 6 months of age are resistant to severe disease and high parasitaemia (Doolan & Dobano 2009). This protection is probably provided by passively acquired maternal IgG (McConkey et al. 2003; Amaratunga et al. 2011). These antibodies continue to decline during the first year of life. Secretory IgA in breast milk may also supply alternative protection for infants (World Health Organization 2010a; Kassim 2000). In areas of high endemicity, the risk of severe forms of malaria, such as cerebral malaria and death tends to increase with age from birth and it is not until children reach the age of five that the frequency of disease and mortality start to decrease. Severe cases are rare from adolescence onwards. (Richie et al. 2007; Doolan & Dobano 2009). This may suggest that the immune system of young children

is not fully developed, and without passive immunity, they are susceptible to clinical malaria before acquisition of adaptive immunity. It has been reported that acquisition of specific antibody isotypes begins with IgM, followed by IgG1 and IgA respectively. Tongren *et al.*, 2006 suggested that the default pathway of IgG subclass switching in children is towards a mixed IgG1 and IgG3 response (Tongren *et al.* 2006). Some antigens, such as AMA-1, induce high IgG1 responses in all age groups (Saul & Fay 2007; Tongren *et al.* 2006), while acquisition of IgG1 and IgG3 against MSP-1<sub>19</sub> increases with age (Shi *et al.* 1996) and the change in proportion between IgG1 and IgG3 antibodies specific to MSP-2 is age-dependent (Pye *et al.* 1997; Taylor *et al.* 1998; Duah *et al.* 2010). As subclass switching from IgG1 to IgG3 is not possible in the same B cell clone, but requires formation of a new B cell clone that switches from IgM to IgG3, this change in subclass specificity of IgG responses is not caused by any age-related delay in the class-switching mechanism. This delay in IgG3 response is more likely to be the result of an alteration in immune response preference, which is dependent on the maturation stage of the immune system (World Health Organization 2010c; Achtman *et al.* 2005; Duah *et al.* 2010).

### **2.1.2 Exposure**

Malaria infections in naïve individuals of any age are generally symptomatic even if they have low parasitaemia levels (Carvalho *et al.* 2002; Doolan & Dobano 2009). In endemic areas, the cumulative exposure to malaria is influenced by both the intensity of malaria transmission and the duration of exposure (which is related to the age of the individual) (World Health Organization 2010c; Tongren *et al.* 2006).

Transmission rate has, of course, been shown to have a clear effect on the development of immunity against malaria (McCallum *et al.* 2008). In highly endemic malaria regions immune responses against severe disease mature after only few infections; however, this is not the case for children who have been living in areas of low transmission, where individuals remain susceptible into teenage and even adult years (S. Gupta *et al.* 1999). In the areas where the risk of infection is high, older children and adults rarely suffer severe forms of malaria and high-density parasitaemia, while those who develop severe disease are usually young children, infants, malaria-naïve visitors and pregnant women (Richie & Saul 2002; Doolan & Dobano 2009).

Transmission intensity is also linked to forms of severe malaria. In young children, cerebral malaria is more frequent in areas of low transmission intensity while malarial anemia is more prevalent in higher transmission areas (Marsh & Snow 1999). Acquisition of IgG1 antibodies against MSP-1<sub>19</sub> and AMA-1 are dependent on cumulative antigen exposure (Tongren *et al.* 2006), and the levels of growth-inhibitory antibodies is influenced by malaria



transmission intensity (McCallum et al. 2008). Repeated malaria exposure, especially at high-transmission rates, may also increase the chances of contact with a wider range of antigenic variation in the parasite population thus providing protective immunity to a broader spectrum of parasite strains (Aravind et al. 2003; Rich et al. 2000; Takala & Plowe 2009).

### **2.1.3 Pregnancy**

Women who are pregnant become susceptible to severe malaria despite the fact that they have previously developed protective immunity. This may be caused by physiological immunosuppression, which may alter concentration of cytokines and hormones, but is also the result of the presence of a placenta which forms a new niche where *P. falciparum* parasites can accumulate in the placenta by adhering to chondroitin sulfate A (CSA) (Beeson et al. 1999).

## **2.2 Naturally acquired immune responses against malaria**

### **2.2.1 Innate immunity**

Although innate immune responses has not yet been extensively studied for malaria infection, it has been postulated to be important for host defense as well playing a role in priming adaptive immunity (Perlmann & Troye-Blomberg 2002). It was suggested that innate immunity is initiated when parasite density reach threshold (Daubersies et al. 2008), which results in partial clearance of infected cells. Activation of dendritic cells (DCs) and macrophages may be one of the earliest events in the innate response to malaria (Beck et al. 1997; Stevenson & Riley 2004). Mechanisms such as activation of Toll-like receptors, which subsequently induce the phagocytic activities of macrophages (Capone et al. 2010; Gowda et al. 2011), may be important in parasitaemia control and lead to protection. Apart from function in opsonisation, macrophages may also be involved in antibody-dependent cellular inhibition, production of nitric oxide, and TNF $\alpha$  (Good & Doolan 1999; Hensmann & Kwiatkowski 2001). And It was reported that merozoite, but not infected erythrocytes, can trigger a phagocyte oxidative metabolism activation in polymorphonuclear cell and mononuclear cell (Lunel et al. 1990). DCs are responsible for the production of cytokines that are important for protection (Pouniotis et al. 2004). NK-cell which is activated by parasitic red blood cells and cytokines was reported to produce IFN- $\gamma$  which is involved in parasite killing (Artavanis-Tsakonas & Riley 2002). In addition, the unconventional population of T cells known as  $\gamma\delta$ T cells, was reported to be activated by the infected erythrocytes during the acute disease (Hviid et al. 2001). They can recognize schizont antigens (Pichyangkul et al. 1997), produce large amounts of IFN $\gamma$  (Hviid et al. 2001;

Hensmann & Kwiatkowski 2001) and shown to have an anti-parasite function (Elloso et al. 1994).

### 2.2.2 Adaptive immunity

It has been postulated that the immune responses are evoked as early as the injection of sporozoites through skin. Processed sporozoite antigens can be presented on MHC class I by DCs or by infected liver cells to CD8<sup>+</sup> T cells. Cytotoxic T cells (CTLs) detected in humans protected by immunisation with irradiated *P. falciparum* sporozoites showed the potential to lyse infected liver-cells in vitro, expectedly by IFN $\gamma$ -induced nitric oxide (NO) (Doolan & Hoffman 2000). The study on RTS,S vaccine showed that CD8<sup>+</sup> T cells are able to produce IFN $\gamma$  that is associated with the protection from sporozoite challenge (Sun et al. 2003). In addition, CTLs may also release perforin and granzyme or bind to apoptosis-inducing receptors on the infected hepatocyte, which sequentially leads to cytolysis of infected cells (Kwiatkowski 1995). The pre-erythrocytic stage is relatively short compared with other stages of the parasite life-cycle, therefore memory cells of CD4<sup>+</sup> and CD8<sup>+</sup> T cells must be maintained as effector cells at quite high frequencies, otherwise they must have ability to rapidly differentiate into effector-killer cells (Langhorne et al. 2008).

The erythrocytic stage of the parasite lifecycle is the major cause of malaria disease symptoms. Therefore, it is expected to be a key target for protective immune responses (Marsh & Kinyanjui 2006). Cell-mediated immune responses are proposed to be important for induction of protection against both pre-erythrocytic and erythrocytic malaria parasite stages. T cells may be involved in protection mechanisms, both as helper cells in antibody responses and as effector cells (Good & Doolan 1999). In protected individuals who had been exposed to low-dose infection, proliferative CD4<sup>+</sup> and CD8<sup>+</sup> T cell responses, cytokine responses including IFN- $\gamma$ , and high nitric oxide synthase activity in peripheral blood mononuclear cells were observed. The immune mechanisms of these responses are unclear, but it was postulated that they are induced by parasite toxins such as GPI anchors via TLR2 (Pombo et al. 2002). It has been suggested that CD4<sup>+</sup> T cells are important for production and maintenance of protective antibody responses to the parasite, but the prevalence of naturally exposed people who contain measurable levels of malaria-specific CD4<sup>+</sup> T cells is usually low. Either these CD4<sup>+</sup> T cells have only a minor role in immune protection or only few T cells are required (Good & Doolan 1999). Data on longevity of T cell in malaria infection is not conclusive, however, it seems to depend on age, persistence of antigens and exposure level (Achtman et al. 2005; Deloron & Chougnet 1992).

Humoral immune responses are thought to be important against blood-stage parasites (Langhorne et al. 2008). Naturally transfer of antibodies from mother to foetus via

the placenta showed a protective effect against clinical disease and parasite density in children younger than 6 months (Branch et al. 1998). The passive transfer of IgG from immune donors has also shown dramatic reductions in parasitaemia and clinical symptoms in malaria infected adults (Sabchareon et al. 1991) as well as in children (Cohen et al. 1961).

### **2.3 Antibodies response to malaria**

Since erythrocytes have no MHC, it is unlikely that cytotoxic T cells can efficiently kill malaria parasites in their intra-erythrocytic form; although one study has proposed a possible T cell induction mechanism via TLR (Pombo et al. 2002). Accordingly, antibodies are thought to be the major effector mechanism in immunity against *P. falciparum* merozoites (Marsh & Kinyanjui 2006). Specific anti-parasite antibodies are generally a marker of recent or current infection, and because *P.falciparum* infection is chronic and immunity almost never sterile, it can be postulated that antibodies are continuously produced.

Primary malaria infections elicit a variety of antibody isotypes and IgG subclasses, which are thought to provide early protection against disease (al-Yaman et al. 1997). By contrast, subsequent re-infections induce only some IgG subclasses and this leads to improved protective efficacy of the antibodies (Garraud et al. 2003).

### **2.4 Ig isotypes**

The effect of antigen, age and, exposure have been shown to be associated with differences in human IgG subclass responses to malaria parasites. One study by our lab observed differential IgG subclass responses to regions of the same antigen, MSP-1 (Cavanagh et al. 2001). In this paper it was proposed that soluble, processed, and intrinsically unstructured antigens such as MSP-1 Block 2 and MSP-2 elicit IgG3 due to their likely uptake as immune complexes via follicular dendritic cells or DCs, influencing the subsequent Ig class switch, whereas insoluble, polymeric membrane bound antigens on the surface of the merozoite (e.g. MSP-1<sub>19</sub>, AMA-1) may be taken up by different APCs and elicit a more IgG1 biased response. Others have reported that antigens such as MSP-2 (Tongren et al. 2006; Metzger et al. 2003; Taylor et al. 1998), MSP-3 (Cavanagh et al. 2001; Oeuvray et al. 1994), MSP-7 (Wang et al. 2003), which contain polymorphic or repetitive sequences, and the polymorphic region of MSP-1 Block 2 (Cavanagh et al. 2001), seem to mainly induce IgG3, while conserved antigens such as AMA-1 (Tongren et al. 2006), MSP-1<sub>19</sub>, (Egan et al. 1995; Tongren et al. 2006), RAP-1 (Stowers et al. 1997), MSP-5 and MSP-6 (Wang et al. 2003), which do not have polymorphic repeats, induce IgG1.

The protective antibodies to malaria are restricted to a subset of IgG subclasses. IgG1 and IgG3 are most frequently reported to be associated with protection, whereas IgG4 is indicated as non-protective (Bouharoun-Tayoun & Druilhe 1992; Rzepczyk et al., 1997; Taylor et al. 1998; Oouvray et al. 1994; Cavanagh et al. 2004; Conway et al. 2000; Polley et al. 2006; Polley et al. 2003; Metzger et al. 2003; Sarthou et al. 1997). IgG2 was reported to interfere with protective antibody responses (Bouharoun-Tayoun & Druilhe 1992), although the study by Aucan *et al.*, 2000 showed that it is associated with protection in some individuals who carry the H131 allele of the gene encoding FcγRIIA, which has IgG2 binding ability (Aucan et al. 2000). There was a report on the ability of the R131 allelic form of FcγRIIA in IgG2 binding (Norris et al. 1998), however the population studied by Aucan *et. al* mostly have H131 allele (Aucan et al. 2000). According to this, more study on different populations should provide more inclusive information on the relationship between protective IgG2 and the Fc receptors. Fewer studies have investigated protective IgM responses in malaria. It was not associated with protection in one seroepidemiological study (Branch et al. 1998). Parasite specific IgE was postulated to be able to cross-link CD23 on monocytes and endothelial cells, resulting in overproduction of TNF and nitric oxide (Perlmann et al. 1997). Significantly higher concentrations of IgE were also observed in patients with cerebral malaria compared to non-malaria control sera (Perlmann et al. 1997; Perlmann et al. 2000).

IgG3 have been demonstrated for their correlation with the protection against clinical malaria (Roussilhon et al. 2007; Soe et al. 2004) and some showed inhibitory effect in *in vitro* antibody-dependent monocyte mediated growth inhibition assays (Tebo et al. 2001). This is supported by the fact that most protective IgG to MSP-2 and MSP-3 antigens are IgG3 (Aucan et al. 2000; Metzger et al. 2003; Rzepczyk et al., 1997; Taylor et al. 1998; Oouvray et al. 1994; Polley et al. 2006). The mechanism of antibody-associated clearance of parasites is not totally understood. Both IgG1 and IgG3 have high affinity to FC receptors. They are able to fix complements and can be induced in Th1-type responses (Norris et al. 1998; Garraud et al. 2003). These two cytophilic isotypes, especially IgG3, can mediate FC receptor cross-linking, involved in opsonisation (Aucan et al. 2000; Bredius et al. 1994), and act synergistically with human monocytes in parasite killing *in vitro* through binding to Fc receptor (Branch et al. 1998; Oouvray et al. 1994). This functional activity of antibody, observed in the *in vitro* assay is called antibody-dependent cellular inhibition (ADCI) (Bouharoun-Tayoun et al. 1995; Groux et al. 1990; Theisen et al. 1998; Tebo et al. 2001). The IgG3 is suggested to be short-lived with a catabolic half-life of approximately 7 days

(Morell et al. 1970). According to this, re-infection may require triggering of the proliferation of memory B cells and therefore production of specific IgG3

In malaria, it is not known which cytokines induce these subclass switching mechanisms and from which cells they are produced. However, in the light of this knowledge, an ideal malaria vaccine should be able to trigger IgG1 and IgG3 responses and stimulate cytokines associated with antibody of the IgG1 and IgG3 subclasses (Garraud et al. 2003).

## **2.5 Functional roles of antibodies**

A number of functional roles have been suggested for antibodies in protection against clinical symptoms of malaria as well as reduction in parasitaemia (Marsh & Kinyanjui 2006; Garraud et al. 2003)

### **2.5.1 Adhesion blocking**

During the asexual blood stage, many erythrocyte-associated malarial antigens are produced and function as a hook to endothelial cells. Antibodies against these antigens, therefore, are expected to prevent sequestration in small vessels, enhance clearance of infected erythrocytes from the circulation and allow infected cells to be eliminated by the spleen (Doolan & Dobano 2009). Naturally acquired IgG antibodies to VAR2CSA (Beeson et al. 2006; Dahlbäck et al. 2006) and their correlation with protection from clinical malaria were reported (Taylor et al. 2004; Duffy & Fried 2003). These antibodies were proposed to provide protection against placental malaria by interfering with the binding of infected red blood cells to CSA (Dodoo et al. 2001; Duffy & Fried 2003).

### **2.5.2 Opsonising**

Macrophages and monocytes can perform their function in phagocytosis more efficiently when infected erythrocytes are opsonised by antibodies. In malaria, studies also showed that high level of antibodies promoting merozoite phagocytosis is associated with low risk of clinical symptoms (Druilhe & Khusmith 1987). Normal monocytes that were incubated with IgG from immune subjects enhanced uptake of the merozoite, but not the schizont of *P. falciparum* (Khusmith & Druilhe 1983). Neutrophil was also reported to be able to opsonise merozoite in the presence of malaria-immune sera, of which the rate of its effect can be enhanced by cytokines such as IFN $\gamma$ , TNF $\alpha$ , and IL-1 (Kumaratilake & Ferrante 2000).

### 2.5.3 Inhibiting merozoite invasion or parasite growth

Some antibodies to parasite surface protein antigens are reported for parasite inhibitory effect. For example, monoclonal IgM to pseudopeptide antigen based on N-terminal of MSP-2 (Lozano et al. 2007), antibodies to PfMSPDBL1 (Sakamoto et al. 2012) and antibodies to conserved regions of MSP-11 (Obando-Martinez et al. 2010) demonstrated neutralizing properties inhibiting parasite invasion *in vitro*. Surface proteins that have been extensively investigated as targets of parasite inhibitory antibodies are MSP-1 and AMA-1. Naturally acquired antibodies that interfere with the processing of the MSP-1 such as, antibodies against MSP-1<sub>19</sub> (Florens et al. 2002; Blackman et al. 1990; Nwuba et al. 2002; Branch et al. 2000; O'Donnell et al. 2001; Uthaipibull et al. 2001; Dekker et al. 2004) can inhibit erythrocyte invasion and are associated with protection from clinical malaria in field studies (Branch et al. 1998; Conway et al. 2000). However, one study suggested that correlation between the level of anti-MSP-1<sub>19</sub> inhibitory effect and protection from infection can be varied according to the measurement methods (John et al. 2004). Antibodies that raised against MSP-1<sub>42</sub> were reported to show an inhibitory effect on parasite growth (Angov et al. 2003; Singh et al. 2006b; Woehlbier et al. 2006) and studies in *Aotus* monkeys, showed protective effect against challenge of blood-stage parasite (Singh et al. 2006b). Interestingly, these antibodies were shown to penetrate through infected erythrocyte and bind to intracellular parasites, suggesting that they have an inhibitory effect on development of schizont, or releasing process of merozoites (Singh et al. 2006b). The antibodies raised in rabbits or mice that can block formation of AMA-1 complex (Collins et al. 2009) or modification of AMA-1 can prevent parasite invasion in *in vitro* assay (Dutta et al. 2005; Dutta 2003). Purified IgG from malaria naïve volunteers who were immunized with AMA-1 (FVO) vaccine was reported for the parasite growth inhibition effect *in vitro* (Remarque et al. 2012).

However, parasite inhibition of specific antibodies or immune sera *in vitro* does not necessarily correlate with clinical protection. For example, the immunization of the mixture of both allelic types of AMA-1 (3D7 and FVO) in rabbits yielded the antibodies that showed the growth inhibition effect *in vitro* (Kennedy et al. 2002) and shown to be immunogenic in Phase I study in Malian children (Dicko et al. 2008). But, the Phase II trial showed that the group which received AMA-1 vaccine and the control group which received vaccine against *Haemophilus influenzae* type b have similar time to first parasite density >3000/μL/day, which was set as the endpoint of this trial. Therefore, the effect of vaccine is not significant (Sagara et al. 2009). This observation has lead to the hypothesis that these antibodies may have parasite killing effect in monocyte-dependent manner (Druilhe & Pérignon 1997).

#### 2.5.4 Antibody-dependent cellular inhibition

Antibody-dependent cellular inhibition (ADCI) has been proposed as an important mechanism in controlling malaria parasitaemia and clinical symptoms (Bouharoun-Tayoun et al. 1990). The ADCI effect is initiated when a specific merozoite surface component and cytophilic antibodies bound to monocyte, which releases a soluble mediator and, sequentially blocks the division of parasites. By microscopic investigation, ADCI does not equally affect all stages of parasites. The maturation of rings and trophozoites was not a main target point of this effect, but the late trophozoite and schizont stage where an uninucleated or picnotic form of parasite have been observed (Bouharoun-Tayoun et al. 1995).

It was reported that only a single antigen is required to initiate this mechanism and the effect is of the same magnitude as using the combination of antigens. However, on these target antigens, at least two epitopes for cytophilic IgG are required (Jafarshad et al. 2007). Surface antigen are thought to be critical in triggering the ADCI effect (Bouharoun-Tayoun et al. 1995). Antigens reported as triggering ADCI reactivity include MSP-1 Block 2 (Galamo et al. 2009), MSP-3 (Pleass & Holder 2005; Oeuvray et al. 1994), and GLURP (Wykes et al. 2007; Soe et al. 2004; Theisen et al. 1998).

*In vitro* studies showed that only monocyte, but not other macrophages derived from monocyte, polymorphonuclear, lymphocytes or platelets could mediate this effect (Bouharoun-Tayoun et al. 1990; Lunel & Druilhe 1989). Antibodies are reported to trigger monocyte activation via both Fc $\gamma$ RIIa and Fc $\gamma$ RIIIa, of which their roles in ADCI is not really understood in detail. Other immune cells such as NK cells and polymorphonuclear cells are able to express these receptors. Nevertheless, there was no ADCI activity reported for these cells and this is may due to their structural differences (Edberg et al. 1989). The antibodies that have this feature are only reported for cytophilic IgG subclasses, where IgG3 was more active than IgG1. This agrees with the immunoepidemiological finding that IgG3 is linked to protection (Druilhe & Pérignon 1994). It has been postulated that this is may due to the fact that the IgG3 has a more flexible hinge region which may allow it to link antigen and the Fc receptor more efficiently (Redpath et al. 1998; Jafarshad et al. 2007). This mechanism only requires a small amount of antibody compared to those that have a neutralizing property (Jafarshad et al. 2007). Cytokines were reported to play their role in this mechanism. Treatment of IL-4 resulted in a decrease in ADCI effect, while contrasting ADCI effect can be yielded when monocytes were treated with IFN $\gamma$ . TNF $\alpha$  was shown to be an essential mediator (Bouharoun-Tayoun et al. 1995). The oxidative stress may be created by means of ADCI, and lead to parasite killing. Even though, according to one study, there was no

supporting evidence for the possible role of NO radicals (Bouharoun-Tayoun et al. 1995), it may still be too soon to make a conclusion, for under the condition of the ADCI, source is limited and all the mediators that essential for this mechanism are yet to be discovered.

### 2.5.5 Involve in complement pathway

Oxygen radicals can be created both by parasite and the host immune system. The antibodies can be induced by the oxidative stress in parasitised red blood cell, which is the result of haemoglobin degradation (Becker et al. 2004). The observation showed that infected red blood cell contains more than normal amounts of haemichromes, which generated by oxidative stress, that cause the aggregation of band3 (Giribaldi et al. 2001). This abnormal formation of band3 allows significant binding of anti-band3 IgG. This can lead to the establishment of the alternative complement pathway, resulting in production of C3b. This fragment of complement has ability to bind to complement receptor type1 (CR1), anti-band 3 cluster, normal band3 ligand, and aminophospholipids. These alterations of infected erythrocyte surface allow them to be recognised by CR1 of macrophage via C3b or by FC receptor via bound anti-band3 IgG (Morell et al. 1970; Becker et al. 2004). (Figure 1.3)

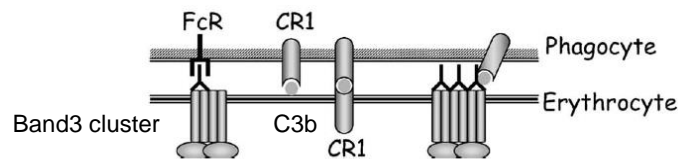


Figure 1.3: Diagram illustrates role of antibodies and oxidative residues in phagocytosis. In infected erythrocytes, oxidative stress can lead to aggregation of band3 which can be recognised by antibodies, and generation of complement factor C3b, which can bind to surface of infected erythrocyte, CR1 receptor of erythrocyte, or mostly to anti-band 3 cluster. Phagocytosis by macrophage can be initiated when it is stimulated via Fc receptor, or via CR1 (Becker et al. 2004).



### **3. Malaria vaccines**

Despite many available approaches for malaria protection and cure, an effective vaccine is also required and is considered an important tool for the global fight against malaria. Since natural acquired protective immunity can be slow in maturing and short lived if boosting is discontinued, malaria vaccines have to do more than natural infection by providing lasting long sterile protection (Carvalho et al. 2002) and hastening the development of the immunity to limit mortality and morbidity rate (Richie & Saul 2002).

#### **3.1 The development of malaria vaccine and strategies**

##### **3.1.1 Obstacles**

There are many factors suggested to be the obstacles in malarial vaccine development. First of all, *Plasmodium falciparum* has extremely complicated biology. Throughout its life cycle, the parasite produces different stage specific antigens which establish sophisticated interactions between the parasite and host immune system (Good & Doolan 1999). Limited knowledge of the relationship between the immune system and malaria the parasite antigens also present a challenge in malaria vaccine research (Crompton et al. 2010). Immuno-epidemiological studies have provided useful data, but so far we are still unable to identify a stable correlation between immune responses and protection. Conventionally, vaccine antigens are identified by immunological techniques. The major limitation of this method is the chance of missing out the less abundant, poorly immunogenic antigens that trigger short-lived immunity, which actually may relate to the protection (Richie & Saul 2002). Thus, some considered that selecting malarial vaccine candidates according to this basis is non-systematic and alternative methods are urgently needed (Carvalho et al. 2002).

Using vaccines that represent only one form of the antigen could provide protection against only some strains of parasite because protein antigens of malaria parasite have extensive antigenic polymorphisms. In addition, this may select nonvaccine serotypes and allow new recombination forms of parasites to be emerge (Takala & Plowe 2009). Antigenic variation also enables the parasite to dodge host immune recognition (Craig & Scherf 2001).

Malaria vaccines are facing challenges in the manufacturing process. For the whole parasite vaccine, there has been a lot of effort trying to produce irradiated sporozoites on a large-scale, while the expressing of conformationally correct sub-unit protein antigens was problematic (Hill 2011).

Each of the established *in vitro* assays using in malaria vaccine research has limitations and has to be standardized in order to compare the results (Holder & Holder 2009). Nevertheless, they can only supply limited predictive value and cannot guarantee effects of the vaccines *in vivo* (Carvalho et al. 2002). More over, the comparative immunology between human host and animal models of malaria, is not very well understood and can caused difficulty in the interpretation of the experimental data (Carvalho et al. 2002; Richie & Saul 2002).

### **3.1.2 Approaches for malaria vaccine development**

Approaches for malaria vaccine development have been developed based on different principals. Injection of irradiated sporozoite, was shown to induce sterile protection against malaria in humans (Hoffman et al. 2002). Although, the production of sufficient sporozoites to supply a large number of vaccinations is unrealistic (Carvalho et al. 2002), it was believed to be a very effective vaccine. Aseptic products under good manufacturing practices (GMPs) have been developed (Luke & Hoffman 2003). The trial was performed in malaria-naïve adults and it was reported that the irradiated sporozoite was safe, but the immunogenicity and protective efficacy of the vaccine were considered as suboptimal (Epstein et al. 2011). Subunit vaccines have gained more popularity for they allow more flexible design and are easily mass produced, (Wang et al. 2009). The DNA vaccine is based on the knowledge that the foreign proteins, encoded from introduced DNA, can be produced in the host and trigger immune responses (Carvalho et al. 2002). Heterologous prime-boost vaccination regime was later developed, and showed to enhance the immunogenicity of the DNA vaccine (McConkey et al. 2003).

### **3.1.3 Development of subunit vaccines**

The development of subunit vaccine can be divided into three steps; research and development (R&D), pre-clinical testing and clinical trials (World Health Organization 2010a).

For the design of subunit vaccines, ideally, the antigens that have been identified for their important roles in parasite survival and the epitopes that are able to trigger protective immune responses are chosen and included in the construct This rationale is not only allows well-identified antigens and/or epitopes to be included in the vaccine, but also minimizes the chance that interfering antibodies may be produced and benefits the production step by limiting the size of the protein antigen (Richie et al. 2007). It has been suggested that multi-immune responses to a range of antigens may provide better protection. Thus, the idea of multistage and multi-antigen vaccine has been proposed as the rationale for modern vaccine

design. The synthetic multi-component protein antigens generally include epitopes of B-cell and/or T-cell which are known to be associated with protection. (Jones & Hoffman 1994). Although they do not increase the overall efficiency of the vaccine, according to the prediction from mathematical models, the multi-component vaccines were suggested to be useful in reducing the proportion of poor responders. And as long as it is targeting a single organism, the immune interference is unlikely to be a problem (Saul & Fay 2007).

Even more challenging is the need to increase the efficiency of the vaccine. Compared to irradiated sporozoite, many of the peptide antigens are poorly immunogenic and can only be effective when used with Freund's adjuvant, which cannot be used in humans (Carvalho et al. 2002). To overcome this negative point, many adjuvants have been developed. These are expected to enhance immunogenicity of the antigen and raise higher antibody titres. Various adjuvants licensed for human use, which have been formulated with different malarial vaccine candidates and tested in different species of animals, have given promising results (Pye et al. 1997).

Antigens that have been selected are produced and validated for their production protocols as well as the formulations, safety, and immunogenicity in animal models. Once they have been approved in small animals such as rodents and then in non-human primates, they are progressed to human clinical trials. In this stage, the procedures must meet a higher standard called as Good Manufacturing Practice (GMP). Clinical trials can be further divided into four phases. In the Phase I trial, vaccines are tested for safety and immunogenicity, as well as investigated for immune responses in non-immune volunteers or small group of people in an endemic area. Phase II can be divided further into sub-phases. In Phase IIa, non-immune volunteers who had received vaccine were challenged with the sporozoites via mosquito bites. Subjects are monitored until the blood-stage form of parasite is detected, which is when the procedure is terminated and the individuals are treated with drug (World Health Organization 2010c). Although the test is intended to evaluate the ability of vaccine to delay infection, it was commented that low parasitaemia may be needed to boost essential immune responses against blood-stage parasites. Therefore, the early elimination of the parasite from bloodstream can lead to the underestimation of the blood-stage vaccine performance (Carvalho et al. 2002). Instead of testing in naïve individuals, subjects from endemic areas were tested in Phase IIb trial. Time intervals between infections of the immunized group are compared with the control group and the protective property of the vaccine is assessed. More subject groups from different endemic areas are tested in the Phase III trial. The candidates that are satisfied by these three stages of testing are further tested in the final stage. The Phase IV requires larger populations, in which the vaccine is tested for

its long-term efficacy and may also be investigated for non-inferiority of immune responses when tested in areas that other vaccines have already been deployed (World Health Organization 2010c). So far, SPf66 (Valero et al. 1996) and RTS,S (Bejon et al. 2008) have progressed into Phase III trial.

## **3.2 Targets of malaria vaccine**

The malaria vaccines targeting antigens of pre-erythrocytic, asexual erythrocytic and sexual stages are expected to raise different immune responses and provide different values of protection.

### **3.2.1 Pre-erythrocytic stage vaccines**

By targeting sporozoite or infected hepatocyte, pre-erythrocytic stage vaccine is expected to prevent the infection. Evidence from irradiated sporozoite immunization suggested that T-cell responses are important if directed against parasite and lead to strong protection (Richie & Saul 2002). However, protection from pre-erythrocytic vaccine will not be successful, if any of the invading sporozoites evade the immune system and progress to the erythrocytic stage, especially in naïve individuals.

The most developed pre-blood stage vaccine is RTS,S. This vaccine is made up of recombinant protein antigen which is based on a combination of tandem repeat tetra-peptide and C-terminal T-cell epitopes regions of CS antigen (circumsporozoite protein), which is the most abundant antigen of sporozoite, and hepatitis B surface antigen S, as well as an unfused S antigen. Formulated with AS02 adjuvant, which is an oil-in-water emulsion that contains immune-stimulants, it was shown to provide protection against natural infection in semi-immune adults in Gambia (Bojang et al. 2001). In Phase IIb trials, which were conducted in children in African countries, it was also confirmed that the vaccine is safe, able to induce high antibody response, which was associated with the reduction of time to new infection (Alonso et al. 2005; Aponte et al. 2007; Bejon et al. 2008). And recently, first results of Phase III trial showed that RTS,S/AS01 was able to reduce both clinical and severe malaria by half in young children during the 12 months after vaccination (Agnandji et al. 2011). With this reported efficiency and acceptable safety, it is expected that the RTS,S will become the world's first licensed malaria vaccine.

Apart from the CSP based antigen, other liver-stage antigens such as LSA1, LSA3, SPf66, TRAP, SALSA and STARP are identified and studied for their potential as vaccines. Liver-stage antigen 1 (LSA1), has consistently been shown by studies of malaria-exposed humans to be a target of specific immune responses that is linked to the protection against *P.falciparum* infection (Kurtis et al. 2001). However, a recombinant LSA1 protein based

vaccine, Ad35-LSA1 vaccine, failed to protect individuals from parasite-infected mosquito bites in a challenge trial (Cummings et al. 2010). LSA3 has been developed into different form of vaccines. The recombinant LSA3 protein antigens showed the promising ability to protect *Aotus* monkeys from *P. falciparum* sporozoite challenge (Perlaza et al. 2008), while in a different study the immunization of DNA vaccine encoding LSA3 was reported to protect chimpanzees against sporozoite infection (Daubersies et al. 2008). The chemically synthetic SPf66, the merozoite-sporozoite-based antigen is another pre-erythrocytic stage vaccine tested in Phase III trials. Its potential to activate protective responses was reported in some area (Beck et al. 1997). Thrombospondin-related adhesion protein (TRAP) is known to contain CD8+ T-Cell epitopes. A prime-boost regime of simian adenovirus (AdCh63) which is encoded for ME-TRAP and boosted with modified Vaccinia virus Ankara (MVA) which is encoded for the same antigen, showed strong CD8+ T cell responses (Capone et al. 2010).

### **3.2.2 Erythrocytic stage vaccine**

The asexual blood stage vaccines have been researched intensively. This type of vaccine aims to reduce symptoms of malaria, decrease parasitaemia and eventually eliminate parasites (Richie & Saul 2002). Blood-stage vaccine is mainly focused on antibody production and generates antibody-related responses, based on the evidence that transfer of antibodies from naturally acquired immunity can protect malaria naïve from clinical symptoms (Richie & Saul 2002).

Vaccines against blood-stage parasite can be divided into two main categories include (1) invasion-blocking, and (2) anti-disease vaccine. Invasion-blocking vaccines aim to disrupt parasite invasion, focusing on parasite surface proteins or erythrocyte surface proteins. Among popular candidate antigens are MSP-1 and AMA-1, which are believed to have roles in initial attachment (Goel 2003) and parasite entry (Mitchell et al. 2004) respectively. PfEMP1 is the focus of anti-disease vaccine research for it allows the parasite to sustain chronic infection by accumulate in the brain or placenta causing severe forms of malaria.

The big challenge in erythrocytic stage vaccine development is the polymorphism of the antigens. Also, it was commented that current blood stage vaccines are focused on only a small number of candidate antigens. Thus, the better target antigens need to be identified and included in the vaccine (Hill 2011). More information about candidate antigens will be further discussed.

### **3.2.3 Transmission-blocking vaccine**

In contrast to other types of malaria vaccine, transmission-blocking vaccine targets diminishing the rate of infection, prevent the development of gametocytes in the mosquito and therefore lead to overall reduction of malaria transmission. Antigens such as Pfs25, the ookinete antigen, have undergone Phase I clinical trials (Malkin et al. 2005; Collins et al. 2006). Even though it could induce transmission-blocking activity, systemic adverse events such as erythema nudosum which is associated with the formulation of Pfs25/ISA51 was reported (Wu et al. 2008).

### **3.2.4 Multi-stage vaccine**

The vaccines containing antigens from many stages of parasite are expected to provide more hitting target for immunity, which then elicit more types of protective immune responses and therefore, provide better protection (Hill 2011; Carvalho et al. 2002). FALVAC-1, for example, is made up of a long series of antigen domains including CSP, LSA1, MSP-1, SSP2, AMA-1, RAP1, EBA175 and Pfg27. Immunisation of this vaccine was reported to produce immunity to different stage of the parasite. The synthetic polymer, SPf66, which consists of blood-stage antigens and the peptide derived from CSP was tested in Africa. The results suggested that it has low protection efficiency against *P. falciparum* (Graves & Gelband 2009). The multi-stage vaccine, NIIMALVAC-1, which contains B-cell and T-cell epitopes from different antigens can be produced in insect cells and was reported for its immunogenicity in mice (Shi et al. 2000). NYVAC-Pf7 is a highly attenuated vaccinia virus containing seven different genes (including CSP, TRAP, LSA1, MSP-1, SERP, AMA-1 and Pfs25) from all stages of *P. falciparum* into the virus genome. The Phase I/IIa trial of this vaccine reported that only one volunteer was protected against challenged with bite of *P. falciparum*-infected mosquitoes.(Ockenhouse et al. 1998).

## **3.3 Surface antigens of erythrocytic stage parasite**

Many blood-stage antigens have been identified, some of which have been further developed into vaccines, but not many of them have been characterized for their functions. The antigens of blood-stage can be categorised into (1) erythrocytic antigens, which presented on the surface of infected erythrocyte; (2) merozoite surface antigens, which present on surface of merozoite; and (3) apical proteins, which present only at the apical end of the merozoite.

### **3.3.1 Erythrocytic antigens**

Erythrocytic antigens are the antigens, which are expressed by the malaria parasite and then presented on an infected erythrocyte membrane. The molecular mechanism of

protein extra-parasitic trafficking network is not totally understood, but it is likely to involve the Maurer's clefts (MC) (Taraschi 1999; Wickham et al. 2001). It was reported that the motif called Plasmodium export element (PEXEL) is required for transportation (Marti 2004). Using this gene sequence, protein products of stevor, rifin, and Pfmc-3TM gene families were predicted to be transported to the erythrocyte membrane (Lavazec et al. 2006).

The antibodies against these antigens may prevent infected erythrocyte binding to host cells, mediate antibody-dependent cellular cytotoxicity (ADCC), play a role in opsonisation, or complement-mediated lysis (Carvalho et al. 2002). The immunogenic antigens of this stage have been identified and have been developed as anti-disease vaccines, which aim to prevent development of severe morbidity.

### **3.3.2 Erythrocyte membrane protein 1 (PfEMP1)**

Erythrocyte membrane protein 1 (PfEMP1) is coded by the multigene family called *var* genes. *Var* genes are located mostly at the telomeric regions of the parasite's chromosomes (Gardner et al. 2002). In *P. falciparum*, PfEMP1 is synthesised early and slowly transported to the surface of infected erythrocyte (Kriek et al. 2003). Regulation of *var* gene transcription and switching is not only dependent on negative feed back from *var* gene product (Kyes, Kraemer, et al. 2007b) but also on non-coding sequences histone hypoacetylation (Freitas-Junior et al. 2005), nuclear repositioning, and there may be other mechanisms which are yet to be discovered (Kyes, Kraemer, et al. 2007b). PfEMP1 is encoded in two exons; the first one produces the variable extracellular binding region and a transmembrane domain, while the second produces a conserved cytoplasmic tail (Su et al. 1995). Important domains of this protein include the KAHRP, PEXEL sequence, which is responsible for transportation of this protein out from the parasitophorous vacuole (Marti 2004). Other extracellular regions such as Duffy binding like (DBL) and cysteine-rich interdomain region (CIDR), are used to categorize structures of PfEMP1 (Kyes, Kraemer, et al. 2007b). Different groups of PfEMP1 can have various functions due to these domains. For example, the PfEMP1 containing CIDR1 was showed to interact with host receptor CD36 (Baruch et al. 1997), while the PfEMP1 that lacks CIDR1 domain, encoded by *var2CSA*, was reported to bind to the placenta (Salanti 2004).

The VAR2CSA, which is a type of PfEMP1 binds to low-sulfated chondroitin sulfate A (CSA) in the placenta (Salanti et al. 2003; Duffy et al. 2005; Gamain et al. 2005). This large (around 350 kDa) and conformational protein is technically difficult to produce. Thus, individual domains are developed as vaccines (Oleinikov et al. 2008; Dahlbäck et al. 2006). It was reported to be recognized by natural acquired antibodies which are associated with a low risk of low weight of newborn babies (Harnyuttanakorn et al. 1992; Salanti

2004). Further studies on the IgG against this protein showed that they are mainly targeting polymorphic regions (Beeson et al. 2006; Dahlbäck et al. 2006). And the polymorphic conformational targets that are associated with the protection were also reported (Barfod et al. 2006). Polymorphisms could be an obstacle to this vaccine's development (Hviid 2011). However, the study in rats showed that the adhesion-inhibitory antibodies raised by immunization of one of the most conserved domains of the VAR2CSA can be achieved (Nielsen et al. 2009). According to these data, further studies on the PfEMP1 domains and their relatedness to protection will help in selecting the potential domains to be produced as vaccines.

### **3.4 Merozoite surface proteins**

The merozoite antigens are considered as a viable targets for antibodies and have been developed as vaccine candidates. Vaccines based on these antigens are expected to raise the titer of antibody, which have ability to kill or inhibit merozoite invasion.

#### **3.4.1 Merozoite surface protein 1 (MSP-1)**

MSP-1 is the most abundant surface protein, covering around 40% the parasite's surface. MSP-1 contains repetitive amino acid sequences (Cavanagh et al. 2001; Ferreira et al. 2003). These sequences are different in length and genetic content between allelic groups, but showed low inter-population variance and allelic types are well maintained within and between parasite populations, suggesting strong balancing selection on this region of the *mSP-1* gene (Conway et al. 2000). The MSP-1 can be divided into 17 blocks by the degree of nucleotide homology between strains as conserved, divergent and highly divergent regions. The dimorphic sequence rule can be used to categorize variation into two allelic types, which named K1-type and MAD20-type, except for the Block 2 region (Figure 1.4).



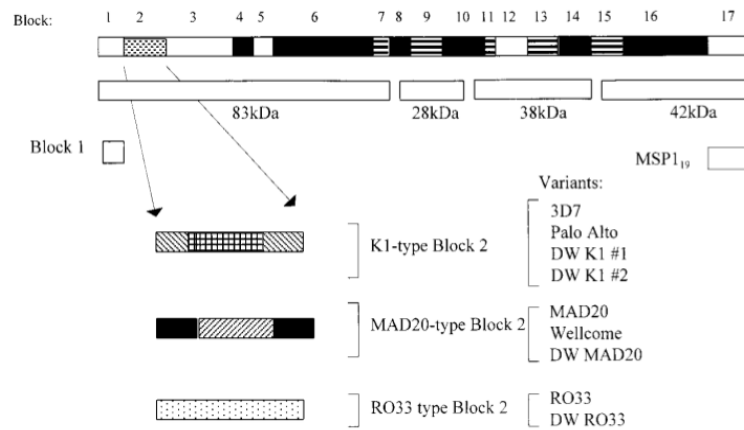


Figure 1.4: Schematic diagram of the *P. falciparum* MSP-1 represents the location of 17 blocks as described by Tanabe *et al.*, 1987 (Tanabe *et al.* 1987). They can be divided according to the degree of polymorphism as conserved (open boxes), dimorphic (full), semi-conserved (hatched boxes), and polymorphic (speckled box). Processed products of MSP-1 precursor at sizes of 83, 28, 38 and 42 kDa are shown. Block 2 of MSP-1 can be further categorised into three types named as K1 type and MAD20 type, each of which contains iconic-type-specific flanking regions and type-specific repetitive region, and RO33 type which does not contain repetitive sequences. Examples of Block 2 variants belonging to each Block 2 types are given. (Figure from (Cavanagh *et al.* 1998))

The MSP-1 precursor, a 190-195 kDa GPI-linked protein, is produced during the schizogony stage, transported to the surface of the erythrocytic merozoite, where the signal sequence at N-terminus is cleaved off (Polley *et al.* 2003; Blackman *et al.* 1990; Cavanagh *et al.* 2001). At the late schizont stage, the precursor undergoes proteolytic processes by a parasite-derived protease, giving four protein fragments known as p83, p28, P30, p38 and p42 (Figure 1.5). These units and other two surface proteins, MSP-6 and MSP-7, bind together non-covalently as a complex on the surface of the merozoite (Jiang *et al.* 2000; Kauth *et al.* 2006). Just before the invasion, another proteolytical event is taking place. The C-terminal of p42 is cleaved into p33 and p19 (Figure 1.5). Finally, most of the complex is removed, except for the MSP-1<sub>19</sub>, which is left to be the only part carried in to the new erythrocyte (Holder *et al.* 1999; Holder & Blackman 1994). The cleavage and shedding of this protein have been shown to be critical steps for the invasion of the parasite (Blackman *et al.* 1990).

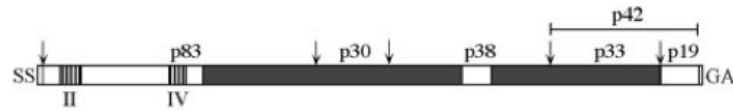


Figure 1.5: Schematic diagram of the precursor of MSP-1 *P. falciparum* 3D7 shows signal sequence (SS), GPI moiety (GA), products after cleavages (p83, p30, p38, p33, and p19) (Figure from (Woehlbier et al. 2006)).

MSP-1 was proposed to be involved in invasion of host cells or cell-to-cell adhesion as determined by bioinformatics analysis of the *P. falciparum* genome database (Florens et al. 2002; Gardner et al. 2002). Based on studies of the MSP-1 ternary complex, the stability and efficiency of MSP-1 in anchoring the parasite to erythrocytes could be beneficial to the merozoite (Kariuki et al. 2005). The importance of MSP-1 for the parasite's survival is also supported by experiments attempting to modify this molecule (Cowman et al., 2002; Blackman et al. 1990). MSP-1 may interact with neighbor proteins to perform their functions. A complex of MSP-1<sub>42</sub> and another merozoite protein, MSP-9, may function in Band 3 binding (Oh & Chishti 2005). The specific interaction between two domains of MSP-9, MSP-1<sub>19</sub> and Band3 was proposed. It was suggested that the two MSPs are able to bind different receptor domains of a Band3 homodimer during the equilibrium stage (Kariuki et al. 2005). The structure of the complex between MSP-1 components and other gene products, such as MSP-6<sub>36</sub> and MSP-7 has also been reported (Kauth et al. 2006).

Antibodies to MSP-1 are present in naturally malaria-exposed individuals and some studies show correlation with protection from malaria symptoms. It was shown that this protection is in an allele-specific manner (Galamo et al. 2009; Lyon et al., 2008). And the epitopes for protective B-cells and T-cells in MSP-1 were reported (Diggs et al. 1993; Woehlbier et al. 2006). The antibodies of MSP-1 fall rapidly to the detection limit soon after antimalarial treatment, suggesting that humoral immunity response against malaria is short-lived (Cavanagh et al. 2001). This may be due to the short half-life of IgG3. Also, it was predicted that the memory B cells and long lived plasma cell do not survive as long as those in other infections, or they are fewer in number (Achtman et al. 2005).

A number of studies have supported MSP-1 as potential vaccine candidate. The antibodies raised by this vaccine are expected to prevent the progression of parasites into the intraerythrocytic state and may also inhibit the maturity of the liver stage parasites, which then diminish the number of parasites released into the circulation. Naturally acquired

cytophilic antibodies can be activated by all blocks of the MSP-1 (Gardner et al. 2002), but for malaria vaccine development, only the protective epitopes should be included in the vaccine construct rather than use length of MSP-1 (Diggs et al. 1993). The most developed MSP-1 vaccine is based on C-terminal of the MSP-1<sub>42</sub> fragment. However, the N-terminal region of the MSP-1 has also been proposed as immune targets (This will be review in chapter 3) (Tetteh et al. 2005; Cavanagh et al. 2004; Conway 1997; Locher et al. 1996). The antibodies to the MSP-1<sub>42</sub> component showed that they can inhibit *P. falciparum* in strain-specific manner (Lyon et al., 2008). The Phase I trial of this vaccine presented hopeful results, reporting that it was immunogenic and safe. However, it conferred no protection in a Phase IIb trial, which is may be due to allelic polymorphism (Ogutu et al. 2009). The MSP-1<sub>19</sub> was also defined as the main target of immunity. Immunization of the recombinant C-terminal region of MSP-1<sub>19</sub> formulated with Freund's adjuvant was reported to provide protection against *P. falciparum* in the Aotus monkey (Kumar et al. 2000). Multi-compartment vaccines based on MSP-1 based antigens such as AdCh63/MVA MSP-1 have been studied. This vaccine contains dimorphic forms (3D7 and FVO) of C-terminal, N-terminal and the MSP-1<sub>19</sub>. It was reported that this protein showed high CD4/CD8 T cell induction and some IgG induction in a Phase I trial study (Sheehy et al. 2011).

### **3.4.2 Merozoite surface protein 2 (MSP-2)**

MSP-2 is an 44-55 kDa protein presented through blood stage of parasite from ring stage to mature schizonts and merozoites (Smythe et al. 1988; Ramasamy 1987). It contains glucophosphatidylinositol domain (GPI anchor), which is the domain that attached to merozoite's surface. Like MSP-1, the MSP-2 is shed from the surface of merozoite upon invasion (Smythe et al. 1988).

To date, at least 170 allelic sequences have been published for MSP-2, suggesting that it is the most polymorphic gene of the *P. falciparum* parasite. The central region of MSP-2 is very polymorphic (Heidari et al. 2007) and varies in length, number and content between isolates (Smythe et al. 1990). It is flanked by N- and C- terminal regions, both of which are more conserved. The central polymorphic region contains allelic tandem repeated motifs flanked by non-repetitive sequences. According to the non-repetitive variable sequence, MSP-2 can be distinguished into two allelic families (Fenton et al. 1991; Smythe et al. 1991; Smythe et al. 1990). These are serogroup A (IC-1/3D7-type) of which CH150/9(5/6) is a member, and serogroup B (FC27-type), in which the Dd2 strain of *P. falciparum* is a member (Smythe et al. 1990). The idiosyncratic of dimorphism of these two *P.falciparum* MSP-2 alleles is suggested as a result of a proliferation of repeats in two different regions of the molecule which leads to loss of other regions (Rich et al. 2000).

The MSP-2 was reported as to be largely unstructured (Yang et al. 2007; Adda et al. 2009) and form amyloid-like fibrils via the conserved N-terminal region (Low et al. 2007). Like MSP-1, function of MSP-2 is suggested to be involved in erythrocyte invasion by studies of invasion-inhibition antibodies (Epping et al. 1988; Miettinen-Baumann et al. 1988; Clark et al. 1989). MSP-2 is immunogenic (Taylor et al. 1995; Aubouy et al. 2003) and interestingly, statistical analysis showed that MSP-2 is under natural positive selection (Conway 1997).

The allelic family specific antibodies were detected in naturally immune infections (Felger et al. 2003; Tami et al. 2002) and shown by two different studies that they lack ability to cross-react between the two allelic forms of MSP-2 families (Taylor et al. 1995; Franks et al. 2003). The hot spot of anti-MSP-2 targets is located within the polymorphic centre part of the antigen (Taylor et al. 1998; Metzger et al. 2003), where the antibodies to constant N and C terminal regions are produced as a minority (Zhang et al. 2008). Anti-MSP-2 subclasses are generally IgG1, especially in young children, and IgG3, where its higher prevalence is correlated with increasing age of host (Polley et al. 2006; Taylor et al. 1998; Tongren et al. 2005).

Anti-MSP-2 antibodies were shown by different studies that they were associated with protection (al-Yaman et al. 1995) and low risk of clinical malaria (Polley et al. 2006). *In vitro* studies for the activities of the MSP-2 antibodies showed different results. The merozoite invasion inhibiting property of the mouse monoclonal anti-MSP-2 antibodies was reported (Clark et al. 1989). The purified human IgG to this antigen showed growth inhibitory effect in a dose-dependant manner (Courtin et al. 2009). However, it was reported by another study that the human anti-MSP-2 has no growth inhibiting property *in vitro*, and only showed parasite killing effect in the presence of monocytes (ADCI) (Flueck et al. 2009). The trial of the vaccine based on one form (3D7) of MSP-2 in Papua New Guinea showed that it can reduce parasite densities in children and lower prevalence of parasites. However, another allelic form (FC27) was suggested to be selected by this immune pressure (Genton et al. 2002). Later, the Phase I trial of MSP-2-C1, which contains both allelic forms, in malaria naïve volunteers showed that the immunisation could raise the specific IgG to both forms of MSP-2, which showed the ADCI activity but not the GIA activity (McCarthy et al. 2011).

### **3.4.3 Merozoite surface protein 3 (MSP-3)-superfamily**

MSP-3 superfamily protein members on chromosome 10 of *P. falciparum*, including MSP-3 (MSP-3.1), MSP-6 (MSP-3.2), PF10\_0347 (H101 or MSP-3.3), PF10\_0348 (MSP-3.4), PF10\_0352 (H103 or MSP-3.7) and PF10\_0355 (MSP-3.8) were identified based on

their common small stretch of amino acid (NLRNA/G) near the N-terminal, called “signature sequence”. Unlike other protein families such as *var*, genes of MSP-3 family members are single exon, located on the same chromosome (Singh et al. 2009).

Strikingly, C-terminal conserved sequences of MSP-3, MSP-6 and other paralogs are similar, very conserved among parasite isolates and reported to be cross-recognised by antibodies raised by each of six members of the MSP-3 family. It was commented that this is very unusual for malaria parasite genes encoding for surface protein antigens, suggesting that these domains should be very important for the parasite. Alternatively, these epitopes may be selected by immune pressure through evolution and play a role in the homeostasis between host and parasite, allowing the host to survive the infection so the parasites can be further transmitted (Singh et al. 2009). Parasite knockout lines of some MSP-3-family member are established and showed that gene disruptions did not cause significant effect on parasite survival (Cowman et al., 2002; Pearce et al. 2005). to this, it was commented that members of the MSP-3 family may compensate for the loss of the others. However, each of these antigens has unique domains, such as the MSP-3.4 and MSP-3.8 which contain a DBL-like cyctein-rich domain, similar to those of the *var* gene products. So before their functions will be further investigate, the fact that these proteins may provide diverse functions cannot be excluded (Singh et al. 2009).

Hyper-immune sera of individuals in malaria endemic area showed different patterns of antibody subclass responses to member of the MSP-3-family proteins. The IgG1 and IgG3 were shown to have strong reactivity compared to other subclasses (Singh et al. 2009). The C-terminal domain contains the antigenic domains, which are targets of natural acquired immunity associated with parasite killing (Singh et al. 2005). Antibodies to the antigens based on the C-terminal region of the MSP-3 family members were also reported for their efficiency in ADCI (Singh et al. 2009).

#### **3.4.3.1 MSP-3**

MSP-3 is a 48 kDa protein which associates with other surface proteins on merozoite surface (LaCount et al. 2005; Oeuvray et al. 1994) MSP-3 is a polymorphic antigen, which can be categorised into two major allelic types (3D7-like and K1-like) (Huber et al. 1997). It comprises heptad repeats, a hydrophilic region and a putative leucine zipper sequence at the C-terminal (McColl et al. 1994). The study of MSP-3-truncated parasite showed that this mutant parasite had lower invasion efficiency. Thus, the role of MSP-3 in red blood cell invasion was proposed (Mills et al. 2002).

The IgG3 was reported to be the major subclass IgG to the MSP-3, followed by IgG1 (Osier et al. 2007). Cohort study in Ghana reported that the level of IgG3 to MSP-3

increase with age until the children reach 7 years old (Dodoo et al. 2008). It was showed that the IgG3 to the MSP-3 is significantly associated with a reduced risk of clinical malaria (Dodoo et al. 2008; Osier et al. 2007; Soe et al. 2004; Roussilhon et al. 2007).

The immunization of MSP-3 recombinant protein was reported to provide immune protection against the parasite challenging in non-human primate (Hisaeda et al. 2002). The IgGs to MSP-3 showed the parasite killing property in the ADCI assay (Jafarshad et al. 2007; Oeuvray et al. 1994). And two studies reported that the targets for the antibodies with ADCI activities are within the conserved region of the MSP-3 (Soe et al. 2004; S. Singh et al. 2004; Druilhe et al. 2005). MSP-3 vaccine trials showed promising results. Clinical trial phase I reported that the antibodies raised by MSP-3 antibodies were able to kill *P. falciparum*, both *in vitro* and *in vivo* (Druilhe et al. 2005). Safety and immunogenicity of the formulation were further confirmed in Phase Ib studies in African population, which showed that MSP-3 vaccine was able to boost strong IgG1 and IgG3 in vaccinated children (Lusingu et al. 2006; Sirima et al. 2009).

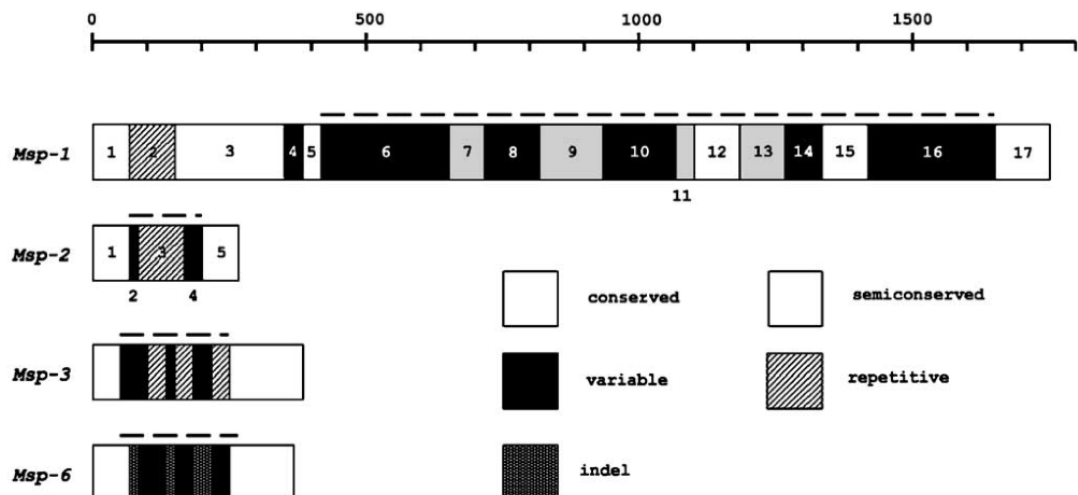


Figure 1.6: This schematic diagram compare genes of major *P. falciparum* merozoite surface antigens. MSP-1 can be divided into 17 blocks where Block 2 has exceptional polymorphism and generally contains a repetitive sequence (stripe). MSP-2 contains a polymorphic repetitive sequence at the central region, flanked by variable regions (black) and conserved regions (white). MSP-3 as well as MSP-6 contain conserved regions at both the 5' and 3' of the genes. While they both contain non-repetitive variable regions, repetitive sequence is reported only in MSP-3. In the other hand, the indel region (dark speckled) or region which is reported for insertion and deletion, is reported only in MSP-6 (Roy et al. 2006).

### 3.4.3.2 MSP-6

The overall sequence organization of MSP-6 is related to MSP-3 (Pearce, Triglia, et al. 2004b; Trucco et al. 2001). Both of them contains N-terminal signal sequence (Singh et al. 2009). Unlike the C-terminal which is highly conserved, the N-terminal part of MSP-6 is polymorphic. It is likely to be less antigenic than the C-terminal part and may be cleaved by proteolytic process (Pearce, et al. 2004b).

MSP-6 was expected to be a target of protective antibodies and can be developed into a vaccine candidate (Singh et al. 2005). Analysis for the specific antibodies to each region of MSP-6 showed that overall, IgG1 and IgG3 are dominant subclass species, while IgG2 antibodies was also detected against some region of MSP-6 (Singh et al. 2005).

Similarly to MSP-3, The C-terminal part of the MSP-6 was identified as a target of the antibodies with ADCl activity (Singh et al. 2009; Singh et al. 2004; Oeuvray et al. 1994). Apart from the epitopes that are cross-reactive by the antibodies that recognise MSP-3, MSP-6 also provides extra epitopes for the naturally induced antibodies which showed the ability to inhibit malaria parasite growth in the ADCl manner (Singh et al. 2005).

Another recently identified MSP-3-superfamily member is PF10\_0347 (referred as H101 or MSP-3.3 in some studies). Based on amino acid content, this protein is more similar to the MSP-3 than the MSP-6 and it may have redundant functions as other MSP-3-protein-family members (Singh et al. 2009). More detail about this protein will be discussed chapter 3.

## 3.5 Apical organellar proteins

The apical end of malaria merozoite has a high protein content, showing as electron dense matrix on electromicroscopy. The proteins reported to be associated with invasion of merozoite are such as merozoite apical membrane antigen 1 (AMA-1), rhoptry associated proteins (RAP), reticulocyte-binding proteins (Rh), and erythrocyte-binding antigen (EBA).

### 3.5.1 Apical membrane antigen 1 (AMA-1)

AMA-1 is extensively studied in malaria research. It is a low abundance protein that firstly is expressed at late schizont stage as a 83 kDa precursor protein. Just before invasion, the precursor protein undergoes several proteolytic processes into the final 66 kDa product, which is then translocated within micronemal organelle (Bannister et al. 2000; Howell et al. 2001; Narum & Thomas 1994; Healer et al. 2002). It was presented in sporozoite of *P. falciparum* (Florens et al. 2002) and showed to have a function in hepatocyte invasion (Silvie 2004). AMA-1 is highly polymorphic (Chesne-Seck et al. 2005) and it is under positive balancing selection (Cortés et al. 2003; Garg et al. 2007; Polley & Conway 2001).

AMA-1 was proposed to be necessary for parasite survival. This idea was supported by the reports on the function of AMA-1, which is experimentally proved to be associated with invasion of erythrocytes (Healer et al. 2004; Healer et al. 2005; Triglia et al. 2000; Treeck et al. 2009), the mode of actions of anti-AMA-1 antibodies (Dutta 2003; Dutta et al. 2005), and the fact that the knockout parasite line has never been successfully established (Triglia et al. 2000).

AMA-1 is very immunogenic in natural infections (Polley et al. 2004; Thomas et al. 1994; Cortes et al. 2004). Isotypes of anti-AMA-1 antibodies are biased for IgG1 and IgG3, whereas IgG2 and IgG4 are also detected (Riley et al. 2000; Polley et al. 2004; Metenou et al. 2007; Stanisic et al. 2009). It has been shown by different studies that both conserved and allele-specific epitopes regions are the targets of antibodies (Hodder et al. 2001; Polley et al. 2004; Cortes et al. 2004). The association between naturally induced anti-AMA-1 antibodies and protection was reported (Courtin et al. 2009). The *In vitro* assay showed that the anti-AMA-1 antibodies have strongly inhibitory effect against invasion of homologous strain of parasite, but have less effective against heterologous strain, suggesting that the inhibitory effect is mainly strain-specific (Hodder et al. 2001; Kocken et al. 2002).

Vaccines based on AMA-1 antigen in combination with aluminum hydroxide, Montanide ISA720, and AS02, were tested in Phase Ia/Ib clinical trials. It was proven to be safe. The antibodies produced by the immunisation showed high GIA activity in one study (Roestenberg et al. 2008) but failed in another one (Geels et al. 2011). According to these results, it was suggested that antibodies to a broad spectrum of AMA-1 form is necessary for the efficiency of the vaccine. Therefore, for the vaccine design, polymorphism of the AMA-1 should be taken into account. AMA-1-DiCo which is a construct containing AMA-1 sequences both from the 3D7 and FVO strains has been developed. This antigen when formulated with CoVaccine HT or Montanide ISA51 was shown to be safe and immunogenic in rhesus macaques (Kusi, Remarque, Riasat, Walraven, Thomas, Faber & Kocken 2011b).

### **3.5.2 Rhoptry-associated protein**

Rhoptry-associated protein 1 (RAP1) and RAP2 have their location at apical organelles. Rhoptry is involved in erythrocyte invasion. RAP1 is produced as 86 kDa protein (Howard & Schmidt 1995) and then cleaved to yield an 82 kDa fragment called p82. Later in schizogony stage, the p82 is further processed at the N-terminal to generate 67 kDa protein (p67) (Bushell et al. 1988). The p82 and p67 fragments are dominant in mature schizont and were reported to form a heterooligomeric complex with RAP2 and RAP3 (Howard, Narum, et al. 1998b). Although processing of p82 and p67 is barely understood, it was proposed to



play a crucial function during merozoite invasion (Howard, Jacobson, et al. 1998a) and the antibodies to the N-terminal of p67, which are supposed to interfere with their processing, were shown to inhibit parasite growth (Harnyuttanakorn et al. 1992). According to this, RAP1 and RAP2 are interesting as vaccine candidates. Unlike many other antigens, the polymorphism of RAP1 is quite limited (Howard 1996).

The study in Tanzanian children age younger than five showed that the IgM antibodies, but not the IgG, to the recombinant RAP1 increased with age. Also, it showed that IgG to the antigen RAP1 is negatively correlated with parasite density, suggesting that anti-RAP1 IgG has a role in parasitaemia control and resistance to infection (Jakobsen et al. 1996). Different longitudinal study of human antibody responses in adult Gambians reported that the IgG to RAP1 was very short-lived. However, a stronger RAP1 antibody response in subsequent infection was observed. This suggested that presence of parasites is necessary to initiate and then to maintain the specific antibodies (Fonjungo et al. 1999).

The RAP1 and RAP2 have been showed to have elicited protection responses against *P. falciparum* infection in Saimiri monkey (Ridley et al. 1990). The *in vitro* assay also suggested that the antibodies to RAP1 may be able to inhibit replication of parasites (Harnyuttanakorn et al. 1992; Howard, Jacobson, et al. 1998a). Nevertheless, some anti-RAP1 antibodies were reported as growth-promotion antibodies, which may enhance binding of merozoite to red blood cells (Howard, Jacobson, et al. 1998a).

### **3.5.3 Reticulocyte-binding homolog protein (Rh) family**

The Rh proteins were postulated to have a function in erythrocyte invasion and play a role in host selection (DeSimone et al. 2009; Duraisingh, Triglia, et al. 2003b; Gao et al. 2008; Gaur et al. 2007; Triglia et al. 2001b; Tham et al. 2010; Rayner et al. 2001). They are located in the apical organelles of merozoite and released to the surface membrane during invasion (Duraisingh, Triglia, et al. 2003b). So far, the identified PfRh family proteins are PfRh1 (Rayner et al. 2001), PfRh2a, PfRh2b, (Rayner et al. 2000) PfRh4 (Kaneko et al. 2000), PfRh5 (Hayton et al. 2008) and PfRh3, which was commented to be a pseudogene (Taylor et al. 2001). These PfRh proteins can be characterized by their ability to mediate merozoite invasion via the sialic acid-dependent pathway (PfRh1 (Rayner et al. 2001)) or sialic acid-independent pathway PfRh2a (DeSimone et al. 2009), PfRh2b (Duraisingh, Maier, et al. 2003a) and PfRh4 (Gaur et al. 2007)). PfRh1 was also reported to involved in a trypsin independent pathway of merozoite invasion (Rayner et al. 2001). According to the study of Rh2a and Rh2b, they were reported to be produced during late schizont stage and transferred to the apical end of the merozoite (Triglia et al. 2001b). The change in expression

of these proteins suggested that they may be involved in the immunity evasion mechanism (Gao et al. 2008; Persson et al. 2008).

The complement receptor 1 (CR1) was reported as a receptor for Rh4 (Tham et al. 2010), while receptors for other Rh proteins family, (such as Rh1 binding ligand, receptor Y (Rayner et al. 2001) or Rh2 candidate receptor Z (DeSimone et al. 2009; Duraisingh, Triglia, et al. 2003b).) are yet to be characterized. Sizes of the Rh proteins are big and vary from around 200 kDa to more than 350 kDa (Duraisingh, Triglia, et al. 2003b; Triglia et al. 2001a; Rayner et al. 2001). The Rh2a and Rh2b are similar and slightly different only in the C-terminal region (Rayner et al. 2000; Triglia et al. 2001a). The knockout line of these two genes can be achieved, and there was no effect on the survival of the mutant *P. falciparum* lines (Duraisingh, Triglia, et al. 2003b)

Rh proteins were reported targets for antibodies. The polymorphism region in the C-terminal of PfRh2b was showed to be a target of humoral immunity (Ahouidi et al. 2010). The antibody raised to the Rh1 binding region was reported to inhibit merozoite invasion (Gaur et al. 2007). The anti-Rh4 antibody was reported to disrupt function of this ligand in invasion (Tham et al. 2009), however another report showed that the antibodies to Rh4 failed to block parasite invasion (Gaur et al. 2007).

#### **3.5.4 Erythrocyte-binding antigen (EBA) family**

Like reticulocyte-binding protein, the erythrocyte-binding antigens (EBA) have been showed to play an important role in the invasion of *P. falciparum* parasite (Lopaticki et al. 2011). The EBA proteins can bind to sialic acid, but also can mediate erythrocyte invasion via alternative receptors (Duraisingh, Maier, et al. 2003a; Stubbs 2005). The genes encoding for EBA175, EBA181, EBL1 and EBA140 were identified. EBL1 is not presented in all parasite lines, which may be due to the missense mutations in the coding region (Mayer et al. 2009). EBA165, which also has missense mutation, it was suggested as a pseudogene and its protein product has not yet been detected (Triglia et al. 2001b). EBA175 and EBA140 were reported to be found only in merozoite and trophozoite (Florens et al. 2002).

Some of the EBA-binding ligands on erythrocytes were identified. EBL1 was reported to bind glycophorin B (Mayer et al. 2009), while EBA175, and EBA140 can bind to erythrocyte receptors glycophorin A and C respectively (Orlandi et al. 1992; Lobo 2003).

The EBL and Rh proteins are functionally interact and cooperative. The function of these two proteins family overlaps, for the loss of function of some *eb1* genes leads to increased transcription of the Rh family gene (Lopaticki et al. 2011). Under certain condition, a selected gene can be activated, resulting in phenotypic variation. Since EBA and Rh may perform the same function, it is possible that these two protein families bind to red

blood cells via a specific receptor, verify the target cell for invasion and then activate the signals including rhoptry release. All these properties of the EBA and Rh proteins allow parasites to have alternative invasion pathways (Duraisingh, Maier, et al. 2003a), adapt to host polymorphic (Duraisingh, Triglia, et al. 2003b) ,and also escape immune responses (Persson et al. 2008).

It has been shown that the EBA proteins are targets of human invasion-inhibitory antibodies. Although EBA175 gene disruption does not effect parasite survival (Duraisingh, Maier, et al. 2003a; Kaneko et al. 2000), the anti-EBA175 was reported to inhibit merozoite invasion in vitro (Orlandi et al. 1992).

### **3.6 Adjuvants and immunization regimes**

One of the challenges in malaria vaccine development is to achieve last-long memory and protective immunity that can persist without natural boosting. An adjuvant is an immunoenhancer, expected to assist in enhancing specific immune responses. They may increase the ability of antigen uptake by antigen-presenting cells (APCs), allow slow-release of the antigen, and improve the stability of the antigen, thus, enabling better antigen presentation to the immune system (Leroux-Roels 2010). Adjuvants can be divided into two main groups; (1) immunostimulants that enhance immune response (such as TLR ligand, cytokines, exotoxin, etc.) and (2) vehicles that present vaccine antigens (such as mineral salts, emulsion, virosome, etc.) (Coler et al. 2009).

In nature, molecules such as lipoproteins, proteins, LPS or DNA, unmethylated deoxycytidy-deoxyguanosines (CpGs) are called pathogen-associated molecular pattern (PAMPs), which are recognised by pattern-recognition receptors (PRRs) such as TLRs. This knowledge has been used as a basic of adjuvant development (Coler et al. 2009). The efficiency of adjuvants can be varied from one antigen to another. The formulation of the adjuvant is another factor that affects the efficiency of the immunogen. Ideal formulated vaccine should be stable, safe, cost effective, immunogenic and induce desired specific immune responses. Unfortunately, our knowledge both of adjuvant functional activities and correlation between types of immune responses and protection is still very limited. Thus, choosing the appropriated adjuvants for potential vaccine antigen is problematic, and normally it is done based on historical data of similar antigens (Coler et al. 2009).

In this study, adjuvants that are approved for human clinical trial used have been tested. Background information including the expected immune responses is given here in short review.

### **3.6.1 Alum**

Alum or Aluminium salt-based adjuvants are components of several vaccines approved for human use. They are used as non-crystalline gels. Alum has a good safety profile record and is simple to formulate. It is known to provide high antibody titer and long-lasting antibody responses (Coler et al. 2009). The molecular mechanism of action of alum is still unclear. It has been proposed that alum may improve the formulated antigen availability at the injection site, and allows the antigen to be taken up by antigen-presenting cells (APCs) (Hem & HogenEsch 2007) and maybe also by dendritic cells (DCs) (Morefield et al. 2005). The alum mainly enhances Th2 responses and has a little effect on Th1 responses. This is maybe a drawn back of using this adjuvant if the Th2 responses are also required for protection against intracellular pathogens such as intraerythrocytic malaria parasite (Leroux-Roels 2010). Nevertheless, Alum was commented as a useful adjuvant for peptide vaccine against many pathogens. It can be used with immunostimulatory molecules such as IL-12 or CpG, which were shown to enhance Th1 responses (Su et al. 2003).

### **3.6.2 CpG**

Nonmethylated CpG oligonucleotide (CpG) is used as a vaccine adjuvant in both preclinical and clinical studies (Mosca et al. 2008). The motifs are considered as PAMPs and act as immunostimulants. The CpG is reported to be recognised by TLR9 of B-cells and dendritic cells (Rothenfusser et al. 2002). It was reported to induce a type1 cytokine production, especially IL-12 and IFN- $\gamma$ , while low secretion of type2 cytokines was detected (Su et al. 2003).

### **3.6.3 Montanides ISA-51 and Montanides ISA-720**

Montanides are water-in-oil emulsions which share some physical characteristics with incomplete Freund's adjuvant. However, they have been developed with more concern for safety and are biodegradable (Aucouturier et al. 2006; Scalzo et al. 1995). Montanides have been extensively tested in different vaccine trials including malaria (Coler et al. 2009). Montanide ISA 51 is a mineral oil-based adjuvant. The mineral oil used for this adjuvant is a white medicinal oil whose toxicology has been well recorded. Local reactions caused by this adjuvant such as flu like symptoms and local pain were reported (Aucouturier et al. 2006). To improve safety, metabolisable oil was used. Squalene is a natural product from plant or animal used in Montanides ISA 720. It has been reported for its ability to enhance strong immune responses. The drawback of this adjuvant is that storage in formulated form is not possible due to the weak stability of the adjuvant (Aucouturier et al. 2006).

### **3.6.4 Oil-in-water emulsion**

Emulsions are a powerful enhancer of antibody production, considered as the best adjuvants for flu and intracellular pathogen, and promising candidates for human vaccines (Mosca et al. 2008). The CoVaccine HT, according to the manufacturer, is an oil-in-water vaccine adjuvant containing Sucrose Fatty Acid Sulphate Esters (SFASE) on the oil droplets of an emulsion of squalane in water. This adjuvant was designed, mimicking that it contains LPS, to elicit TLR4 signaling (Bodewes et al. 2010). It was claimed to be responsible for the magnitude of both T helper 1 and 2 responses, and up-regulation of pro-inflammatory cytokines production by dendritic cells, but not CD8+ cell response (Bodewes et al. 2009). In this study, another three forms of oil-in-water emulsion adjuvants were tested.

### **3.6.5 TLR agonists.**

Toll-like receptor (TLRs) are receptors, of which function is recognition of PAMPs. TLR agonists are used as adjuvants to enhance APCs, resulting in increase of cytokine secretion. They also enhance expression of MHC class II, and draw DCs to T cell area in lymph node (Schiller et al. 2006). It was also shown that signaling of the TLRs that are expressed on B or T cells can support antibody class-switching, memory B-cell activation, and suppression of the regulatory T-Cell response (Duthie et al. 2011).

TLR-4 was reported to recognize LPS from most Gram-negative bacteria (Carl 2002). It is only TLR type that uses two signaling pathways known as MyD88/MAL and TRIF/TRAM which are responsible for IL-12 (Th1-type response) and IL-6 production (Th2 type response) respectively (Krummen et al. 2010). TLR-4 agonists, among the TLR agonists, are the most developed adjuvants. Monophosphoryl lipid A (MPL), one of the TLR-4 agonist, was reported for its safety profile to be similar to that of alum and also showed potential in induction of Th1 immune responses to different pathogens, in different animal models (Casella & T. C. Mitchell 2008). In this study, early-developed TLR-4 agonists were tested alone or in combination with Alum as a vaccine adjuvant.

Both TLR-7 and TLR-8 are found on endosomes of neutrophils, monocytes, and endothelial cells. While T cells have TLR-8, B cells and plasmacytoid DC have TLR-7. They were reported as RNA segments enriched for GU or poly-U sequences as an agonist and induce Th1 type response (Chang 2010)

TLR-9 is a receptor for bacterial DNA, CpG. This Toll-like receptor can be found in the endosome of neutrophils, monocytes, eosinophils, T cells, B cells, NK cells, plasmacytoid DC and endothelial cells (Chang 2010). The signaling that is triggered by agonist such as CpG can be initiated after it is translocated into endosomes (Hemmi et al. 2000). Study in mice showed that this receptor, in response to CpG, can mediate Th1 signals.

TLR-9 of the macrophage was reported for its role in the production of inflammatory cytokines such as TNF $\alpha$ , IL-6, and IL-12. Surface expression of CD40, CD80, CD86 and MHC class II, was reported to be up-regulated. When this receptor is triggered, ability in DNA binding of the macrophage is increased (Hemmi et al. 2000). Although the TLR-9 is important for immune responses mechanism against bacterial infection, in absence of this receptor, mice were reported to be also susceptible to parasitic infection, due to lack of IFN $\gamma$  expression (Foureau et al. 2010).

## **4. Intrinsically Unstructured Protein**

### **4.1 Disordered proteins: Good or Bad?**

It has been accepted that the behavior of functional proteins is essentially determined by protein conformations. Alteration of protein folding, may result in the change of protein's rigid 3D structure and sequentially affect protein functions.

Through different diseases studies, the disordered proteins have been implicated to be associated with various pathogenesis of human diseases (Uversky 2011a). Some disordered proteins were associated with formation of amyloid-like fibrils (Uversky et al. 2009). This has been considered as a nonnative quaternary structure (Kelly 2003), which is the cause of toxic aggregation and leads to pathology in many diseases such as Alzheimer's disease (Uversky & Dunker 2008). However, it has recently been reported that not all disordered proteins form aggregation which could be the result of well orchestrated protection mechanisms in cells as well as the extreme Isoelectric point (pI) of the IUP that make it unlikely for them to reach zero net charge in a physiological environment. (Dunker et al. 2002; Turoverov et al. 2010). In addition, fibril formation is an evolutionary conserved mechanism and the disordered proteins were reported to have biological functions (Kelly 2003).

The discovery of functional disordered domains and functional disordered proteins has challenged the traditional paradigm protein structure and function and more studies of the disordered proteins have been carried out (Dunker et al. 2001; Tompa 2002; Wright & Dyson 1999). Currently, these functional disorderd proteins are called by many terms such as "natively unfolded" (Uversky 2002), "intrinsically unstructured protein (IUP)" (Wright & Dyson 1999; Tompa 2002), and "intrinsically disordered protein (IDP)" (Dunker et al. 2001). All of these names mean the proteins that are unable to gain stable 3D structure independently in their native form under physiological conditions. (Dyson & Wright 2005)

## 4.2 Intrinsically unstructured protein *in vivo*

IUP is wide-spread and can be found in every kingdom of living organisms (Uversky 2002; Uversky et al. 2005), but more common in eukaryotes (Dunker et al. 2000). It was suggested that approximately 33% of eukaryotic proteins contain long disorder regions (Ward et al. 2004; Dunker et al. 2000), which are considerably longer in the early-branching eukaryotes (Mohan et al. 2008).

The disordered regions of proteins have different amino acid compositions from the ordered protein. Generally, the disordered proteins contain few hydrophobic amino acid contents, but are enriched in polar and charged residues. They also has fewer aromatic amino acids (Romero et al. 2001; Williams et al. 2001), which generally have lower substitution rate than the charged amino acid (Brown et al. 2002). These differences of the amino acid composition between ordered and disordered protein may explain why, in general, the disordered proteins evolve more rapidly (Brown et al. 2002). Particular amino acid sequences are linked to IUPs. Amino acids seen as disorder-promoting amino acid residues such as Ala, Arg, Gly, Gln, Ser, Glu, Pro and Lys, were enriched in IUPs, while the order-promoting amino acids such as Trp, Cys, Phe, Ile, Tyr, Val, Leu, and Asn, were be depleted in these proteins (Dunker et al. 2001; Williams et al. 2001). Some IUPs were reported to contain multiple tandem repeats of these amino acids (Dyson 2011; Uversky et al. 2000). The IUPs are likely to have low complexity regions (LCRs), a biased composition region, such as short-period repeats, or a region that consists of few types of amino acids (Romero et al. 2001; Ferron et al. 2006). These unique amino acid compositions of IUPs induced their flexible conformations and also reflect some interesting behaviour. IUPs are respond to extreme treatment such as increase in temperature in an opposite way from the traditional globular proteins (Uversky 2002). Based on their intrinsic indifference to denaturing conditions, high temperature or extreme pH treatment can be used to isolate them from crude protein extracts (Csizmok et al. 2006).

IUPs are tightly regulated at different levels. Comparing the synthesis and degradation rates, the mRNAs of both ordered and disordered have comparable transcription rates, however, the mRNA coding for the IUPs were reported to be degraded more rapidly. At the protein level, the IUPs have shorter half-lives, and are more sensitive to protein kinases (Gsponer et al. 2008). Although there are some IUPs which have long half-lives, they were proposed to be protected from proteolysis by chaperone activity (Dunker et al. 2001) or by association with a partner (Dunker et al. 2002). According to these facts, they are generally less abundant than ordered proteins (Dunker et al. 2002).

### 4.3 Structures, protein interactions and functions

The structure of IUPs is very variable ranging from totally unstructured to compact disordered ensembles (Uversky 2002; Dunker et al. 2001). Three proposed dynamic structures include (1) molten globule, which has no rigid tertiary structure, and is described as folding intermediate of globular proteins, (Uversky & Dunker 2010), (2) pre-molten globule, and (3) coil-like, both of which are also known as extended disordered protein, are considered as unfolded polypeptide chains which have less density than a molten globule protein (Uversky 2002) (Figure 1.7).

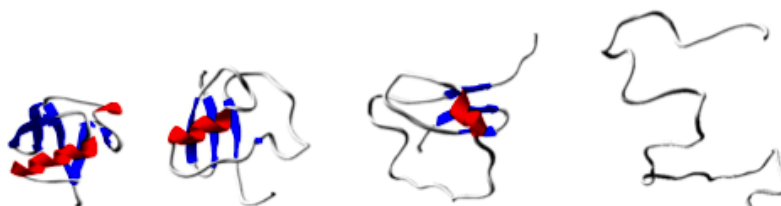


Figure 1.7: Different forms of IUPs known as (from left to right) Molten globule-like (collapsed disorder), pre-molten globule (extended), and coil-like respectively (Uversky & Dunker 2010).

According to the bound stage, the IUPs can be categorised into 4 groups, which show different degrees of disorder (Tompa & Fuxreiter 2008) (Figure 1.8). Although they are natively unfolded, many of IUPs have been shown to gain good 3D structure in the bound stage (Dyson & Wright 2002) but in some cases, the IUP-partner complexes may not be able to be described as a single conformation stage. This phenomenon was coined by Tompa *et al.*, 2008 as “fuzziness”. Different kinetic mechanisms of binding have been proposed. The functional model called “fly-casting mechanism” suggested that the IUPs, which have a larger capture radius than ordered proteins, first bind weakly to a distant binding site and then folds as the protein gets closer to the partner (Shoemaker et al. 2000). Upon binding, the linear motifs of the IUPs, which is known as molecular recognition features (MoRFs), undergo a phenomenon known as disorder-to-order transition (Mohan et al. 2006), which transforms the unstructured region into helix, beta-strand or other forms (Wright & Dyson 2009). Another binding mechanism suggested that IUPs use the primary contact site (PCS), which is more exposed than other regions to initiate interaction event (Csizmok et al. 2005). The regions that performed these functions are usually relatively short parts of the IUP (Wright & Dyson 1999). In some cases, IUPs can also bind to the partners



without disorder-to-order transition. This was recently reported by the study of a new family of the IUPs involved in immune signalling which can bind to different partners such as IUP, well-folded proteins, and lipid bilayers (Sigalov 2011).

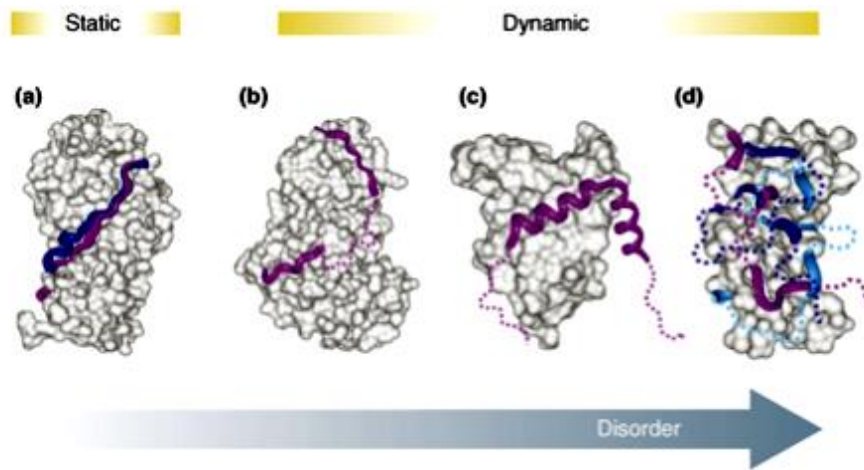


Figure 1.8: Fuzziness of IUPs (in color) in bounding stage with partner (grey). Alternative from having a well-defined 3D structure, the IUP structures in the complex can be described as static (a) or dynamic (b-d). The degree of disorder is increased from left to right. In the polymorphic model, the IUP may gain multiple alternative conformations (magenta and blue ribbons) upon binding to the same partner (a). The IUP may serve as a linker (magenta dotted line) between binding domains in the clamp model (b). It may serve as the over-hang part of the binding region (magenta dotted line) in the flanking model (c) or remain totally unstructured in the random model (d). From (Tompa & Fuxreiter 2008)

The functions of IUPs can be grouped into four broad categories including (1) molecular recognition (as involved in signalling pathways), (2) molecular assembly, (3) protein modification (e.g. phosphorylation, methylation, etc.) and (4) entropic chains (also referred to as linkers and spacers) (Dunker et al. 2002).

The intrinsically unfolded structure of the IUPs allows them to have many functional advantages. Disordered regions are flexible and can be easily induced into different conformations by environment (Wright & Dyson 1999). The IUPs can highly-specifically bind to targets with low affinity (Huang & Liu 2009). This interaction is unstable and can be dissociated rapidly (known as “binding staccato”). This allows the interaction to be performed quickly and reversibly (Uversky 2011b), which is an ideal property for signal transduction (Dunker et al. 2002). Therefore, the IUP can act as a hub protein, which can

interact with a number of targets (one-to-many) or bind to a structured hub protein (many-to-one) (Dunker et al. 2005). In addition, the IUPs also have the ability to bind the same partner in alternative conformations. Although functional information for this alternative binding mode of IUPs is unclear, some have suggested that different forms of the complex might give different outcomes, and as a result, may allow the IUPs to be a multitasking (moonlighting protein) (Jeffery 2009; Tompa et al. 2005). Apart from their role as functional sites, unstructured regions can serve as linkers between domains. These linkers enable two domains to move close together and some also control distance between functional domains (Pearce et al. 2005; Dunker et al. 2002).

#### **4.4 Studies of intrinsically unstructured protein**

Methods that are used for protein structure analysis such as X-ray crystallography, high-resolution NMR, hydrodynamic methods, proteolytic sensitivity and circular dichroism (CD) spectroscopy can be used to detect for disordered protein by searching for residues with missing backbone coordinates in 3D structures. (Dunker et al. 2001). Far-UV circular dichroism (CD) spectroscopy is often used for IUP study. Although it does not give as much specific structural detail as X-ray crystallography and NMR spectroscopy, it is considered as a rapid method that provides useful data and requires only small amounts of sample. CD result of IUPs is shown as a spectrum which has negative peak at around 200 nm, distinct enough from ordered structures (Tompa 2002). This method is suitable for soluble protein, which is favourable for IUPs study since generally they are hydrophilic (Dyson 2011).

Because of the anomalous sequence composition of the IUPs, development of algorithms for disordered region in proteins is possible and currently plays an important role in IUP discovery and study (Turoverov et al. 2010). Many computational prediction methods have been developed, some of which have been applied for more specific prediction such as prediction of amyloid formation (Dyson 2011). Amino acid compositions of the IUPs have been used as a predictive parameter of the disorder, yet some study reported that it cannot be used independently to get accurate results (Ferron et al. 2006) similar to prediction based on low secondary structure content (Liu et al. 2002) or LCR (Romero et al. 2001). Another indicator of the disorder is high-sequence variability, which is based on the knowledge that the disorder regions are, comparatively, more variable than those of the ordered proteins (Brown et al. 2002). Sequence alignment analysis that is based on nucleotide substitution rate can be used to identify the IUPs, on this basis (Ferron et al. 2006). Different physicochemical parameters and the databases, on which the programs have been trained, have resulted in different outcomes from different predictors. The informatics tools such as PONDR, DisEMBL, and RONN are based on datasets of disorder proteins, while predictors

such as Foldindex and IUPred rely on different principles (Ferron et al. 2006). In the case of the IUPred, it uses the algorithm that can identify the IUP based on the their low interresidues interaction energy, which reflects the lack of a well-defined structure (Garbuzynskiy et al. 2008).

Although each of these prediction methods has a different error rate and there are pitfalls in these analyses, it is recommended they be primarily used before more complex analysis be further performed (Ferron et al. 2006). Using these analytical tools in combination has shown to provide relatively rigorous results.

#### **4.5 Intrinsically unstructured protein in the malaria parasite**

In comparison to free-living microbes, pathogenic parasite proteomes were reported to have a larger abundance of long disordered proteins (Mohan et al. 2008). The IUPs are highly abundant in malaria parasite proteomes, and many of them were suggested to be encoded by genes that are unique to this parasite (Romero et al. 1999; Brocchieri 2001; Pizzi & Frontali 2001; Gardner et al. 2002; Aravind et al. 2003), Malaria parasites are enriched in low complexity regions (LCR) (Aravind et al. 2003; Pizzi & Frontali 2001; Brocchieri 2001), a typical marker to identify IUPs (Mohan et al. 2008). Based on computational studies, around 35% proteins encoded from chromosome 2 and 3 of *P. falciparum* was shown to contain long disordered regions (more than 40 consecutive residues) (Dunker et al. 2000). However, later studies showed that this result was underestimated and these long disordered regions may be harbored in 52-67% of the *P. falciparum* proteome (Vucetic et al. 2003). This abundance of LCRs in *P. falciparum* was strongly correlated with genomic A+T content. And the molecular mechanisms such as DNA replication slippage and unequal crossover recombination were suggested to be responsible for their expansion (DePristo et al. 2006). The repetitive sequences which are enriched in *P. falciparum* were also reported to be in correlation with the abundance of IUPs (Feng et al. 2006).

Interestingly, human/primate malaria parasites are more enriched in IUPs than those of rodent malaria parasites (Feng et al. 2006). And interestingly, most of the proteins expressed during sporozoite stage are IUPs (Feng et al. 2006). IUPs of malaria parasites can be frequently found both as hub proteins and non-hub proteins (Mohan et al. 2008). The IUPs of malaria parasites has been proposed to have role in facilitation of host cell invasion and may be hamper host immune mechanisms (Feng et al. 2006). They were also proposed to be involved in pathogenesis, antigen diversification and immune invasion (Ferreira et al. 2003; Hughes 2004). This idea was proposed based on the *P. falciparum* sequence analysis study which showed that LCRs were under the selective force of the host immune system (Hughes 2004) and therefore these repetitive regions for insufficient immune response and

immune evasion of the parasite (Hughes et al. 2010; Schofield 1991). Since the IUPs generally contain LCRs, these proposed roles of LCR's may possibly overlap with some of the IUPs (DePristo et al. 2006). Arguably, these protein may serve as natural spacers between functional domains (Huntley & Golding 2000) or may have some functions that have not yet been reported (Pizzi & Frontali 2001).

Many of the known and well-characterised malaria proteins, initially studied for their potential as vaccine candidates, have been reported for unstructured regions or entire lack of tertiary structure. The MSP-2 is shown to be largely unstructured and the N-terminal is responsible for forms amyloid-like fibrils under physiological pH (Yang et al. 2007; Yang et al. 2010; Low et al. 2007; Adda et al. 2009). C-terminal of MSP-3 and Glutamate-rich protein (GLURP) are also vaccine candidates, reported for unstructured regions (Feng et al. 2006). AMA-1, though generally known as a well-structured molecule (Pizarro et al. 2005), contains disordered N-and C-terminal regions, as well as disordered loops within the molecule (Nair et al. 2002).

#### **4.6 Immunity to intrinsically unstructured protein antigens**

There are numbers of studies suggested that the conformational epitopes are crucial for recognition of the protective antibodies. These lead to the idea that the 3D structures of the protein antigens should be considered in vaccine design (Hodder et al. 2001; Blackman et al. 1990; Crewther et al. 1996; Pan et al. 2004), while the flexible domains has been considered to be involved in immune evasion in pathogenic organisms (Dunker et al. 2001). For example the study of V3 loops of HIV1 showed that it is flexible and able to gain different conformations during the binding stage (Stanfield et al. 1999), which tended to complicate the vaccine design and development against HIV (Dunker et al. 2001).

Despite the data suggesting that they are not ideal targets for antibodies, the idea of binding affinity of antibodies to disordered region might have come as early as in 1984 when there was a study which showed that combining sites of many antibodies were highly flexible. Interestingly, it showed that anti-peptide antibodies to highly mobile regions react strongly with the native protein when compared with antibodies that recognize well-ordered regions (Tainer et al. 1984). Another study showed that peptide-mimics of linear epitopes, not those of discontinuous epitopes, dominate in affinity selection; and therefore, may possibly be a target for peptide vaccine strategies (Craig et al. 1998). For example, IUP of HIV-1 called Trans-activator of transcription (Tat) (Foucault et al. 2009) has been shown to provide epitopes for specific antibodies of which their presence frequencies are associated with slow, or in some cases, non-progression to AIDS (Campbell & Loret 2009). Another example supporting the idea that the IUPs are important immune targets was shown by the

study in hepatitis vaccine antigen, NS5A of which the intrinsically disordered domain was reported to form a fuzzy complex with the host ligand (Gupta et al. 2012) and the vaccine developed on this protein was proved to be a good immunogen (Masalova et al. 2010).

In the malaria parasite, many of IUPs have been reported as highly immunogenic (Feng et al. 2006). The epitopes in the variable region of the MSP-2 were reported to be more accessible than the constant terminal regions (Zhang et al. 2008). The Loop II of AMA-1, which has been identified as an unstructured domain, was reported to provides epitopes for parasite-inhibitory monoclonal antibodies (Collins et al. 2009). Based on the studies of these well-known malaria vaccine candidates, it supports the idea that the surface IUPs of malaria parasite are important for its survival and they are potentially targets of protective immunity. The recent identified surface IUP antigens of malaria such as PFF0165c, has also emphasized this idea by showing that it contains the epitopes for antibodies that have inhibitory effect in ADCI assay (Olugbile et al. 2009).

In the light of present data, it is convincing that malarial surface IUP antigens provide epitopes for protective immunity and should be developed as vaccine candidates.

## Chaptor 2: Materials and methods

### 1. DNA preparation

Genomic DNA from *P. falciparum* was extracted from schizont pellets. Parasite cultures were washed in PBS twice and then purified using a QiaAmp DNA mini kit (Qiagen) according to the manufacturer's instructions. The DNA was recovered in 100µl of elution buffer from the kit. All parasite DNA extracted were stored at -20°C until use.

Plasmid DNA. In this study, pET24a (Novagen, UK) was used as the expression vector for MSP-3.3C and MSP-1 hybrid, and pET28a (Novagen, UK) for the MSP multihybrid construct. Plasmid DNA was purified using QiaAmp DNA mini kit (Qiagen) according to the manufacturer's protocol.

### 2. PCR

PCR was used to amplify individual gene fragments which were then used in cloning reactions (e.g for MSP-3.3C) and to join gene fragments together by overlap PCR (e.g. for the MSP-multihybrid construct). Enzymes and buffers were mixed with primers and DNA fragments as recommended in the manufacturer instructions (Fermentas). The details of PCR steps and conditions, which vary depending on gene to be amplified, are given in each chapter. Desired PCR product bands were cut from agarose gels and purified using a QiAEGel extraction kit (Qiagen). Stock DNA fragments were stored at -20°C before use.

### 3. Agarose gel electrophoresis

PCR products or DNA fragments were separated by electrophoresis on 1.2% agarose gels in TAE buffer (40 mM Tris acetate, 1 mM EDTA). Gels were visualized on UV transilluminator (BioRad Gel Doc 1000).

### 4. Cloning of *P. falciparum* genes

DNA fragments were prepared, purified and checked for concentration using a NanoDrop system before cloning reactions. For sequencing of DNA fragments, PCR fragments were cloned into TOPO TA cloning vector kits (pCR4-TOPO, Invitrogen). Transformants were selected by growing culture on LB agar with kanamycin overnight, before plasmid DNA from selected clones was extracted using a QIAprep spin miniprep kit (Qiagen) and sent for Sanger sequencing via the Genepool (<http://genepool.bio.ed.ac.uk/>).

Bacterial clones with plasmids of the desired sequence were then grown in 500 mL volumes of kanamycin selective LB media (containing kanamycin at concentration of 50  $\mu\text{g mL}^{-1}$ ) overnight. Cultures were then harvested, and plasmid DNA was extracted using QIAfillter Plasmid Maxi prep Kit (Qiagen). Plasmid DNA was stored at  $-20^{\circ}\text{C}$  until use.

DNA fragments were cloned into expression vectors using restriction enzyme digestion and ligation reactions. Donor gene fragments from pCR4-TOPO plasmids and the acceptor expression plasmid were sequentially cut with restriction enzymes (*NdeI* and *BamHI* for MSP-3.3C, *NcoI* and *NotI* for the MSP-multihybrid, New England Biotech, UK) Between each restriction enzyme reaction, DNA fragments were resolved by electrophoresis on 1.2% agarose gels, before being purified by QIAquick Gel extraction kit (Qiagen). Ligation reactions were performed by incubating digested DNA gene fragments and the expression vector with T4 DNA ligase (Promega). Ligation reactions were performed from  $16^{\circ}\text{C}$  to  $4^{\circ}\text{C}$  overnight. Ligation mix was either then used immediately or stored at  $-20^{\circ}\text{C}$  until use.

Recombinant plasmids from ligation mixes or plasmid preparations were used for transformation into competent *E. coli* cells. Host strains included XL-1 Blue (Stratagene) for plasmid amplification, or BL21(DE3)pLysS or BLR(DE3)pLysS (Novagen) as expression hosts. Transformations, were performed according to the manufacturer's instructions. Transformants were grown on LB agar containing 50  $\mu\text{g mL}^{-1}$  of kanamycin at  $37^{\circ}\text{C}$  overnight, and cultures were kept at  $4^{\circ}\text{C}$  until use.

## 5. Screening for clones with inserts

To screen for inserted genes in recombinant plasmids, colony-PCR techniques and restriction enzyme digestions were used. From selective LB agar plates, colonies were picked and dispersed into PCR mixture, including cloning primers. PCR reactions were performed using the same conditions as used for gene amplification. PCR products of the correct insert size were then detected by agarose gel electrophoresis. In parallel, small overnight cultures of selected colonies were grown in selective media. Cultures were then harvested by centrifugation, and plasmid DNA purified using QIAprep spin miniprep kits (Qiagen). Inserts were detected by restriction enzyme digestion of flanking *EcoRI* sites for detection of insert in pCR4-TOPO plasmids, or the two restriction enzymes used in cloning of genes into expression plasmids (*NdeI* and *BamHI* or *NcoI* and *NotI*). DNA fragments from restriction enzyme reaction were separated by electrophoresis. Linear forms of vector plasmid and PCR products of insert genes, which were also separated on the same agarose gel were used as size references.

## 6. DNA sequencing

Plasmid pCR4-TOPO derivatives containing inserts of the correct size were DNA sequenced with T7 sequencing primer (5' TAATACGACTCACTATAGGG 3' ) and T3 sequencing Primer (5' ATTAACCCTCACTAAAGGGA 3' ). For sequencing of inserted gene fragments in pET expression vectors, T7 sequencing primer, T7 Terminator Primer (5' GCTAGTTATTGCTCAGCGG3' ) and the original PCR amplification cloning primers were used. All sequence data was aligned and analysed by comparison with the original gene sequences using Geneious R5 software (<http://www.geneious.com/>).

## 7. Growth media for DNA preparation.

XL-blue cells containing recombinant plasmids were grown in Luria broth (1% (w/v) Bacto-tryptone, 0.5% (w/v) Bacto-yeast, and 1% (w/v) NaCl) supplemented with 50  $\mu\text{g mL}^{-1}$  of kanamycin. For LB agar, LB media with 1.5% (w/v) of Bacto-agar and appropriate antibiotics was used.

## 8. Bacterial culture methods

Freshly transformed BL21(DE3) or BLR(DE3) *E.coli* were used for each round of protein expression. A single transformant colony was grown at 37°C in LB media supplemented with 0.5% (w/v) glucose and 50  $\mu\text{g mL}^{-1}$  overnight. The inoculum was diluted 1:200 into a flask containing 1 litre of LB broth, 0.5% w/v glucose and 50  $\mu\text{g mL}^{-1}$  kanamycin. Cells were grown at 30°C in a shaking incubator (200 rpm) until the OD<sub>600nm</sub> of the culture reached log phase (OD range 0.4-0.6). Protein expression was induced by addition of isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) to the culture to a final concentration of 1mM. Induced cultures were incubated in a shaking incubator (200 rpm, 30°C) for a further 4 hours. To monitor culture growth and protein expression, small samples were taken hourly after induction. Cells were harvested by centrifugation at 4,225 g for 5 minutes at 4°C. Cell paste was frozen at -80°C until required.

Alternatively, Terrific broth or LBE5052 medium were used instead of LB (in studies involving expression of MSP-3.3C or the MSP-multihybrid). Terrific broth contains 12g L<sup>-1</sup> Tryptone, 24g L<sup>-1</sup> yeast extract, 4ml L<sup>-1</sup> Glycerol with addition of 100mL L<sup>-1</sup> of a filter sterilized solution of 0.17M KH<sub>2</sub>PO<sub>4</sub> and 0.72M K<sub>2</sub>HPO<sub>4</sub>. LBE5052 medium is a rich auto-induction media that induces protein expression by *E. coli* cultures via *lac*-derived promoters. Protein expression is induced in cultures when then exhaust the glycerol and glucose carbon sources in the medium as they approach stationary phase and begin



metabolizing the lactose carbon source (Studier 2005). A litre of this medium contains 10g tryptone, 5g yeast extract, 5g glucose, 10g NaCl, 1ml of 2M MgSO<sub>4</sub>, filtered sterilized potassium phosphate mix (20ml of 1M KH<sub>2</sub>PO<sub>4</sub> and 80ml of 1M K<sub>2</sub>HPO<sub>4</sub>) and 1ml of 100x metals mix. To make the metals mix, 50 ml of FeCl<sub>3</sub> stock (0.1M FeCl<sub>3</sub>-6H<sub>2</sub>O in 0.1M HCL) is mixed with 50 ml of filter sterilized metals solution (1 ml of 1M MnCl<sub>2</sub>-4H<sub>2</sub>O, 1 ml of 1M ZnSO<sub>4</sub>-7H<sub>2</sub>O, 1 ml of 0.2M CoCl<sub>2</sub>-6H<sub>2</sub>O, 1 ml of 0.2M NiCl<sub>2</sub>-6H<sub>2</sub>O). Both these media were supplemented with appropriate antibiotics as for LB based cultures.

Large scale bacterial growth protocols, used for process-scale production of MSP-1 hybrid, are provided in Chapter 3.

## 9. Small scale protein purification

Fusion proteins used in this study include GST-3D7 MSP-1 Block 2, GST-Palo Alto17 MSP-1 Block 2, GST-MAD20 MSP-1 Block 2, GST-Wellcome MSP-1 Block 2, GST-RO33 MSP-1 Block 2, GST-MSP-2A (CH150/9), GST-MSP-2B (Dd2), and His-MSP7. Some of these antigens were provided by Drs. David Cavanagh and Anthony Holder, otherwise they were prepared according to the protocols below:

Washed overnight *E. coli* cultures (10 mL) harbouring GST fusion protein expression vectors were used to inoculate 1 litre of LB supplemented with 100µg mL<sup>-1</sup> ampicillin. Cultures were grown at 30°C with shaking to OD<sub>600nm</sub> 0.6 and then induced with 1mM IPTG. Cultures were incubated for a further 4 hours to allow protein expression. Cells were harvested by centrifugation at 4,225 xg for 5 minutes at 4°C. Cell paste was resuspended in 50 mL PBS and frozen at -80°C until required. Cells were lysed by freeze-thawing and lysates treated with 1U mL<sup>-1</sup> Benzonase (Novagen). Lysate was separated from cell debris by centrifugation at 13,000 xg and 0.45 µm filtered (Acrodisc® Syringe Filters, Pall Corporation, USA) before purification on PBS pre-equilibrated glutathione Sepharose columns (GSTrap, GE Healthcare) using an AKTA prime system. After sample application, columns were washed with PBS and the GST fusion protein was then eluted with a buffer containing 50mM Tris pH8, 10mM reduced glutathione. Eluted proteins were collected and analysed by SDS-PAGE and concentration measured by Bradford Assay as described in section 14.2.

For non-tagged proteins (MSP-1 hybrid, MSP-multihybrid, and MSP-3.3C), conventional column chromatography purification protocols were used. For all three proteins, cell pellets were resuspended in 25mM Tris, pH8.0 (50ml of buffer per 1 litre culture) and then lysed by three cycles of freeze-thawing. Lysates were treated with 1U mL<sup>-1</sup>

of Benzonase (Novagen). Soluble lysate proteins were separated from cell debris by centrifugation at 13,000 xg and was stored at -80°C until use.

High temperature treatment was used as a second step of purification. NaCl at the final concentration of 250mM was added to lysates. This solution was then incubated for 20 minutes at 70°C to denature host proteins and then incubated at 4°C for further 20 minutes. Denatured proteins were removed by centrifugation at 13,000 xg for 30 minutes at 4°C. The supernatant was desalted by extensive dialysis against 25mM Tris pH8.0 (buffer changed 3 times). This partially purified protein solution was stored at -20°C until use.

The final step of purification for each of the proteins was anion exchange chromatography. For the MSP-1-hybrid, protein was filtered through 0.22µm Millex-GP syringe filters (Millipore) before applied into column. Proteins were purified by sequential step-wise increases in NaCl concentration. For all three proteins, purification steps included application of protein mixtures to the column in zero NaCl buffer (25mM Tris, pH8.0), extensive washing (>5 column volumes) with the same buffer, an elution of contaminants at a low NaCl concentration, and a final elution step of the target protein at a higher NaCl concentration. A Q-Sepharose FF column (XK 16/20, GE healthcare) was packed as suggested by the manufacturer's protocol and equilibrated with 10 column volumes of starting buffer (25mM Tris, pH8.0) before purification. Details of the purification protocol for each protein purification is given in each results chapter. Purified protein fractions were assessed for purity and protein concentration by SDS-PAGE and BCA assay respectively. High purity fractions were pooled, dialysed against PBS using Thermo SnakeSkin Dialysis Tubing (Thermo, Uk), and concentrated using centrifugal ultrafiltration (Amicon, Millipore, UK or Vivaspin, Sartorius UK).

A similar protocol with modifications for process-scale production was used for purification of the MSP-1 hybrid at large scale. The protocol for this is part of the results in Chapter 3.

## **10. Immunisations**

Before used purified protein antigens were filtered through a 0.1µM 25 mm Acrodisc syringe filter with Supor membrane (Pall Corporation, USA) using aseptic technique. For most immunogenicity tests, female MF1 mice used in this study. In the MSP-1 hybrid study, they were either injected subcutaneously or intraperitoneally. Each immunising dose contained 20 µg of protein antigen, which was mixed with adjuvant immediately before immunisation. Three doses of vaccine were given at 28 day intervals. At day 70, 14 days after the 3<sup>rd</sup> dose, all animals were exsanguinated under terminal anaesthesia and serum

collected for immunogenicity testing. All mouse experimentation was carried out in accordance with the Animals (Scientific Procedures) Act 1986 and conforms to the Recommendation from the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals. The University of Edinburgh Ethical Review Committee approved the project license under which all mouse experimentation was performed on 3rd July 2006, reference number PL 13-06. Mice were humanely killed by Schedule 1 methods.

Eight New Zealand White rabbits were immunized intramuscularly, using 50 µg per dose of MSP-1 Hybrid antigen formulated with CoVaccine HT, following the manufacturer's instructions. A 500 µL vaccine dose contained 50 µg of antigen and 10 mg of SFASE. Each animal was immunized 3 times at 28 day intervals, with blood samples taken at days 0, 28, 56 and 70. Rabbit housing and immunization were at BioGenes GmbH (Berlin, Germany), and were in accordance with national and international animal welfare regulations. Rabbit immunization at this facility was under approval from NIH/OLAW (ID number #A5755-01).

Two rabbits (code numbers 6066 and 6067) were immunized intramuscularly with five doses each of 100 µg of the recombinant MSP-3.3C protein antigen, formulated with Freund's complete/incomplete adjuvant (Biogenes GmbH, Berlin, Germany). Animals were immunized on days 0, 7, 14, 28 and 42. Blood samples were collected for serum on days 0, 8, and 49.

## **11. Formulation of protein in adjuvants**

Formulation of vaccine in this study was done according to the manufacturer's instructions. Adjuvants that were tested in mice for this study included:-

A) Alhydrogel™, or Alhydrogel™ with CpG in combination with 10 µg mL<sup>-1</sup> of CpG ODN 7909. For each mouse, a final concentration of 20µg of MSP-1 hybrid or MSP-3.3C was used for each dose. Antigen was mixed with adjuvant by gentle pipetting, then incubated at room temperature for 30 minutes. Unbound antigen was removed by centrifugation at 10,000 g for 10 minutes. The pellet was resuspended in 0.9% (w/v) NaCl solution, at 100 µl per 20µg dose per mouse.

B) CoVaccine HT™ (Protherics Medicines Development Limited, a BTG International Group Company, London, UK) following the manufacturer's instructions and as previously described (Studier 2005; Mahdi Abdel Hamid et al. 2011). CoVaccine HT contains 40 mg mL<sup>-1</sup> of Sucrose Fatty Acid Sulphate Esters [SFASE] in a squalane o/w emulsion). A 100 µL vaccine dose contained 20 µg of antigen and 2 mg of SFASE. Briefly, CoVaccine HT was mixed gently with 20 µg of MSP-1 hybrid or MSP-3.3C. For all groups of mice, three doses were given at 28 day intervals.

C) Montanide ISA 51. Groups of five mice were immunized with 20µg doses of MSP-1 hybrid formulated by emulsification with Montanide ISA51 adjuvant (ratio 1:1) (Seppic, France), following the manufacturer's formulation instructions. A final volume of 100 µl per dose was administered per mouse.

D) Montanide ISA 720. Groups of five mice were immunized with 20µg doses of MSP-1 hybrid formulated by emulsification with Montanide ISA720 adjuvant (ratio 7:3) (Seppic, France), following the manufacturer's formulation instructions. A final volume of 100 µl per dose was administered per mouse.

E) Formulated Aqueous TLR-4 Agonist (IDRI-AQ019). AQ019 was formulated with 20 µg of the MSP-1 hybrid at a ratio of 1:4, to obtain a final volume of 100 µl dose per mouse. This adjuvant was also tested in combination with Alhydrogel. In this case, MSP-1 hybrid was formulated first with the Alhydrogel as described earlier before being formulated with AQ019. The mixtures were vortexed gently for a few seconds and were stable at 4°C before being used within 2-4 hours.

F) Formulated TLR-4 agonist oil-in-water emulsion (IDRI-EM031). For formulation of this adjuvant with the MSP-1 hybrid, the same formulation protocol as for Formulated Aqueous TLR-4 Agonist was used.

G) Formulated TLR-7/8 Agonist (IDRI-AQ002). For formulation of this adjuvant with the MSP-1 hybrid, the same formulation protocol as for Formulated Aqueous TLR-4 Agonist was used.

H) Formulated Oil-in-Water Emulsion (IDRI-EM030). For Formulated Oil-in-Water Emulsion, the adjuvant was diluted 1:5 with 20 µg MSP-1 hybrid antigen to obtain a final volume of 100 µl dose per mouse. This adjuvant-antigen mixture was vortexed for a few seconds and was stable at 4°C before being used within 2-4 hours.

I) Formulated TLR-9 Agonist (IDRI-AQ006). For Formulated TLR-9 Agonist, for each 100 µl dose per mouse, 16 µl of the adjuvant was mixed with 20 µg of MSP-1 hybrid to obtain a final volume of 100 µl dose per mouse.

## **12. Antibody purification**

To purify total IgG, sera were diluted 1:10 in PBS, filtered (Acrodisc® Syringe Filters, Pall Corporation, USA) and loaded onto pre-equilibrated HiTrap Protein G columns using an AKTA Prime system (GE Healthcare). For each purification, the column was then washed extensively (~10 column volumes) with PBS before bound antibodies were eluted using 0.1 M Glycine-HCl pH 2.7. 1 mL fractions were eluted into 200µL of 1M Tris pH9.0

neutralization buffer. Antibodies were then dialysed extensively into PBS using Side-A-Lyzer Dialysis Cassettes (Thermo, UK) before use.

### **13. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was routinely performed using gels consisting of 4.5% stacking gel (0.63 mL 30% acrylamide, 0.56 mL 1M Tris pH 6.8, 50 $\mu$ L 10% SDS, 50 $\mu$ L 10% APS, 3.5 mL dH<sub>2</sub>O, 5 $\mu$ L TEMED) and 12% resolving gel (2 ml 30% acrylamide, 1.3 mL 1.5 M Tris pH8.8, 50 $\mu$ L 10% SDS, 50 $\mu$ L 10% APS, 1.6 mL dH<sub>2</sub>O, 4  $\mu$ L TEMED), unless otherwise specified (protocol provided by Dr. David Cavanagh). NuPage Bis-Tris gels (Invitrogen) were also used where necessary. Proteins were resolved on these gels at 200V constant voltage for 45 minutes in Tris-Glycine buffer (7.55g Tris base, 47g glycine, 25m<sup>L-1</sup> 10%SDS).

Gels were stained with silver nitrate solution (MSP-1 hybrid and MSP-multihybrid) according to Blum *et al.*, 1987 (Studier 2005; Blum et al. 1987) or Coomassie Blue (for MSP-3.3C).

### **14. Protein quantitation assays**

#### **14.1 BCA assay**

Concentrations of the protein from cell lysate, MSP-1 hybrid, MSP-3.3C and MSP-multihybrid were measured by BCA assay (Pierce) according to the manufacturer's instruction. 25  $\mu$ L of protein samples and diluted BSA solution at concentration of 0, 25, 125, 250, 500, 750, 1000, 1500, 2000  $\mu$ g mL<sup>-1</sup> were apply into a microplate well (Microlon high protein binding plates, Greiner, UK). Reagent A and B were mixed at ratio 50:1 then the mixture was applied into each well (sample to mixed reagent ratio is 1:8). Plate was covered and incubated at 37°C for 30 minutes then absorbance was measured at OD 570 nm by Multiskan Ascent plate reader program (Thermo Labsystems, UK). Protein concentrations were calculated by interpolation from a standard curve of BSA diluted in the same buffer as used for test samples.

#### **14.2 Bradford assay**

Bradford assay (BioRad, UK) was used to measure concentration of GST proteins. For microassay procedure, 160  $\mu$ L of protein samples and diluted BSA solution at concentration of 0, 40, 80, 120, 160, 200  $\mu$ g mL<sup>-1</sup> were apply into a microplate well

(Microlon high protein binding plates, Greiner, UK). 40  $\mu$ L Diluted Bradford assay reagent (1 in 5 with ddH<sub>2</sub>O) was applied into each well. The sample and reagent were mixed thoroughly and incubated at room temperature for 5 minutes. Absorbance was measured at OD 595 nm by Multiskan Ascent plate reader program (Thermo Labsystems, UK). Protein concentrations were calculated using the same method as used for the BCA assay.

## 15. Western blotting

Protein transfer from SDS-PAGE gels to Schleicher and Schuell BA83 nitrocellulose membranes (Whatman) was performed at 100V, 350 mA for 90 minutes, using a Mini TransBlot Cell (BioRad, UK). Blotted membranes were incubated in Blocking buffer (5% (w/v) milk powder in PBS containing 0.05% Tween 20) at 4 °C for at least 1 hour. The membrane was then incubated for 1 hour with primary antibody, either monoclonal or polyclonal, which was diluted to appropriate concentrations in Blocking buffer. The membrane was then washed four times for 5 minutes each in PBS, before a conjugated secondary antibody was added and incubated for 1 hour. For horseradish peroxidase-conjugated antibodies, blots were developed with 0.6 mg mL<sup>-1</sup> 4-chloronaphthol/0.003% H<sub>2</sub>O<sub>2</sub> in a solution of 40mM Tris pH8.0, 20% methanol. Bound antibody was detectable as a black precipitate on the membrane. For detection of MSP-3.3C, the secondary antibody used was Alexa Fluor 680 conjugated goat anti-rabbit IgG (Invitrogen), while Alexa Fluor 700 conjugated goat anti-mouse IgG (Invitrogen) was used in the MSP-multihybrid study. Binding of both of these secondary antibodies was detected in the 700nm emission channel using a Li-Cor Odyssey imaging system (LI-COR Biosciences, Ltd., UK).

## 16. *In silico* analysis

Molecular weights and pI values of the MSP-1 hybrid, the MSP-multihybrid and MSP-3.3C were predicted using the Compute pI/Mw tool ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)) (Studier 2005; Mahdi Abdel Hamid et al. 2011; Gasteiger et al. 2005). IUPred (<http://iupred.enzim.hu/>) was used to predict unstructured domains in the MSP-1 hybrid, MSP-3.3C and the MSP-multihybrid. Further *in silico* analyses on MSP-3.3C are described in Chapter 4.

## 17. Mass spectrometry

Soluble forms of the proteins in PBS, or protein bands on SDS-PAGE gels stained with silver nitrate were used for mass spectrometric analysis. Mass spectrometric analysis of

purified proteins was carried out by the SIRCAMS (Department of Chemistry, University of Edinburgh, <http://www.sircams.ed.ac.uk>). For LC-MS, an Ultimate 3000 HPLC system (Dionex Corporation, Sunnyvale, CA), equipped with a monolithic PS-DVB (500  $\mu\text{M}\times 5$  mm) analytical column (Dionex Corporation), was used. Samples containing  $\sim 1$   $\mu\text{g}$  of protein were centrifuged (16,100 g for 2 min) immediately prior to injection onto the column. Solutions B and C comprised of 2:97.95 and 80:19.95 acetonitrile:water with 0.05% formic acid respectively. Samples were injected onto the analytical column, washed with buffer B for 5 min, followed by a 20 min linear gradient elution (20  $\mu\text{L min}^{-1}$ ) into buffer C. MS data was acquired on a Bruker 12 Tesla Apex Qe FT-ICR (Bruker Daltonics, Billerica, MA) equipped with an electrospray ionization source. Desolvated ions were transmitted to a 6 cm Infinity cell<sup>®</sup> penning trap. Trapped ions were excited (frequency chirp 48–500 kHz at 100 steps of 25  $\mu\text{s}$ ) and detected between  $m/z$  600 and 2000 for 0.5 s to yield a broadband 512 Kword time-domain data. Fast Fourier Transforms and subsequent analyses were performed using DataAnalysis (Bruker Daltonics) software. Multiple charge states could be observed in this way for each of the major species.

## **18. Circular Dichroism (CD)**

Circular dichroism analysis was carried out according to the protocol provided by Centre for Translational and Chemical Biology, University of Edinburgh (<http://ctcb.bio.ed.ac.uk>). Protein samples (MSP-3.3C or MSP-1 hybrid) were dialysed against 10mM  $\text{NaH}_2\text{PO}_4$  pH8.0 at a concentration of 2 mg  $\text{mL}^{-1}$ . The CD spectrum of the proteins was evaluated on a JASCO J-810 spectropolarimeter. The measurements were taken over a wavelength range of 185 to 285 nm at 25°C pH 8.0 with a 1 mm path length cuvette.

## **19. ELISA**

Individual MSP-1 recombinant proteins based on the 3D7, Palo Alto 17, MAD20, Wellcome and RO33 MSP-1 Block 2 serotypes have been described elsewhere (Studier 2005; Cavanagh & McBride 1997; Mahdi Abdel Hamid et al. 2011; Cavanagh et al. 2004). Human sera, monoclonal antibodies and sera from immunized animals were tested by previously described ELISA methods from the Cavanagh Lab for recognition of the MSP-1 hybrid, MSP-3.3C and Block 2 GST fusion proteins (Blum et al. 1987; Cavanagh & McBride 1997; Cavanagh et al. 2001).

Antigens were coated onto the wells of a 96-well plate (Microton high protein binding plates, Greiner, UK) with 50ng of each antigen in 100 $\mu\text{l}$  of coating buffer (15mM  $\text{Na}_2\text{CO}_3$ , 35mM  $\text{NaHCO}_3$ , pH 9.3) added per well. Plates were kept humidified and

incubated at 4°C overnight. Plates were washed with washing buffer (PBS (1.9mNaH<sub>2</sub>PO<sub>4</sub>, 8.1mM Na<sub>2</sub>HPO<sub>4</sub>, 150mM NaCl, pH to 7.2-7.4) plus 0.05% Tween 20) 3 times using ELISA plate washers (Ultrawash Plus, Dynex, UK or SkanWasher 400, Skatron, UK). Wells were then blocked with 200µL of blocking buffer (1 % (v/w) non-fat skimmed milk in washing buffer) and incubated for 5 hours at room temperature. Plates were washed again and 100µL volumes of antibodies or sera (diluted in blocking buffer) were then added to each well. All sera were tested across a range of doubling dilutions (1:500 to 1:128,000) against each antigen in duplicate wells, with a standard pool of positive sera also tested on each plate, and across the same dilution range. After overnight incubation at 4°C, plates were washed three times and 100µl of horseradish peroxidase conjugated secondary antibodies (Dako Ltd, High Wycombe, UK ) were added to each well at an appropriate concentration. Plates were incubated at room temperature for 3 hours and then washed again 3 times. Antigen specific antibodies were detected by addition of 100µl of substrate (0.1 mg mL<sup>-1</sup> O-phenylenediamine and 0.012% H<sub>2</sub>O<sub>2</sub>) in development buffer (24.5 mM citric acid monohydrate and 52 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 5.0) per well and incubating for up to 15 minutes at room temperature. The reaction was stopped by the addition of 25µl of 2M H<sub>2</sub>SO<sub>4</sub> to each well and absorbance was measured at 492nm using a Multiskan Ascent microplate reader (Thermo Labsystems, UK).

ELISA titres were calculated by interpolation from the fitted standard curve on each plate using polynomial logistic regression. Negative antigen control wells in ELISA were either coating buffer alone for the MSP-1 hybrid, or GST-coated wells for Block 2 GST fusion proteins. For the MSP-1 hybrid study, a pool of sera from mice immunized with individual MSP-1 Block 2 antigens were used as a standard when mouse sera were tested. Similarly, a pool of anti-MSP-1 hybrid rabbit sera was used as a standard for the MSP-1 studies in rabbits. The K-1 Block 2 type-specific monoclonal antibody 12.2 was used as a positive control in some assays (Wilson et al. 1987). For human serum assays using the recombinant MSP antigens, human sera from Ghana, Malawi, Gambia and Sudan were used. All human sera were diluted at 1:500 in blocking buffer and assayed as described unless specified. Naïve European sera and naïve animal sera were used as negative antibody controls for each of these assays.

### **19.1 IgG subclass ELISA**

To analyze for human IgG subclasses a similar ELISA assay was used, which is described elsewhere (Cavanagh et al. 2001; Cavanagh et al. 2004). For mouse IgG subclass ELISA, plates were coated overnight with 100µl of 0.5 µg mL<sup>-1</sup> antigen in coating buffer. Plates were washed twice with washing buffer, then wells were blocked for 2 hours with



100 $\mu$ l of 1% (w/v) BSA in PBS at room temperature. Plates were washed three times before 50 $\mu$ l of doubling serial dilutions from 1:100 downwards of each serum sample was added to duplicate wells. After incubation for 2 hours or overnight, plates were washed and 50  $\mu$ L Alkaline Phosphatase-labelled anti-IgG subclass antibodies were added at appropriate dilutions (1:2000 for anti-IgG1, 1:1000 for anti-IgG2a, 1:2500 for anti-IgG2b and 1:1000 for IgG3). After incubation for 1 hour at room temperature, plates were washed and PNPP substrate was added. PNPP (para-Nitrophenylphosphate) tablets (Thermo, Uk) were dissolved in substrate buffer (61.25mg/l of  $MgCl_2 \cdot 6H_2O$ , 120ml diethanolamine, adjusted to pH 9.8) to a final concentration of 1mg  $mL^{-1}$ . As antigen standards, plates were coated with mouse IgG1, IgG2a, (BD Pharmingen) and IgG3 (Biolegend) at doubling dilutions from 0.5 $\mu$ g  $mL^{-1}$  downwards. Monoclonal antibody 12.8 (Wilson et al. 1987) was used as a standard for IgG2b, diluted from 1 $\mu$ g  $mL^{-1}$ .

## 19.2 Peptide epitope mapping

A set of 133 biotinylated dodecapeptides covering all possible linear epitopes contained within MSP-1 hybrid sequence were synthesised by Mimotopes Pyt. Ltd. (Clayton, Australia). ELISA plates (Immulon 4 HBX, Thermo Dynex) were coated with 100  $\mu$ L of 5  $\mu$ g  $mL^{-1}$  streptavidin (Sigma) and incubated at 37°C until dry. Plates were stored in heat sealed foil pouches with 1 g silica gel at room temperature until use. Reactivity of sera against the peptide library was determined by ELISA. Streptavidin-coated plates were washed in washing buffer (PBS/0.05% Tween20) and blocked with blocking buffer (1% ByCoA, Croda Healthcare, UK dissolved in PBS) for 5 hours at room temperature. Peptide library plates were prepared by addition of 300 ng peptide per well, in duplicate, and plates were incubated overnight at 4°C. Sera were added to each well (100  $\mu$ L at 1:500 dilution) and incubated overnight at 4°C, then washed with washing buffer. Dilutions of a species-specific HRP-linked secondary antibody (Dako, UK), appropriate to the serum being tested, were added to each well and plates were incubated at room temperature for 3 hours. Plates were washed three times with washing buffer and OPD substrate was added to each well. Reactions were stopped by addition of sulfuric acid and absorbance was read at 492 nm using a microplate absorbance reader (Multiskan Ascent, Thermo Scientific, UK). Background reactivity was calculated as the mean of all OD values in the lowest two quartiles (i.e. below median) plus 6 standard deviations peptides.

## 20. IFA

In IFA assays of MSP-3.3C-specific sera, antibodies were tested by IFA on parasites of the Wellcome isolate, while antibody reactivity with other strains (3D7, Palo Alto17, RO33, and MAD20) were also tested in the MSP-1 hybrid study. IFA slides were prepared from standard parasite cultures, which had been synchronized and were checked for the desired lifecycle stage by light microscopy. Parasite cultures were washed with RPMI twice. Cells were then resuspended in PBS to approximately 3% haematocrit. Aliquots of 25 $\mu$ L were pipetted onto the wells of PTFE-coated multispot microscope slides (C.A.Hendley (Essex) Ltd, UK). Slides were dried at room temperature and stored in air-tight containers at -20°C with silica gel desiccant before use.

Slides were removed from the freezer and immediately fixed with acetone for 5 minutes, then allowed to dry. Serial dilutions of the tested sera or antibodies were prepared in IFA sample buffer (PBS containing 1% bovine serum albumin (BSA) and 0.01% Sodium azide). Duplicate spots on each slide were incubated with 25 $\mu$ l of each working dilution of antiserum for 30 minutes at room temperature in a humidified chamber. Unbound antibodies were removed by immersing slides three times in PBS, for 5 minutes per wash. Slides were gently dried for 5-10 minutes at 50°C. Aliquots of 15 $\mu$ l of FITC-conjugated secondary antibodies (either swine anti-rabbit or rabbit anti-mouse, Dako, UK) were added to wells at the manufacturer's recommended dilutions. Slides were again washed three times with PBS. Slides were immersed in staining solution for five minutes (1:100,000 (w/v) of 4',6'-diamino-2-phenylindole (DAPI) and 0.1% (w/v) Evans blue in PBS). Slides were washed once with PBS and mounted under coverslips in Citifluor (City University, London). Slides were stored at 4°C before examination with a fluorescence microscope using 50X objective lens. Incident light of 390-440 and 450-490 nm were used for DAPI fluorescence and FITC fluorescence respectively. Endpoint titres against parasite strains were determined as the highest serum dilution at which clear schizont-specific fluorescence could be observed.

## 21. Statistic analysis

ELISA titres between groups were analysed using the non-parametric Kruskal-Wallis test, with Dunn's post-hoc test. To compare titres between two groups, a Mann-Whitney U-test was used. Correlation plots of human antibody reactivity between MSP-1 hybrid and MSP-1 Block 2 antigens or between MSP-3.3C and MSP7 or MSP-1<sub>19</sub>, were analyzed using Spearman's rank correlation coefficient. GraphPad Prism software (GraphPad Software, Inc, USA) was used to produce Figures and perform statistical analyses.

## 22. Parasite culturing methods

*Plasmodium falciparum* strain Wellcome was used for most *in vitro* parasite growth experiments. Blood stage *P. falciparum* cultures were grown at 37°C, 5% CO<sub>2</sub> in complete RPMI media at 5% haematocrit, containing O+ blood and 10% heat inactivated human serum from the Scottish National Blood Transfusion Service. Culture medium was changed daily and cultures were monitored by microscopy of thin blood smears stained with 10% Giemsa stain. Parasitaemia was routinely maintained below 5% throughout all experiments.

Parasite cultures were synchronised by resuspending pelleted cells in the original culture volume of sterile 5% Sorbitol. The mixture was incubated at room temperature for 5 minutes, then an excess volume (3-5 fold excess) of culture medium minus serum was added. Cultures were then centrifuged at 830 g for 5 minutes. The pelleted cells were then resuspended in complete medium at 5% haematocrit and cultured as before.

## 23. Parasite Growth Inhibition Assays (GIA)

*In vitro* parasite growth Inhibition assays (GIA) were carried out on freshly thawed clones of the *P. falciparum* Wellcome strain according to standard protocols (Bergmann-Leitner et al. 2006; Darko et al. 2005) using purified IgG from immunized animals. Total IgG purified from a naive non-immunized animals were used as negative controls, or from day 0 (pre-immunization) serum samples in the case of Rhesus macaques.

All IgG samples were tested in triplicate at 2-fold serial dilutions, from 6 mg mL<sup>-1</sup> (rabbit, sheep) or from 10 mg mL<sup>-1</sup> (Rhesus) in 96-well cell culture plates (Greiner BioOne, UK). Parasites were cultured under standard conditions (Arnot et al. 2008). Parasite cultures were mycoplasma-free and synchronized at least twice with 5% sorbitol before use in assays. Late trophozoite/early schizont stages at a parasitaemia of ~0.3% and 2% final haematocrit were used in all assays. The final culture volume was 100 µL/well and parasites were incubated for 42 hours. After 42 hours, cultures were tested for growth inhibition using pLDH measurement, flow cytometry and Giemsa-stained slide microscopy. GIA was performed for testing of the antibodies against both the MSP-1 hybrid and for MSP-3.3C. Specific protocols for this assay can be found in Chapters 3 and 4.

## Chapter 3: MSP Block 1/2 hybrid

### 1. Introduction

Malaria vaccine research has not been focused on the N-terminal of MSP-1, but many studies have indicated that the region of the molecule is a target of protective immunity. There is evidence that the N-terminal Block 1 region of MSP-1 contains T-cell epitopes in both humans and mice (Pearce et al. 2005; Quakyi et al. 1994; Parra et al. 2000). Immunisation with MSP-1 p83, an MSP-1 proteolytic component containing the N-terminal part of the MSP-1, showed that this antigen was immunogenic. Moreover, the antibodies elicited by this immunization showed a strong *in vitro* growth inhibitory effect of up to 95% (Marti et al. 2005; Woehlbier et al. 2006). And in other studies, vaccination of New World monkeys with constructs derived from the N-terminal part of MSP-1 provided partial protection from *P. falciparum* challenge (Pearce et al. 2005; Cheung et al. 1986; Etlinger et al. 1991; Herrera et al. 1990; Herrera et al. 1992).

Near the N-terminus of MSP-1, located next to Block 1, is MSP-1 Block 2. Block 2 is very polymorphic, with more than a hundred different Block 2 sequences recorded. Despite this extensive diversity, Block 2 sequence polymorphisms can be categorised into 3 allelic families known as the K1- MAD20- and RO33-like types. The allelic frequencies of the 3 types of *msp-1 block 2* are maintained at similar levels throughout the global parasite population (Pearce et al. 2005; Conway et al. 2000). The K1 and MAD20 Block 2 types, but not the RO33 type, contain internal tripeptide repeat sequences and non-repetitive serotype specific flanking sequences. The tripeptide repeat sequences of these serotypes are semi-conserved and vary in both length and amino acid content. Interestingly, each of the tripeptide motifs always begins with a serine residue (Musto et al. 1999; Jiang et al. 2000; Miller et al. 1993; Cavanagh et al. 2004). The K1 type of MSP-1 Block 2 consists of tandem repetitive sequences that are variable in length, (between 5 to 25 tripeptide repeats) and allele-specific-conserved sequences on the N-terminal and C-terminal side of the variable region. Variation in the repeat of K1 type is limited to only four tripeptide motifs; SAQ, SGA, SGP and SGT (Bergmann-Leitner et al. 2009; Jiang et al. 2000). The coding for serine in these repeats is always AGT; and SGT is always the last tandem repeat. For MAD20 type Block 2 sequences, the C-terminal sequence is SVASGG with the TCA codon used for serine in the MAD20 repeats (Jiang et al. 2000), which is frequently used in highly expressed genes of *P. falciparum* (Musto et al. 1999). The tripeptide motifs found in the MAD20 type are SGG, SVA, SKG, and SVT (Sallenave-Sales et al. 2000; Jiang et al. 2000;

Zwetyenga et al. 1998; Ferreira et al. 2003). Contrasting with K1 and MAD20-types, the RO33 type has no repetitive amino acid sequences and is less variable (Ferreira et al. 2003; Certa et al. 1987; Cavanagh & McBride 1997; Miller et al. 1993). RO33-type sequences are almost identical when observed in parasites collected from different countries (Cheung et al. 1986; Ferreira et al. 2003; Etlinger et al. 1991; Herrera et al. 1990; Herrera et al. 1992). However, several polymorphic residues have been reported in some areas (Sallenave-Sales et al. 2000; Zwetyenga et al. 1998). A single non-synonymous nucleotide replacement was discovered at codon 64, according to the alignment of Miller *et al.*, 1993, resulting in 2 dimorphic variations; RO33-type (Aspartic acid) and CSL2-type (Glycine). In this case, the polymorphism was reported to be biased in its geographical distribution (Ferreira et al. 2003).

The diversity in length of Block 2 repeats might be a consequence of intragenic recombination, a mechanism which either increases or decreases the number of repetitive sequences in these genes (Locher et al. 1996; Rich et al. 2000). The strong linkage disequilibrium at MSP-1 Block 2 of *P. falciparum* is maintained in the non-repetitive sequences, suggesting that these repeats are cold spots for homologous recombination. This was proposed to be a result of less possibility of helical pairing and crossing over due to the repeat sequence polymorphisms or the negative selection of recombinants by the host (Cavanagh et al. 1998; Ferreira et al. 2003).

MSP-1 Block 2 is a target of human immune responses to *P. falciparum*. The level of Block 2 specific antibodies is thought to be a marker of recent infection rather than immune responses that associated with susceptibility to disease (Cavanagh et al. 1998). A positive correlation between the presence of Block 2 specific IgG and reduced incidence of clinical malaria in exposed children has been clearly shown in several studies (Conway et al. 2000; Cavanagh et al. 2004; Polley et al. 2003; Mawili-Mboumba et al. 2003). The protective association of Block 2 specific antibodies is additive, as children who have antibodies to multiple Block 2 types have even fewer malaria episodes (Woehlbier et al. 2006; Conway et al. 2000). Protective immunity to Block 2 appears to be short-lived in the absence of re-infection (Cheung et al. 1986; Da Silveira et al. 1999; Etlinger et al. 1991; S. Herrera et al. 1990; Herrera et al. 1992). However, in another cohort, a positive association with protection was present for up to 8 months after sampling (Conway et al. 2000; Cavanagh et al. 2004). IgG3 is the predominant subclass elicited against MSP-1 Block 2, suggesting that the soluble Block 2 part of the MSP-1 protein, which is shed from the parasite during invasion, may be taken up by dendritic cells and trigger a T-cell-dependent response (Musto et al. 1999; Cavanagh et al. 2001; Jiang et al. 2000; Cavanagh et al. 2004;

Miller et al. 1993). *In vitro* studies showed that human antibodies against Block 2 are effective at parasite killing in ADCI assays (Jiang et al. 2000; Galamo et al. 2009). In an earlier study, mouse monoclonal antibodies to Block 2 were able to inhibit parasite growth *in vitro* (Jiang et al. 2000; Locher et al. 1996).

In developing MSP-1 Block 2 as a vaccine, antigenic diversity is the most challenging problem, as ideally any vaccine based on Block 2 should provide protection against all parasite strains. Recombinant polypeptides derived from Block 2 representing different parasite isolates, including 3D7, Palo Alto, MAD20, Wellcome and RO33, along with some isolates from Daraweesh, Sudan were produced. These Block 2 antigens elicited serotype-specific antibodies in immunised mice, and were recognised in a serotype-specific manner by serum from individuals with malaria infection history (Musto et al. 1999; Cavanagh & McBride 1997). Block 2 is therefore immunogenic and contains serotype specific epitopes recognized by human antibodies (Sallenave-Sales et al. 2000; Cavanagh & McBride 1997; Jiang et al. 2000; Cavanagh et al. 1998; Zwetyenga et al. 1998; Ferreira et al. 2003). In an unpublished study, protection from parasite challenge was observed in MSP-1 Block 2 vaccinated *Aotus* monkeys who had high anti-MSP-1 Block 2 antibody titres (Cavanagh; unpublished data).

The variation existing in the Block 2 region revealed a limited number of patterns of the serine-containing repeat sequences found in the K1 and MAD20 alleles, which is very important information for vaccine design (Ferreira et al. 2003; Jiang et al. 2000; Certa et al. 1987; Cavanagh & McBride 1997; Miller et al. 1993). In order to overcome sequence diversity, amino acid sequences of all known Block 2 sequences were collated and repeat sequences simplified using the numerical coding used by Jiang *et al.*, 2000 (Cheung et al. 1986; Jiang et al. 2000; Ferreira et al. 2003; Etlinger et al. 1991; Herrera et al. 1990; Herrera et al. 1992). Based on the knowledge gained from the previous studies, a synthetic protein construct named MSP-1 Block 1/Block 2 Hybrid (MSP-1 hybrid) was designed. This synthetic hybrid was based on combinations of all 12 amino acid repeat sequence motifs identified in the K-1 and MAD20 types respectively, together with an RO33 Block 2 sequence, whilst maintaining the natural similarity of amino acid order and composition within wild-type MSP-1 Block 2 sequences as far as possible (Figure 3.1). At the N-terminal of this synthetic sequence, the sequence of Block 1 (K-1 type) of MSP-1 was included in order to provide cognate help in the form of known human CD4+ T cell epitopes, (Sallenave-Sales et al. 2000; Quakyi et al. 1994; Zwetyenga et al. 1998; Parra et al. 2000). The synthetic gene, called MSP-1 hybrid, encodes a 348 amino acid protein with a predicted molar mass of 31.1 kDa.

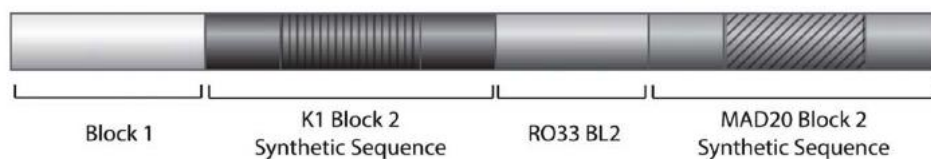


Figure 3.1: Schematic representation of the MSP-1 hybrid vaccine construct, based on the polymorphic N-terminal region of MSP-1. The construct encodes the N-terminal MSP-1 Block 1 region, the K1 Block 2 synthetic sequence, the RO33 Block 2 sequence and the MAD20 Block 2 synthetic sequence of MSP-1 of *P. falciparum*. Synthetic Block 2 repeat sequences of the K1 and MAD20 serotypes are indicated by vertical and diagonal hatched markings respectively (Figure from (Ferreira et al. 2003; Cowan et al. 2011)).

In general, expression of malaria proteins in heterologous hosts can be problematic (Locher et al. 1996; Gustafsson et al. 2004; Rich et al. 2000), not only because the codon preferences of the expression host, *E. coli*, and the malaria parasite are different, but also because some *P. falciparum* genes, including the MSP-1 hybrid gene sequence, have high repetitive sequence content. To overcome these problems, the DNA sequence of the MSP-1 hybrid was codon optimized to be suitable for expression in *E. coli* hosts, and repetitive DNA sequences were minimised by use of multiple abundant serine codons within the repeat sequences. The MSP-1 hybrid sequence was synthesised and then cloned and expressed as an un-tagged protein in *E. coli*, and a purification protocol developed. This yielded a product of high purity which could be used for pre-clinical studies including animal immunization. The MSP-1 hybrid is immunogenic in animals, eliciting specific antibodies that recognize all three Block 2 serotypes of *P. falciparum*. The MSP-1 hybrid contains multiple Block 2 epitopes recognised by serum antibodies from malaria exposed individuals (Cavanagh et al. 1998; Cowan et al. 2011; Ferreira et al. 2003).

In this chapter, I describe (1) how the MSP-1 hybrid can be produced on a large scale, using a modified lab-scale production protocol (in collaboration with Dr. Graeme Cowan) and (2) characterisation of the MSP-1 hybrid's biochemical and immunological properties.

## 2. Methods and Results

### 2.1 Flask-based MSP-1 hybrid expression

The DNA sequence of the MSP-1 hybrid was codon optimized for expression in *E. coli*. The original coding sequence was sent to GeneArt AG, Regensburg, Germany, and optimized using the company's GeneOptimizerH software. The codon-optimized sequence contained no purification tag sequences, and consisted entirely of MSP-1-derived sequences, with the single amino acid addition of an N-terminal methionine initiation codon. The synthesized gene was then cloned into the pET24a expression vector (Novagen, UK). The expression plasmid was transformed into chemically competent BL21 (DE3) pLysS *E. coli* (Agilent Technologies Ltd, Stockport, UK) and plated on LB agar plates containing 50  $\mu\text{g mL}^{-1}$  kanamycin. Starter cultures of 10 mL LB broth containing 50  $\mu\text{g mL}^{-1}$  kanamycin and 0.5% w/v L-glucose were inoculated with a single colony from a freshly streaked agar plate. Erlenmeyer flasks containing 1 litre LB broth, 0.5% w/v L-glucose and 50  $\mu\text{g mL}^{-1}$  kanamycin were inoculated with 5 mL starter culture. Culture growth was monitored until the optical density reached 0.6  $\text{AU cm}^{-1}$ , then protein production was induced by addition of IPTG to 1 mM final concentration. Cultures were incubated for a further 4 hours shaking at 30°C then cells were harvested by centrifugation at 5000 g and 4°C for 15 minutes. Cell pellets were stored frozen at -80°C until further processing.

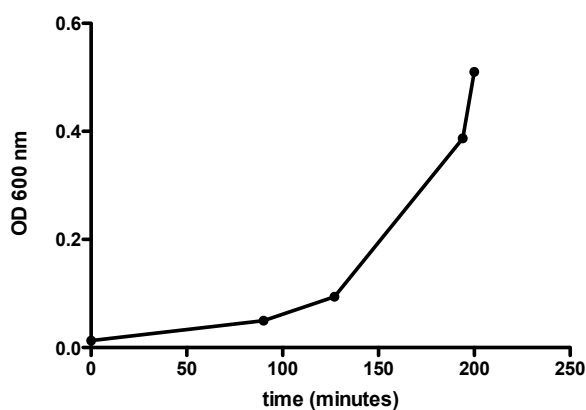


Figure 3.2: Bacterial growth curve before induction of protein expression in flask-based culture of *E. coli* BLR (DE3) pLysS containing the MSP-1 hybrid recombinant plasmid. Optical density of the culture is plotted in the Y-axis against time since culture inoculation on the X-axis.



## 2.2 Fed-batch fermentation

In collaboration with Dr. Graeme Cowan, the small scale production protocol was optimised for MSP-1 hybrid production at large scale (Cavanagh et al. 1998; Cowan et al. 2011). The same MSP-1 hybrid expression vector and expression host was used as described earlier. A 15-litre stirred bioreactor (Applikon Technologies, UK) was filled with 8 litres of modified YT medium. pH control was set to maintain pH at 7.2 by addition of 4N H<sub>2</sub>SO<sub>4</sub> or 30% v/v NH<sub>4</sub>OH. Dissolved oxygen was monitored and maintained at 20% by manually increasing air flow or agitation speed, and temperature was maintained at 37°C. The bioreactor was inoculated by addition of 2% v/v starter culture and culture density was monitored throughout the experiment by measurement of optical density (OD) and wet pellet mass. Using modified YT media, the culture grew rapidly and within 5 hours had reached OD<sub>600nm</sub> of 1.2 (Figure 3.3). At this point, the culture had a wet weight of 13.47 g L<sup>-1</sup> (0.13 g L<sup>-1</sup> dry weight) in total volume of 8 litres. Upon reaching OD<sub>600nm</sub> 1.2, batch feeding commenced by pumped addition of feed medium at an initial rate of 6.48 mL L<sup>-1</sup> h<sup>-1</sup> with an exponential increase in feeding rate ( $\mu = 0.1$  h<sup>-1</sup>). A litre of this feed medium consisted of 45g yeast extract, 90g Tryptone, 540g Glycerol 75  $\mu$ l PPG2000 and 10% of 10X salts solution (per litre. This solution has 65g KH<sub>2</sub>PO<sub>4</sub>, 50 g K<sub>2</sub>HPO<sub>4</sub>, 32g NaHPO<sub>4</sub>.12H<sub>2</sub>O, 32g MgSO<sub>4</sub>.7H<sub>2</sub>O, 1g NH<sub>4</sub>Cl, 900mg FeSO<sub>4</sub>.7H<sub>2</sub>O, 900mg CaCl<sub>2</sub>.2H<sub>2</sub>O, 225mg MnSO<sub>4</sub>.1H<sub>2</sub>O, 125mg CoCl<sub>2</sub>.6H<sub>2</sub>O, 97.5mg ZnSO<sub>4</sub>.7H<sub>2</sub>O, 50mg AlCl<sub>3</sub>.6H<sub>2</sub>O, 45mg Na<sub>2</sub>MoO<sub>4</sub>.2H<sub>2</sub>O, 40mg CuCl<sub>2</sub>.2H<sub>2</sub>O, and 2.5mg H<sub>3</sub>BO<sub>3</sub>). Protein expression was induced by addition of 1 mM final concentration of IPTG once a wet pellet weight equivalent to 75 g L<sup>-1</sup> was achieved. Fermentation continued for a further 4 hours with the continued maintenance of the parameters described above. Cells were harvested by centrifugation at 5,000 g and the cell pellet was stored in aliquots at -80°C until further processing. At the end of this fermentation process, the total volume of culture was 8.9 litres and the wet cell pellet weighed 107.4 g L<sup>-1</sup> or 18.87 g L<sup>-1</sup> of dry pellet.

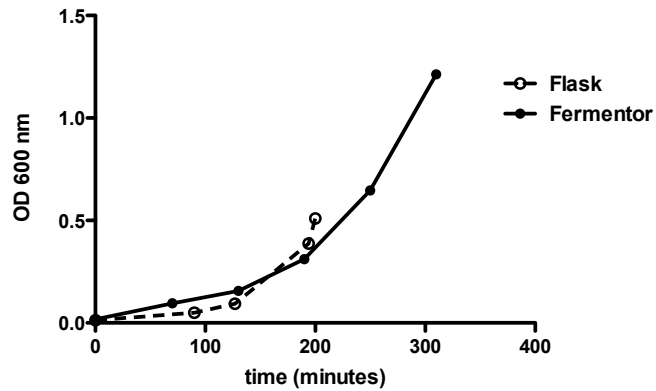


Figure 3.3: Growth rates of fed-batch fermentation culture (dark line) and flask-based culture (dashed line) before induction. Optical density of cultures (Y-axis) is plotted against time since inoculation (X-axis).

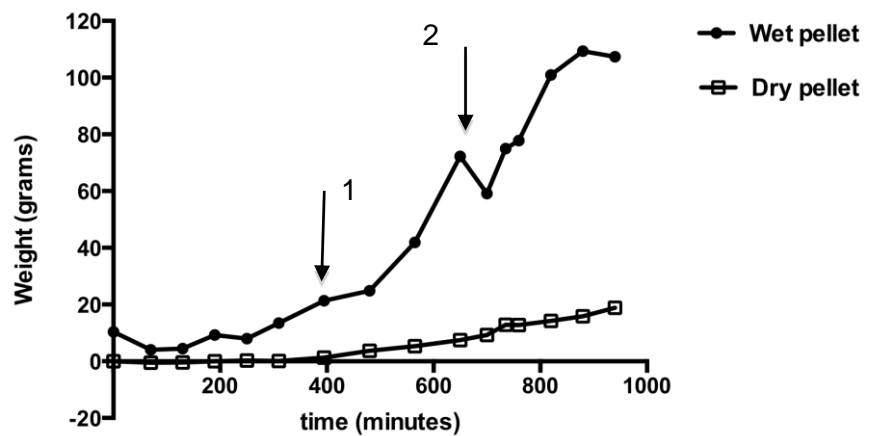


Figure 3.4: Growth rate of fed batch fermentation culture by wet cell weight (circles) and dry cell weight (squares). The first arrow indicates the time point where introduction of fed-batch media was started. The second arrow indicates the time of induction of expression by addition of IPTG.

### 2.3 Development of protein expression and purification methods

Pilot expression tests were carried out to establish optimal small-scale expression conditions. Expression and induction conditions were modified, with cell growth at 30°C in Luria-Bertani medium containing 1% glucose and induction at 25°C with 1 mM IPTG for 4 hours (Figure 3.2). This protocol resulted in high-level expression of MSP-1 hybrid on induction as a soluble protein at ~55 kDa in the cell lysate (Fig. 3.5A). The identity of the protein was confirmed by Western blotting using a mouse monoclonal specific for the K1 Block 2 serotype of MSP-1 (mAb12.2, Fig. 3.5B) and with polyclonal sera raised against individual MSP-1 Block 2 recombinant proteins (not shown). The major soluble protein in the cell lysate was MSP-1 hybrid, with minor degradation products as detected by mAb 12.2 reactivity (Fig. 3.5B, Lane 2). In 1-liter shaking-flask culture, MSP-1 hybrid accounted for an estimated 15% of the total soluble protein, as assessed by densitometry of silver stained gels.

The protein was then further purified, firstly by heat treatment and secondly by anion exchange chromatography. Clarified cell lysate, in 25mM Tris pH8.0 buffer containing 250 mM NaCl was incubated in a water bath at 70°C. This step denatured a large proportion of the host proteins, which were observed as a light-coloured precipitate, and which were removed by centrifugation. This heating step yielded around 80% pure MSP-1 hybrid product (Figure 3.5 lane 4). This was estimated to be 210 mg L<sup>-1</sup> of cell culture, based on BCA assays and protein band intensity. The resulting protein mixture was dialysed against column buffer A (25mM Tris pH8.0). An anion exchange chromatography step gradient was then performed to further purify the MSP-1 hybrid. Low molecular weight contaminants were eluted with 20 mM NaCl in column buffer A and MSP-1 hybrid eluted by application of 50 mM NaCl in column buffer A (Figure 3.6). Based on ImageJ analysis of a silver stained SDS-PAGE gel, purified MSP-1 hybrid was estimated to be at least 97% pure. One litre of culture yielded 197.6 mg of purified MSP-1 hybrid protein.

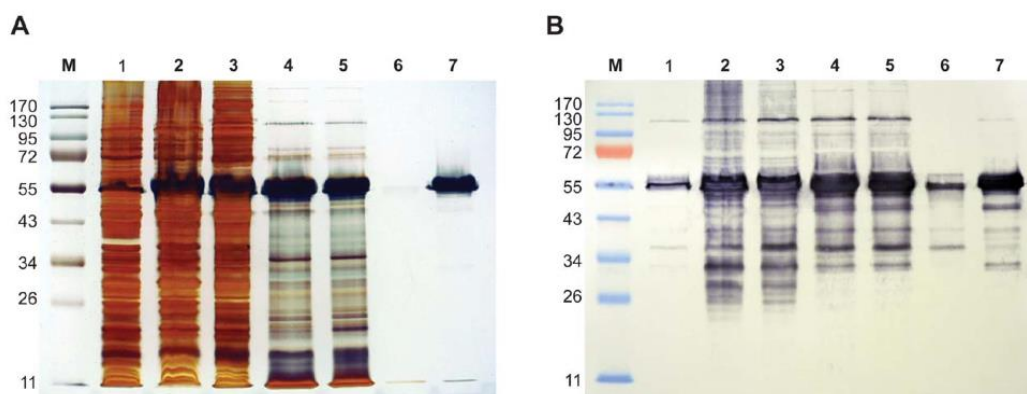


Figure 3.5: MSP-1 hybrid from cell culture and at different steps of purification is shown on A) SDS-PAGE stained by silver nitrate and B) Western Blot of an identical gel probed with a monoclonal antibody against K1 type Block 2 of MSP-1 (mAb12.2). Samples analysed are:- lane 1, cells before induction; lane 2, cells after 4 hours of induction; lane 3, soluble cell lysate; lane 4 soluble fraction after heat-treatment; lane 5, dialysed soluble fraction from lane 4; lane 6, flow through fraction from anion exchange; lane 7, anion exchange purified MSP-1 hybrid protein.

## 2.4 Scale up of protein purification

This purification protocol, used for small-scale production was modified for large-scale production of the MSP-1 hybrid. Cell paste recovered from bioreactor culture was resuspended in column buffer A (25mM Tris buffer pH8.0) at a ratio of 10 mL per gram of frozen cells. The same freeze-thaw lysis method as used for small-scale purification was performed in 50ml batches. After lysis, DNase (Benzonase, Novagen, UK) was added at 2U mL<sup>-1</sup> of lysate. The soluble protein lysate was then recovered by centrifugation and stored at -20°C until use. For the heat treatment step, a range of different salt concentrations in column buffer A and different temperatures were tested on batches of cell lysate from the fed batch fermentation culture. All the samples were heated for 20 minutes followed by cooling for 30 minutes on ice. Soluble fractions from these treatments were investigated for their purity on SDS-PAGE gels and measured for protein concentration by the BCA assay. Results of these tests showed that the optimal condition for the best yield and purity was heat treatment at 90°C without addition of NaCl to the buffer (data not shown).

To ensure that the protein was evenly and thoroughly heated, the heating step was performed at 90°C for 20 minutes by passing the lysate through a 250ml heat exchanger,

which was constructed by Dr. Graeme Cowan from a stainless steel coil immersed in a water bath, attached to a Watson-Marlow peristaltic pump. The clarified lysate solution was pumped through the heat exchanger with a total passage time at 90°C of 20 minutes, and into a collecting beaker on ice. The denatured lysate was clarified by centrifugation at 5,000x g for 30 minutes and dialysed against column buffer A. This process provided 80% pure MSP-1 hybrid product (Figure 3.7), yielding 1724 mg protein L<sup>-1</sup> of culture, as estimated by ELISA.

The partially purified protein was then finally purified by anion exchange chromatography using a 200 mL CptoQ Bioprocess grade Sepharose column (GE Healthcare, UK). The same stepwise elution protocol as described for lab-scale purification method was used to purify the MSP-1 hybrid. The final product was a highly pure product (>97%) with a yield of approximately 550mg L<sup>-1</sup> cell culture.



Figure 3.6: Anion exchange chromatography purification of the MSP-1 hybrid. Conductivity (red line), elution buffer percentage (green line), and protein concentration as measured by absorbance at 280 nm (blue line). Red numbers on the X-axis represent the fraction number of 50 mL eluted fractions from the CptoQ anion exchange column. MSP-1 hybrid absorbs UV light poorly due to its low extinction coefficient, and thus peak elution height is correspondingly low.

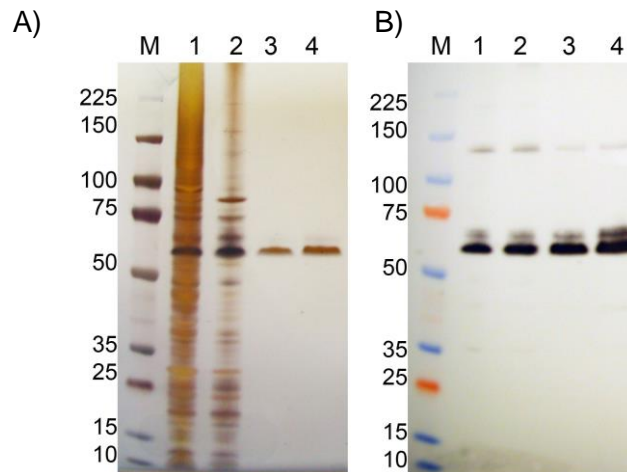


Figure 3.7: MSP-1 hybrid production at large scale from bioreactor cell culture. Individual purification steps are shown on A) SDS-PAGE stained with silver nitrate and B) Western Blot probed with mAb12.2. Samples shown are:- lane 1, soluble cell lysate; lane 2, heat treated and clarified lysate; lanes 3-4, eluted fractions from CaptoQ column.

Purified MSP-1 hybrid from bioreactor production was tested for the level of endotoxin contaminants. Freshly purified MSP-1 hybrid and MSP-1 hybrid sample that had been dialysed against PBS were heated at 70°C for 20 minutes. Samples were then filtered and tested using the Rapid Endotest kit (Lonza). The results suggested that the level of endotoxin was low, giving readouts of 72.1 EU mL<sup>-1</sup> and 8.50 EU mL<sup>-1</sup> for samples before and after final dialysis respectively.

## 2.5 Properties of the MSP-1 hybrid

By mass spectrometry, the MSP-1 hybrid was estimated to have a molecular mass of 31,113 Da, which is very similar to the predicted molecular weight based on the amino acid content (30,987 Da). However, on SDS-PAGE gels the protein resolved at about twice the size of its actual molecular weight (Figures 3.5 and 3.7). MSP-1 hybrid also stained weakly with Coomassie blue dye, but stained strongly with silver nitrate. The MSP-1 hybrid was shown to be a soluble and heat-resistant protein. In column buffer A, the protein was stable at temperatures as high as 90°C without any significant degradation detected by SDS-PAGE.

Using a bioinformatics tool, IUPred (Dosztányi et al. 2005) which predicts the disorder tendency of a protein from its amino acid sequence, the algorithm predicts that the

MSP-1 hybrid is generally unstructured with a high disorder tendency in the middle part of the molecule (Figure 3.8).

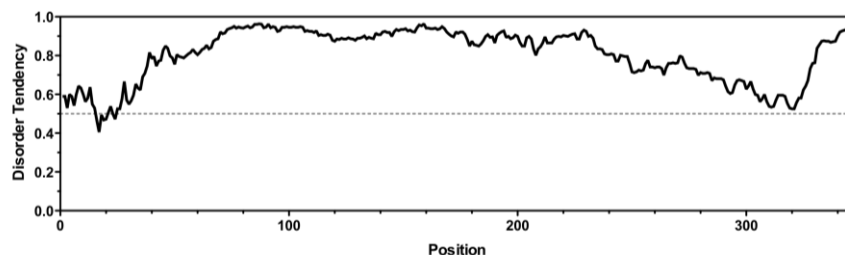


Figure 3.8: IUPred prediction of disorder tendency in the MSP-1 hybrid. Plot shows the disorder tendency of the MSP-1 hybrid protein (Y-axis) along the length of the 348 amino acid sequence. X-axis represents amino acid position in the MSP-1 hybrid protein.

Using circular dichroism, the CD spectrum profile of the MSP-1 hybrid in a low salt buffer showed that it had single deep minimum at round 200 nm (Figure 3.9), which is a typical pattern for proteins with random coil conformation (Kelly et al. 2005). This suggests that the recombinant protein lacks any stable 2D or 3D structures, such as alpha helices or beta sheets.

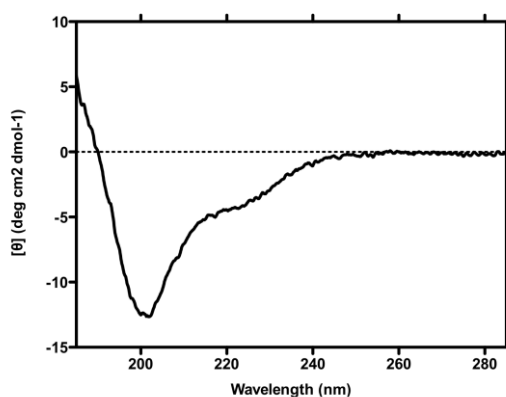


Figure3.9: Figure shows the circular dichroism spectrum of purified MSP-1 hybrid protein, assessed over a 185 to 285nm wavelength range at 25°C in 10mM NaH<sub>2</sub>PO<sub>4</sub> pH 8.0. The distinct valley groove at 200nm is typical of a random coil structure.

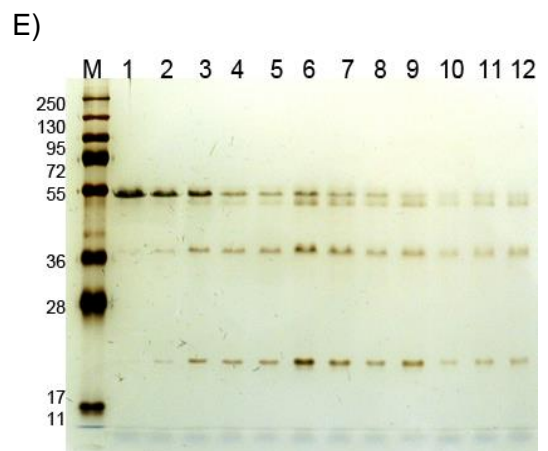
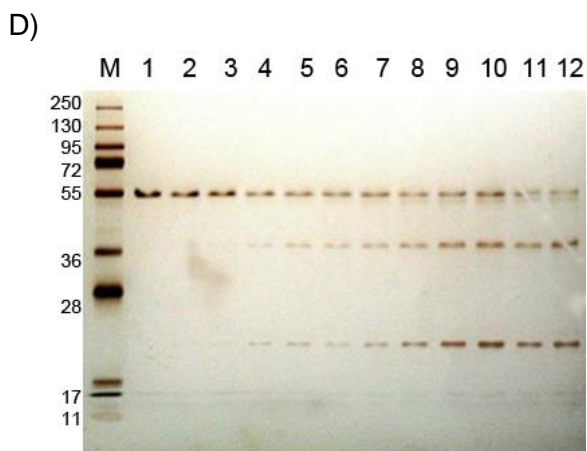
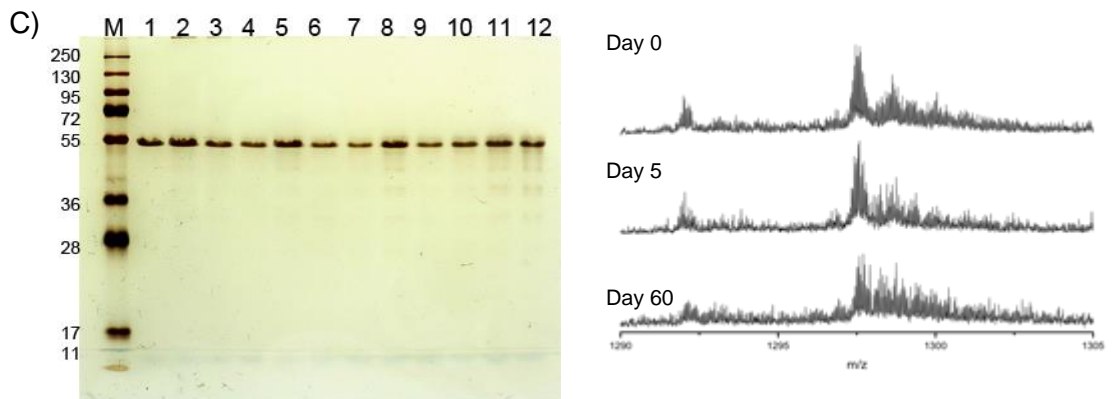
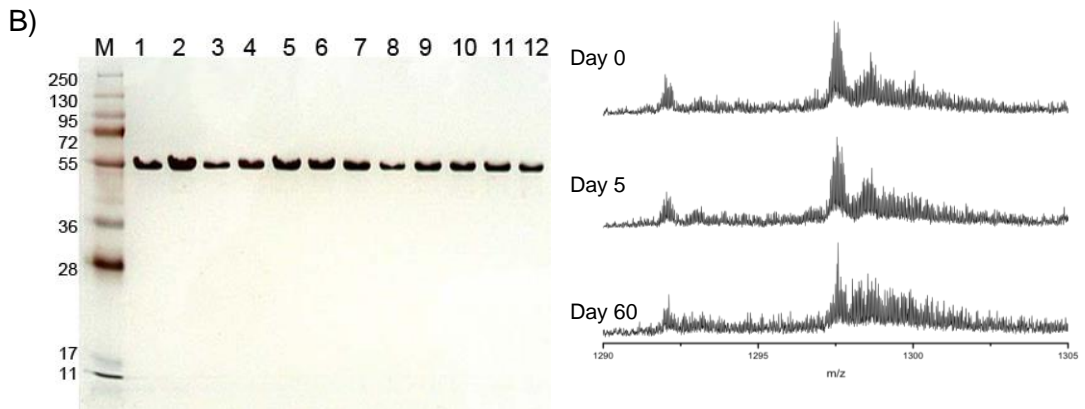
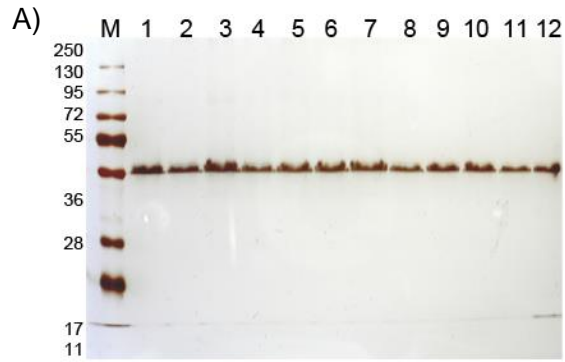
## 2.6 Protein stability testing

For testing the stability of the native MSP-1 hybrid, protein in PBS buffer at concentration of  $1.26 \text{ mg ml}^{-1}$  was incubated at  $-70^{\circ}\text{C}$ ,  $-20^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$ ,  $25^{\circ}\text{C}$  and  $37^{\circ}\text{C}$  with samples taken and frozen at  $-70^{\circ}\text{C}$  every five to seven days for 60 days. Samples were measured for concentration and investigated for degradation by silver-stained SDS-PAGE gels. Results showed that the MSP-1 hybrid could be stored at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$  for at least 60 days without any significant detected degradation (data not shown). All samples at all temperatures were tested for possible degradation by silver stained SDS-PAGE. At room temperature, the MSP-1 hybrid showed minor degradation at 14 days, with two lower molecular weight proteins of 36 kDa and 20 kDa observed from day 20 (Figure 3.10, panel D). A faster but near identical degradation profile was observed in the sample incubated at  $37^{\circ}\text{C}$  (Figure 3.10, panel E).

The MSP-1 hybrid stock was stable for longer periods if “Pasteurised” by heating to  $70^{\circ}\text{C}$  for 20 minutes after the final purification step. Stability tests were repeated on MSP-1 hybrid stocks which had been heated. The protein solution was incubated, sampled, and investigated in the same way as described above. At room temperature, the pre-heated MSP-1 hybrid could be stored up to 60 days without any visible signs of degradation as determined by silver stained SDS-PAGE (Figure 3.10, Panel B). The protein concentration of these samples was also very similar and remained stable throughout the whole incubation period (data not shown). Mass spectrometry of day 0, day 5 and day 60 samples showed a single dominant peak at  $m/z$  of 1297, which is the intact MSP-1 hybrid protein. The minor  $m/z$  species at 1292 is the MSP-1 hybrid without the N-terminal methionine residue, which is a result of post-translational production that commonly occurs in *E. coli* (Hirel et al. 1989). The minor species of higher  $m/z$  ratios which accumulate over time are the result of salt adducts which increase over the incubation period. Low temperature incubation of the MSP-1 hybrid (at  $-20^{\circ}\text{C}$ ) showed comparable profiles to the starting material both on SDS-PAGE and Mass spectrometry (Figure 3.10, Panel A), while high temperature incubation ( $37^{\circ}\text{C}$ ) showed similar resistance to degradation. Minor levels of degradation of MSP-1 hybrid can be detected at days 56-60 of the incubation period (Figure 3.10, Panel C).



Figure 3.10: Silver stained SDS-PAGE and mass spectrometric profiles to assess stability of MSP-1 hybrid incubated for up to 60 days at -20°C, 25°C and 37°C. Sampling time points were: lanes 1-12 = day 5,10,14,20,24,28,30,35,42,49,56, and 60 respectively. Panels A, B, C = heat-treated MSP-1 hybrid incubated at -20°C, 25°C and 37°C respectively); Panels D and E = MSP-1 hybrid without final heat treatment incubated at 20°C and 37°C respectively.)



## 2.7 Vaccine stability and immunogenicity testing

MSP-1 hybrid was also tested for its stability after formulation with CoVaccine HT. The protein at the recommended concentration was formulated with adjuvant according to the manufacturers protocol and then stored at 4°C and 20°C for up to seven weeks. Protein antigen was extracted from vaccine every week according to the protocol from Miles *et al.*, 2005 (Hay *et al.* 2004; Miles *et al.* 2005). Benzyl alcohol was added to vaccine formulation to a final concentration of 10%. It was mixed by vortexing for 20 minutes followed by centrifugation at 16,000g for 10 minutes. The mixture separated into three layers, of which the protein antigen is in the middle aqueous phase. Recovered protein antigen MSP-1 hybrid was removed and stored frozen at -70°C until use. Extracted samples were analysed by silver stained SDS-PAGE.

MSP-1 hybrid was stable when formulated with the adjuvant for at least 21 days stored at 4°C. These protein samples showed similar band intensity as the original MSP-1 hybrid material on SDS-PAGE and were of similar concentration to the same amount of MSP-1 hybrid protein not formulated with CoVaccine HT. After 35 days, the concentration of the protein appeared to be slightly reduced. However, there was no degradation that could be detected by SDS-PAGE.

MSP-1 hybrid formulated with with CoVaccine HT and incubated at room temperature was stable for up to 21 days, showing very similar band intensity and pattern to the unformulated protein on SDS-PAGE. After day 28, some of the MSP-1 hybrid from the formulated extract had undergone multimerization. As a result, the amount of 55 kDa product was reduced and two bands of ~120 kDa and ~200 kDa could be detected. Western blots showed that these larger protein forms were recognised by mAb 12.2 and by polyclonal sera raised to the MSP-1 hybrid (data not shown).

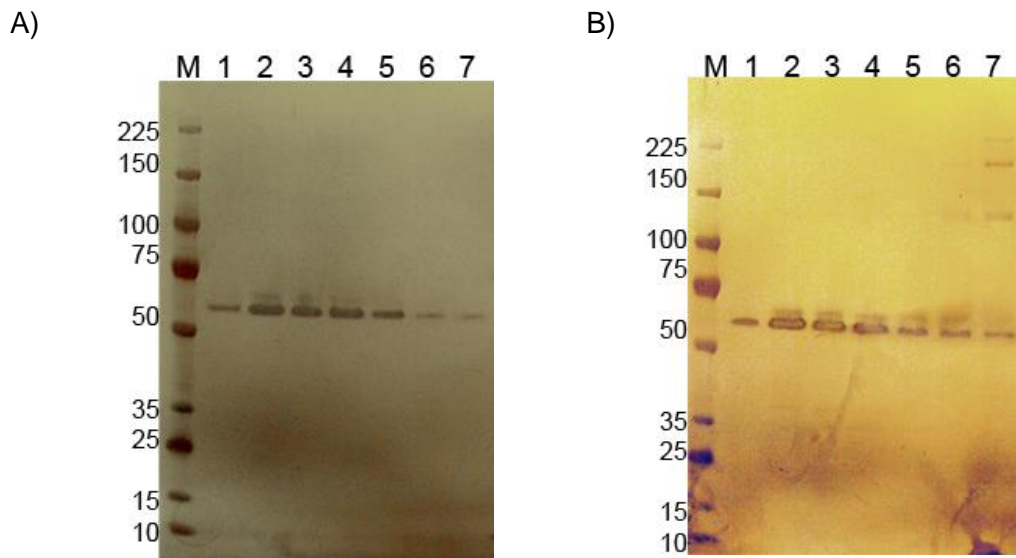


Figure 3.11: Characterization of MSP-1 hybrid extracted from vaccine following storage at 4°C (A: 2-7) and at 20°C (B: 2-7). Samples were sampled at days 7, 14, 21, 28, 35, 42, and 49 (lanes 2-7 respectively). Unformulated MSP-1 hybrid was run on each gel as a control (lane1).

Immunogenicity of MSP-1 hybrid incubated at room temperature for 60 days was performed in mice. Protein samples were mixed with CoVaccine HT immediately prior to immunisations. Control material consisted of MSP-1 hybrid that had not undergone any incubation prior to use (i.e. day 0 sampled). Each mouse received three doses of the immunogen and the sera were collected at the end of the experiments. The sera were tested against the MSP-1 hybrid antigen by ELISA. Incubation conditions did not significantly decrease the immunogenicity of the MSP-1 hybrid. Most of the mice from both experimental and control groups were able to produce specific antibodies to the MSP-1 hybrid; two mice of the non-incubated MSP-1 hybrid group (day 0 sample) failed to produce specific antibodies (Figure 3.12). Both immunisation of day 0 and day 60 MSP-1 hybrid yielded antigen specific antibodies that were detected by ELISA, giving median titres per group of  $1.48 \times 10^5$  and  $1.74 \times 10^5$  respectively. Antibody titres of the two groups were not significantly different from each other (Mann-Whitney U test,  $p=0.7336$ ).

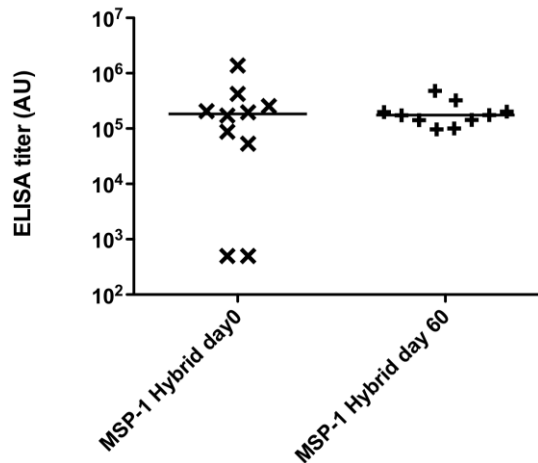


Figure 3.12: Antibody titres in mice immunised with MSP-1 hybrid as measured by ELISA. MSP-1 protein that had been stored at -20°C (day0) and had been stored at room temperature for 60 days (day 60) were both formulated with CoVaccine HT. Mice that were vaccinated with these formulations showed no statistically significant difference between the 2 groups in specific antibody titre to the MSP-1 hybrid. Horizontal bars represent the median titre for each group.

These sera also recognised all five MSP-1 Block 2 antigens from all three Block 2 serotypes. Furthermore, there was no statistically significant difference between the in the median titre of the two immunisation groups to 3D7, Palo Alto17, MAD20, and Wellcome Block 2 antigens (Mann-Whitney U test,  $p=0.1852$ ,  $p=0.5204$ ,  $p=0.1476$ , and  $p=0.07815$  respectively). However, RO33 specific antibody titres of the day 60 group were significantly higher than in the day 0 group ( $p=0.0017$ ) (Figure 3.13 Panels A and B).

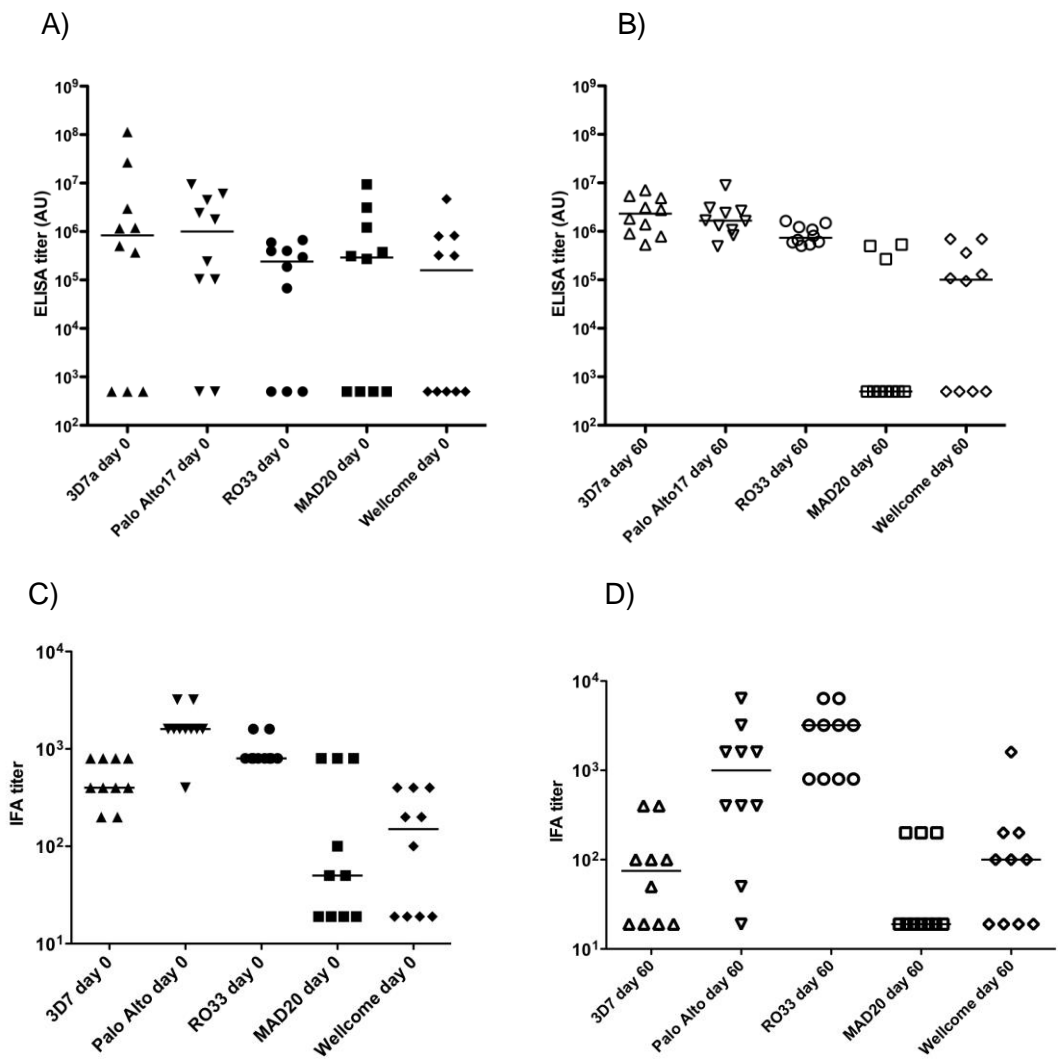


Figure 3.13: MSP-1 Block 2 serotype specificity and titres of sera from day 0 (Panel A) and day 60 (Panel B) immunisation groups assessed by ELISA. Each serum was tested against the recombinant proteins indicated on the X-axis. Corresponding IFA titres of the same sera to five strains of parasite are shown in Panels C (day 0) and D (day 60) respectively. Horizontal bars indicate the median titre of each group.

The same serum samples were used in IFA against five different strains of parasite, namely 3D7, Palo Alto17, MAD20, Wellcome and RO33, representative of all three MSP-1 Block 2 serotypes. IFA reactivities agreed closely with ELISA results, confirming that antibodies to five serotypes of the MSP-1 Block 2 could be elicited by immunisation with the MSP-1 hybrid. This confirmed that these antibodies recognise natural protein targets on the parasite surface. Comparing antibody reactivities between the groups, the median IFA titres against parasite strains Palo Alto17, MAD20 and Wellcome were not significantly different (Mann-Whitney U test,  $p=0.2079$ ,  $p=0.2283$ , and  $p=0.6361$  respectively). However, IFA titres against 3D7 and RO33 serotypes of the day 0 and day 60 samples were significantly different ( $p=0.0013$  and  $p=0.0287$  respectively, Figure 3.13 C and D).

The isotype distribution of antibodies produced by these immunisations were also determined by ELISA. Subclasses IgG1, IgG2a, IgG2b and IgG3 were all detected against the MSP-1 hybrid, to a greater or lesser extent. Surprisingly, median ELISA titres of the day 60 group appeared to be higher for all subclasses than those of the day 0 group. However, these were not statistically significant differences (Mann-Whitney U test,  $p=0.4710$ , and  $p=0.1485$  for IgG2b and IgG3 respectively), except in the case of the IgG1 ( $p=0.0022$ ). The IgG2a level of the day 0 group was higher than in the day 60 group, but again it was not statistically significant (Mann-Whitney U test,  $p=0.7910$ ) (Figure 3.14)

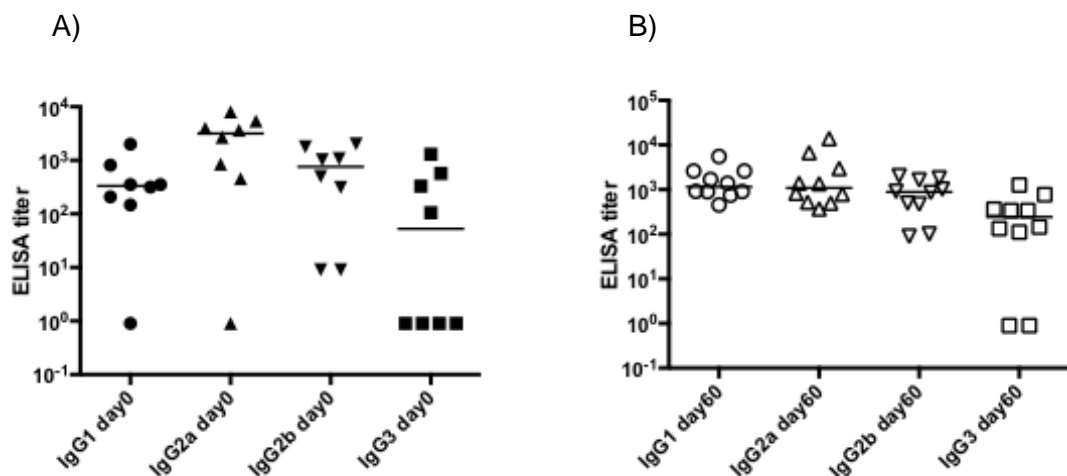


Figure 3.14: Isotype distribution of specific IgG antibodies against the MSP-1 hybrid. IgG subclass titres from all mice immunised with A) day 0 MSP-1 hybrid and B) 60-day incubated MSP-1 hybrid showed similar profiles of anti-MSP-1 hybrid antibodies.

## 2.8 Routes of immunisation

Not all routes of immunisation are suitable for the MSP-1 hybrid antigen. Groups of mice that were injected subcutaneously (s.c) with the MSP-1 hybrid formulated with Montanide™ ISA51, Montanide™ ISA720, Alhydrogel™, Alhydrogel™ with CpG ODN 7909, or CoVaccine HT™ elicited higher antibody titres than those immunised intraperitoneally (i.p) with the same formulations (Figure 3.15). IFA testing of the same sera had correspondingly ranked titres to the ELISA titres (data not shown).

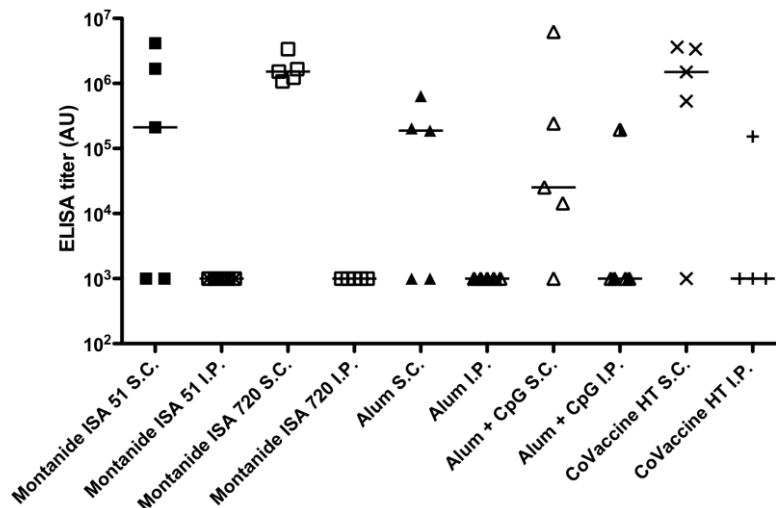


Figure 3.15: Antibody titres of sera from mice immunised with MSP-1 hybrid in combination with different adjuvants. For each adjuvant, two groups of mice were injected either subcutaneously (s.c.) or intraperitoneally (i.p.). Adjuvants and immunisation routes are displayed on the X-axis. ELISA titres (Y-axis) against the MSP-1 hybrid in the i.p. groups were low or undetectable, whereas much stronger responses were detected in the majority of MF1 outbred mice immunised with the same vaccine formulation injected s.c.

## 2.9 Potency testing of MSP-1 hybrid

The potency of the MSP-1 hybrid was tested in inbred mouse strains BALB/c and C57/BL6. Each group of ten mice were immunised by s.c. injection of either 10 or 50 µg MSP-1 hybrid in Alhydrogel™. Immunisations were carried out seven days apart for a total of three doses. The mice were exsanguinated 14 days after the last immunisation. The sera were collected and tested against MSP-1 hybrid by ELISA. A pool of sera from five mice



immunised with MSP-1 hybrid in combination with CoVaccine HT from another experiment was used as a positive control to produce a standard curve to assess titres between groups.

Specific antibodies to the MSP-1 hybrid were detected in all four groups of mice. BALB/c mice immunised with 50µg MSP-1 hybrid had the highest median titre ( $3.78 \times 10^3$ ). BALB/c mice in the 10 µg group had lower levels of specific antibody response (median titre  $1.71 \times 10^3$ ), but the titres of two groups were marginally not statistically significantly different ( $p = 0.063$ ; Mann-Whitney U test). For the C57/BL6 mice, the 10 µg group responded well to the MSP-1 hybrid, (median titre  $2.06 \times 10^3$ ), with two mice not responding. By contrast, most of the C57/BL6 mice from the 50µg group failed to respond to the MSP-1 hybrid. Although the ELISA titres of the responding mice were as high as most of the mice from 10µg group, the two groups were significantly different by statistical analysis ( $p = 0.032$ ; Mann-Whitney U test). (Figure 3.16)

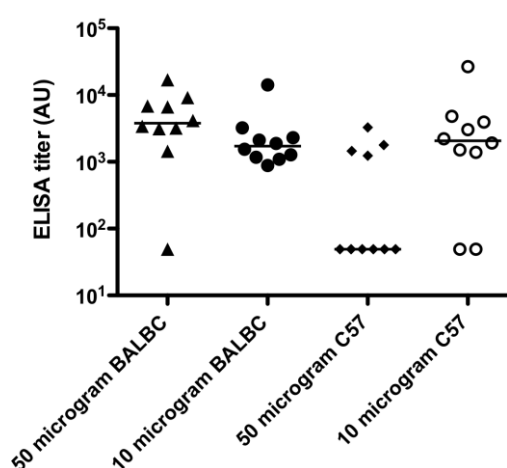


Figure 3.16: Potency test antibody titres against the MSP-1 hybrid antigen assessed by ELISA. Sera from mice immunised with either 10µg or 50 µg of MSP-1 hybrid in combination with Alhydrogel™ were tested. Horizontal bars represent the medians of each group.

## 2.10 Adjuvant formulation, compatibility and immunogenicity testing

Adjuvants that were used with the MSP-1 hybrid in this study were compatible with or have been in human use, including 1) Montanide™ ISA51, 2) Montanide™ ISA720, (both from Seppic, France) 3) Alhydrogel™ (Brenntag Biosector, Denmark) (with or without CpG 7909 ODN), 4) Covaccine HT™ (Protherics Medicines Development Limited, A BTG International Group Company, London, UK), 5) Formulated Aqueous TLR-4 Agonist (with

or 6) without Alhydrogel™), 7) Formulated TLR-4 agonist in oil-in-water emulsion, 8) Formulated Oil-in-Water Emulsion, 9) Formulated TLR-7/8 agonist aqueous formulation and 10) Formulated TLR-9 agonist aqueous formulation (adjuvants 5,7,8,9,10 are provided by Dr. Darrick Carter of IDRI (Seattle, USA). All these adjuvants were tested by immunisation of MF1 mice. Sera from immunisations were tested against MSP-1 hybrid by ELISA and checked for their ability to cross-react with parasite surface antigens by IFA. Details of immunisation protocols are given in Materials and Methods.

Different adjuvants elicited different levels of antibodies in each immunisation group. The immunisation group with the highest median ELISA titre was the CoVaccine HT formulation. All 8 mice in this group had antibodies against MSP-1 hybrid and their titres were similar, producing a median titre of  $8.90 \times 10^6$ . Montanide ISA51 and Montanide ISA720 showed very similar median titres of  $2.11 \times 10^5$  and  $1.52 \times 10^6$  respectively. Using Alhydrogel, either with or without CpG, also elicited specific antibodies against MSP-1 hybrid, giving ELISA titres of  $1.87 \times 10^5$  and  $2.52 \times 10^4$  respectively. However, the titres of individual mice from each of these groups differed widely from each other. Stronger antibody titres were observed in groups of the Aqueous TLR-4 Agonist, either with or without Alhydrogel. These two groups had median titres of  $\sim 1.8 \times 10^6$  and  $3.00 \times 10^6$  respectively. Similar median titres were observed with the Formulated Oil-in-Water Emulsion and Formulated TLR-4 agonist oil-in-water emulsion adjuvant groups ( $1.56 \times 10^5$  and  $1.58 \times 10^5$ ). Most of the mice failed to respond when aqueous TLR7/8 agonist was used as adjuvant, while TLR-9 agonist aqueous group showed a low median for the specific IgG to MSP-1 hybrid ( $3.57 \times 10^4$ ).

Comparing Montanide ISA51, Montanide ISA720, and Alhydrogel with CpG, aqueous TLR-4 agonist (both with and without Alhydrogel), the antibody titre of the CoVaccine HT group was not statistically different ( $p < 0.05$  or less; Kruskal-Wallis test). Nevertheless, a non-parametric Mann-Whitney U test suggested that median titre of the CoVaccine HT group was higher than other groups (Figure 3.17)

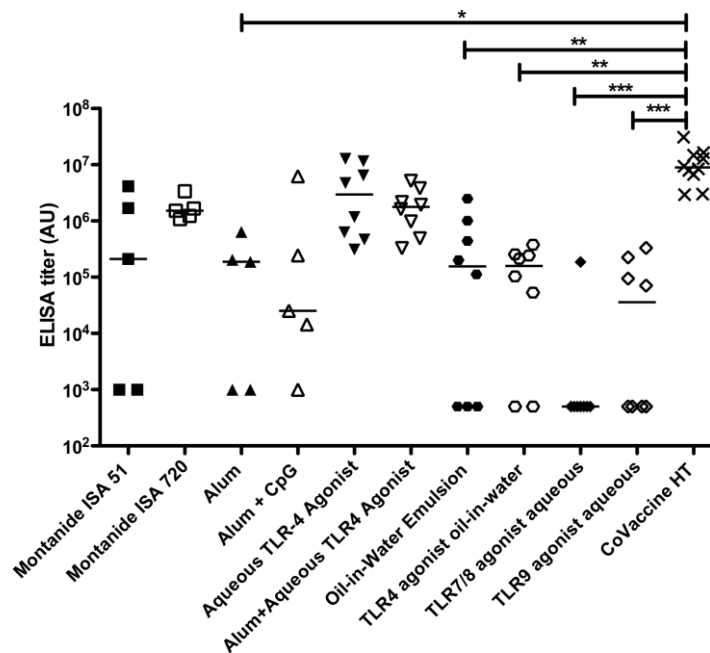


Figure 3.17: ELISA titres of sera from mice that were immunized with MSP-1 hybrid in combination with different adjuvants. Differences between the CoVaccine HT group and Alhydrogel, oil-in-water emulsion, TLR-4 agonist oil-in-water, TLR7/8 agonist aqueous, and TLR-9 agonist aqueous were statistically significant (Kruskal-Wallis test) Asterisks represent statistical significance; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.005$ .

Sera were further tested against individual MSP-1 Block 2 antigens by ELISA. The CoVaccine HT group had antibodies to all three serotypes of Block 2, including K1-like type (3D7 and Palo Alto), RO33 type and MAD20-like type (MAD20 and Wellcome) (Figure 3.20, Panel A). Block 2- specific antibodies were also observed in the Montanide ISA51 and Montanide ISA720 group (Figure 3.18 Panels A and B). Specific antibodies to 3D7, Palo Alto17 and RO33 were detected in a group of mice that were immunised with MSP-1 hybrid in combination with Formulated Aqueous TLR-4 Agonist, however this ELISA was unable to detected specific antibodies to MAD20 and Wellcome antigens (Figure 3.18 Panel C). Similar results were obtained in the Aqueous TLR-4 Agonist plus Alhydrogel formulated group. Most sera from this group were able to recognise 3D7, Palo Alto and RO33 antigens. Only one serum recognised the MAD20 and Wellcome antigens (Figure 3.18 D).

IFAs were performed to test cross-reactivity of these sera to the same panel of five parasite strains. IFA results showed the same general trend as the ELISA results (Figure 3.19). The sera from the groups immunised with antigen plus Alum, and Alum+CpG also

showed positive signals against parasites, although the signals were very low and sometimes almost undetectable (data not shown).

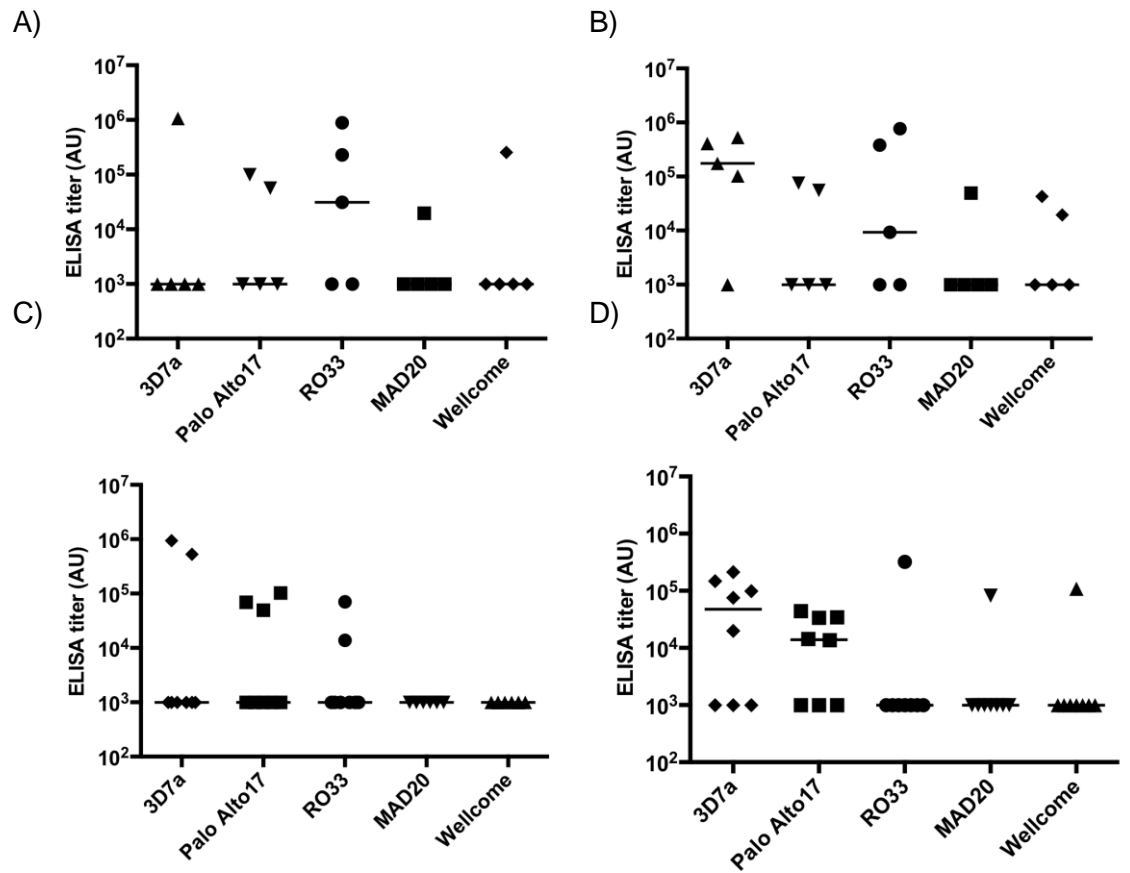


Figure 3.18: ELISA analysis of serotype specificity of antibodies yielded from immunization of MSP-1 hybrid formulated with different adjuvants. A=Montanide ISA51, B=Montanide ISA720, C= Aqueous TLR-4 agonist, D= Aqueous TLR-4 agonist with Alum).

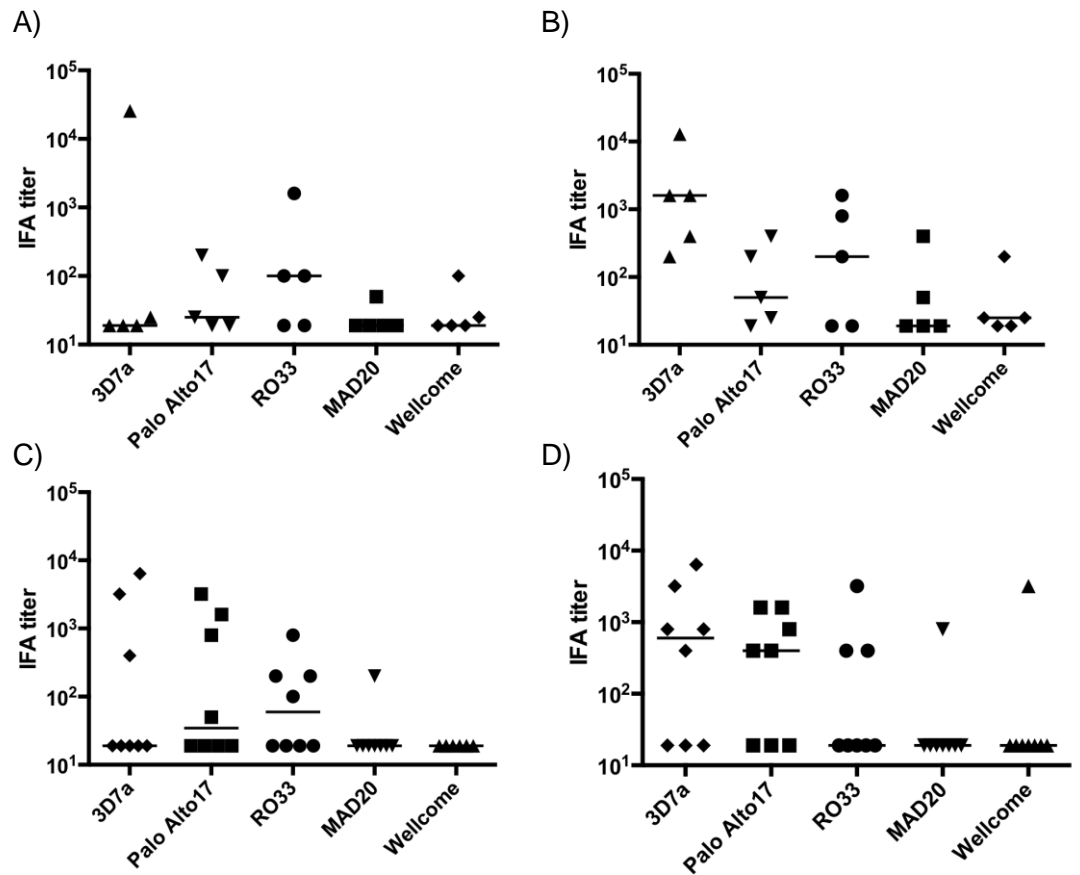


Figure 3.19: IFA-reactive serotype specificity of mouse antibodies from immunization with MSP-1 hybrid formulated with different adjuvants. A = Montanide ISA51, B = Montanide ISA720, C = Aqueous TLR-4 agonist, D = Aqueous TLR-4 agonist with Alum. Titres (Y-axis) are plotted against parasite strain on the X-axis for each serum sample.

## 2.11 Immunogenicity and serotype specificity in different animals

Immunisation of the MSP-1 hybrid in combination with CoVaccine HT raised consistently high titre and multiple serotype reactivities in the serum of mice. This adjuvant/antigen combination was further tested as a vaccine formulation in different animals. ELISA and IFA assays were performed as described earlier to assess the immunogenicity of the MSP-1 hybrid in larger animals and non-human primates.

### 2.11.1 Mice

Immunisation of the CoVaccine HT/MSP-1 hybrid formulation in mice elicited antibodies to all five Block 2 antigens tested. Medians ELISA titres to each Block 2 protein were very similar and as high as the antibody titre to the MSP-1 hybrid (median of

8.90X10<sup>6</sup>). Median ELISA titres for each Block 2 antigen ranged from 1.15 X10<sup>7</sup> (RO33) to 7.11 X10<sup>5</sup> (Wellcome). Titres of 3D7 and Palo Alto17 were similar, giving medians of 2.35 X10<sup>6</sup> and 1.12 X10<sup>7</sup> respectively (Figure 3.20 A). Although all of sera were able to recognise the immunogen, some sera failed to recognise some of the Block 2 antigens.

IFA testing of the same antisera again showed that the sera from the CoVaccine formulated MSP-1 hybrid immunised mice had specificity to all five parasite serotypes. There were differences in the titres observed between individual Block 2 serotypes. In comparison to 3D7, Palo Alto and RO33, median IFA titres to MAD20 and Wellcome strain of parasite were lower (Figure 3.20 B).

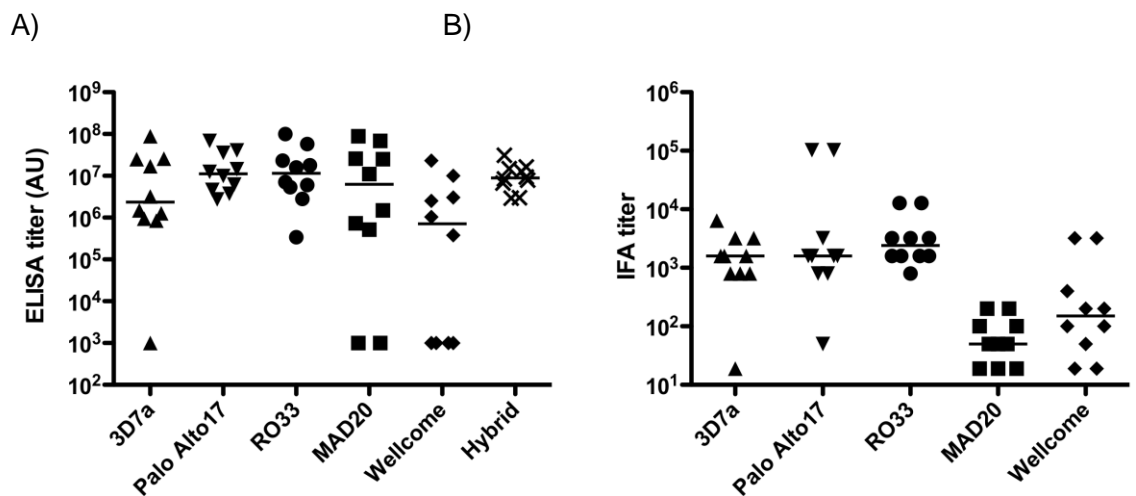


Figure 3.20: Block 2 serotype specificity of sera from mice that were immunized with MSP-1 hybrid in combination with CoVaccine HT. Titres (Y-axis) are plotted against individual MSP-1 Block 2 antigens on the X –axis (Panel A) or individual parasite serotypes (Panel B).

ELISA was performed to investigate the IgG subclass distribution of the antibodies elicited by MSP-1 hybrid in combination with CoVaccine HT. Strong reactivity of IgG1, IgG2a and IgG2b to MSP-1 hybrid was observed, giving median titres of 1.03x10<sup>3</sup>, 5.96x10<sup>2</sup>, and 3.23x10<sup>2</sup> respectively, while IgG3 reactivity was low or undetectable in some sera (median 6.55x10<sup>1</sup>) (Figure 3.21).

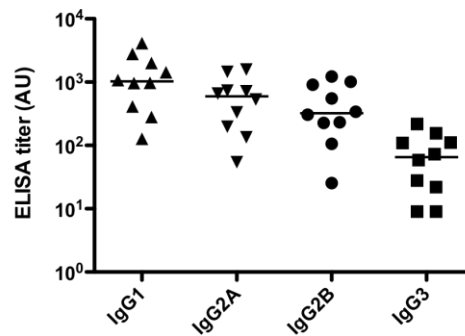


Figure 3.21: IgG subclass titres in mice immunised with MSP-1 hybrid in combination with CoVaccine HT. Titres (Y-axis) for individual mice are plotted against IgG subclass on the X-axis. Horizontal bars represent median titres of each group.

### 2.11.2 Rabbits

Serum samples from each animal were collected after each of three immunisations with MSP-1 hybrid formulated in CoVaccine HT. Animals were immunised as described in Materials and Methods (section 12). The serum sample from each time point was tested by ELISA and by IFA. MSP-1 hybrid specific antibody titres increased over the course of the immunisations and reached a median of  $3.87 \times 10^6$  at day 70 (Figure 3.22 Panel A). However, there was one rabbit that failed to respond to any Block 2 antigens or the MSP-1 hybrid antigen (data not shown). Sera from the 7 rabbits that were able to recognise all the Block 2 antigens tested and the titres between individual rabbits were consistent. Both ELISA and IFA antibody reactivity to each Block 2 serotype increased with repeated immunisation. Sera collected after the last immunisation showed similar antibody titres to 3D7 and Palo Alto (median of  $6.60 \times 10^5$  and  $6.57 \times 10^5$  respectively), while the median titre to RO33 was slightly lower ( $6.02 \times 10^5$ ). The median Ab titre to the Wellcome Block 2 antigen was the highest, giving a readout of  $9.05 \times 10^5$ , while anti-MAD20 titres were  $8.54 \times 10^5$  (Figure 3.22 B) IFA reactivity with parasites correlated broadly in specificity and titre, as seen in the mice (Figure 3.22 C).

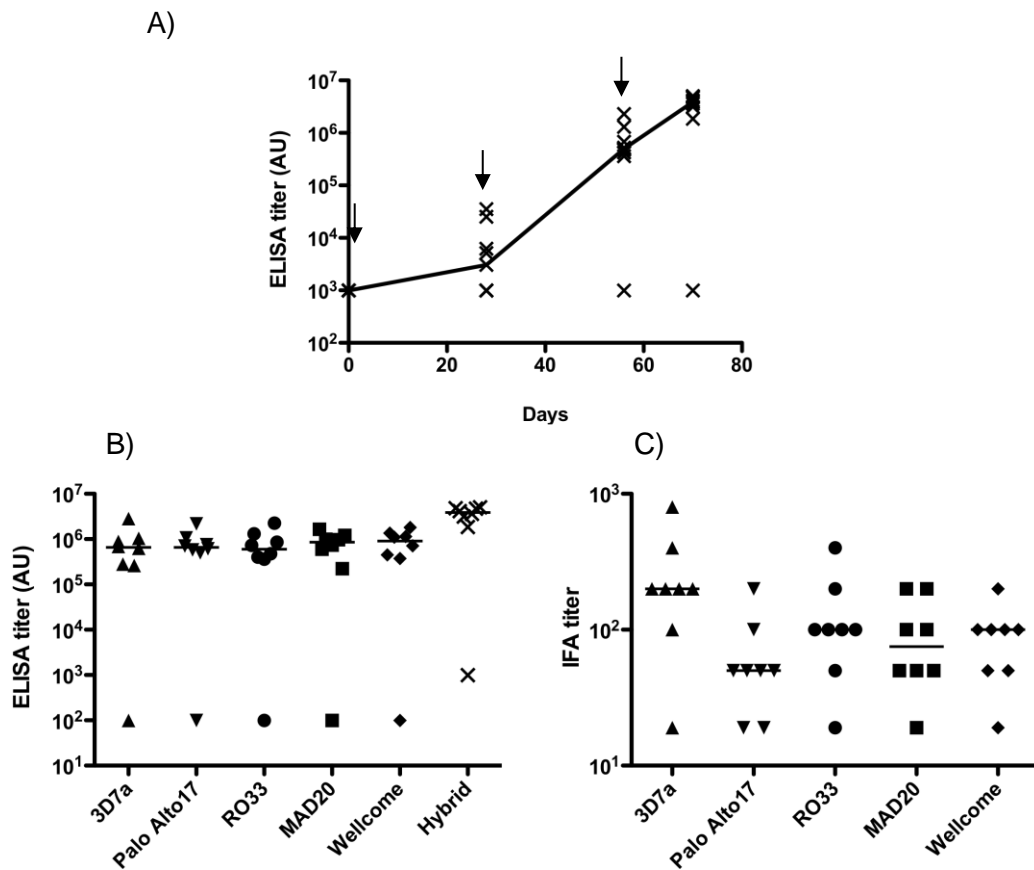


Figure 3.22: Immunogenicity testing of the MSP-1 hybrid in rabbits. A) ELISA titres (Y-axis) are plotted against days since the beginning of the immunisation schedule. Arrows show immunisation points. Sera from the final bleed were tested against B) five Block 2 antigens by ELISA and (C) by IFA with five different strains of *P. falciparum*.

## 2.12 Epitope mapping of anti-MSP-1 hybrid sera

A panel of 132 N-terminal biotinylated 12-mer peptides, representative of all major linear epitopes in the Block 2 region of MSP-1 were used to fine-scale map antibody responses in all immunized and responding animals. This panel included all known repetitive sequences from the K1 and MAD20 serotypes, peptides from all non-repetitive regions of all three serotypes, and a panel of 19 peptides covering the junctions between the individual Block 2 serotypes. Due to the reduced sensitivity of this assay with small volumes of available sera, animals that were seronegative were not tested. In the remaining 25 mice, 6 rabbits and 5 rhesus monkeys, reactivity with peptide epitopes was observed across all three



Block 2 serotypes, with some exceptions. Generally, mice showed a more oligoclonal response to Block 2, with a narrower number of reactivities, and with less broad recognition of individual variant peptides. The pattern of peptide recognition of individual mouse sera were similar. However, compared to other animals, fewer peptides were recognised by sera from mice. Repetitive regions and middle part of the RO33 sequence clearly provided epitopes that were recognised by the majority of sera from mice, while some also showed reactivity to flanking sequences of the K1-like region and the MAD2- like region (Figure 3.23).

Mapping of sera from rabbits showed a broader reactivity, recognising epitopes from all three serotypes, although more epitope reactivities in MAD20-like type region were detected. Using different adjuvants also resulted in different serum recognition patterns. It was noted that the sera from the group immunised with the CoVaccine HT formulation recognised more peptides than the group with Freund's adjuvant (Figure 3.24 A and B). Reactions of the sera from a sheep that had been immunised with Freund's formulated MSP-1 hybrid was similar to reactivity in rabbits and mice (Figure 3.24 C). Similarly, in rhesus monkeys, reactivity of the antibodies to all three serotypes of Block 2 was observed (Figure 3.24 D).

Recognition of neo-epitopes was also observed in some of these immunised animals. The sera that were reported to be positive to the junction peptides were found more in mice and less in rabbits and monkeys (Figure 3.24). However, almost all antibody reactivity in individual serum samples that showed reactivity with the 'non-natural' junctional peptides was explained by antibody specificities in the same sera to peptides from individual 'natural' Block 2 sequences.

Figure 3.23: Peptide epitope recognition of sera from mice that were immunized with MSP-1 hybrid in combination with A) Montanide ISA51, B) Montanide ISA720, (C) Alhydrogel™ (D) Alhydrogel™ with CpG, and (E) CoVaccine HT. Reactivity of each group of mice for each peptide is plotted as a column against peptide sequence on the X-axis. Whiskers on each bar represent the standard deviation (SD) of ELISA readings. Dashed lines represent the mean background signal for all peptides. The small Figure on the right of each panel shows serum recognition to the neo-epitope peptides.

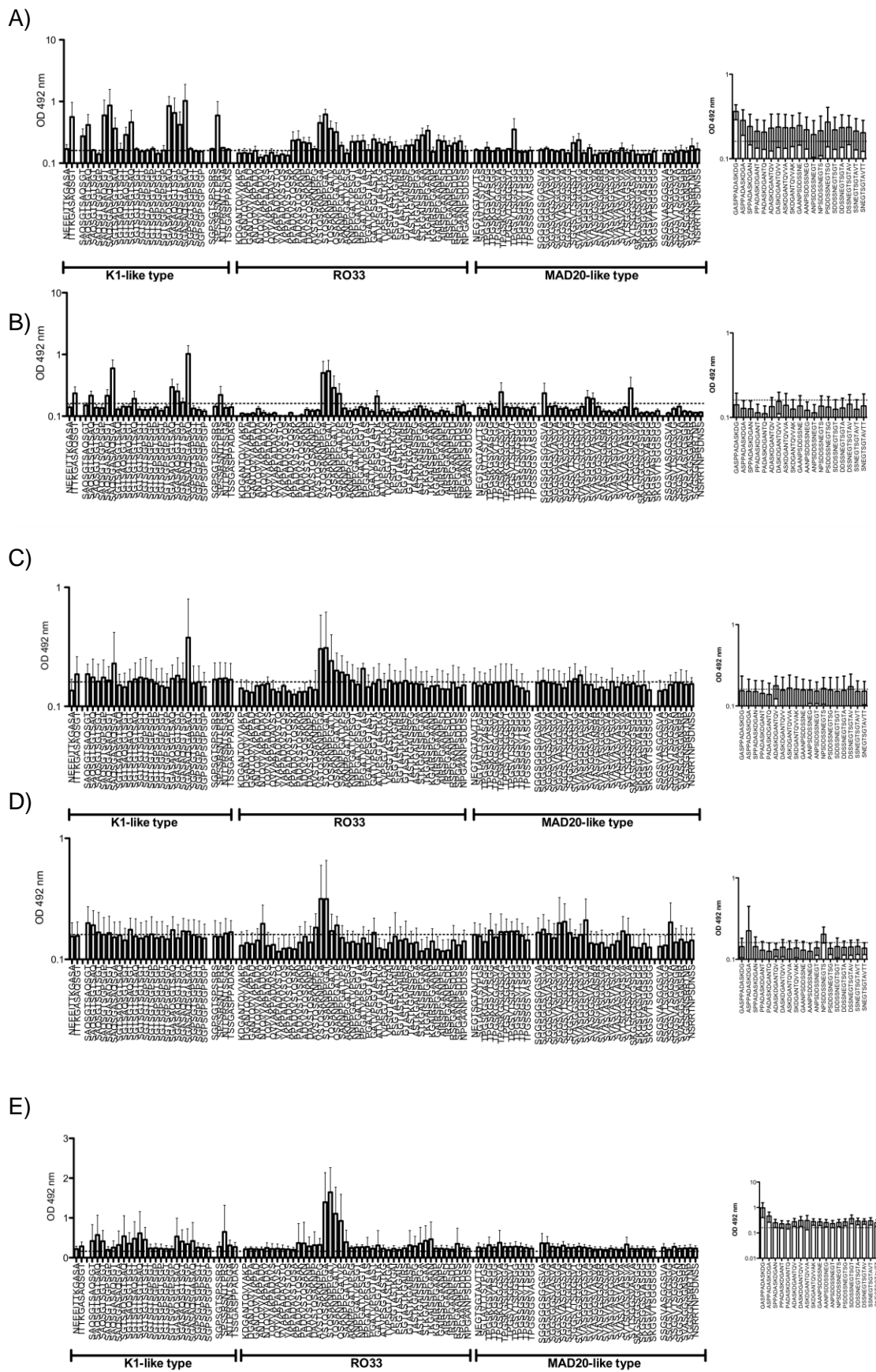
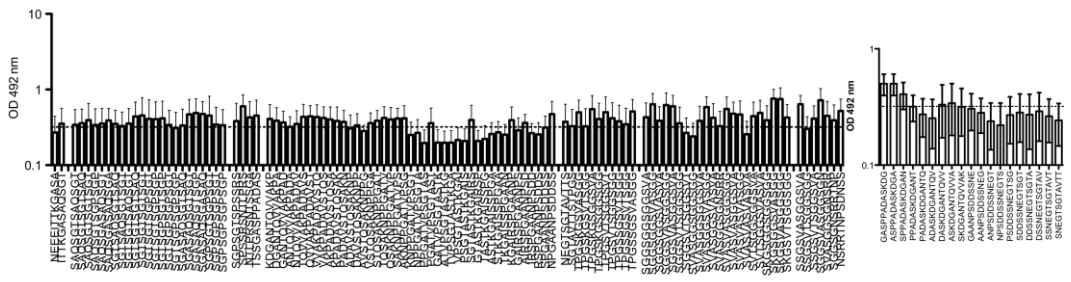
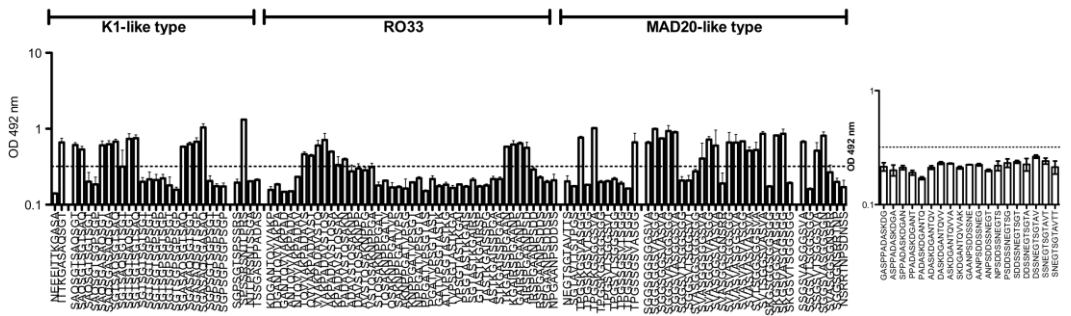


Figure 3. 24: Peptide epitope recognition patterns of sera from A) rabbits that were immunized with MSP-1 hybrid antigen in combination with CoVaccine HT and B) Freund's adjuvant. Peptide recognition of sera from C) sheep immunized with MSP-1 hybrid with Freund's adjuvant and D) Rhesus monkeys immunized with MSP-1 hybrid formulate with CoVaccine HT. Dashed lines represent mean background signal for all peptides. Small Figure on the right of each panel shows serum recognition to neo-epitope peptides.

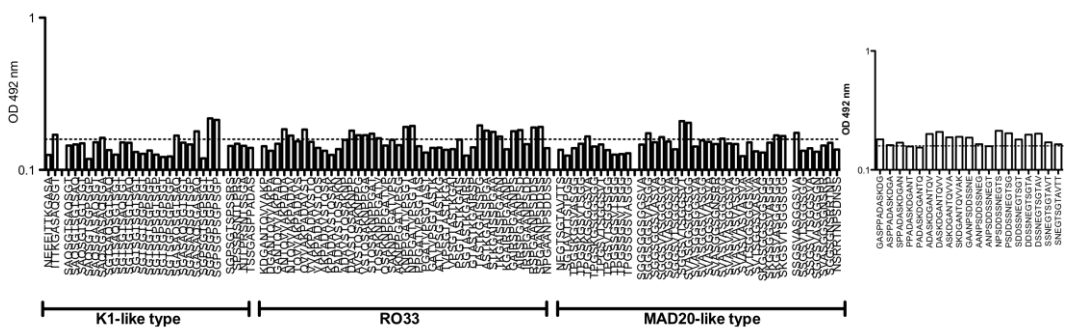
A)



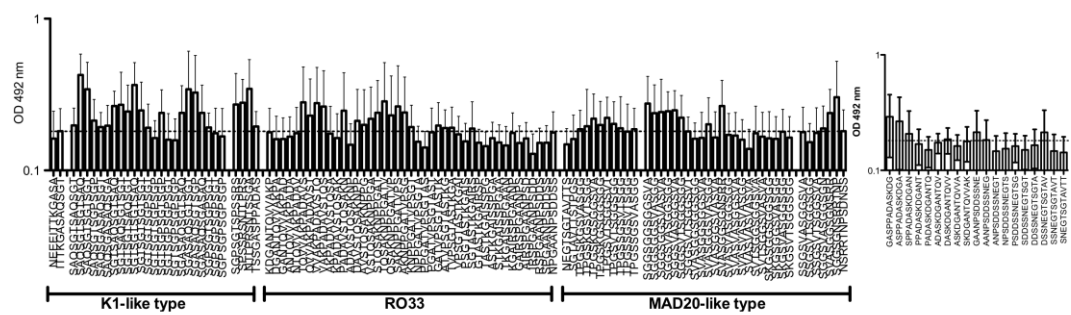
B)



C)



D)



### 3. Discussion

#### 3.1 Properties and production of the MSP-1 hybrid

MSP-1 hybrid was codon-optimised construct containing the sequence and antigenic polymorphism present within the majority of MSP-1 Block 2 alleles found within the *P. falciparum* parasite population. It contains all polymorphic Block 2 sequences known to be recognised by human and mouse antibodies elicited by natural infection and immunisation respectively. Apart from MSP-1 Block 2 sequences, this antigen also contains T-cell epitopes from MSP-1 Block 1 region, which have been demonstrated to be recognised by T cells from humans exposed to malaria and essential for immunogenicity of this antigen in pre-clinical testing (Parra et al. 2000; Quakyi et al. 1994; Cowan et al. 2011).

As an IUP, the MSP-1 hybrid antigen is thermostable because it has a high frequency of small, polar, hydrophilic amino acids and lacks a hydrophobic core. The MSP-1 hybrid exhibits several properties of IUPs, as observed in this study, where the MSP-1 hybrid migrated on SDS-PAGE as a protein of around twice its actual molecular weight, because IUPs are known to bind poorly to SDS and therefore cause the anomalous migration of these proteins on SDS-PAGE gels (Tompa 2002). The hydrophilic amino acid-rich sequence also made this protein antigen bind poorly to Coomassie Blue dye, which binds aliphatic amino acid residues (Tompa 2002).

The MSP-1 hybrid was easily expressed in the heterologous host *E.coli* with high yield in laboratory flasks and in a bioreactor using modified Luria Bertani media, and could be easily purified by the simple three-step protocols that have been developed during this work. This is mainly due to the particularly unstructured (IUP) properties of the protein, its lack of disulphide bonds and its thermostability. Codon optimisation of the MSP-1 hybrid construct also enabled even higher-level expression than has been seen with non-optimised Block 2 proteins (Cavanagh & McBride 1997). The heat stability of the protein not only made this antigen easy to handle during purification steps, but also allowed it to be partially purified by high temperature treatment. In addition, refolding steps are not required for the MSP-1 hybrid.

In this project, the lab scale and bioprocess scale protocols developed have established ideal conditions for cell growth and are suitable for expression of the MSP-1 hybrid protein as a soluble intact protein on a large scale. The DNA sequence of the MSP-1 hybrid plasmid in the starting culture and the harvested bioreactor culture were checked by DNA sequencing and found to be identical, suggesting that this DNA sequence is stable during the period of large scale protein expression, which is substantially longer than that of the original flask-scale protocol. Cycles of freezing and thawing proved to be the most

suitable method for lysing host cells. In a previously attempted protocol, a French press, which is commonly used to process large-scale cell cultures, was used. Although more of the soluble MSP-1 hybrid could be harvested using this method, the sample contained more proteins and biomaterial contaminants as estimated when lysates were run on SDS-PAGE. Downstream purification steps were also more problematic and less efficient, including the heating step. It provided a partially purified sample that contained more contaminants than the heat-purified sample prepared from the freeze-thaw process. This less pure sample from the French press also created additional difficulties in the development of the anion exchange chromatography step, resulting in lower yield and a less pure end product. The final purification protocol for the MSP-1 hybrid is simple, requires relatively unsophisticated equipment and chemicals and is easily scalable. The heating step is a very efficient purification step for small-scale as well as large-scale purification. Heating excluded the majority of host cell contaminants without significant loss of the MSP-1 hybrid product. More importantly, this process did not damage the primary structure of the MSP-1 hybrid. Anion exchange chromatography, which is used as the final step of purification, can be adapted to accommodate larger sample sizes, and a scaled-up protocol was easily developed based on the small-scale purification method. The dialysis step, which is performed before the chromatography step, is time consuming. Nevertheless, it proved to be essential for the efficiency of the chromatography step. This is because the MSP-1 hybrid binds very weakly to the anion exchange column and can be lost with other contaminants if the ionic strength of the sample is too high.

Endotoxin levels in the final purified product were assayed by Lonza, using the LAL kinetic chromogenic technique. The MSP-1 hybrid product was shown to have  $72.1 \text{ EU mL}^{-1}$  in the eluted fractions from the anion exchange step and  $8.50 \text{ EU mL}^{-1}$  in the final dialysed product. One limitation of this assay is that the sample has to be soluble, however this was not a problem for the MSP-1 hybrid. The final MSP-1 hybrid product was reported to have less endotoxin than the protein after the last anion exchange chromatography step, given that the concentration of MSP-1 hybrid in the two samples was the same. Since endotoxins are heat-resistant, and therefore not destroyed during the purification process, it is possible that the dialysis step was responsible for eliminating endotoxins from the final sample during buffer exchange, as endotoxins may bind to dialysis tubing membrane. Endotoxins are lipopolysaccharide (LPS) complexes of gram-negative bacterial cell walls, which are common contaminants in bacterial based biological products and should always be kept at a low level in vaccine production. They are pyrogens, which can mediate pathological disorders (Blatteis et al. 2000). Clinically, endotoxins have been identified as agonists of

TLR-4, and can trigger the signal cascade for cells such as macrophages to secrete pro-inflammatory cytokines, leading to endotoxic shock. As little as  $4\text{ng kg}^{-1}$  of endotoxin (approximately  $40\text{-}80\text{ EU mL}^{-1}$ ), can precipitate inflammatory stimuli such as TNF- $\alpha$  and IL-6. This, sequentially, can cause physiological changes in human blood such as hyperactivation of leucocytes (Wilson et al. 2001). For drugs and vaccines quality control, regulatory agencies such as the United States Pharmacopeia (USP) and the European Medicines Agency (EMA) set strict guideline for acceptable pyrogen contamination levels in final products. Although there is no clear recommendation on acceptable endotoxin level, previous data of the endotoxin levels in commercial vaccines can give a rough idea of what could be acceptable endotoxin levels. The recommended endotoxin levels of recombinant subunit vaccines as well as polysaccharide vaccines should not exceed  $20\text{EU mL}^{-1}$  in animal studies, since high purity is expected from this kind of vaccine. Guidelines for acceptable endotoxin level in vaccines may also differ according to each animal study and licensed vaccine. The readout of immune responses is critical data in evaluating the antigen in vaccine studies. Therefore in general, the level of endotoxin should be as low as possible to reduce immune responses to endotoxin which might interfere with immune responses induced by the antigen itself (Brito & M. Singh 2010). With this purification process, we have achieved endotoxin levels close to those needed for a viable human vaccine.

## **3.2 The potential of MSP-1-hybrid as vaccine**

### **3.2.1 Antigenicity**

The MSP-1 hybrid is recognised by serum antibodies and mAbs from animals that have been immunised with Block 2 antigens, as well as serum antibodies from naturally infected Africans. Antibody reactivity in malaria exposed children to the MSP-1 hybrid was as strong as, or stronger than the reactivity observed to individual MSP-1 Block 2 antigens. This confirms that this synthetic protein contains epitopes which are recognised by antibodies from naturally infected humans (Cowan et al. 2011). MSP-1 Block 2 epitopes are the target of antibodies that are significantly associated with protection against clinical malaria (Conway et al. 2000; Polley et al. 2003; Mawili-Mboumba et al. 2003; Cavanagh et al. 2004). In this project I showed that in a cohort of Ghanaian children there was a statistically significant correlation between possession of antibodies against the MSP-1 hybrid and reduced risk of experiencing clinical malaria symptoms in the following transmission season. Although the statistical significance of this association was confounded by age, it remained close to significance. This is because the tested sera were collected from



a small cohort of 278 children with a wide age range (3-15 years) where Block 2 antibody positivity frequency is relatively low (Cavanagh et al. 2004; Cowan et al. 2011).

### **3.2.2 Immunogenicity**

Despite its relatively small size (31 kDa), MSP-1 hybrid elicited polyclonal antibodies reactive with *P. falciparum* parasites of all three Block 2 serotypes in mice, rabbits, and Rhesus monkeys. Sera from animals immunized with this antigen display a broad reactivity with all three MSP-1 Block 2 serotypes in both IFA, ELISA and peptide mapping. In this study, adjuvants licensed for human use were tested in mice and found to be capable of raising specific antibodies against the MSP-1 hybrid. Montanide adjuvants were expected to raise strong immune responses, since they share some physical characteristics with incomplete Freund's adjuvant. However, some studies reported that Montanide ISA-51 and ISA-720 were the cause of sterile abscesses in recent human trials (Wu et al. 2008; Roestenberg et al. 2008). Because of this, other human compatible adjuvants should be used. Alhydrogel (Alum) is an adjuvant with a good profile, which has been shown to enhance Th2 responses in animals and humans (Leroux-Roels 2010). Nevertheless, it did not provide the high and consistent antibody titres by comparison with other adjuvants in this study. CpG adjuvant was expected to stimulate B-cells through TLR-9 signalling, but when used together with Alum in this study it did not improve specific antibody titres. Similarly, mice that were immunized with another TLR-9 agonist gave as high a median titre as the group with CpG plus Alum, although the level of reactivity within the group seemed to be more consistent. Agonists of TLR-4, uniquely, are thought to elicit both Th1 and Th2 responses (Duthie et al. 2011), and thus to enhance specific immunity against both intra-erythrocytic malaria parasites and merozoites, which are present outside the red blood cells. Using the TLR-4 agonist, both alone and in combination with Alhydrogel, elicited similar antibody titres to the MSP-1 hybrid in mice. Since high antibody titre is likely to be an important property of any invasion-blocking-erythrocytic-stage vaccine, these two adjuvants are interesting adjuvant candidates which are able to trigger stronger antibody responses. The TLR-7/8 agonist formulated with the MSP-1 hybrid was tested, but this group produced the lowest antibody titre of all the adjuvant groups. However, this experiment was inconclusive since it has been suggested that TLR-8 is non-functional in mice. This makes any extrapolation from mice to humans more difficult (Jurk et al. 2002; Hemmi et al. 2002). The CoVaccine HT is thought to be a good antibody enhancer (Mosca et al. 2008). When immunizing together with the MSP-1 hybrid, it raised specific antibodies as high as those in the group with Alhydrogel™. A similar level of antibody titre was seen in the group with the TLR4 agonist in an oil-in-water emulsion. Of the tested panel of adjuvants, CoVaccine HT

was the most efficacious adjuvant for the MSP-1 hybrid. The CoVaccine HT is an oil-in-water adjuvant consisting of synthetic sucrose fatty acid sulphate ester (SFASE) immobilised in the oil droplets of a submicron emulsion of squalene in water. It has been shown in a number of studies to provide TLR-4 signalling (Bodewes et. al, 2010; Bodewes et al. 2009). Statistical analysis of the data from each immunisation group showed that the antibody levels in the CoVaccine HT group were as high as the groups containing aqueous TLR4 agonist (both with and without Alum) and higher than the groups immunised with oil-in-water based adjuvants (oil-in-water emulsion and TLR-4 agonist oil-in-water). Using MSP-1 hybrid formulated with CoVaccine HT, MSP-1 hybrid specific polyclonal antibodies were produced at high titres. Antibodies elicited by the MSP-1 hybrid formulated with CoVaccine HT recognised MSP-1 Block 2 protein antigens of all three serotypes and recognised the surface MSP-1 antigen on all five strains of *P. falciparum* parasite tested. In addition, peptide epitope mapping confirmed that the MSP-1 hybrid elicits antibodies against epitopes in both repetitive regions and non-repetitive serotype-specific regions of all three Block 2 serotypes. The experiments in outbred mice (MF1 mice) and inbred mice (BALB/c and C57/BL6) showed that similar titres of specific antibody to all three serotypes could be elicited in each strain of mouse. The studies in rabbits and monkeys showed that MSP-1 hybrid was immunogenic in larger, outbred animals including non-human primates, and that boosting of antibody responses to the MSP-1 hybrid protein occurred with each of three immunisations.

Interestingly, intraperitoneal immunisation of animals with the MSP-1 hybrid proved to be much less efficient than when animals were immunised subcutaneously. An explanation of this result has not yet been experimentally determined, but it is possible that the protein may be susceptible to the degradative environment in the peritoneum, due to the abundance of macrophages in this cavity. Secondly, the intrinsically unstructured nature of the antigen could make it more susceptible to digestion by proteases. Both factors could result in lower immunogenicity and therefore lower antibody titres. By contrast the MSP-1 hybrid elicited much higher antibody titres when delivered subcutaneously. It is possible that the MSP-1 hybrid is taken up by dendritic cells in this environment and subsequently processed in lymphoid tissue to stimulate antibody production.

### **3.2.3 Stability**

An ideal blood stage malaria vaccine would be highly stable and require minimal cold chain costs. This study has shown that the MSP-1 hybrid is stable at ambient temperatures (at 25°C and 37°C) over incubation periods of up to 60 days without any significant degradation or decrease in antigenicity or immunogenicity. This is a significant

factor for vaccine transport as well as storage at sites where the refrigeration is sometimes difficult or unreliable.

The MSP-1 hybrid was also shown to be very stable in combination with the adjuvant CoVaccine HT: it can be maintained at ambient temperature for around 20 days without significant change in protein concentration and/or primary structure. The protocol used in this study to extract the protein antigen from vaccine was initially developed for characterisation of vaccine antigen from water-in-oil adjuvants, such as Montanide ISA720. However, it was also efficient in extracting MSP-1 hybrid from an oil-in-water adjuvant formulation such as CoVaccine HT. This vaccine formulation has not yet been tested for immunogenicity in animals following this long incubation period. Future studies on the potency, stability and immunogenicity of this vaccine formulation should be completed to assess the viability of this combination.

#### **3.2.4 Immune responses raised by immunisation with MSP-1-hybrid**

Sera from these immunisations showed antibody specificity for all Block 2 antigens tested and reactivity with *P. falciparum* parasites of five different strains, representing all three Block 2 serotypes. Using CoVaccine HT, all four isotypes of IgG were detected in mouse sera. These included the cytophilic antibody subclasses IgG2a and IgG2b. These two antibody subclasses in mice are counterparts of the human IgG1 and IgG3 according to their similarities in biological and functional activities. It has been reported that they share functions in fixing complement and binding protein antigens (Carvalho et al. 2002; M. R. Clark 1997). They have also been reported to be involved in opsonisation which mediates phagocytosis by macrophages (Stevenson & Riley 2004). In humans, the two cytophilic subclasses are the most abundant antibody isotypes in individuals who are protected from malaria (Bouharoun-Tayoun & Druilhe 1992). IgG1 and especially IgG3 are also reported to be the most effective isotypes for antibody-dependent cellular inhibition (ADCI) and immune phagocytosis assays (Bouharoun-Tayoun et al. 1995; Groux et al. 1990). IgG1 and IgG3 anti-MSP-1 hybrid antibodies were also detected in immunised mice. Mouse IgG1 was considered to be similar to human IgG4 in its ability to bind to mast cells, while mouse IgG3 and human IgG2 share the ability to recognise carbohydrate epitopes (Hussain et al. 1995). However, there has so far been no evidence that these two antibody isotypes correlate with protection from malaria (Bouharoun-Tayoun & Druilhe 1992; Rzepczyk et al., 1997; R. Taylor et al. 1998; Oeuvray et al. 1994).

An important role for antibodies against MSP-1 Block 2 in protection from malaria has been suggested by a number of studies. However, there has yet to be evidence of any parasite inhibitory effect of these antibodies in *in vitro* GIA, either against individual Block

2 serotypes or by antibodies against the MSP-1 hybrid (Valero et al. 1996; Cowan et al. 2011). However, human antibodies to Block 2 have shown activity in parasite inhibition in the presence of monocytes in the ADCI assay (Galamo et al. 2009), in a manner similar to several other vaccine candidates such as MSP-2 and GLURP (Theisen et al. 1998; Stubbs et al. 2011).

In this project formulations of the MSP-1 hybrid with adjuvant were expected to induce high antibody titres to all three serotypes of the MSP-1 Block 2. Experiments were designed to measure and compare levels of specific antibodies elicited by formulation with different adjuvants, and their IgG subclass distribution. However, different adjuvants may trigger other immune responses that may link to protective immunity. For example, this study showed that specific antibodies could be produced by immunisation of the MSP-1 hybrid with Alum. However, in other studies, Alum has been reported not to have special potential in the boosting of the Th1 and cytotoxic T-lymphocyte (CTL) responses, which are thought to be important in the controlling of intracellular pathogens such as malaria parasites (Coler et al. 2009). Further study on cytokine production, B-cell differentiation and other immune responses to these immunizations will provide more informative data on the mechanisms by which these adjuvants work, which hopefully will help in assessment of these adjuvants' potential as well as allow us to learn more about immune responses to vaccination.

One of the goals of Malaria Vaccine Technology Roadmap is to “pursue multi-antigen, multi-stage, and attenuated whole-parasite vaccine approaches” ([www.malariavaccine.org/files/Malaria\\_Vaccine\\_TRM\\_Final\\_000.pdf](http://www.malariavaccine.org/files/Malaria_Vaccine_TRM_Final_000.pdf) -page 4). This is due to the fact that blood stage malaria parasite antigens are often polymorphic and using of mono-allelic vaccines have been shown to be ineffective (Ogutu et al. 2009) or effective against only homologous parasite strains or serotypes (Flück et al. 2004; Genton et al. 2002). Therefore, the vaccine targeting multiple antigens and/or multiple stages of parasite lifecycle is needed to be developed. Instead of focusing on the conserved domains of candidate antigens, the MSP-1 hybrid antigen overcomes the polymorphism issue by design. As a multimeric vaccine constructs, it is strongly immunogenic in all three species of tested animals, including non-human primates, able to elicit immune responses that recognize multiple parasite strains, and contains a target of antibody-dependent cellular parasite inhibition activity *in vitro* (Galamo et al. 2009). These promising evidence and its unique biophysical properties of the protein antigen support the further development of this blood stage (and theoretically liver stage (Szarfman et al. 1988; Krzych et al. 1995)) vaccine based on MSP-1 Block 2. A vaccine that target the extensive antigenic polymorphism and multiple

stages of lifecycle of *P. falciparum* could essentially support the goals of the Global Malaria Action Plan.

## Chapter 4: MSP-3.3C

### 1. Introduction

Characteristics of the MSP- hybrid IUP, described in an earlier chapter in this thesis, encouraged us to speculate that other IUPs in the *Plasmodium* genome might be suitable candidates for a vaccine against malaria. Using the available genomic data and bioinformatic tools, we looked for putative parasite surface proteins that contained unstructured regions. One of the IUP containing proteins identified was a member of the MSP 3/6 family, expressed by gene PF10\_0347, previously known as H101 (Pearce et al. 2005). This protein family was of interest to us, as this region of the parasite genome, including GLURP and LSA-1, is specific to *Plasmodium falciparum*. Since the beginning of my work on MSP-3.3, the gene products of the MSP-3/6 family have subsequently been proposed as a possible family of target molecules which merit development as malaria vaccines (Singh et al. 2009; Bang et al. 2011). The originally identified prototype member of the MSP-3/6 family, MSP-3 (now known as MSP-3.1, (Singh et al. 2009) was predicted to have unstructured regions (Burgess et al. 2005) and some members of this family are already being investigated as potential vaccine candidates (Oouvray et al. 1994; Druilhe et al. 2005; Sirima et al. 2011; Hisaeda et al. 2002). We decided to investigate and characterise the intrinsically unstructured part of the protein expressed by gene PF10\_0347 to see whether another parasite IUP, from a conserved, *P. falciparum*-specific gene family would have similar beneficial vaccine properties of ease of expression, thermostability and potential immunogenicity to that of the much more polymorphic IUP, the MSP-1 hybrid antigen (Cowan et al. 2011).

Many proteins of malaria parasites contain extensive regions of so-called 'low complexity' sequence (Gardner et al. 2002; Pizzi & Frontali 2001; Brocchieri 2001). Some are encoded by genes that lack orthologues in other organisms, which causes difficulty in predicting their structure and function from the data of homologous proteins. So far little is known about the structure or function of the newly identified protein encoded by PF10\_0347, MSP-3.3. The MSP-3.3 protein, like other MSP-3/6 family proteins, has been reported to be expressed during the asexual stage of the parasite as a merozoite surface protein (Singh et al. 2009; Pearce et al. 2005). While any functional or structural data on MSP-3.3 remain to be generated, it has been suggested that MSP-3 family members may have an important role in the biology of *P. falciparum* (Singh et al. 2009). It has been reported that MSP-3.3 is recognized by human antibodies elicited by natural infection (Pearce et al. 2005) and that human IgG to the C-terminal region of this protein has

monocyte-dependent parasite inhibition effects in ADCI assays *in vitro* (Demanga et al. 2010).

In this part of the project the gene sequence of PF10\_0347 was analysed and a non-tagged recombinant protein derived from the C-terminal unstructured region of PF10\_0347 gene (hereafter called MSP-3.3C in this thesis) was expressed and purified as a potential malaria vaccine candidate. This protein has been investigated for its biochemical characteristics, and its interactions with biomolecules of the immune system, in the hope that it can be further developed as a novel malaria vaccine.

## **2. Materials and results**

### **2.1 Sequence analysis**

#### **2.1.1 Gene sequence of the PF10\_0347**

The gene sequence of PF10\_0347 3D7 and the single nucleotide polymorphisms between different isolates were available in the Plasmodium genome resource, PlasmoDB (<http://plasmodb.org>). Sequence homologies between PF10\_0347 and other MSP-3 family members were determined using BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and ClustalW (Larkin et al. 2007; S. Singh et al. 2009). The Simple Modular Architecture Research Tool (SMART) (<http://smart.embl-heidelberg.de/>) and ProSite (<http://expasy.ch/prosite>) were used to look for known protein domain sequence homologies within this amino acid sequence.

The PF10\_0347 gene is located on chromosome 10, in a region of the genome that is unique to *P. falciparum*, which contains all the other MSP-3 family members in one contiguous. MSP-3.3 shows little variation in sequence between parasite isolates. The PlasmoDB online malaria genome database describes only four non-synonymous single nucleotide polymorphisms (SNPs) in PF10\_0347 and only two of these are within the C-terminal-unstructured region. Of these two, one is located at residue 282 where Adenine is changed into Thymine, causing an amino acid change from Glutamine (Q) to Proline (P), the other is located at residue 349, where Thymine is mutated into Adenine resulting in change of Asparagine (N) into Lysine (K) (Figure 4.1).

MKKIVNIIFY	ILYLYIYKRN	LVQENENVNKS	NLRKGLSTNN	SENGIKSLKD
EDEHINIIGD	DFSAFSYGGY	PIYETTGSLG	TGVESVKAID	GESG/ATSMDSK
PKENKISTEP	GADQVSIQGLV	NESDSSLEND	KKKKENVKKE	MLGTEKEGSP
DSHDSSKEKL	NLNDNSKWSL	FLKNI/MVTFGG	FGPTVVHDVS	DTLSDISKDE
VTQKTTKDIG	STLLDFFLPL	PTKNTNTYEK	KNENKNVSNV	DSKTKSNEKG
RPPTYSPILD	DGIEFSGGLY	FNEKKSTEEN	KQ/PKNVLESVN	LTSWDKEDIV
KENEDVKDEK	DEDDEEEEEK	YENEIIKQPE	DILDEEEVLE	EEILEENKN/KD
TVDTSDLEKK	NIPDLSNDNN	YYSLIYKNYK	DNDKSEKTAQ	TLITALISLL
NGKNELDATI	RRLKHRFMEF	FTYN		

Figure 4.1 Amino acid sequence of *P. falciparum* (3D7A) PF10\_0347. The shaded area represents the amino acid sequence of the cloned unstructured region of MSP-3.3, hereafter referred to as MSP-3.3C. Coloured letters represent the identified non-synonymous SNPs (Data from PlasmoDB).

### 2.1.2 Predicted protein domains and homologies to other *P.falciparum* proteins

The PF10\_0347 gene is present only in *P.falciparum* and no true orthologue has been found in any other *Plasmodium* species, based on sequence alignments. Bioinformatic analysis shows that the derived amino acid sequence contains several different domains. MSP-3.3 contains a very short low complexity domain at the N-terminal end and a SPAM domain (secreted polymorphic antigen associated with merozoites) in the C-terminal half. Other domains such as a leucine zipper domain and other low complexity domains are also present. These domains and regions, such as the Glutamic acid-rich domain and the Glycine-rich domain that were identified by us and by Singh *et al.* (Singh *et al.* 2009) are shown in Figure 4.2.

ClustalW was used to produce a multiple alignment of the MSP-3-like ORFs namely, MSP-3 (recently redesignated as MSP-3.1), MSP-6 (MSP-3.2), PF10\_0347, (MSP-3.3), MSPDBL1 (MSP-3.4), MSP-11 (MSP-3.7) and MSPDBL2 (MSP-3.8). Other proteins that contain an MSP-3-like ORF have similar N-terminal signal domains to that of MSP-3.3. The MSP-3.3 sequence also shows a signature asparagine-arginine-leucine (NRL) sequence typical of other members of the MSP-3 protein family. Interestingly, a C-terminal region of these proteins is relatively conserved between the proteins (Figure 4.3). MSP-3.3 has one unique feature not found in all other MSP-3 family members, namely a PEXEL motif, usually only found in proteins exported from the intra-erythrocytic parasite into the cytoplasm or onto the surface of the host red blood cell (Marti 2004).



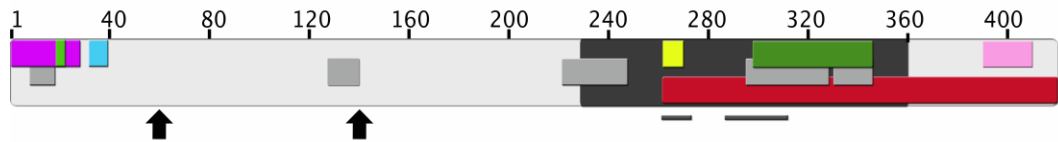


Figure 4.2: Schematic diagram of MSP-3.3 showing predicted structural domains and sequence homologies. N-terminal signal domain (bright pink), PEXEL motif (Bright green), NRL signature sequence of MSP-3 family (Blue), Glycine-rich region (yellow), Glutamic acid-rich region (green), putative leucine-zipper domain (pink), SPAM domain (red), and low complexity domains (grey). Two black arrows indicate enzyme SUB1 cleavage sites. Two black horizontal lines underneath the diagram indicate the regions which have been predicted by Singh *et al.*, to be targets of protective antibodies of MSP-3 (Pearce *et al.* 2005). The dark shaded background area represents the region of PF10\_0347 cloned and expressed in this study (MSP-3.3C).

CLUSTAL O(1.1.0) multiple sequence alignment

```

MSP-3.3      MKKIVNIIFYIL---YLYIYKRNLVQNEENVNK--SNLRKGLSTNNSSENGIKSLKDEDEHI
MSP-3.1      MKSFINITLSLFL-LHLYIYINNVASKEIVKKNLNLNRNAILNNSQIENEE--NV--NT
MSP-3.2      MNKIYNITFLFIL-LNLYINENNFIRNELINEKNHNLNRNGSMYNNDKILSKN--EV--DT
MSP-3.4      MKKIYSIFFSLFI-LNLHIYIKNIKCNLDLINYNDSNLRNGLLNSLDLTNGL-NN-K---
MSP-3.7      MNKFLNIIFYIFLILNFSFFQSNATSKEIQKDEQKNLRNGSSINNNKNIENK--NDNIET
MSP-3.8      MIYILSIVFYIFF-LHIDIYVNIYSTCFVVNEGNPNLRNNIINDELK GKAY-NN-TIDA

*   :  .*  :  ::   :  :           :   ***:   :.       :

```

MSP-3.3 NIIGDDF-----SAF-----SYG  
MSP-3.1 TITGND-----  
MSP-3.2 NIESNE-----NSIHESG  
MSP-3.4 ---DNSF-----ID--SKIEEHENKSYQNKDNNISI-----VGQDVPITSVYS  
MSP-3.7 QYEASEY-----IEKQNDILNMYN  
MSP-3.8 NNQNIIEYNKLNKHNVNSSHISKFSDIMDQEDKGDNENSHDIKFEEKKNINKSLDAESNYG

.

MSP-3.3 GY-----PIYETTGSLGTGVE SVKAIDGESGTS-M-D-----  
MSP-3.1 -----FSGGEFLWPGY-----T-----  
MSP-3.2 -----HKIDGEEVLKANV-----D-----  
MSP-3.4 SKIINANDLEGNSIDDTKGLSVTNSGFDDGSAFGGGLPFSGYSPLQGNHNKCPDENFCKG  
MSP-3.7 DE-----KEKNMNSLDTNVTKN TVIDNSNKFQSIED-----  
MSP-3.8 IN---EISITGN---DSNSDNSQNI FPDGSELAGGI PRSIYTI N-LGFNKCPTTEEICKD

:

MSP-3.3 -----SKPKENKISTEPGADQV SIGLVNESDSSLENDKK  
MSP-3.1 -----EELKAKKAS-----EDA EKAANDAEN-----  
MSP-3.2 -----DITYKKNV-----DDSEIPFS-----  
MSP-3.4 IKNVLS CPPKNSTGRNGDWISVAVKESST TNKGVLVPPRR TKLCLRNIN---KVWHRIKD  
MSP-3.7 -----NNVYNKGIF-----VGTGIKLNDSQT-----  
MSP-3.8 FSNLPQCRKN--VHERNNWLGSSVKNFSSDNKGVLVPPRRQSLCLRITL---QDFRTKKK

:

MSP-3.3 KKENVKKEML-----GTEKE---GSPDS  
MSP-3.1 -----ASKEA  
MSP-3.2 -----  
MSP-3.4 EK-NFKKEEFVKVALGESNALMKHYKEKNLNALTAIKYGFSDMGDIKGTDLIDYQITKNI  
MSP-3.7 -----TS---  
MSP-3.8 KEGDFEKFIIYSYASSEARKLRTIHNNNLEKAHQAIRYSFADIGNIIRGDDMMDTPTSKET  
  
MSP-3.3 HDS-----SKEKLNLDNSKWSDFLKN-----I--  
MSP-3.1 EEA-----AKEAVNLKESDKSYTKAK-----  
MSP-3.2 -----GYDI---QATYQF-----  
MSP-3.4 NRALDKILRN-ETSNDKIKKRVDWWEANKSAFWDAFMCGYKVHIGNKPCPEHDNMDRIPQ  
MSP-3.7 DNY-----KNERYQIDDEKLKYGGSFD-----  
MSP-3.8 ITYLEKVLKIYNENNDKPKDAKKWWTENRHHVWEAMMCGYQSAQKDNQCTGYGNIDDIPO  
  
MSP-3.3 --VTFGGFGPTVV-----HDVSDTLSDISKD-EV-----  
MSP-3.1 -----EA-----C-TA---ASKAKKA-----  
MSP-3.2 -----PS---TSGGNNV-----  
MSP-3.4 YLRWFREWGTYVCSEYKNKFEDVIKLCNIQQFTNQDDSQLLEISKKDKCKEALKHYEEWV  
MSP-3.7 --TIFSGFVNLLT-----PSS-PTQNDGSTGRNV-----  
MSP-3.8 FLRWFREWGTYVCEESEKNMNTLKAVCFPKQPRTEANPALTVHENEMCSSTLKKYEEWY

MSP-3.3 -----T

MSP-3.1 -----V

MSP-3.2 -----I

MSP-3.4 NRRRPEWKGQCDKFEKEKSKYEDTKSITAEKYLKEICSECDCKYKDLDNTPFKEFKDNVTL

MSP-3.7 -----

MSP-3.8 NKRKTEWTEQSIKYNNDKINYTDIKTILSPSEYLLIEKCPECKCTKKNLQDVFELTFDGKAL

MSP-3.3 QKTTKD-----IGSTLL--DFFLPLP---TK-----

MSP-3.1 ETALKA-----KDDAEK--SSKADSISTKTK-----

MSP-3.2 PLPIK-----QSGEN--QYTVTSISGIQK-----

MSP-3.4 LKAVIDNKKNQDSLTTTSLSTSINSVRDSSNLD-----

MSP-3.7 PPPSEP-----NVDTPD--PPTAPAP-----

MSP-3.8 LEKLEKKEESP-----VNSVNALPEPGQITLPDPSLKQTTQENQPVVETPVTTAVI

MSP-3.3 -----NTNT--YE**KKNENKNVSN-VDS-KTKSN**----**E-KGRPPTYSPILDDGI**

MSP-3.1 -----E--YAEKAKNA--YEKAKNAYQKANQAVLK-AKEASSYDYI--LGW

MSP-3.2 -----GANG--LTGATENITQVVQANSETNKNPTSHSN-STTSLNINI--LGW

MSP-3.4 --QRGNITTSQGNH--R-----ATVVQQV-DQTNRLDNVNSVTQRGNINY----NNNL

MSP-3.7 -----VKVPEDAKLSSSPRPEGPRANNRNEN-NQNTDPYNHY--FAW

MSP-3.8 NEHQGQTEPNKGDNNNERENHESNVGSIQEV-NQGSVSEESHK-TIDPSKI----DDRL



```

MSP-3.3      SLNKGKNEIDATIRK LKHRFMEFFTYN
MSP-3.1      GLIKGNNQIDSTLKD LVEELSKYFKNH
MSP-3.2      GLFNEKNEIDSTIN NLVQEMIHLSNN
MSP-3.4      SLFNDNNNLETIFK GLTEDMTDLFQK-
MSP-3.7      NFLQTNQLDPSLKD LENEELTFFLNYY
MSP-3.8      SLLEEGNGSDSTL NSLSKIDITNLFKN-

      ::: * : :. * . : :

```

Figure 4.3: Multiple alignments of MSP-3 like ORFs produced by Clustal Omega. Predicted domains were showed; N-terminal signal domain (Bright Pink), PEXEL motif (Bright green), Signature sequence of MSP-3 family (Blue), Glycine rich motif (yellow), Glutamic acid rich (green), Leucine-zipper domain (Purple).

### 2.1.3 Prediction of unstructured regions in MSP-3.3 and selection of sequence for

For prediction of unstructured regions in MSP-3.3, the derived amino acid sequence of PF10\_0347 from *P. falciparum* (3D7 strain) was analysed for unstructured domains using IUPred (<http://iupred.enzim.hu/>) (Dosztányi et al. 2005). IUPred analysis of the protein expressed by PF10\_0347 gene showed that it was predominantly unstructured; only 41 residues at the C-terminal end are predicted to have any stable secondary structure. In this project, I cloned and expressed part of the C-terminal region of MSP-3.3 that showed high disorder tendency. This spanned the region from nucleotide 1,409,962 to 1,410,358 on *P. falciparum* chromosome 10. This unstructured region contains part of the predicted SPAM domain, the glutamic acid-rich region and several regions of predicted low-complexity. All these domains contain amino acid residues that are also common in intrinsically unstructured protein regions.

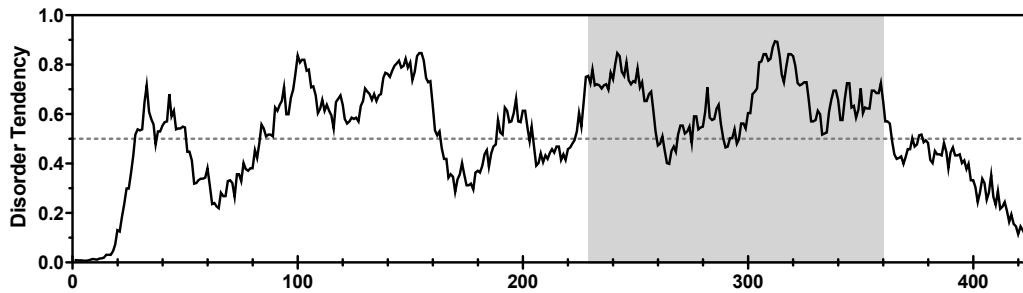


Figure 4.4: Disorder tendency prediction of the putative protein PF10\_0347 (MSP-3.3) according to IUPred (Dosztányi et al. 2005). Numbers on the x-axis represent the position of amino acid residues in MSP-3.3. The shaded area shows the region of MSP-3.3 that was cloned and expressed in this study.

## 2.2 Gene cloning

Since the PF10\_0347 gene has no intron, it can be directly amplified from genomic DNA by PCR and then cloned and transformed into an expression host. A 300bp gene fragment of the unstructured-region of PF10\_0347 was amplified from 3D7a genomic DNA using the forward primer (5' ATCATATGGAGAAGAAAAATGAAAATAAAAATGTAC 3' and the reverse primer 5' GTGATTTAGAAAAGAAATAAGGATCCCAT 3'. Cycling conditions for PCR were performed as follows: initial denaturisation 94°C for 2 minutes, followed by 30 subsequent cycles with 1) denaturing at 94°C for 30 seconds, 2) annealing 50°C for 30 seconds, and 3) primer extension at 72°C for 30 seconds. The resulting PCR product of approx. 300bp was then purified and cloned into pCR4-TOPO using the TOPO TA kit for cloning (Invitrogen, UK), and transformed into *E.coli* TOP10 cells. The insert was sequenced to ensure that it was free of mutations. This fragment of PF10\_0347 was then cleaved from pCR4-TOPO plasmid by sequentially cutting with restriction enzymes *NdeI* and *BamHI* (NEB). The purified fragment was then cloned into pET24a plasmid (Novagen, UK). The inserted fragment was again checked by restriction enzyme analysis and DNA sequencing before a recombinant plasmid stock was made.

## 2.3 Protein expression

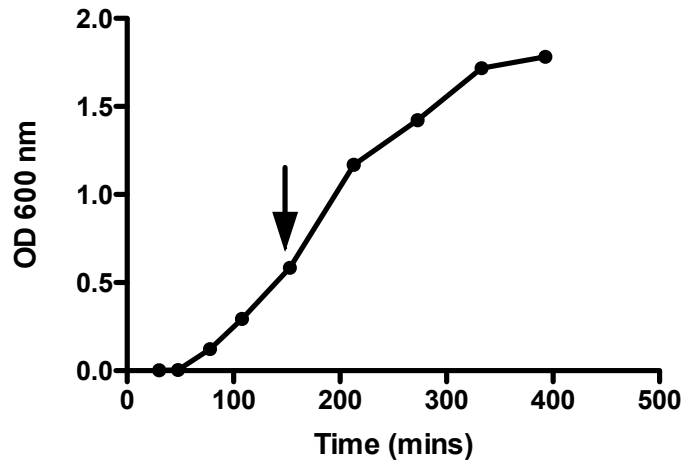
Fresh transformation of recombinant plasmid of MSP-3.3C into the expression host, *E.coli* BLR (DE3) pLysS (Agilent Technologies Ltd, Stockport, UK) was performed for each round of protein expression. Cells were grown in selective media with 1% (w/v) glucose supplementation overnight and diluted the next morning 1:200 in culture media (LB or Terrific broth with 0.5% glucose). Cultures were grown at 37°C in a shaking incubator at

200 rpm. Culture density was monitored until the  $OD_{600nm}$  was 0.6, before Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) was added at a final concentration of 1mM to induce protein expression. Induced cultures were incubated at 30°C for either 4 hours or overnight (12-15 hours) before being harvested by centrifugation at 5,000 G at 4°C for 15 minutes at 4°C. Culture samples were taken at appropriate time points and use to detect the expressed protein and to measure the level of expression by resolution on SDS-PAGE.

MSP-3.3C protein was expressed readily in *E. coli* BL21 (DE3) LysS using standard LB media supplemented with glucose. Cell cultures grew exponentially and reached mid-log phase ( $OD_{600nm} = 0.6$ ) 6 hours after incubation (Figure 4.5 Panel A). Two hours after induction, it could be visualised on SDS-PAGE as a band of just under 28 kDa in size (Figure 4.5 Panel B). Cell cultures were usually harvested 4 hours after induction, yielding around 7 grams of wet cells per litre of culture. Alternatively, cell cultures could be further incubated overnight (16 hours). This longer expression period did not increase biomass of the culture, although a MSP-3.3C band of somewhat higher intensity could be detected on SDS-PAGE (data not shown). Cells cultured in Terrific broth produced similar growth curves. However, the biomass from cultures grown in Terrific broth was slightly lower than those grown in LB.



A)



B)

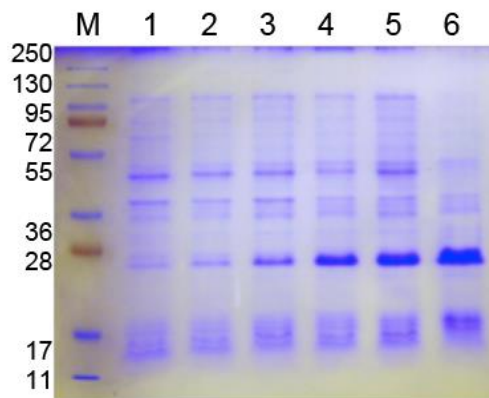


Figure 4.5: A) Growth curve of MSP-3.3C before and after induction. Cell culture grew exponentially until it reached log phase, when the culture was induced (see arrow). B) Amount of MSP-3.3C protein, shown as a protein product just under 28 kDa in size: 1=cell lysate before induction, 2-5 = cell lysate from culture at 1 hour intervals post-induction respectively, 6= cell lysate 16 hours (overnight) after induction.

## 2.4 Protein purification

Cell pellets were resuspended in 25mM Tris-Cl pH8.0 at a ratio of 10ml buffer per 0.5-1g of cells. Three cycles of freeze-thawing were performed to rupture the cells. Soluble cell lysate proteins were then separated from the insoluble fraction by centrifugation at 15,000 G for 15 minutes at 4°C. As the solution became viscous, 1 unit per ml DNase (Benzonase, Novagen) was added and the lysate incubate on ice until viscosity decreased.

### **2.4.1 Heat treatment**

NaCl was then added to the lysate to a final concentration of 250mM, and then incubated in a water-bath at 90°C for 20 minutes, transferred to ice and incubated for further 20 minutes. Denatured proteins were removed by centrifugation at 15,000 G at 4°C for 20 minutes. The heat-treated supernatant protein solution was dialysed extensively against 25mM Tris-Cl pH 8.0 (SnakeSkin Dialysis Tubing, 3.5K MWCO; Thermo Scientific).

High temperature treatment (90°C) provided partially purified MSP-3.3C. This method allowed the denaturation of globular protein contaminants and other non-soluble complexes to be excluded, while MSP-3.3C remained in the soluble fraction. By empirical experimentation, I found that a concentration of 250mM NaCl in the buffer was essential for MSP-3.3C solubility. Without additional salt in the heat treatment buffer, more of the MSP-3.3C protein was observed in the non-soluble fraction and less was recovered as soluble protein (data not shown). After this heat treatment, the MSP-3.3C could be seen as the major soluble protein, which was visualised by Coomassie blue stained SDS-PAGE (Fig 4.6, lane 1).

### **2.4.2 Anion exchange chromatography**

An Anion Exchange Chromatography CantoQ FF column (GE Healthcare, UK) was used as a final polishing step to purify MSP-3.3C protein to homogeneity. The column was equilibrated with five column volumes of binding buffer (25mM Tris-Cl pH8.0) before applying the clarified, heat-treated lysate. The column was then washed with twice the column volume of binding buffer. Protein fractions eluted out at the different concentration of salt were measured using the BCA protein assay (Pierce) and examined for quality and purity on SDS-PAGE and Western Blotting. Details of the BCA Assay, SDS-PAGE and Western blotting are described in Chapter 2 (Materials and Methods section 15). The ImageJ program was used to estimate purity of protein samples.

For the first trial, a continuous gradient of NaCl from 0 to 1M in binding buffer was used in order to optimise the purification. A continuous gradient of NaCl concentration in the buffer ranging from 0 to 1 M was applied to the column continuously during elution step. The eluted fractions were measured for protein concentration at 280 nm absorbance. These eluted samples were also subjected to further analysis. They were measured for protein concentrations by the BCA assay and run on SDS-PAGE gels in order confirm its identity, size and purity. The initial results showed that MSP-3.3C was eluted from anion exchange column by the eluting buffer that contains approximately 300 to 400 mM NaCl (Figure 4.6).

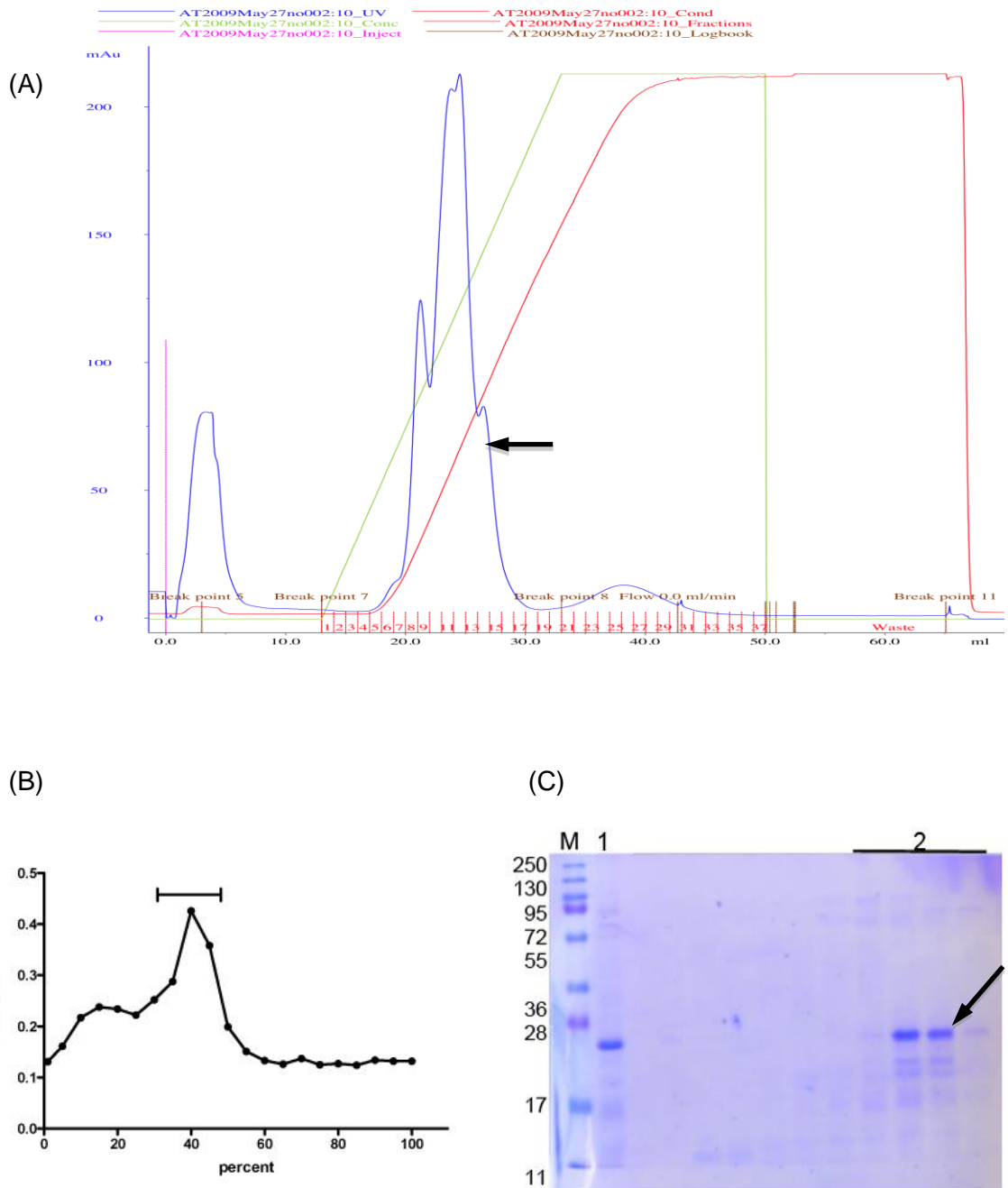


Figure 4.6: (A) Anion exchange chromatography profile of gradient elution for MSP-3.3C; Blue line represents OD at 280 nm, Red line represents conductivity of elution buffer. MSP-3.3C was found in the 3<sup>rd</sup> peak of the eluting sample, which can be collected during application of approximately 30-40% of elution buffer. (B) BCA assay of eluted samples showing that most of the proteins can be collected during 30% elution and 40% elution respectively. (C) SDS-PAGE shows protein samples collected at different points during elution (M=marker, 1=lysate, 2=fractions contain MSP-3.3C product). Black arrow indicates samples containing MSP-3.3C

Based on this information, the protocol was further developed to determine conditions that provided the best yield of MSP-3.3C with the lowest level of contaminants. Using step-wise elution conditions, buffers containing NaCl concentrations ranging between 150 to 500 mM were tested sequentially. Samples from each elution step were again analysed using the same methods as described earlier. The results from each purification were used to adjust the eluting conditions to narrow down the ranges of concentrations required for the greatest elimination of contaminants and the purest MSP-3.3C elution profile. In this study, we developed a successful 3-step-elution method for anion exchange chromatography and purification of MSP-3.3C which involved (1) contaminant elimination with 200mM NaCl, (2) 350mM NaCl elution of the MSP-3.3C protein and (3) a column washing step using 1M NaCl (Figure 4.7).

This optimised protocol was suitable for shaking-flask culture, yielding almost 10 mg of MSP-3.3C per 1 litre of cell culture or approximately 2.45 mg per each gram of wet cells. Using the ImageJ program (Image J <http://rsb.info.nih.gov/ij/index.html>), the purity of MSP-3.3C in each purification step including cell lysate, heated lysate and final purified product from chromatography column was estimated. It was established that the final product is at 92.64% pure. Even though this purification method provided high purity protein, there was a considerable loss of the desired protein during each step of purification (Table 4.1).

Purified MSP-3.3C protein was dialysed extensively against PBS. The protein was concentrated, using a Vivaspin 6 concentrator (Vivaproducts, to a suitable storage concentration (around  $1\text{mg mL}^{-1}$ ) before being filtered through a 5 kDa NMWL Amicon filter (Millipore, UK), heated to 70°C for 20 minutes before storage at -20°C until use.

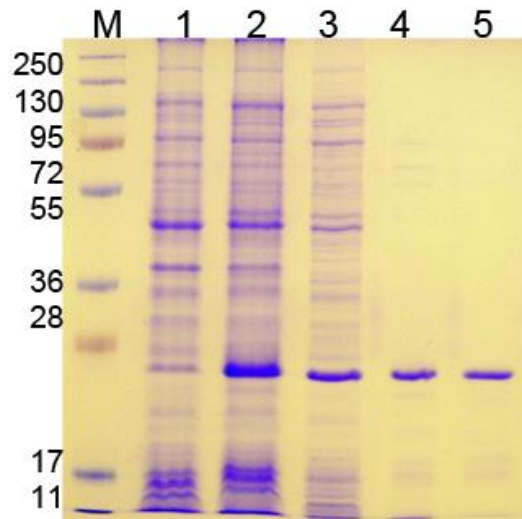


Figure 4.7: Coomassie Blue stained SDS- PAGE from each step of purification of MSP-3.3C. M=marker, 1, culture before induction; 2, culture at 4hours after induction; 3, soluble proteins from cell lysate; 4, heat-treated lysate; 5, anion exchange 350mM elution step

	Total protein (mg)/ litre	Total protein (mg/g wet cells)	% Purity	MSP-3.3(mg/g wet cells)	% Yield recovered from previous purification step
Lysate	130.96	34.92	26.55	9.27	N/A
Heat treatment product	27.04	7.21	76.83	5.54	59.76
AEX product	9.96	2.65	92.64	2.45	44.22

Table 4.1: Amount of MSP-3.3C recovered during each step of purification. Concentrations of protein were measured by BCA assay. Percentage of purity was analysed from intensity of bands on SDS-PAGE and calculated using ImageJ.

## 2.5 Properties of MSP-3.3C

### 2.5.1 Molecular weight and pI

Protein products from each step of purification were sampled and investigated for purity and quantity by SDS-PAGE and BCA protein assay. MSP-3.3C is visualised as ~25 kDa protein on Coomassie Blue stained SDS-PAGE gels (Figure 4.6). Interestingly, this observed molecular weight estimated by migration in SDS-PAGE was significantly different from the results of *in silico* analysis and mass spectrometry.

Molecular weight and pI of MSP-3.3C were predicted by the Compute pI/Mw tool ([http://web.expasy.org/compute\\_pi/](http://web.expasy.org/compute_pi/)) (Gasteiger et al. 2005). Based on the amino acid sequence of this molecule, MSP-3.3C was predicted to have the molecular weight of 15.41 kDa and a theoretical pI of 4.22. Mass spectrometry confirmed this prediction with an observed molecular weight of 15.57 kDa (Figure 4.8).

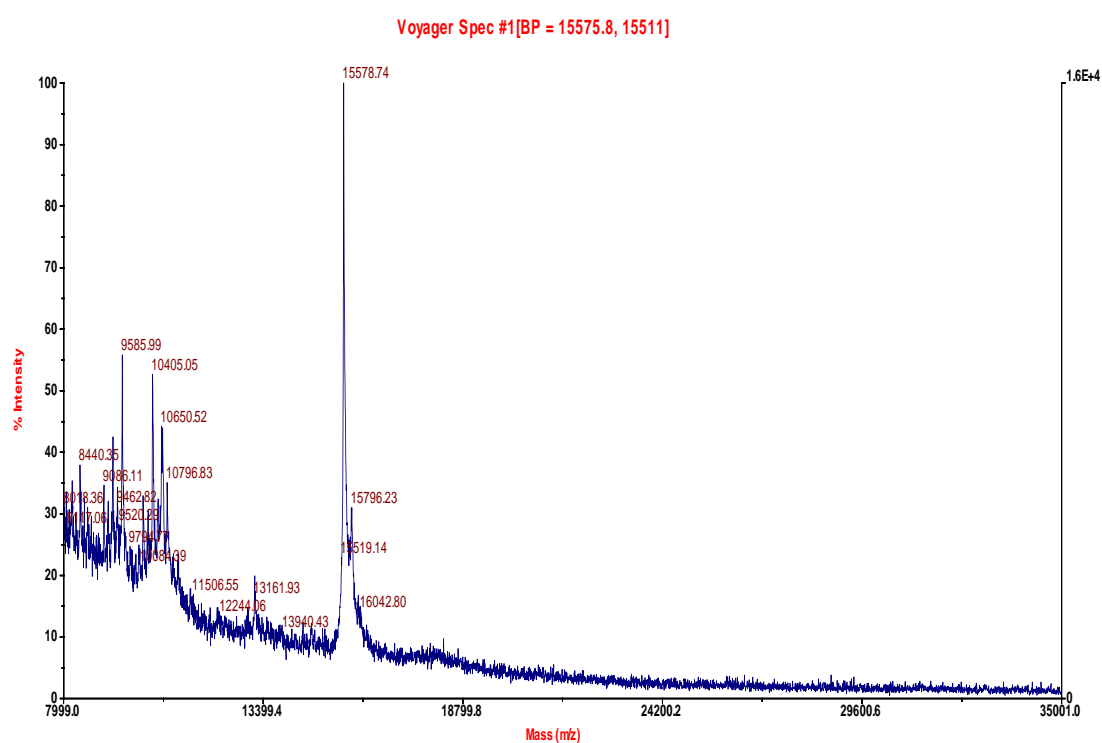


Figure 4.8: Diagram of mass spectrometry result showed that major product of final purified MSP-3.3C. was 15.57 kDa protein. MSP-3.3C protein was prepared in PBS. The protein mass was analysed by ESI (electrospray ionisation) mass spectrometry. X-axis represents a relationship between the mass to charge ratio (m/z) and Y-axis represents signal intensity of the ions (%intensity).

### 2.5.2 Circular Dichroism (CD)

The lack of predicted 3D structure in MSP-3.3C protein was confirmed by circular dichroism. MSP-3.3C was dialysed in 10mM NaH<sub>2</sub>PO<sub>4</sub> pH8.0 to a concentration of 2 mg/ml and the CD spectrum evaluated on a JASCO-810 spectropolarimeter. The measurements were taken over a wavelength range of 185 to 285 nm at 25°C, pH 8.0 with a 1 mm path length cuvette (Centre for Translational and Chemical Biology, University of Edinburgh).

The CD spectra obtained from MSP-3.3C showed a single deep spectral minimum around 200 nm, a Figure close to zero at 220nm, and no maxima observed in the far UV, which corresponds to a protein with a lack of secondary structure (Tompa 2002) (Figure 4.9). The spectrum shown indicates no evidence for the presence of any significant amount of secondary structure, which is typical of a random coil protein conformation.

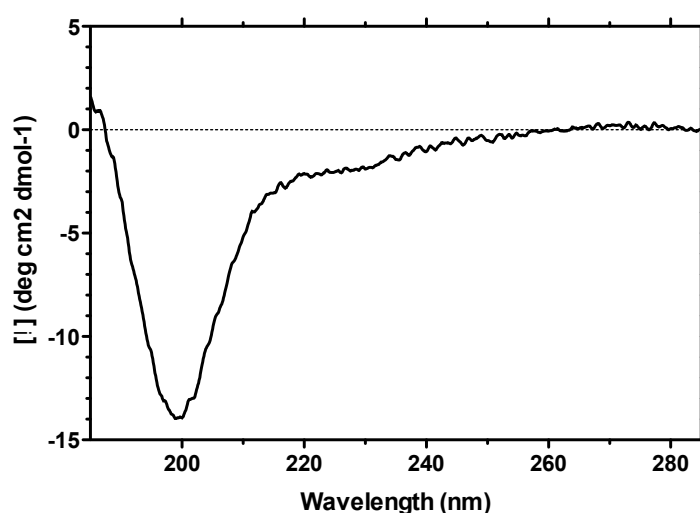


Figure 4.9: Circular dichroism profile of MSP-3.3C showing the deep valley at 220nm, characteristic of unfolded proteins.

### 2.6 Antigenicity of MSP-3.3C

ELISA and IFA protocols used are as described in Chapter 2. (Materials and Methods section 19 and 20). For ELISA, MSP-3.3C was coated onto plates at a concentration of 0.5 µg µl<sup>-1</sup> in coating buffer. Sera of rabbits and mice were tested across a range of doubling dilutions from 1:1000. Human sera were tested at 1:500 dilution.

Human sera from malaria-endemic regions (Sudan, Malawi, Ghana, and Gabon) were used in ELISA to assess the antigenicity of the MSP-3.3C antigen. A panel of 98 sera selected as positive for at least one other merozoite surface proteins (MSP-1 or MSP-2) were used to assess the frequency and strength of recognition of MSP-3.3C in naturally exposed

individuals. Serum IgG reactivity with MSP-3.3C was weak in all sera tested, with ELISA readouts <OD 1.0 in all samples tested at 1:500 dilution. Frequency of antibody positivity was lower than for other conserved antigens such as MSP-1<sub>19</sub>. Of the 98 sera tested, 41 sera were also positive for MSP-3.3C.

Correlation between immune recognition of MSP-3.3C and other parasite surface proteins was also tested. MSP-3.3C-positive sera were also positive for MSP-7 (Spearman's  $Rho=0.4050$ ;  $p<0.0001$ ), and to MSP-1<sub>19</sub> (Spearman's  $Rho=0.5057$ ;  $p<0.0001$ ) (Figure 4.10). However, there was no correlation between antibody responses to MSP-1 Block 2 antigens and MSP-3.3C (Data not shown).

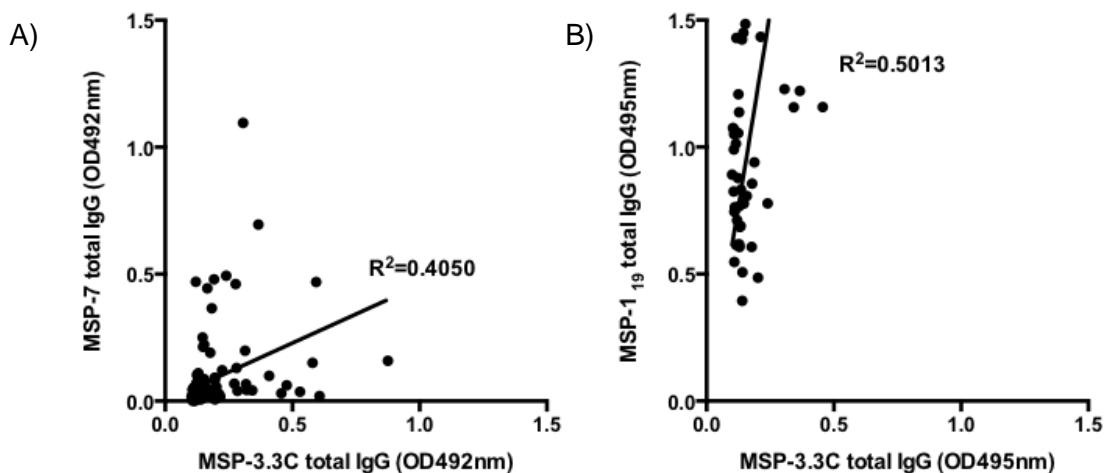


Figure 4.10: Correlation between antibody reactivity of malaria-exposed human sera to MSP-3.3C and reactivity with MSP-7 (Panel A) and MSP-1<sub>19</sub> (Panel B).

Human sera that were antibody positive for MSP-3.3C antigen were further analysed for IgG subclasses. Specific IgG to MSP-3.3C in these sera were biased towards IgG3. There was a correlation between reactivity of IgG1 and IgG3 in ELISA assays (Spearman's  $\rho=0.3450$ ;  $p<0.0001$ ), but this correlation was heavily biased towards the IgG3 subclass (Figure 4.11).



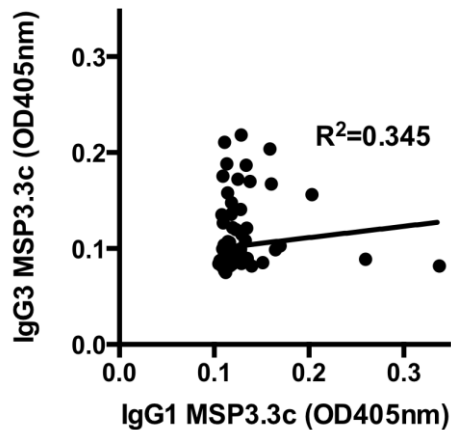


Figure 4.11: Specific IgG3 and IgG1 subclass reactivity in the 98 human sera to MSP-3.3C. Reactivity with MSP-3.3C was positively but weakly correlated. The majority of MSP-3.3C positive antibodies in these selected sera were however biased towards the IgG3 subclass.

## 2.7 Immunogenicity

### 2.7.1 Mice

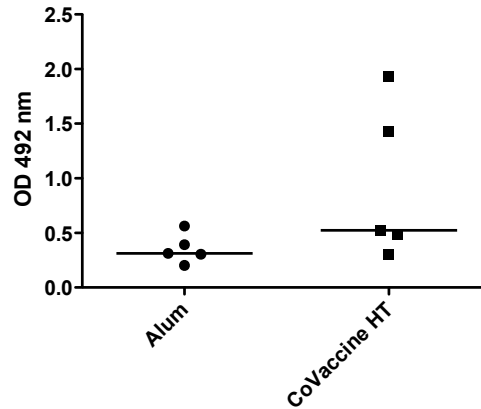
Purified MSP-3.3C was used as an immunogen to test for immunogenicity in mice, in combination with two adjuvants that are licensed for use in humans. Two groups of 5 outbred female MF1 mice (*Mus musculus*) were immunised with the MSP-3.3C protein antigen in combination either with 0.64% Alum (Alhydrogel) or CoVaccine HT (detailed description of these adjuvants can be found in Chapter 2. (Materials and Methods section 12.2). The immunogen was prepared by mixing 20µg of protein antigen with adjuvant at a 1:1 ratio. A total of 3 doses were given subcutaneously at 30 day intervals. Mice were exsanguinated under terminal anaesthesia on day 70.

Results showed that immunisation of this antigen with these two adjuvants yielded specific antibodies that can be detected by ELISA. Sera from only 2 out of 5 mice in the CoVaccine HT group showed distinct reactivity to MSP-3.3C, while sera from all mice in the Alum group showed low reactivity to this antigen. The median ELISA OD for specific antibody responses of the two groups, using the different adjuvants, were very close; 0.313 for the Alum group and 0.5245 for the CoVaccine HT group. There was no statistically significant difference in the ELISA reactivity between these two groups (Mann Whitney test;  $p > 0.05$ ) (Figure 4.12 panel A)

### **2.7.2 Rabbits**

Two rabbits (code numbers 6066 and 6067) were immunized intramuscularly with five doses each of 100 µg of the recombinant MSP-3.3C protein antigen, formulated with Freund's complete/incomplete adjuvant (Biogenes GMBH, Berlin, Germany). Animals were immunized on days 0, 7, 14, 28 and 42. Blood samples were collected for serum on days 0, 8, and 49. Approximately 70 ml of sera was collected from each rabbit at the end of the immunisation schedule. In rabbits, with this stronger adjuvant combination, higher titre antibody responses were observed. Results from ELISA showed that antigen specific titre to MSP-3.3C in each immunised rabbit increased with each immunisation, although reactivity of the sera from rabbit number 6066 (R6066) increased more rapidly than that of rabbit 6067 (R6067). However, the final serum sample from both animals reached OD<sub>492nm</sub> of over 1 in both cases (Figure 4.12B).

A)



B)

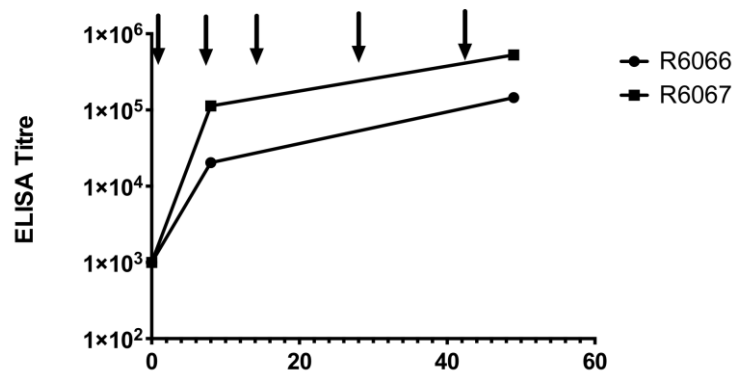


Figure 4.12: ELISA results show reactivity of MSP-3.3C immunised sera. (A) Mice that were immunised with MSP-3.3C in combination with Alum showed lower median of reactivity to the MSP-3.3C antigen comparing with the CoVaccine HT group. (B) Level of rabbit-antisera response to MSP-3.3C had increased though course of immunisation. Both animals were immunised on days 0, 7, 14, 28 and 42 (arrow). Sera were collected three times, on the day 0, 8 and 49.

## 2.8 Purification and Characterisation of Anti-MSP-3.3C antibodies

### 2.8.1 Covalent coupling of MSP-3.3C to an NHS-Sepharose column

MSP-3.3C specific IgG was purified using a NHS-activated Sepharose column, which had been prepared by coupling it with purified MSP-3.3C protein. The column was prepared according to the manufacturer's protocol (GE Healthcare). MSP-3.3C protein at

concentration of  $7\text{mg mL}^{-1}$  in coupling buffer (0.2 M  $\text{NaHCO}_3$ , 0.5 M  $\text{NaCl}$ , pH 8.3) was bound to a 1 mL NHS-activated Sepharose column, incubated at room temperature for 30 minutes, and washed sequentially with Buffer A (0.5M ethanolamine, 0.5M  $\text{NaCl}$ , pH 8.3) and Buffer B (0.1M sodium acetate, 0.5M  $\text{NaCl}$ , pH4) according to the manufacturer's protocol. The column was finally washed with coupling buffer and stored in 0.05 M  $\text{Na}_2\text{HPO}_4$  + 0.1%  $\text{NaN}_3$ , pH 7.0 until use.

### **2.8.2 Antibody purification**

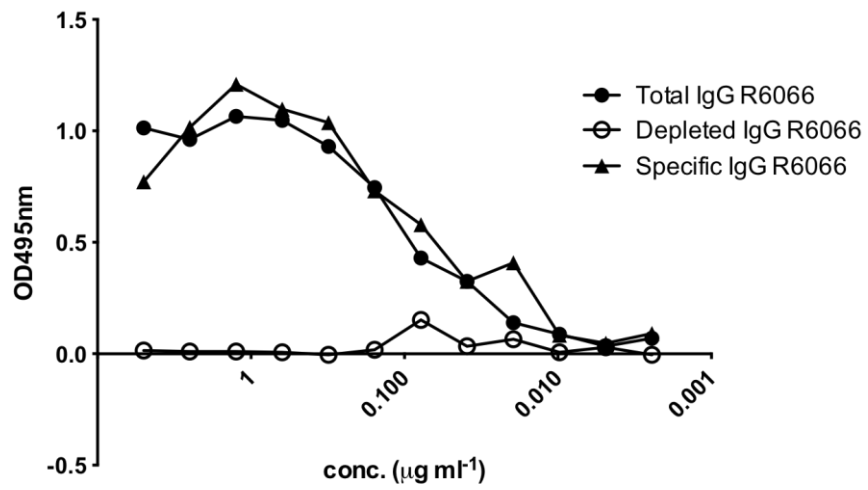
Sera from rabbits R6066 and R6067, immunised with MSP-3.3C (see section 2.7 above) were purified for either total IgG or MSP-3.3C-specific IgG using affinity chromatography protocols described earlier in Materials and Methods section 13.

Total IgG from sera of the rabbits immunised with MSP-3.3 was purified using a Protein G column. Each of ml serum yielded 13 to 14 mg of total IgG. This purified IgG was then further purified for specific MSP-3.3C IgG and total IgG depleted of MSP-3.3C-specific antibodies (hereafter referred to as depleted IgG). An aliquot of 7 ml serum from R6066 yielded 1.5 mg of MSP-3.3C specific IgG and 78 mg of depleted IgG. For R6067, 2mg of MSP-3.3C specific IgG and 96mg of depleted IgG were produced from the same volume of serum.

### **2.8.3 Antibody specificity**

ELISA and IFA were used to confirm the specificity of these IgG preparations for purified MSP-3.3C protein as well as to native MSP-3.3C protein on the surface of fixed parasites. Depleted IgG from both rabbits showed very low reactivity to MSP-3.3C by ELISA and gave negligible fluorescent signals in parasite IFA, whereas total and MSP-3.3C-specific IgG from these two rabbits showed very strong reactivity to both purified protein and native protein on fixed parasites (Figure 4.13 and Figure 1.14).

A)



B)

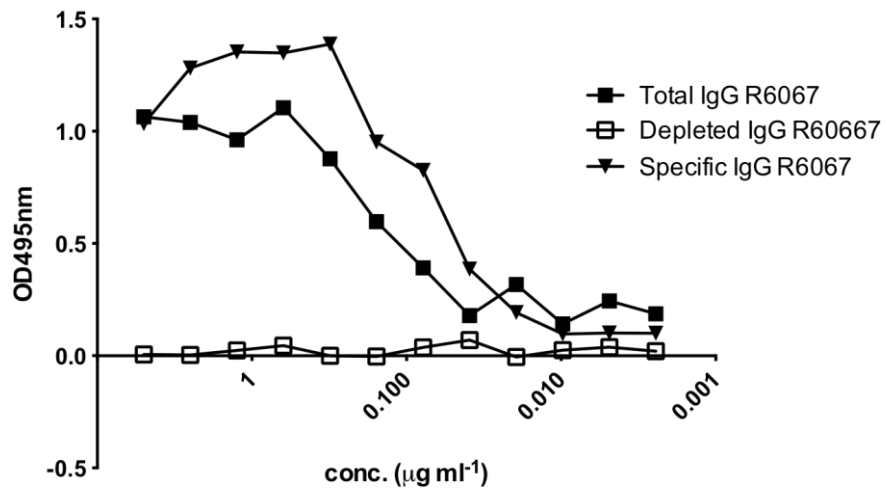


Figure 4.13: ELISA titrations of purified rabbit antibody reactivity with MSP-3.3C protein. Total and MSP-3.3C-specific IgG purified from both rabbits R6066 and R6067 recognised MSP-3.3C protein, while ELISA reactivity of the depleted IgG pools from both rabbits was very low.

Sample	Dilutions					End point concentration (µg/ml)
	1/100	1/200	1/400	1/800	1/1600	
Sera R6066	+++/>++	++/>++	+/+	+/-	-/-	n/a
Sera R6067	+++/>++	++/>++	++/>+	+/-	-/-	n/a
Total IgG R6066	+/+	+/-	-/-			252
Total IgG R6067	+/+	-/-				284
Specific IgG R6066	++/>++	+/-	-/-			4.3
Specific IgG R6067	+++/>++	++/>+	+/-	-/-		2.45
Depleted IgG R6066	-/-	-/-				n/a
Depleted IgG R6067	-/(+/-)	-/-				n/a

Table 4.2: Schizont-rich cultures of *P. falciparum* (Wellcome) were used to make IFA slides to test serum and IgG reactivity to fixed malaria parasites. IFA signals were scored according to the brightness of parasite-specific FITC fluorescence against background, with location of parasites on each slide confirmed by DAPI staining. Each sample dilution was tested in duplicate. IFA signal scores were given as very strong (+++), strong (++) , weak (+), indeterminate (+/-) and no signal (-/-). Concentrations of the tested antibodies were measured by BCA assay and the end point concentrations were calculated.

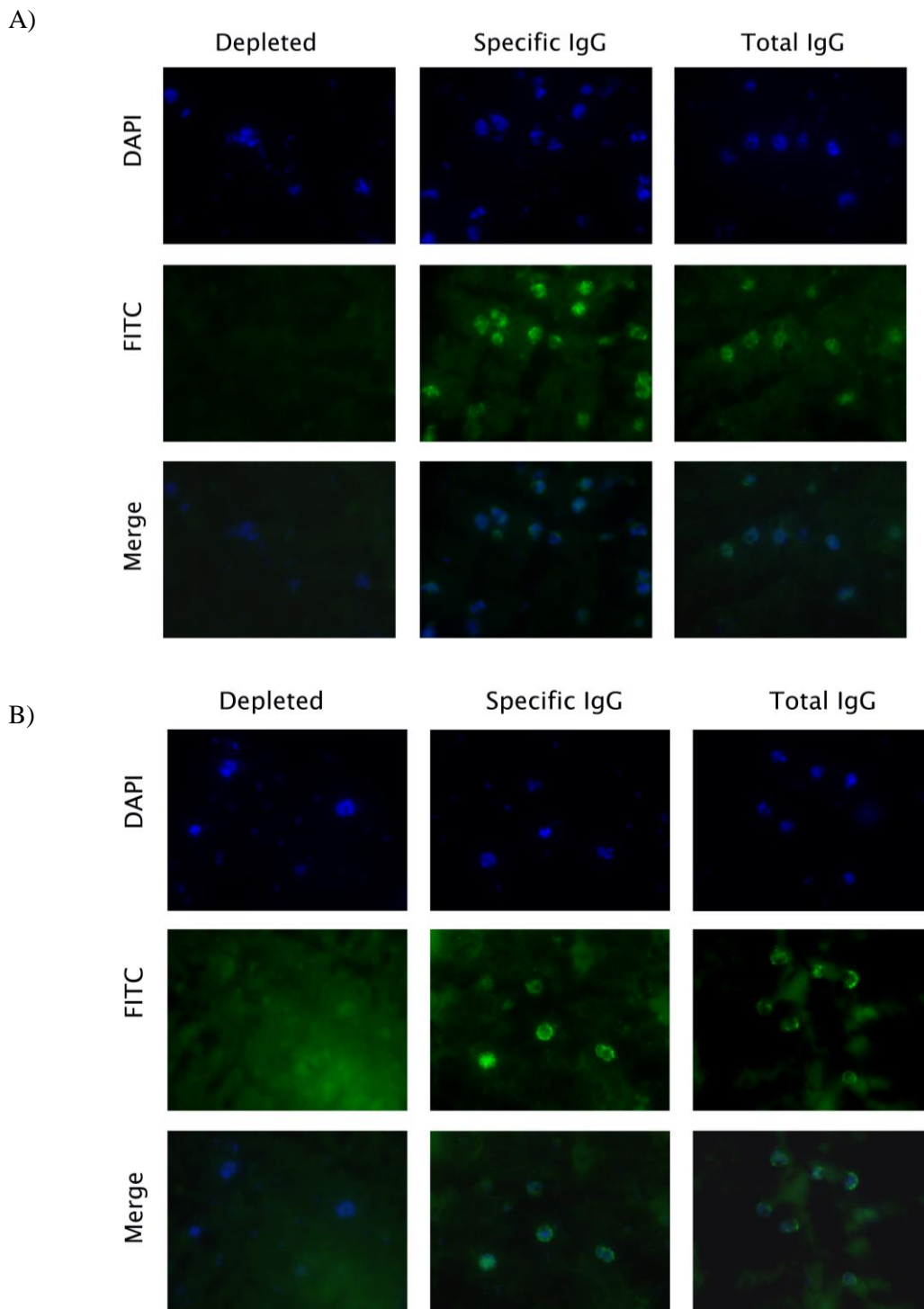


Figure 4.14: IFA reactivity with fixed parasites of purified antibodies from anti-MSP-3.3C rabbit sera. Panel A: MSP-3.3C specific IgG, MSP-3.3C-depleted IgG and total IgG from rabbit R6066 Panel B: rabbit R6067. All the samples showed here were tested at 1/100.

## **2.9 MSP-3.3 stage-specific localisation**

### **2.9.1 Parasite culturing and IFA slide preparation**

Parasites of the Wellcome strain were synchronised at ring stage over several cycles using treatment with 5% sorbitol treatment. Samples were sequentially collected at ring, trophozoite, and schizont stages. In each case the culture was washed twice in RPMI and resuspended in PBS to achieve approximately 5% parasitaemia and 2% haematocrit. 25 µl of this culture was applied to each spot of multispot glass slides (Hendley, Essex) and dried. Slides were wrapped in lint-free tissue and sealed in bags containing silica dessicant and stored at -20°C until use.

### **2.9.2 MSP-3.3 localisation**

Indirect immunofluorescent assays (IFA) showed that the rabbit sera were able to recognise the surface of blood stage parasites. IFA titres of each of the 2 rabbit samples were similar for all blood stages (rings, trophozoite, schizonts and free merozoites). The intensity of the IFA signal was weak at ring stage, and increased as parasites developed in the erythrocyte, with the strongest signal at the schizont stage (Figure 4.15). The target of anti-MSP-3.3C antibodies appeared to be evenly distributed over the surface of the parasite, and could be seen on individual merozoites released from segmenter stage schizonts.



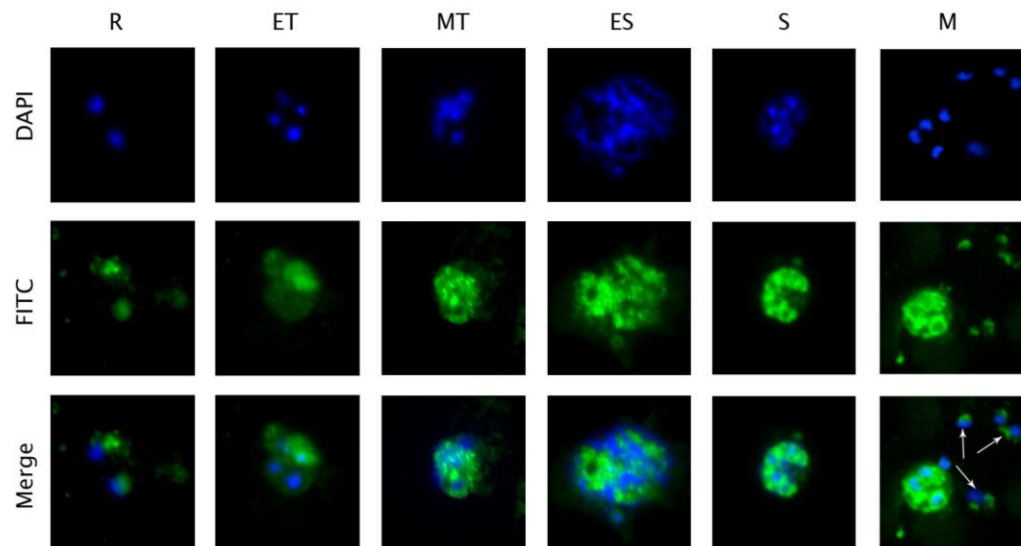


Figure 4.15: IFA with anti MSP-3.3C IgG. MSP-3.3 is present throughout the blood-stage cycle (from left to right: ring (R), early trophozoite (ET), middle stage trophozoite (MT), later stage trophozoite (LT), schizont (S) and free merozoites (M)) and is located on the surface of parasites. IFAs were performed using purified total IgG. DAPI-staining shows the location of parasite DNA.

## 2.10 Growth Inhibition Assay (GIA)

*P. falciparum* (Wellcome strain) parasites were cultured according to the protocol described earlier in Chapter 2. (Materials and Methods section 23). Parasites were synchronized at least twice by sorbitol treatment. GIA assays were started at the mature trophozoite stage. GIA assays were performed in triplicate for each antibody tested. MSP-3.3C total IgG, depleted MSP-3.3C IgG, anti-AMA-1 IgG (positive control) and naïve IgG control (negative control) were titrated from  $6\text{ mg mL}^{-1}$  to  $0.75\text{ mg mL}^{-1}$ , while specific MSP-3.3C IgG was titrated from  $1\text{ mg mL}^{-1}$  to  $0.125\text{ mg mL}^{-1}$ .  $50\mu\text{l}$  of antibody sample at the desired concentration was added to each well in a sterile 96-well plate (Greiner BioOne, UK).  $50\mu\text{l}$  of synchronised trophozoite stage parasite culture (in 2x concentration of normal culture media) were then added to each well to give a final concentration of 0.5% parasitemia and 4% haematocrit and incubated at  $37^{\circ}\text{C}$  in a humidified  $\text{CO}_2$  incubator at 5%  $\text{CO}_2$ . After 40-42 hours, control (i.e. untreated) parasite cultures on each plate were checked to confirm that they had reached trophozoite stage, by making Giemsa-stained thin smears from these wells. Parasite cultures were then harvested for flow cytometry and pLDH

analysis. Thin smears were also taken from each well and Giemsa stained to assess parasite morphology in test and control cultures.

For assessment of pLDH activity assay, 50µl of parasite culture from each well was harvested and washed in ice cold PBS by centrifugation. All but 50µl of the supernatant was removed and then plates were frozen in order to lyse parasites. For the pLDH assay, plates were brought to room temperature, 100µl freshly prepared LDH buffer (100 mM Tris pH 8) containing 40 mM sodium L-lactate, 0.25% Triton X-100, 0.5 µg of 3-acetylpyridine adenine dinucleotide (APAD), 0.1 Unit of Diaphorase and 20 µg of Nitro Blue Tetrazolium) was added into each well. Plates were incubated in the dark at room temperature and read at 650nm (Multiskan Ascent, Labsystems Inc.) after 5, 10, 15, and 30 minutes.

For flow cytometry, 30µl culture from each well was stained with Coriphosphine O at 2µg mL<sup>-1</sup> and parasitaemia measured with a Becton Dickinson LSRII flow cytometer, using 488 nm laser for excitation and FL1 and FL3 channels (525/50nm for green fluorescence and 670/24nm for red fluorescence). Flow cytometry data was analysed using FlowJo software (Tree Star Inc. Ashland, Oregon). Growth inhibition was calculated using the equations:

$$\text{A) pLDH Growth Inhibition (\%)} = 100 - [(A_{650}\text{parasites with test antibody} - A_{650}\text{uninfected red cell control}) / (A_{650}\text{parasites with no antibody} - A_{650}\text{uninfected red cell control})] \times 100$$

$$\text{B) Flow cytometry Growth Inhibition (\%)} = 100 - [(\% \text{parasitaemia with test antibody} - \% \text{uninfected red cell control}) / (\% \text{parasitaemia with no antibody} - \% \text{uninfected red cell control})] \times 100$$

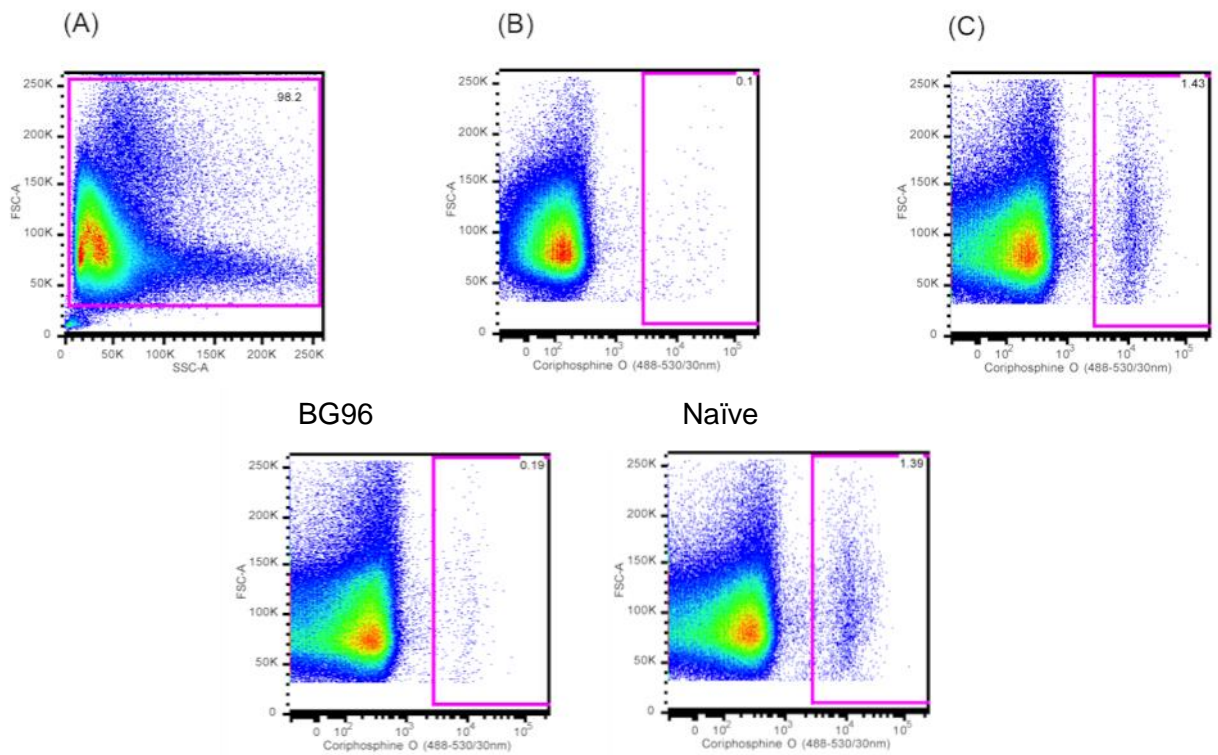


Figure 4.16: Scatter plots from Flow Cytometre. A) The area in which the RBC population was selected is shown in pink box. The number of event is shown as 98.2%. B) Scattergram of *P. falciparum* malaria-negative sample and (Uninfected RBC) C) Scattergram for malaria-positive sample (infected RBC) showing the parasitemia as 1.43%. Scattergram of the positive control and negative control are indicated as BG96 and Naïve respectively.

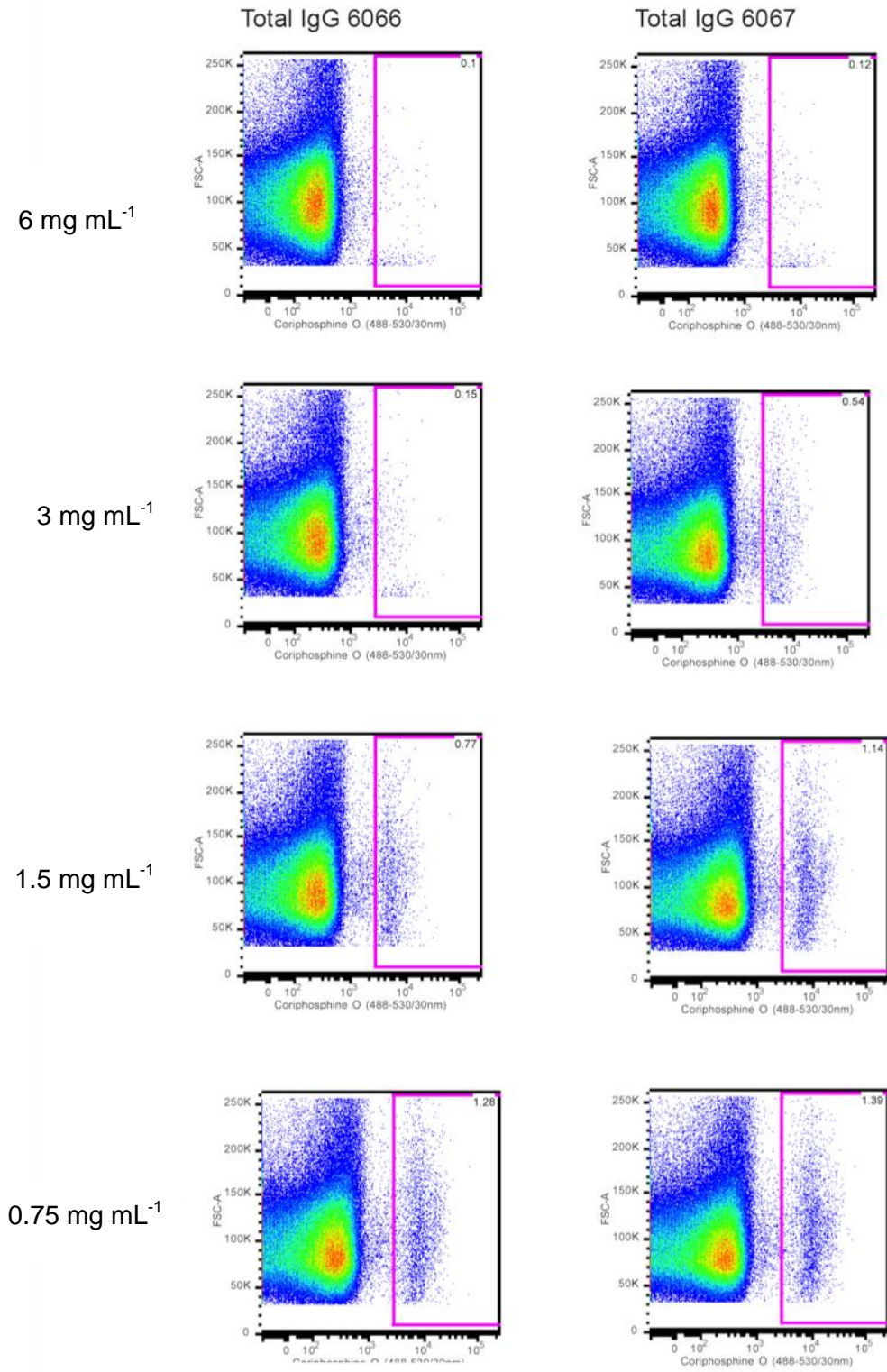
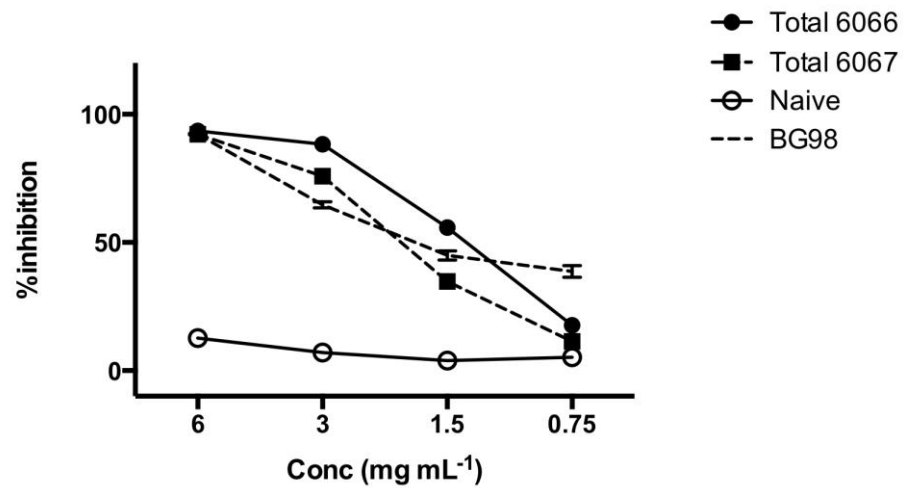


Figure 4.17: *P. falciparum* population when treated with purified anti-MSP-3.3C IgG at the concentration of 6, 3, 1.5, and 0.75 mg ml<sup>-1</sup>. The parasitemias were titrate from 0.1, 0.15, 0.77 and 1.28 respectively (for sample 6066; left panel) and 0.12, 0.54, 1.14 and 1.39 respectively (for sample 6067 (right panel)).

### 2.10.1 *In vitro* parasite inhibition by antibodies to MSP-3.3C

Purified total IgG to MSP-3.3C from two rabbits was tested in *in vitro* parasite growth inhibition assays (GIA). A standard pool of purified rabbit anti-AMA-1 IgG (BG98 standard, BPRC, Netherlands) was used as the positive (i.e. inhibitory) control and purified naïve rabbit IgG as the negative control for all assays. Antibodies from both rabbits showed remarkably potent growth inhibition against Wellcome strain parasites by both measurement of parasite-specific lactate dehydrogenase (pLDH) activity and by flow cytometric analysis of parasitaemia. 90-95% inhibition was achieved when parasite cultures were treated with the total IgG at the highest concentration of 6mg mL<sup>-1</sup>. The effect was titratable down to 0.75 mg mL<sup>-1</sup>, the lowest IgG concentration tested in this assay, where inhibition was 15% (Figure 4.17A). Similar results were also shown in the measurement of pLDH, where 100% inhibition was observed in cultures treated with 6mg mL<sup>-1</sup> total IgG. A titratable effect down to 50% inhibition was observed with IgG at 0.75mg mL<sup>-1</sup> (Figures 4.18B). The specific parasite growth inhibition seen was equivalent in potency to that observed with the control hyper-immune (BG98) anti-AMA-1 IgG (Figure 4.18, both panels).

A)



B)

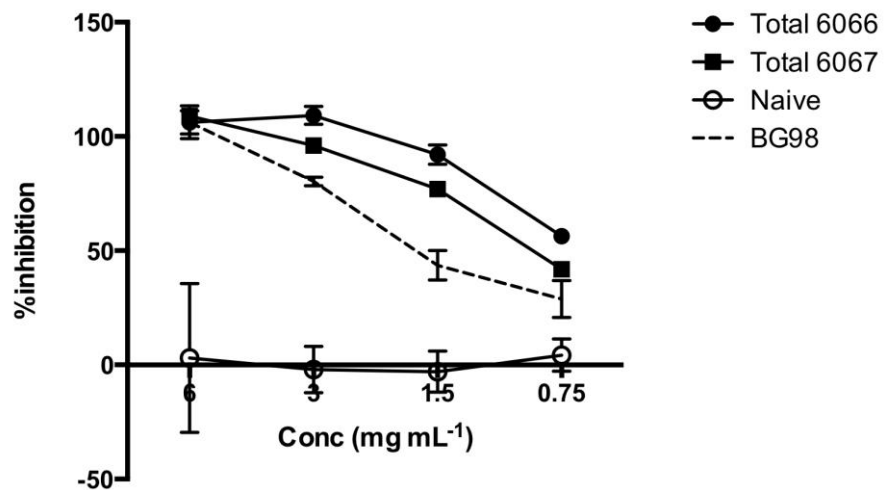


Figure 4.18: GIA of *P. falciparum* (Wellcome) using anti-MSP-3.3C antibodies. Purified anti-MSP-3.3C total IgG (from rabbits R6066 and R6067 are shown as black line with dark circle and dark square symbol respectively). Inhibition by anti-AMA1 total IgG (BG98) is shown as a dashed line. Inhibition by total IgG from naïve rabbit sera is shown in black line with open circle symbol. Panel A shows results obtained using the pLDH assay, Panel B shows flow cytometry measurements of parasitaemia in cultures.

To verify this strong inhibitory effect, the assay was repeated five times by myself, Dr. Alison Creasey, Dr. Graeme Cowan and Mr. Kakra Dickson. Consistently strong parasite killing effects were observed in all assays. At the highest concentration of anti-MSP-3.3C IgG ( $6 \text{ mg mL}^{-1}$ ), 97-100% parasite inhibition was observed, as measured by pLDH assay, which titrated to 25-34% inhibition at  $0.75 \text{ mg mL}^{-1}$  IgG (Figure 4.19A). Similar inhibition, ranging from 83.4%-85.4% at  $6 \text{ mg mL}^{-1}$  to 7.3-9.2% at  $0.75 \text{ mg mL}^{-1}$  was observed using flow cytometry measurements of parasitaemia on aliquots of the same cultured parasite material (Figure 4.20A). To confirm these findings independently, aliquots of anti-MSP-3.3C total IgG were sent for testing to the PATH Malaria Vaccine Initiative Growth Inhibition Assay–Reference Center, NIAID, Rockville, USA. In initial testing, parasite inhibition at  $6 \text{ mg mL}^{-1}$  total IgG was reported as 99-100% by pLDH assay. Antibody preparations were re-dialyzed and independently tested in the NIAID standard pLDH assay at concentrations of  $6 \text{ mg} - 0.67 \text{ mg mL}^{-1}$  IgG. In these assays, inhibition was recorded as 98-99% at  $6 \text{ mg mL}^{-1}$ , titrating to 32-49% at  $0.67 \text{ mg mL}^{-1}$  for the two IgG preparations (Figure 4.19C). Thus, these potent inhibition results were independently confirmed, with a notable degree of similarity, by the GIA Reference Center

To exclude the possibility of a non-specific antibody inhibitory effect in the two total IgG preparations, GIA was repeated using MSP-3.3C antigen-specific affinity-purified IgG, as well as the flow-through IgG (i.e. MSP-3.3C-depleted IgG) collected from an MSP-3.3C-Sepharose conjugate column. As measured by pLDH assay, MSP-3.3C-specific IgG strongly inhibited parasite growth at considerably lower concentrations than total IgG from MSP-3.3C immunized rabbits, giving 100% inhibition at a concentration of  $1 \text{ mg mL}^{-1}$ , which titrated to a mean inhibition of 21.6% at  $0.125 \text{ mg mL}^{-1}$  (Figure 4.19B). By contrast, the MSP-3.3C depleted IgG fraction showed only weak inhibition (mean 41% at  $6 \text{ mg mL}^{-1}$ ), higher than the negative control total IgG from naïve rabbit sera, but this inhibition was not seen at lower concentrations (Figure 4.19B). Similar parasite inhibitory effects were observed using flow cytometry to detect parasitaemia (Figures 4.20A and 4.20B). Notably, as has been observed before in our laboratory (Arnot et al. 2008), flow cytometry measurement of parasite nucleic acids gives consistently lower growth inhibition measurements than the pLDH assay. This difference is particularly marked when using antibodies in GIA against some targets, such as MSP-1<sub>19</sub> and MSP-3.3C, and less so when measuring growth inhibition with anti-AMA-1 IgG (Figures 4.19 and 4.20, panels A and B).

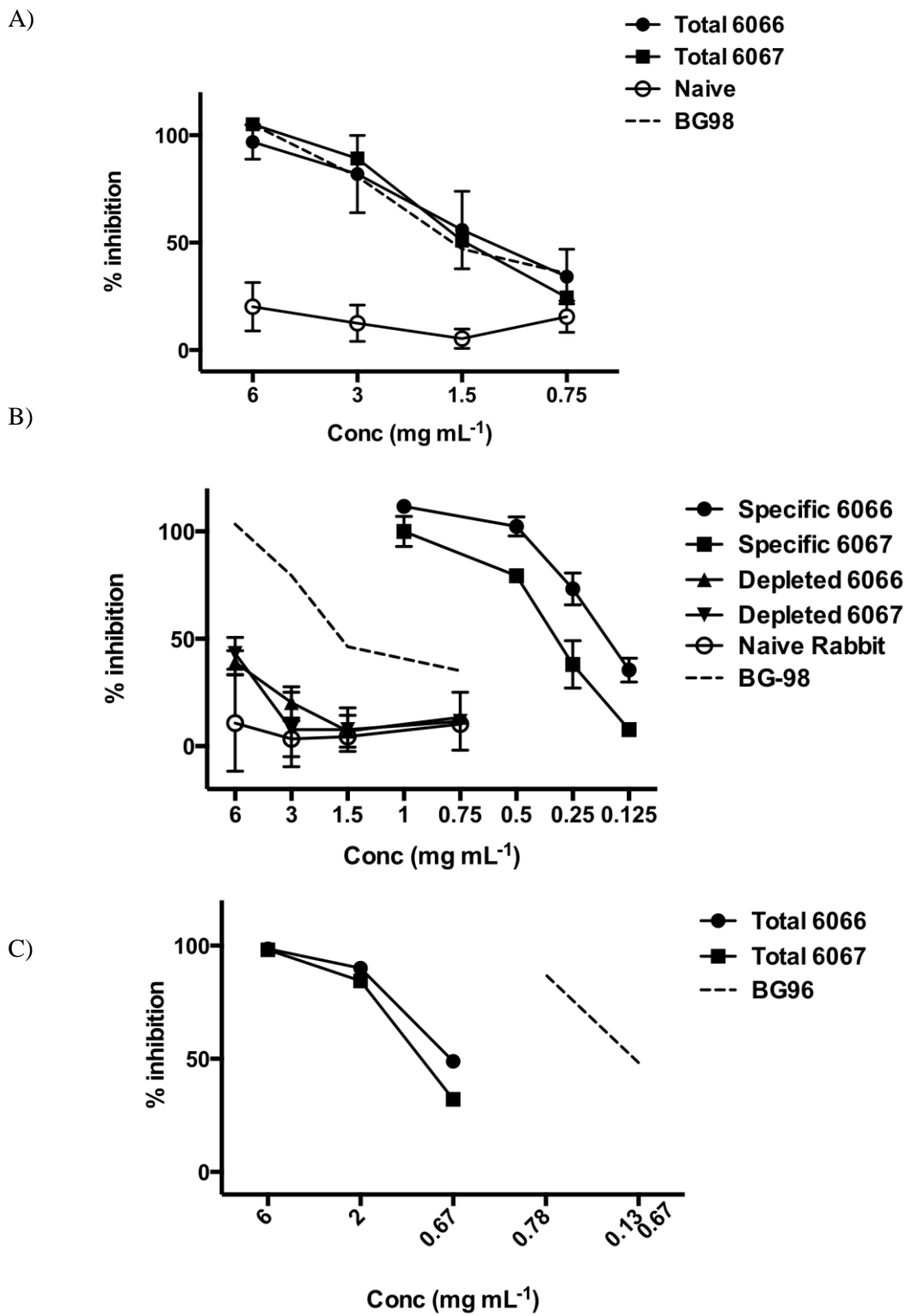
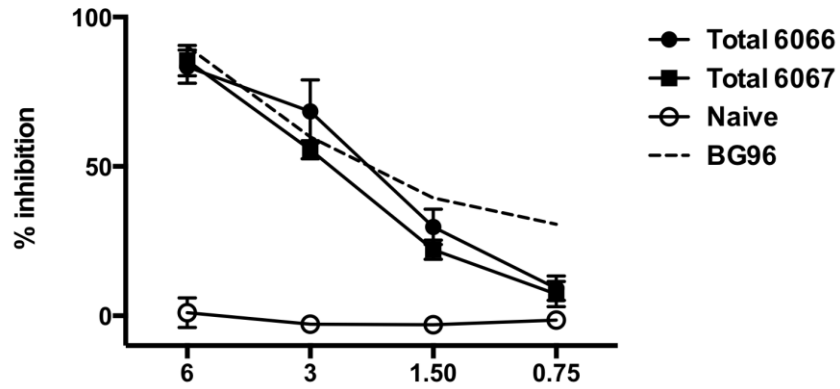


Figure 4.19: GIAs results of *P. falciparum* (Wellcome) as measured by pLDH. Panel A shows the result of the assays using purified anti-MSP-3.3C total IgG, while Panel B shows the result when MSP-3.3C antigen-specific affinity-purified IgG and MSP-



3.3C-depleted IgG were used. Panel C shows the result of the GIA of *P. falciparum* (3D7a) by tested at the NIAID.

A)



B)

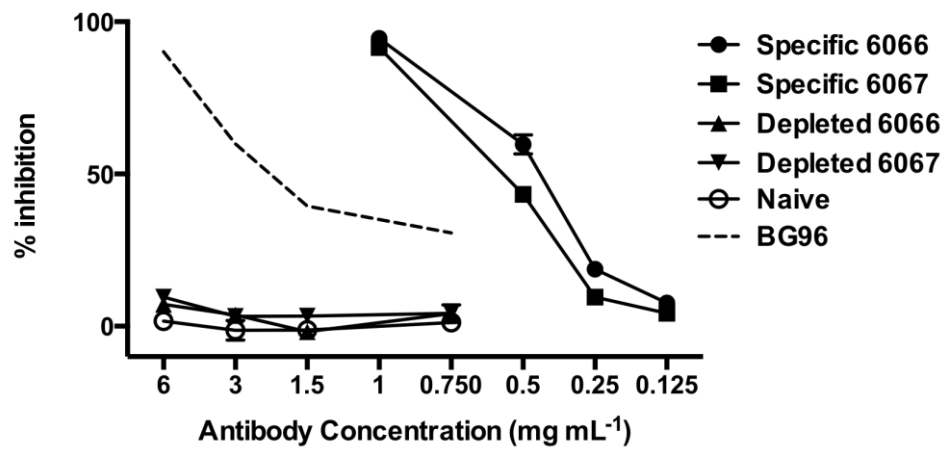


Figure 4.20: GIAs results of *P. falciparum* (Wellcome) as measured by flow cytometry. Panel A shows the result of the assays using purified anti-MSP-3.3C total IgG, while Panel B shows the result when MSP-3.3C antigen-specific affinity-purified IgG and MSP-3.3C-depleted IgG were used

### 2.10.2 MSP-3.3C Antigen Reversal GIA

We attempted to reverse the strong inhibitory effect of MSP-3.3C antibodies by pre-incubating total anti-MSP-3.3C IgG with MSP-3.3C protein prior to the GIA assay. In the course of development of the controls for this assay, we found that the MSP-3.3C protein itself could exert an inhibitory effect on parasitaemia. As measured by flow cytometry, 6 mg mL<sup>-1</sup> of soluble MSP-3.3C protein showed about 65% GIA inhibition. We speculated that these high amounts of protein in the culture medium might be limiting the parasite's access to culture nutrients, or interfering with parasite invasion by blocking unknown erythrocyte receptors usually bound by parasite-associated MSP-3.3 during parasite invasion.

Recombinant MSP-3.3C protein was then coupled to magnetic beads, using the protocol provided by the manufacturer. MSP-3.3C protein at a concentration of 7 mg mL<sup>-1</sup> was dissolved in conjugation buffer (0.1M MES, 0.9% NaCl pH4.7) and mixed with 1mL of a suspension of MagnaBind Carboxyl Derivatized beads (ThermoScientific). 10mg mL<sup>-1</sup> EDC (1-ethyl-3-(3-dimethylaminopropyl) carbodiimide in coupling buffer was freshly made by dissolving 10mg EDC in conjugation buffer. For 1 ml of protein-beads mixture, 10 µl of EDC solution was added. The mixture was incubated for 30 minutes at room temperature, before excess MSP-3.3C was washed off. The magnetic beads were separated from the solution by magnet bar and the supernatant from the coupled beads was aspirated. The beads were washed three times with 1 ml PBS. They were stored at 4°C before use. BCA assay was used to detect non-immobilised MSP-3.3C in the washing solution, and therefore estimate the amount of MSP-3.3C bound to the beads. MSP-3.3C protein coupled beads were used in ELISA to measure binding to anti-MSP-3.3 antibodies.

MSP-3.3C in this immobilized form was expected to neutralise MSP-3.3C specific antibodies but interfere less with parasite cultures. When tested by the ELISA, it was shown that 50 µl of bead suspension can up take at least 5mg mL<sup>-1</sup> of antibodies in 50µl solution or 0.25 mg of antibodies (data not shown). In the antigen reversal assay, 20µl of beads suspension was therefore added to the culture in the presence of the anti-MSP-3.3C IgG, which was titrated as before in GIA from 6mg mL<sup>-1</sup> to 0.1875mg mL<sup>-1</sup>. Culture with the same amount of bead suspension in the absence of antibodies was set up as a control. These beads were expected to decrease the inhibitory effect of the antibodies to MSP-3.3C. However, not only was the expected reversal effect not observed, but the inhibitory effects of antibody were still very strong. The culture that was treated with 6mg/ml of total IgG (from rabbit no. 6066 and 6067) and the antigen-coated beads showed inhibition at 79.5% and 79.24% respectively (Figure 4.21).

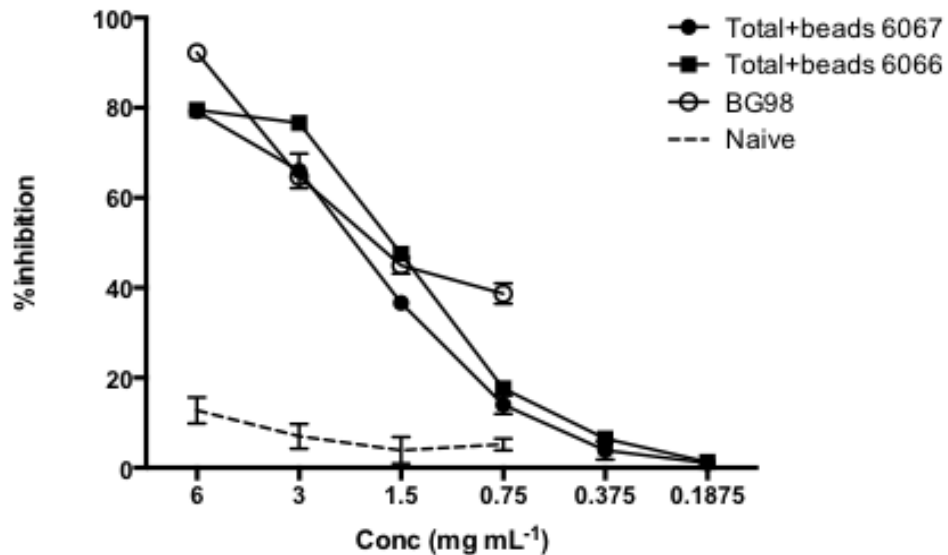


Figure 4.21: GIAs results of *P. falciparum* (Wellcome) as measured by flow cytometry shows the result of the assays using purified anti-MSP-3.3C total IgG from rabbit no.6066 and 6067 in present of MSP-3.3C-coated beads.

### 2.10.3 Morphological analysis

Microscopic observation of Giemsa stained thin smears from all the GIA experiments showed that parasites that had been treated with MSP-3.3C antibodies had different morphology from the control groups. The morphology and size of parasites at schizont stage from anti-AMA1 treatment wells was very similar to both untreated parasite cultures and cultures treated with naïve rabbit IgG. In the presence of anti-MSP-3.3C IgG, parasites were smaller in size and had denser morphologies within infected red blood cells. High concentrations of anti-MSP-3.3C antibodies (3-6 mg mL<sup>-1</sup> total IgG or 0.5-1 mg mL<sup>-1</sup> of MSP-3.3C specific IgG) resulted in pyknotic parasites and poorly-developed ring stage parasites as detected in Giemsa stained smears (Figure 4.22). Normal parasite morphology was evident at lower antibody concentrations (0.5-1.5 mg mL<sup>-1</sup> of total IgG, 0.125-0.25 mg mL<sup>-1</sup> of MSP-3.3C specific IgG). However even at lower antibody concentrations, most parasites developed at a slower rate than those in the cultures with naïve rabbit IgG or anti-AMA-1 control IgG. In addition, some parasites in the cultures that were treated with anti-MSP-3.3C appeared to be free of their surrounding red cells (Figure 4.22).

Treatment with depleted-anti-MSP-3.3C IgG showed little effect on parasite morphology (Figure 4.22). The parasites that were treated with these antibodies were smaller in size to the control culture. Morphology of the culture that was treated with MSP-3.3C

immobilized to beads was also similar to that of the control. However, when the soluble form of the MSP-3.3C was present in the culture, parasites were smaller than those of the control and appeared to have arrested at the trophozoite stage rather than developing into schizonts (Figure 4.22).

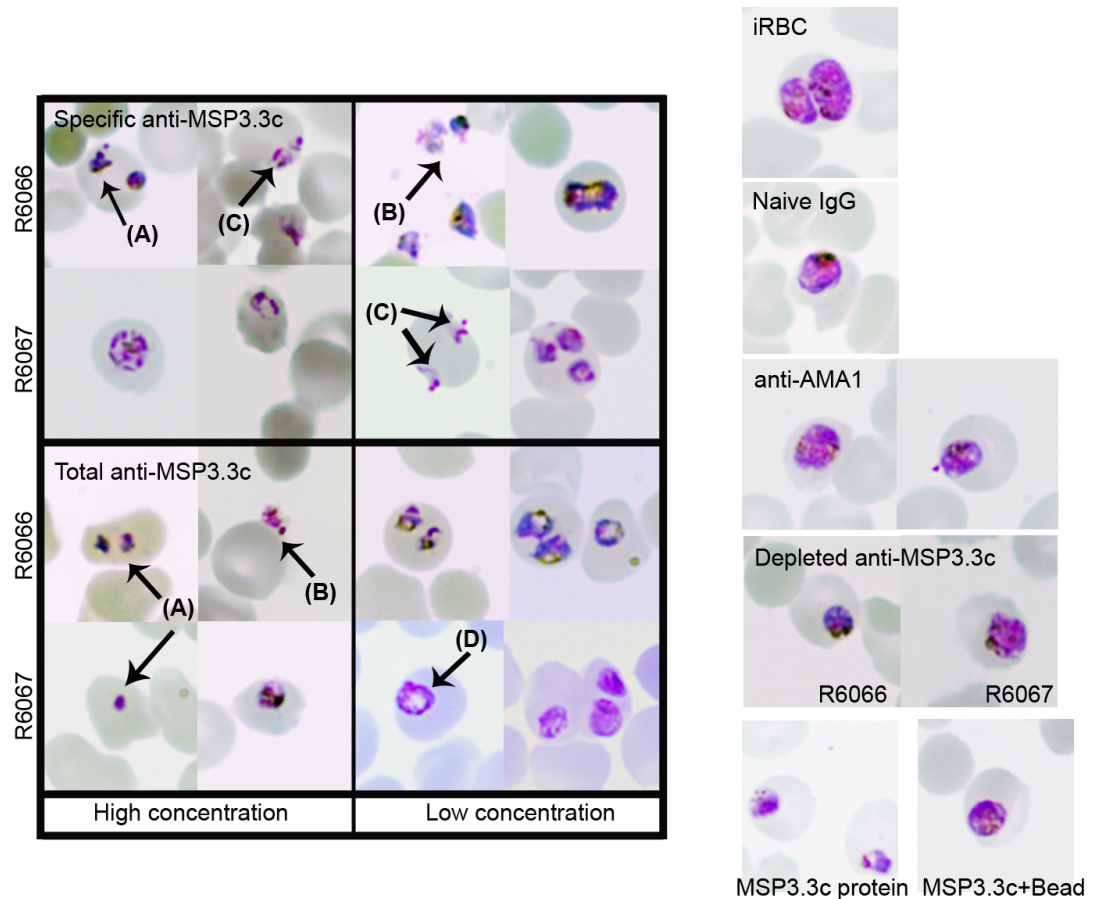


Figure 4.22: Giemsa stained thin smears of parasite cultures from GIA. Morphological abnormalities observed included (A) pyknotic parasites, (B) parasites apparently outside red blood cells, (C) collapsed ring stage parasites and (D) swollen, vacuolar trophozoites in cultures treated with either total purified anti-MSP-3.3c or specific purified anti-MSP-3.3C IgG. Anti-AMA1 IgG-treated cultures had morphology comparable to that of control cultures treated with naïve IgG. Cultures treated with recombinant MSP-3.3C protein, either in solution or on beads are shown for comparison.

### 3. Discussion

#### 3.1 PF10\_0347 is conserved and belongs to the MSP-3-multigene family

The DNA sequence of PF10\_0347 is very conserved between isolates. Published reports show no evidence that the PF10\_0347 gene is under balancing selection (Tetteh et al. 2005). The few mutations reported are neutral mutations (Pearce et al. 2005), possibly created by gene duplication events (Cowman & Crabb 2002). The MSP-3.3 protein has a very small number of amino acid variations. Most of the observed point mutations in PF10\_0347 are synonymous changes, and this high degree of conservation leads to the hypothesis that this protein may be crucial for parasite survival (Singh et al. 2009). Non-synonymous SNPs lead to changes in properties of proteins such as polarity, charge, and hydrophobicity. These mutations may also alter tertiary structure in particular regions or domains, which may result in change of protein conformation as a whole. Disorder tendency is another protein property that likely to be affected by amino acid changes. Using IUPRED, all published mutant sequences of PF10\_0347 were assessed for the disordered tendencies of their protein products. No significant changes in the unstructured regions of the protein were seen, and some of variants had even higher average disorder tendency compared to the sequence of 3D7 (data not shown). It would seem that even though there are variations in PF10\_0347 sequence, the structure and functions of this gene product should remain similar, unless these mutations are involved in post-translational modifications.

#### 3.2 Predicted domains of PF10\_0347

Members of the MSP-3 protein family have neither a transmembrane domain nor a GPI-anchor-associated region and thus may be present on the surface of parasite by interaction with other surface proteins (Pearce et al. 2005). These MSP-3 protein members share 3 major common domains known as the Signature motif domain, the Glutamic acid-rich domain and the C-terminal region.

The Signature motif domain consists of an NLRNA/NLRNG motif and is located near the N-terminal of all MSP-3 family protein sequences. For the PF10\_0347 gene product, there is also a short amino-terminal sequence, RNLVQ, located just before this family signature sequence. This short motif (R/KxLxE/Q) is termed the Plasmodium export element (PEXEL) (Sargeant et al. 2006) and is important for protein trafficking from the parasite into the erythrocyte (Marti et al. 2005).

Toward the C-terminus, there is a Glutamic acid rich region (referred to as the acidic region in Pearce *et. al.*, 2005). This amino acid sequence has also been found in the MSP-3

homologues of *P. vivax*. Although much larger in size, the MSP-3 homologue in *P. vivax* is located at the same position relative to the C-terminal of the PF10\_0347 gene product. This suggests that, for these two species, the function of this region is conserved (Pearce et al. 2005). The role of this domain in the malaria parasite has yet to be confirmed. However, it is hypothesized that it may interact with part of a surface protein which is positively charged. Thus, it may allow the MSP-3-like proteins to stay on surface of the parasite as peripheral membrane proteins (Galinski et al. 1999). Studies on the Glutamic acid rich region in other organisms has suggested another possible role for this region. It may act as a calcium-binding domain (Carafoli 1997) and may be involved in the membrane fusion process (Grishanin et al. 2002). These interesting data suggest that this region may responsible for interaction of MSP-3.3 with other surface proteins of the parasite or may also play a role in interactions with the host cell.

The C-terminus of the MSP-3 protein family is also relatively conserved, according to sequence alignments. It contains heptad repeats of which the first and fourth residues are hydrophobic amino acids. This pattern of amino acid sequence is known to form an  $\alpha$ -helical structure which is also known as a leucine-zipper. According to a biophysical study on the C-terminal region of MSP-3, this leucine-zipper region allows the MSP-3 molecule to be self-assembled, forming a tetrameric structure, suggesting that other proteins that contain this domain may also form this intermolecular structure (Gondeau et al. 2009). The leucine-zipper domain was demonstrated to be essential for the trafficking of MSP-3 to the parasitophorous vacuole (Mills et al. 2002). Apart from this function, it may also have a role in promoting efficient binding of other proteins; using the C-terminal domains as a core, they can be assembled as branches of a homo-oligomer. Oligomeric structures which have binding domains protruding from the core can have high avidity for their targets due to their multivalency (Terskikh et al. 1997).

Sequence alignments and results from cross-reactivity assays of antibodies raised to the glutamic acid-rich regions of these proteins suggest that they have very similar epitopes, except for MSP-3.3 which shows the lowest level of antibody cross-reactivity (Singh et al. 2010; Pearce et al. 2005; Demanga et al. 2010). Apart from these long common domains, there is a small region known as the glycine-rich domain. This short motif is centrally located, just next to the glutamic acid-rich region, in MSP-3, MSP-6, MSP-11 and MSP-3.3. The function of this small domain still remains unknown, but it has been shown to be a target of antibodies in ADCI assays (Oeuvray et al. 1994).

### 3.3 Expression and localisation of MSP-3.3

The members of the MSP-3 family have unique expression profiles compared with other gene families expressed during the asexual blood stage. They are all located continuously on the same chromosome and lack introns, a characteristic of genes that are rapidly regulated (Jeffares et al. 2008). Interestingly, the expression profile of PF10\_0347 is different from other family members. Unlike MSP-3 or MSP-6, which have only one peak of expression at 42 hr (mid-schizonts), the PF10\_0347 expression profile peaks at 14 hours after invasion (mid-late trophozoite) and then again at 36 hours (mid-schizont) (Bozdech et al. 2003). This multiple expression peak profile is characteristic of genes with unusual signal time-modulated expression and PF10\_0347 is one of 21 genes with this expression profile (Fedorov & Leinine.). This unique expression profile raises further questions about the role of MSP-3.3 during the parasite life-cycle. Why do the parasites need to produce MSP-3.3 twice in one cycle? Is there a common use of MSP-3.3 at two different stages of parasite development, or does MSP-3.3 have a different function at different stages of parasite development? All these questions remain to be debated and await further study of this protein.

In this project I have shown that MSP-3.3 is present throughout the intraerythrocytic stage of the parasite and in the free merozoite stage. Western Blots using MSP-3.3C specific antibodies showed that MSP-3.3 is processed during schizont maturation before the release of free merozoites. Full-length MSP-3.3 is ~60 kDa by SDS-PAGE and can be detected in schizont protein extracts. Using merozoite protein preparations, the rabbit antibodies raised to MSP-3.3C detected two smaller MSP-3.3 proteins of approximately 40 and 50 kDa. The processing of MSP-3.3 is assumed to be caused by the enzyme subtilisin-like protease 1 (Sub1), for which there are two recognition sites in the N-terminal region of MSP-3.3 (Ellen Knuepfer, personal communication) (Figures 4.2 and 4.22).

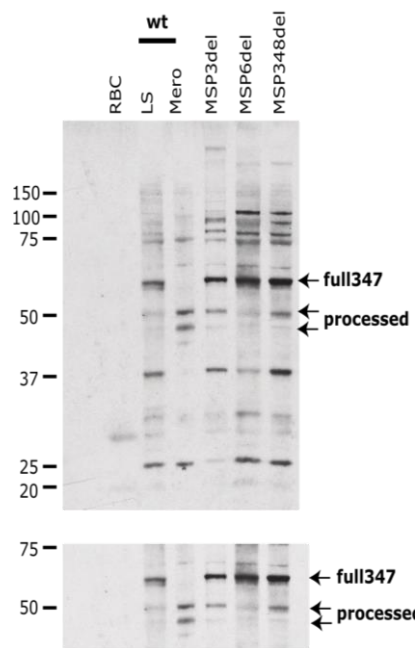


Figure 4.23: Western blot showing reactivity of anti-MSP-3.3C IgG to protein extracts of *P. falciparum* 3D7. Purified IgG from rabbit R6066 immunised with MSP-3.3C was used to detect parasite MSP-3.3. Full length MSP-3.3 has a predicted size of 48 kDa, but migrates at ~60kDa due to its IUP properties. This 60kDa protein can be detected in schizont extracts of wild type parasites (LS=late schizont proteins), as well as other three knockout lines (MSP-3del = MSP-3 knockout line; MSP6del = MSP6 knockout line; and MSP-348del = PF10\_0348 knockout line). However, the full-length protein was undetectable in merozoite extracts (Mero), and smaller processing products of MSP-3.3 ~ 45-50kDa were detected. Antibodies to MSP-3.3C did not show cross-reactivity to protein extracts from normal red blood cells (RBC). (Figure produced by Ellen Kneuper, NIMR, Mill Hill).

Pearce *et al.*, 2005 reported that the MSP-3.3 (referred to therein as H101) could not be detected in merozoite extracts by Western Blotting. In the same publication, another proteolytic cleavage site was identified (Pearce *et al.* 2005) However, the antisera used in that study were raised against full length MSP-3.3 fused to GST. The sensitivity of the blotting technique and the possible differential specificities of the antibodies raised by immunisation may explain these differing results. The antisera raised to H101 reacted with full-length MSP-3.3 but also with the truncated delta-H101 protein produced by KO parasites, indicating a substantial reactivity with the N-terminal part of MSP-3.3, and no



reactivity with the smaller, more C-terminal processing products of MSP-3.3 that we have observed (Figure 4.2). It is not clear why MSP-3.3 is processed, but the cleavage step may expose some functional domain of this protein for a different function in the subsequent stages of parasite development, such as in the schizont maturation step or merozoite invasion step. Another possibility is that MSP-3.3 is processed to be shed along with other MSP-3 family protein complexes that are removed from the merozoite surface during invasion, such as the MSP-1/MSP-6 complex. Again, further studies should allow better understanding of the role of this protein.

Some studies have suggested that certain protein motifs provide a signal that is important for protein transportation. Interestingly, MSP-3.3 contains a short PEXEL motif, which has role in protein translocation to the RBC cytosol via the parasitophorous vacuolar membrane (PVM) (Marti et al. 2005). Whether this protein is further exported to Maurer's clefts and then to the iRBC surface is still unclear, as at least some MSP-3.3 protein is cleaved downstream of the PEXEL motif, as shown in Figure 4.2. It has been reported that disruption of PEXEL motifs can affect protein transportation from the parasitophorous vacuole (PV) to the iRBC membrane (Marti et al. 2005).

### **3.4 Postulated functions of MSP-3.3**

The functions of MSP-3.3 may be similar to those of MSP-3, since they have similar size and share some common domains and sequence structure. It is possible that MSP-3.3 is associated with other MSPs and forms part of the protein coat of the merozoite. MSP-3.3 may be involved in parasite surface protein interactions and may also play a role with other proteins in binding to host RBCs and subsequent invasion steps. However, mutant parasites that contain truncated PF10\_0347 genes demonstrate that this gene is not directly crucial for erythrocyte invasion and growth of parasites, at least *in vitro* (Pearce et al. 2005). The same study proposed that MSP-3.3 could be used as an alternative when other MSP-3 protein family members are blocked or malfunctioning. Nevertheless, these mutant parasites still contain a substantial part of this PF10\_0347 gene and still express a truncated protein product. Thus, there is a possibility that the truncated protein is still able to sustain at least part of its normal function (Pearce et al. 2005).

### **3.5 Recombinant antigen MSP-3.3C**

In this project, an MSP-3.3C recombinant protein was expressed, representing the long unstructured region of MSP-3.3 from the 3D7 strain of *P.falciparum*. MSP-3.3C was produced for the purpose of structural, immunological, antigenic and functional studies of this region of this MSP-3 family member. The ultimate aim of the project was to investigate

MSP-3.3C as a suitable vaccine candidate, and therefore did not contain any non-parasite protein sequences. Protein tags, such as GST or HIS tag were intentionally excluded from this construct in order to preserve antigenic integrity and prevent contamination with toxic residues such as Cu<sup>2+</sup>, Ni<sup>2+</sup>, Co<sup>2+</sup> or Zn<sup>2+</sup>, which can possibly be co-purified with the proteins when metal-chelating resins are used.

It has been postulated that many malaria proteins are poorly expressed in heterologous hosts because of codon usage bias (Mohan et al. 2008). The presence of intrinsically unstructured regions has also been associated with low expression yields, possibly due to genetic instability of repetitive DNA inserts or the proteolytically sensitive nature of these unfolded proteins (Feng et al. 2006). However, this does not seem to be the case for the MSP-3.3C, which showed no genetic instability in *E coli* and was expressed efficiently as a soluble protein. The MSP-3.3C protein can be successfully produced at laboratory scale using a simple purification protocol. It is hoped therefore that this protocol can be further developed for industrial scale production. With optimal growth conditions, and fermentor scale-production, MSP-3.3C can be produced in bulk. To improve yields of expressed product further, codon optimisation (Zhou et al. 2004) or codon harmonisation (Angov et al. 2008) could be considered. Other options such as using alternative expression hosts (Yadava & Ockenhouse 2003) or richer nutrient media may also enhance the production of this recombinant protein.

The purification protocol for MSP-3.3C was developed based on the knowledge obtained from the MSP-1 Hybrid study (Chapter 3). The fact that MSP-3.3C is thermostable made production and purification of this protein straightforward. A two-step purification scheme proved to be very simple and efficient, providing a high quality product. However, there was substantial loss of product during these two steps. To further improve product yield, heating conditions and chromatography protocols could be further improved to reduce losses. It seems probable that, like the MSP-1 Hybrid, MSP-3.3C vaccine production will prove to be scalable.

MSP-3.3C appears to be an IUP, both by biophysical analysis and by its biochemical properties. This allows it to be maintained at ambient temperature without significant loss of immunogenicity. MSP-3.3C migrated anomalously on SDS-PAGE, at almost double its actual size, as IUPs bind poorly to SDS due to their amino acid composition.

### **3.6 Antigenicity and immunogenicity**

The antigenicity of the MSP-3.3C protein was demonstrated by its recognition by human sera from malaria exposed Africans. Thus recombinant MSP-3.3C shares epitopes with MSP-3.3 protein from parasites. Antibodies to MSP-3.3C were of IgG3 and IgG1

subclasses, although biased towards IgG3, like many other parasite IUPs (Cavanagh et al. 2001; Taylor et al. 1998; Singh et al. 2004). This is in agreement with data from Singh *et al.*, 2009 who used hyperimmune sera, where IgG1 and IgG3 were detected to the C-terminal part of MSP-3.3 (Singh et al. 2009). My ELISA assays with human sera from Africa produced important data about MSP-3.3 reactivity in a naturally exposed population, but number of sera tested were not sufficient to draw conclusions about the frequency of positive anti-MSP-3.3C in naturally exposed individuals. The sera used were selected for high reactivity to at least one other merozoite surface protein. Thus, they represented only a subset of infected individuals. For future studies, larger collections of sera from different populations, especially those in which clinical data of the individuals have been recorded, will be needed. Some work has begun in this area with our collaborators in Burkina Faso, where MSP-3.3 specific antibodies are reported to be of low frequency and low titer in most individuals tested (Cherif et al. 2012). Since MSP-3.3C is an IUP, I have assumed that this antigen contains continuous linear epitopes. However, it would be useful to investigate of sub-regions of this protein and map the epitopes recognised by naturally infected sera, with the aim of identifying antibody reactivities that correlate with protection.

I have shown that recombinant MSP-3.3C shares similar epitopes with MSP-3.3 of parasites. Using rabbit antibodies raised against MSP-3.3C, IFA showed specific MSP-3.3 staining patterns around the parasite nucleus. The structure of this staining becomes more organised and denser as parasites mature towards schizont stage. In contrast with a previous study (Pearce, Hodder, et al. 2004a), I found reactivity in one particular area of the free merozoite. MSP-3.3 is confirmed as a merozoite surface protein that may play a role in host RBC binding and/or invasion. More sensitive imaging methods such as confocal microscopy should be used to learn more about this protein's subcellular and stage-specific localisation.

Unstructured regions in malaria proteins are reported to be poorly triggering weak immune responses (Dunker et al. 2002; Feng et al. 2006). In this study, we showed that MSP-3.3C was poorly immunogenic in mice. However, using a stronger adjuvant, higher titre antibodies were elicited in rabbits. Thus, I believe that there is still room for improvement of the immunogenicity of this novel protein antigen. A range of adjuvants that may enhance production of specific antibodies will be considered for future tests. Adjuvants which are derivatives of the Montanides, which were used to raise specific antibodies to C-terminal region of the PF10\_0347 in other studies, may also be considered (Singh et al. 2009). Note that, unlike other previous publications, this is the very first MSP-3.3 recombinant protein without a protein tag in its construct. Using this protein should prevent

undesired immune responses against GST protein and this should make the results easier to interpret.

### **3.7 Function of MSP-3.3C-specific antibodies in *in vitro* assays**

The growth inhibition assay (GIA) has been used in malaria vaccine research as a tool for measuring antibodies' functionality *in vitro*. GIA can assist in the assessment process of malaria vaccine development, because of the limited range of *in vivo* animal models. GIA measures the ability of antibodies to disrupt parasite growth, development and/or invasion. Like other functional assays, it is informative, yet has limitations. In this study, two methods of measurement of parasitaemia were employed to confirm results and to provide a broader understanding of the process of inhibition. The pLDH assay is designed to measure parasite lactate dehydrogenase activity (LDH), which is important for intracellular metabolism of the parasite. The pLDH assay only detects living parasites. Assay of LDH activity can vary depending on accuracy of pipetting, ambient temperature, the density of parasites in culture, as well as high background signal absorbance by RBC components. Alternatively, parasite cultures can be stained with fluorescent nucleic acid binding dyes and analysed by flow cytometry. This more sensitive method allows us to group parasites into subpopulations based on their DNA content and size, so that each stage of the intraerythrocytic parasite can be distinguished. In addition, Giemsa-stained thin smears of parasite cultures were taken in order to observe the morphology of parasites. Combining these measuring methods provided us with very useful information regarding the potential functions of antibodies to MSP-3.3C and the function(s) of MSP-3.3 itself.

Many previous studies have sought to show associations between specific antibodies to malaria antigens and protection from disease, although knowledge of the molecular functions of these antibodies is incomplete. Effects of different antibodies on the parasite have been shown to be diverse, possibly due to variation in antibody affinity (Reddy et al. 2012), isotype (Cowan et al. 2011) or paratope (Bergmann-Leitner et al. 2006). Apical membrane antigen-1 (AMA-1), for example, is known to be a target for antibodies which have the ability to inhibit parasite invasion (Coley et al. 2007) blocking the functional region of AMA-1 (Dutta et al. 2005), and treatment with anti-AMA-1 results in lower numbers of parasites. The GIAs in this experiment showed that the specific antibodies to MSP-3.3C have the ability to kill parasites. The higher the concentration of purified total IgG, the lower the parasitaemia in the treated culture. Affinity-purified antibodies from the same sources confirmed that it was MSP-3.3C specific antibodies rather than background antibodies that were responsible for the parasite killing effect. The stronger GIA activity of affinity-purified

antibodies observed in this experiment may show that the presence of non-specific anti-idiotypic antibodies can hamper the activity of the specific antibodies (Wåhlin et al. 1990). In addition, GIA with MSP-3.3C depleted IgG showed that the non-specific antibodies from the two immunised rabbits did not have a significant inhibitory effect on parasites. The parasite killing effect of purified total IgGs was consistent between individual GIAs. The same IgG samples were also tested in GIAs by Dr. Carole Long's laboratory at the NIH using the *P. falciparum* 3D7 strain. In these experiments, the samples were re-dialysed and filtered before use, as initial GIA results were so strong, Dr. Long's laboratory suspected contamination of the IgG preparation. The result confirmed that antibodies from both the immunised animals had strong killing effects with up to 100% growth inhibition. All these data confirmed that the antibodies against MSP-3.3C could kill the parasite *in vitro*.

As mentioned earlier, although the GIA is an informative assay, it does not provide direct information on how these specific antibodies affect parasites. Importantly, these assays showed us that these antibodies not only decreased parasitaemia, but also caused abnormalities of parasite morphology. Unlike anti-AMA-1 Abs, anti-MSP-3.3C Abs caused parasites that had successfully invaded into new red blood cells to decrease in size. The growth or development rate of anti-MSP-3.3C cultures also appeared to be slower, although this was impossible to quantify in these assays. While the untreated or naïve rabbit IgG cultures had developed into late-trophozoites, parasites that were treated with anti-MSP-3.3C were arrested at early blood-stage forms that contained condensed nuclei. These malformed parasites were not producing pLDH and so were not contributing to measurements in pLDH assays. This suggests that anti-MSP-3.3C antibodies function in a very different way from the function of antibodies to AMA-1.

It has been suggested that some antibodies with functional activity may not show a direct invasion inhibitory effect, but may still cause disorder in post-invasion parasites (Arnot et al. 2008). Malformed morphologies in antibody-treated parasites have been reported in several different studies (Ahlborg et al. 1996; Bergmann-Leitner et al. 2006). Several models of antibody function have been proposed, but the molecular mechanism these antibodies use is not very well understood. However, we speculate that antibodies to the MSP-3.3C are likely to have both an invasion and an intracellular growth inhibition effect. If antibodies only inhibit parasite invasion, there will be fewer parasites in treated cultures than in control cultures, but morphology and lifecycle stage of parasites in both cultures should be very similar. The best example of this model are antibodies to AMA-1, which block parasite invasion (Arnot et al. 2008). In contrast, antibodies that target proteins such as MSP-1<sub>42</sub> have been reported to affect growth of parasites, halting development of

parasites from trophozoite stage onward. As a consequence, these treated parasites were blocked at the schizont stage, and termed “Stalled schizonts” (Bergmann-Leitner et al. 2009). Another vaccine candidate MSP-1<sub>19</sub> has been shown to trigger specific antibodies that have functional activity on post-invasion parasites. Although these antibodies do not show strong invasion inhibition effects, misshapen parasites were seen in the treated cultures: these included collapsed ring forms, pyknotic nucleoli in trophozoites and swollen vacuolar late trophozoites (Pearce et al. 2005; Arnot et al. 2008). Antibodies raised to a megadalton protein called Pf322-C231 have been shown to cause abnormal schizont growth, including an absence of segmenters (Balogun et al. 2011). In anti-MSP-3.3C treated cultures, the majority of ring stage parasites contained crumpled nuclei, especially in cultures with higher concentrations of IgG. At lower IgG concentrations, deformed trophozoites, which were small with packed and darkly stained nuclei, were observed together with the tiny ring forms described above.

Three phenomena of antibody functional activity may be taking place during the blood-stage cycle: I) Antibodies to MSP-3.3C are able to block parasite invasion, possibly by interfering with the binding process of the parasite to the red blood cell and causing a drop in parasitaemia. This phenomenon is obvious at high IgG concentrations, suggesting that it may require quite a lot of specific IgG to opsonise free merozoites; II) Anti-MSP-3.3C antibodies are able to bind to MSP-3.3, do not prevent parasite invasion, but may be carried in with successfully invading merozoites, and continue to affect parasite growth and development (Bergmann-Leitner et al. 2009) presence of anti-MSP-3.3C IgG during schizont stage provides an opportunity for antibodies to enter iRBC (Taraschi 1999). The membrane of the infected erythrocyte is more permeable, especially immediately before rupture (Lyon et al. 1997). Alternatively, antibodies may enter the iRBC via the parasitophorous duct, a pore with a thin tube structure that extends into the parasitophorous vacuole (Goodyer et al. 1997; Pouvelle & Gysin 1997). The fact that MSP-3.3 is expressed throughout the blood-stage of the parasite suggests that any or all these hypotheses may be possible. The antibodies may be able to attack the parasite at any stage if they are allowed to reach the target antigen. Based on our current data, it is not clear what might be the molecular mechanism of the antibodies that affect intra-erythrocyte growth inhibition. These antibodies may bind directly to an active site on MSP-3.3 that is crucial for parasite development, resulting in slowed development and defects in parasite structure integrity.

In GIA, antibodies did not block schizont development when antibodies might have access to infected cells, interfere with parasite development and prevent cell rupture. It may be that longer incubation times may be required for the anti-MSP-3.3C to function

efficiently. GIA is an assay which can only detect whether anti-MSP-3.3C has any inhibitory effect on malaria parasites, as samples were harvested at only one time point, after cultures had been allowed to grow for one cycle. In order to understand the process in a detail, time point measurements during GIA would be very useful. MSP-3.3C used to immunise rabbits in this study were formulated with a strong adjuvant (Freund's), producing a mixture of antibodies that are different in idiotype and serotype. Future production of MSP-3.3C monoclonal antibodies as well as more studies on antibody-protein interactions would help to enhance our understanding of the activity of anti-MSP-3.3C antibodies.

It has been suggested that the flexibility of the IUPs may make them poor targets of immunity and that they might be involved in evasion of host immune responses (Feng et al. 2006). Biologically, antibodies generally act with other immune cells in response to a pathogen. The principal theory of the antibody-dependent cellular-inhibition assay (ADCI) is that antibodies inhibit multiplication of blood-stage malaria parasites in cooperation with monocytes (Bouharoun-Tayoun et al. 1990). This assay has been used by others for human antibodies to MSP-3.3, which have ADCI effects *in vitro* (Singh et al. 2009; Demanga et al. 2010). It will be interesting to see if this MSP-3.3C protein, which shares sequence similarity to C-terminal sequences of other MSP-3 family members will also be a target of antibodies with ADCI activity.

### **3.8 Disordered region of the PF10\_0347 protein: function and relationship with immune responses**

Bioinformatics analysis has shown that long-disordered proteins are enriched in early branching eukaryotes (Mohan et al. 2008). In the malaria parasite genome, they are surprisingly abundant. It has been proposed that IUPs may play important roles in multiple protein-protein interactions. IUPs are proposed to act as hub proteins that can interact with multiple partners (one-to-many model) or to allow a single protein to be associated with many different IUPs (many-to-one model) (Dunker et al. 1998). These abilities can benefit a pathogenic protozoa's fitness by facilitating interaction with host cells (Feng et al. 2006). Another role of IUPs is to act as a junctional sequence between globular domains. The unstructured region that has been studied in this project has no reported functional domain(s). It is possible that instead of acting as a functional site, this region may form a flexible junction that links other functional domains (Olson et al. 2005; Fuxreiter 2011). This kind of junction may assist in rapid binding to host ligands and allow access to many different protein partners.

In this study, some interesting properties of the MSP-3.3C were discovered. In GIA, to confirm that the specific inhibitory effect was caused by antibodies to MSP-3.3C,

recombinant MSP-3.3C in a soluble form and as an immobilised form were introduced into parasite cultures, either in the presence or absence of antibodies to MSP-3.3C. However, the antigen-reversal effect was not achieved by any of these forms of the MSP-3.3C antigens. Conversely, the MSP-3.3C protein showed a growth inhibitory effect alone, although of lower potency than that of anti-MSP-3.3C antibodies. It is possible that the MSP-3.3C antigen competed with parasites for binding the surface of red cells, blocking parasite adhesion. Alternatively, this protein might access infected red blood cells and result in slowed development of the parasite. Since the soluble protein is smaller and more flexible than when it was attached to magnetic beads, the protein could act more rapidly. This may explain why soluble MSP-3.3C inhibited parasite growth to a greater extent than bead-immobilised MSP-3.3C. The sequence of MSP-3.3C does not contain any homology with known RBC binding domains, but experimentation will be required to confirm whether the MSP-3.3C has a function in erythrocyte binding.



# **Chapter 5: Construction and expression of the MSP-multihybrid vaccine antigen**

## **1. Introduction**

Developing a vaccine is a lengthy process and it is even more challenging when the pathogen is as complex as malaria. Recombinant protein-based antigens are widely used because once production and purification protocols have been developed, they can be manufactured on a large scale and their quality can be easily monitored. However, it is unlikely that they will be as immunogenic as a live-attenuated vaccine. This suggests that protective immunity might require the production of antibodies against multiple targets (Bruder et al. 2010). Evidence from a number of studies supports this idea, showing that protective responses are against polymorphic and variable regions. It is possible that a protective immune response relies on several different responses against variable or polymorphic antigen epitopes rather than a response to a single antigen or epitope (Bruder et al. 2010; Marsh & Kinyanjui 2006).

### **1.1 Multi-component vaccines**

A multi-component vaccine is a modified antigen that contains selected multiple regions, domains, allelic types of antigen or epitopes. Depending on the design, the multi-component vaccine may consist of epitopes that represent all known polymorphism of the target antigen or contain more than antigenic type or may be a combination of both. This kind of protein antigen is expected to provide more targets for immune responses, overcome polymorphism of parasite antigens, and therefore increase the efficacy of such vaccine.

Prior knowledge of the polymorphisms of the malaria parasite as well as data from immunological studies, molecular epidemiological studies and subunit vaccine trials is important and can provide very useful information. For example, studies on the diversity of MSP-1<sub>19</sub> in Kenya revealed a bias in the different polymorphisms of this antigen in the parasite population (Takala et al. 2007; Ferreira et al. 2003). The failure of a subunit vaccine trial to provide efficient protection to individuals in this area suggested that the immune responses raised by this vaccination targeted only the parasites with the minority form of the MSP-1<sub>42</sub> in population (Ogutu et al. 2009). Since allele-specific immunity cannot cross-react with other parasite strains, this subunit vaccine was ineffective in that region. Therefore, in such cases, a multi-component vaccine should provide better protection (Takala & Plowe 2009). Moreover, it was suggested that vaccines containing only one allelic type of the

polymorphic antigen may induce a limited spectrum of immune responses which may then select for parasites expressing alternative alleles. Therefore, an effective vaccine against the polymorphic antigens must be able to provide protective immune responses to all the allelic types.

Apart from the potential to raise broad immune responses, multi-component vaccines are reported to be more immunogenic than their individual components. It has been demonstrated that the antibodies raised by a chimeric protein can recognize native antigens on the surface of parasites and also show an inhibitory effect in GIA, while the specific antibodies produced by immunization of the individual components (domain III of AMA-1 and MSP-1<sub>19</sub>) failed to do so (Pan et al. 2004). Or in case of MSP-Fu<sub>24</sub>, which contains the C-terminal part of Pf MSP-1<sub>19</sub> and of PfMSP-3<sub>11</sub>, the ADCI activities of antibodies elicited by MSP-Fu<sub>24</sub>, protein fusion were comparable to those generated by immunization of each of the two component antigens (Mazumdar et al. 2010).

## **1.2 Design of the MSP-multihybrid: a multi-component vaccine**

There is encouraging data to support the idea of creating a multihybrid MSP vaccine based on the MSP-1 hybrid and the 2 allelic forms of MSP-2. Studies on MSP-1 Block 2 have shown that it provides targets for naturally induced antibodies associated with protection against clinical malaria episodes (Polley et al. 2003; Cavanagh et al. 2004; Conway et al. 2000). Antibodies to MSP-1 Block 2 have parasite killing properties as measured by ADCI assays (Jiang et al. 2000; Galamo et al. 2009). A monoclonal antibody to one MSP-1 Block 2 variant has been shown to inhibit parasite growth *in vitro* (Jiang et al. 2000; Locher et al. 1996). These antibody responses are however strain-specific (Conway et al. 2000; Cavanagh et al. 2004; Cavanagh & McBride 1997; Draper et al. 2010; Cavanagh et al. 1998; Sallenave-Sales et al. 2000; Zwetyenga et al. 1998; Ferreira et al. 2003). Since the allelic frequencies of the gene encoding the MSP-1 Block 2 region are under balancing selection throughout the global parasite population (Conway et al. 2000), individuals with antibodies to more than one strain of parasite are likely to be better protected (Conway et al. 2000; Cowan et al. 2011). Vaccine antigens such as the MSP-1 hybrid elicit broadly cross-reactive immune responses covering all three MSP-1 Block 2 serotypes and is a potential vaccine candidate.

MSP-2 is another parasite surface protein antigen under balancing natural selection (Conway 1997), suggesting that it may be an important target of antibodies affecting survival of the malaria parasite. The immunogenicity and target regions for specific antibodies to MSP-2 have been reported in a number of studies (Taylor et al. 1995; Aubouy et al. 2003). Several regions of MSP-2 are recognised by antibodies that have a parasite inhibitory effect

(Clark et al. 1989), parasite killing activity (Flueck et al. 2009), or are correlated with protection from clinical symptoms (al-Yaman et al. 1995; Polley et al. 2006; Metzger et al. 2003; Osier et al. 2010). MSP-2 has N- and C-terminal conserved regions, which are reported to be recognised by sera from naturally exposed individuals (Zhang et al. 2008), some of which have ADCI activity (McCarthy et al. 2011). However, most antibodies against MSP-2 target the variable regions of the molecule (Taylor et al. 1998; Metzger et al. 2003; Felger et al. 2003; Tami et al. 2002). Moreover, antibodies to one type of variable region of MSP-2 do not cross-react with other variable region types (Taylor et al. 1995; Franks et al. 2003; Flück et al. 2004). The effect of this serotype-specific response to MSP-2 was seen in the study by Genton *et. al.*, in 2002 in which the Combination B vaccine, based on only one allelic form of MSP-2 was used to immunize populations in sub-Saharan Africa where both serotypes of MSP-2 can be found. As a result, this vaccine induced immune responses that selected against one allelic form of the parasite and allowed the other parasite serotype to persist in vaccinees (Genton et al. 2002). It has subsequently been suggested that any recombinant vaccine based on MSP-2 should combine different serotypes to provide immune protection against the whole range of parasite strains (Flueck et al. 2009; Flück et al. 2007).

The aims of this part of my PhD project were to 1) produce a multi-component vaccine based on two potential vaccine candidates, the MSP-1 hybrid and the two allelic types of MSP-2 and 2) investigate the resulting recombinant protein for its biochemical properties, antigenicity and immunogenicity.

## **2. Materials and results**

### **2.1 Predicted properties of the MSP-multihybrid**

The MSP-multihybrid was predicted to be 81.17 kDa in size and have theoretical pI of 4.84, based on its amino acid sequence. Since the MSP-1 hybrid and MSP-2 are all IUPs, this MSP-multihybrid was also predicted to have similar properties. The theoretical protein construct was predicted to be largely unstructured using the IUPred prediction tool (<http://iupred.enzim.hu/>) (Figure 5.1).

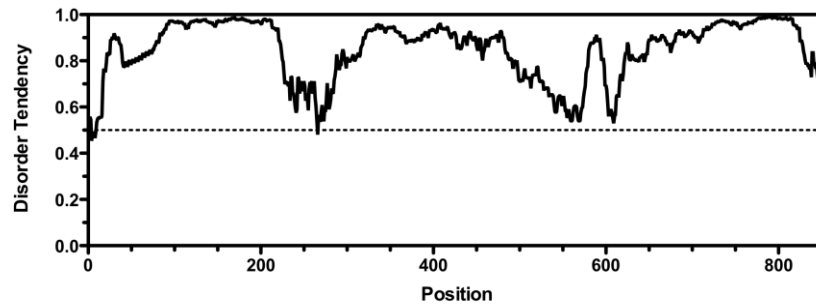


Figure 5.1: Disorder tendency profile of the MSP-multihybrid antigen, predicted by IUPred (Dosztányi et al. 2005), Disorder tendency on the Y axis is plotted against amino acid position in the theoretical protein sequence. High disorder tendency throughout the sequence predicts that this protein antigen is generally unstructured.

## 2.2 Gene cloning

### 2.2.1 PCR

The MSP-multihybrid DNA construct was produced by joining sequences of MSP-1 hybrid, MSP-2A and MSP-2B by PCR. Full-length MSP-2A (CH150/9) and MSP-2B (Dd2) constructs were gifts from Dr. David Cavanagh. Like the MSP-1 hybrid, they were codon optimized for expression in *E.coli*. Initially, three hybrid gene fragments were made;

- (1) MSP-2A with 5' region of the MSP-1-hybrid as an overhang at the 3' end, using MSP-2A as a template, and forward primer MSP-2A (5' CATCCATGAATGAAAGCAAATACAGCAACACC 3') and reverse primer MSP-2A-Hybrid (5' AGATTCGTGGGTAAC TTTGTTGATGCTCGC 3').
- (2) The MSP-1 hybrid with MSP-2 A (5') and B (3') overhangs at each end, using forward primer (5' GCGAGCATCAACAAAGTTACCCACGAATCTTAC 3') and reverse primer (5' GCTATATTTGCTTTTCATTGGAGCTATTATCGCTCGG 3')
- (3) MSP-2B with a 3' MSP-1 hybrid overhang on the 5' end of the gene using the MSP-2B as a template, and forward primer (5' AGCGATAATAGCTCCAATGAAAGCAAATATAGC 5') and reverse primer (5' ctcgcgccgcttattattaatgctggcaatattgctgc 3')

These fragments were made using PCR cycling condition as follows: an initial denaturation at for 94°C 2 minutes 50 seconds, followed by for 19 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 2 minutes

50 seconds, with a final extension at 72°C for 5 minutes. PCR fragments were separated on agarose gels and PCR products of the correct size were purified.

The original plan was to combine the MSP-2A and MSP-1 hybrid fragments by PCR, to fuse the gene fragments of these two genes. This product would then be further fused by PCR with MSP-2B, using the MSP-2A forward primer and the MSP-2B reverse primer. However, the recombinant genes that were achieved by this method were truncated, mostly missing part of the MSP-2 sequences. Some of the clones that contained sequences, of approximately the expected size, showed mutations within the repetitive region. Therefore, another method which relied on more conventional cloning and restriction enzyme digestion was used.

To solve the mutation problem, the fusions of MSP-2A-MSP-1 hybrid and MSP-1 hybrid-MSP-2B were made separately by PCR. The PCR products of MSP-2A and the MSP-1 hybrid were used as a template to produce a gene fusion of MSP-2A and MSP-1 hybrid (M2a-H) using the forward primer of MSP-2A and reverse primer of MSP-1 hybrid, described earlier. Similarly, gene fusion between the MSP-1 hybrid and MSP-2B (H-M2b) was produced using the forward primer of MSP-1 hybrid and reverse primer of MSP-2B. Both PCR reactions were performed as follows: an initial denaturation at 94°C for 5 minutes, followed by 29 cycles of denaturation at 94°C for 30 seconds, annealing for 55°C for 2 minutes and extension at 72°C for 4 minutes, with a final extension at 72°C for 5 minutes. Each fragment was then directly cloned into plasmid pCR4-TOPO using the TOPO TA cloning kit. TOPO plasmids were checked for cloned genes by restriction digest as suggested by the manufacturer's protocol and checked for sequence integrity by Sanger sequencing. Plasmids which contained correct gene constructs were extracted and stored as stock DNA plasmids (Figure. 5.2)

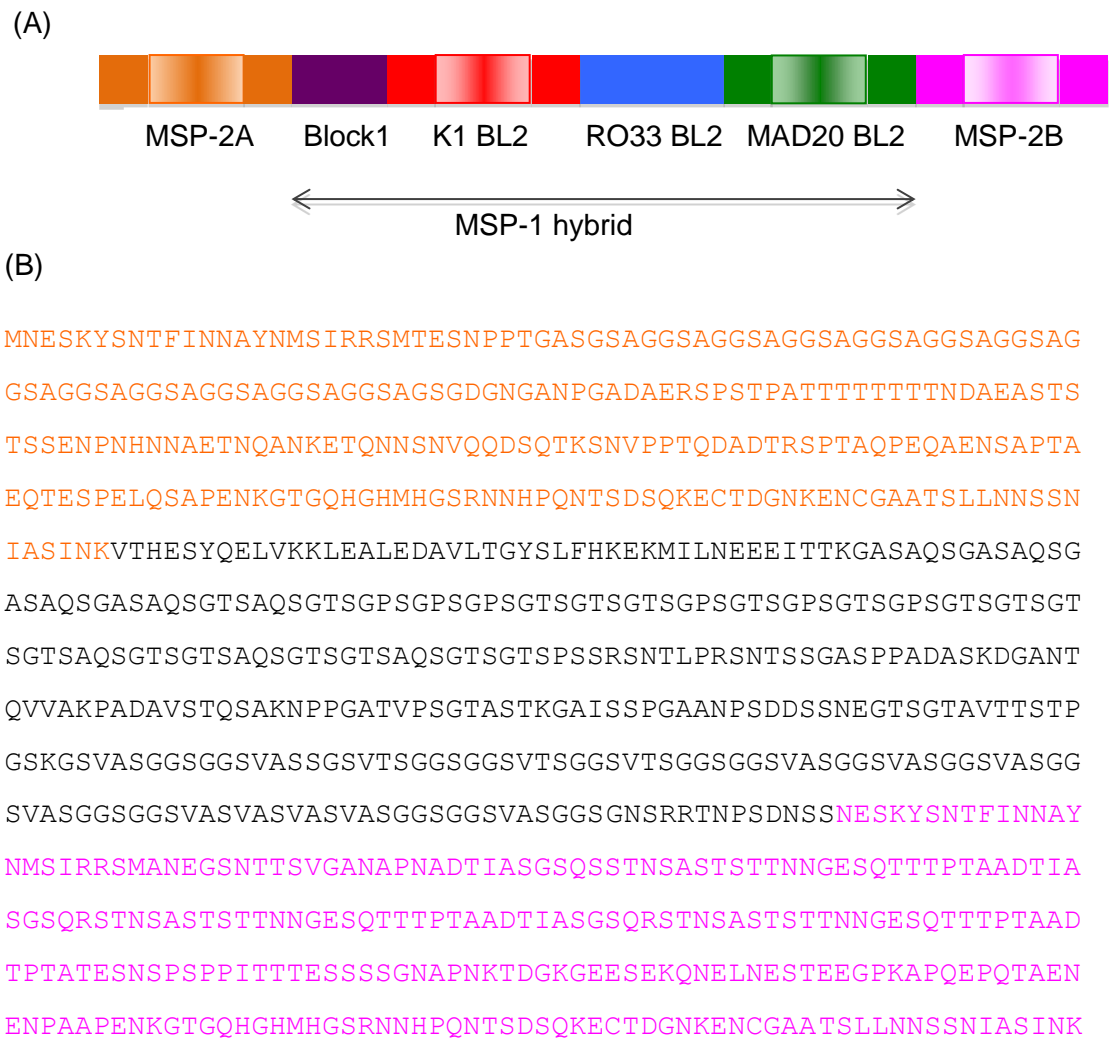


Figure 5.2 (A) Schematic representation of the MSP-multihybrid protein construct. The construct encodes the MSP-2A, the MSP-1 hybrid, and the MSP-2B. (B) Amino acid sequence of the MSP-multihybrid. Sequences of MSP-2A and MSP-2B are presented in orange and pink respectively, while sequence of MSP-1 hybrid is presented in black.

### 2.2.2 Restriction reaction

The two cloned DNA fragments (M2a-H and H-M2b) and the DNA expression vector pET28a were then cleaved by restriction enzymes to make compatible fragments for ligation cloning. The M2a-H and H-M2b genes were excised from the cloning plasmid sequentially by restriction enzyme *NcoI* (for M2a-H) or *NotI* (H-M2b) and then *Bpu10I*. All these restriction enzymes were used with Buffer 3 (NEB). DNA fragments were separated on agarose gels then purified and measured for concentration after each step. Ligation was

performed overnight using purified stocks of both gene fragments and the linearised form of the pET28a plasmid (Novagen, UK), which had been cut sequentially with *NcoI* and *NotI*.

### 2.2.3 Ligation reaction

Finally all gene fragments were all fused together by a ligation reaction. The ligation mix was transformed into *E.coli* XL-1 Blue using the protocol described in Chapter 2 (Materials and Methods, Section 4). Transformants with plasmids containing inserts of the correct size were checked for correct sequence and orientation. A clone which contained a full-length insert of the correct MSP-multihybrid sequence was selected, the plasmid DNA purified and then stored at  $-70^{\circ}\text{C}$  until use as described in Chapter 2, Section 1.

## 2.3 Protein production

### 2.3.1 Protein expression

For each round of expression the recombinant multihybrid plasmid was freshly transformed into *E. coli* BLR (DE3) pLysS cells. Cultures were grown in LB broth, Terrific broth, or LBE 5052 using the same protocols as described in Chapter 2, Section 7). Cultures were induced with 1 mM IPTG at OD<sub>600nm</sub> of 0.5-0.6 (log phase) and then sampled every hour until harvesting.

The fastest growth rate was observed when cells were grown in LB, providing around  $4.5\text{ gL}^{-1}$  of wet cells at harvest 4 hours post induction (Figure 5.3). The highest total wet-cell weight was achieved from the culture in Terrific broth which gave  $4.95\text{ gL}^{-1}$  of cell paste 4 hours post induction, and  $8.08\text{ gL}^{-1}$  at 12 hours post induction.

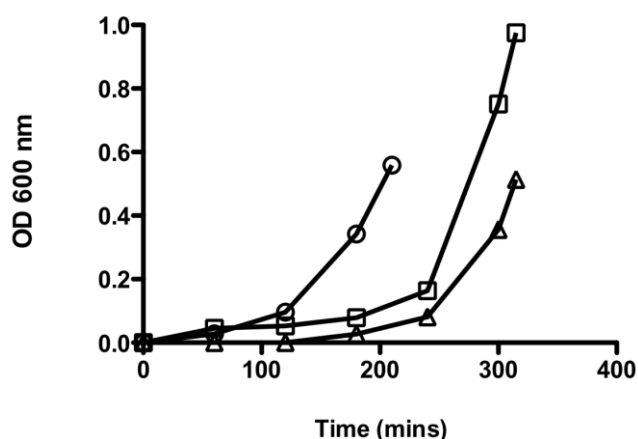


Figure 5.3: Growth curve of expression culture of MSP-multihybrid in different culture media; LB (circle), Terrific broth (square), LB5052 (triangle).

Either after 4 hours or 12 hours (overnight), cell cultures was harvested by centrifugation. Cell pellets were stored at  $-70^{\circ}\text{C}$  until use. To extract soluble protein, cells were lysed by cycles of freeze-thawing. Cell culture samples and lysates were then examined by SDS-PAGE and Western blotting. No clear evidence could be seen of induction of expression of the multihybrid either by silver nitrate or Coomassie Blue staining of cell extracts (Figure 5.4).

### 2.3.2 Heat treatment

Cell lysates were produced by the freezing-and-thawing method. As the first purification step for the MSP-multihybrid protein, heat treatment, as performed for MSP-1 hybrid (Chapter 2, Section 9), was tested to see if it would be suitable. The heated product was investigated by SDS-PAGE and Western blotting using monoclonal and polyclonal antibodies to MSP-1 Block 2 and to MSP-2.

Heat treatment, which was successfully used as a first purification step for the MSP-1 hybrid and MSP-3.3C, was similarly used for the MSP-multihybrid. Using either  $70^{\circ}\text{C}$  or  $90^{\circ}\text{C}$  treatments, host cell proteins were denatured and then removed by centrifugation as before. However SDS-PAGE analysis of the resulting material showed very low concentrations of residual soluble proteins in the supernatant (data not shown).

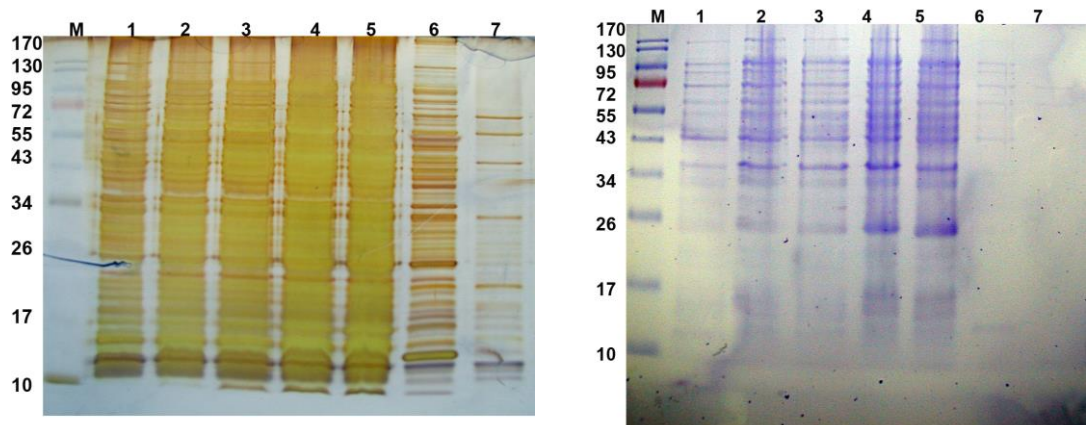


Figure 5.4: SDS-PAGE of the *E. coli* cell pellets and lysates harbouring the MSP-multihybrid plasmid at different production steps (M= marker, lane 1= culture before induction, lanes 2-5= culture 1, 2, 3 and 4 hours after incubation respectively, 6= cell lysate, 7= heated lysate)



### 2.3.3 Anion exchange chromatography

Partially purified soluble proteins from the heat treated lysate were dialysed extensively against 25mM Tris-Cl pH 8.0. Anion exchange chromatography was then used to further purify the MSP-multihybrid product. After loading a Q-sepharose FF column with the dialysed lysate, and washing with 25mM Tris pH8.0, a continuous gradient from 0 to 250 mM NaCl in 25mM Tris pH 8.0 was applied to the column and fractions collected. Strongly bound proteins were then eluted from the column by washing with 1M NaCl.

Eluted samples were analysed by a rapid dot immunoblot. 2  $\mu$ L of each protein sample was spotted on Schleicher and Schuell BA83 nitrocellulose membranes (Whatman). Once the spots were dried, the blot was blocked with Western blocking buffer (Chapter 2, Section 14). Blots were then probed using diluted pooled sera from 8 rabbits that were immunised with MSP-1 hybrid formulated with CoVaccine HT (Chapter 2, Section10). From these blots it was clear that fractions containing proteins reactive with MSP-1-hybrid antisera had eluted from the column at NaCl concentrations as low as 60mM, although antisera-reactive fractions continued up to 250mM NaCl and were also present in the 1M NaCl wash (Figure 5.5). Dot-blots probed with antibodies against MSP-2A and MSP-2B showed similar results (data not shown).

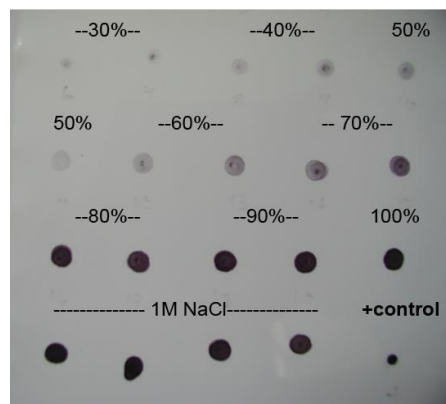


Figure 5.5: Fractions collected from the continuous 0-250mM NaCl gradient elution of Q-sepharose bound material were probed with rabbit sera against the MSP-1 hybrid. The numbers on the blot indicate the percentage of elution buffer concentration (0.25M NaCl) at the point of elution.

Each of eluted fractions was measured for protein concentration by BCA assay and analysed by SDS-PAGE. However, the protein concentrations were very low. Protein bands were not obviously visualised either by silver stained SDS-PAGE or by Western blotting (data not shown). Based on these results, it was assumed that the concentration of the MSP-multihybrid was very low.

#### **2.3.4 Antibody affinity chromatography**

To purify the low levels of multihybrid present in the lysates, an affinity chromatography column (HiTrap NHS activated HP, GE Healthcare, UK) was coupled with the 12.2 monoclonal antibody (gift of Dr. Graeme Cowan) and used to purify the MSP-multihybrid. This purification method was expected to provide more a concentrated and pure MSP-1-multihybrid product, which then could be used for analysis. The lysate was applied to the column in 25mM Tris and the protein was eluted with 1M NaCl in 25mM Tris-pH 8.0. Samples that were collected from this step of purification were then further analysed on SDS-PAGE and Western blot. Monoclonal antibody 12.2, mouse sera to MSP-1 hybrid (from mice that were immunized with MSP-1 hybrid in conjugated with CoVaccine HT; Chapter 2 Section 10), 12.3 monoclonal antibody to MSP-2A and mouse sera to MSP-2B (both provided by Dr. David Cavanagh) were used to detect the product of the MSP-multihybrid, for the Western blots and Dot blots.

The purified product from the 12.2 column was investigated on SDS-PAGE and Western Blot in parallel with MSP-2A, MSP-2B (provided by Dr. David Cavanagh) and MSP-1 hybrid proteins. The silver stained gel of this product showed five distinct bands, ~200kDa (A), ~130kDa (B), ~100kDa (C) and two smaller products of ~70kDa and ~55kDa (Figure 5.6).

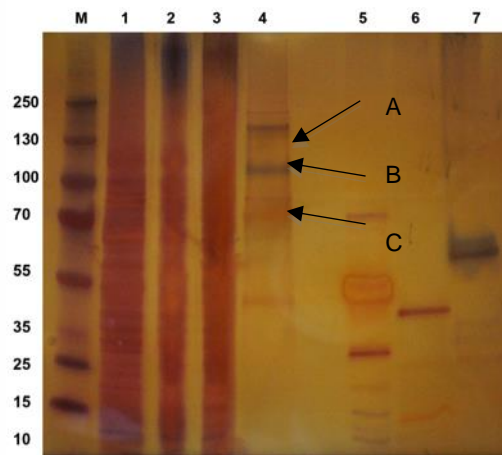


Figure 5.6: Silver stained SDS-PAGE showing proteins in each purification step. M= molecular weight markers, 1) culture before induction, 2) culture 4 hours post induction, 3) cell lysate, 4) affinity purified protein, 5) MSP-2A protein, 6) MSP-2B protein, 7) MSP-1 hybrid protein. Protein bands of ~200kDa, ~130kDa and ~100kDa are indicated as A, B, and C respectively.

Using antibodies to the three MSP components of the multihybrid, different patterns of protein bands were detected. The 12.3 monoclonal antibody, which is specific to MSP-2A, recognised two protein bands. The dominant band was ~200kDa, the same size as product A in SDS-PAGE (Figure 5.7). The second 12.3 reactive band of ~130 kDa, is approximately the size of product B. MSP-2B antisera gave different patterns of reactivity with bands of ~130kDa, ~100kDa, and ~70kDa observed. A band of ~200kDa was also very faintly detected. Using mAb 12.2, specific for an epitope in the K1 type of MSP-1 Block 2, four bands of ~200kDa, ~130kDa, ~100kDa and ~70kDa were detected (Figure 5.6).

Although the molecular weight of the full-length MSP-multihybrid was predicted to be 81.17 kDa, no protein band of that size was detected any antibody on Western Blots, but since IUPs migrate slowly in SDS-PAGE, bands corresponding to proteins of higher molecular weight were expected. The bands observed at ~200kD, ~130kDa and ~100kDa were assumed to be products of the MSP-multihybrid. All three bands were sent to SIRCAMS for mass spectrometric identification. However, due to the low concentrations of the purification products, they were out of detectable range by mass spectrometry and no identification was possible (data not shown).

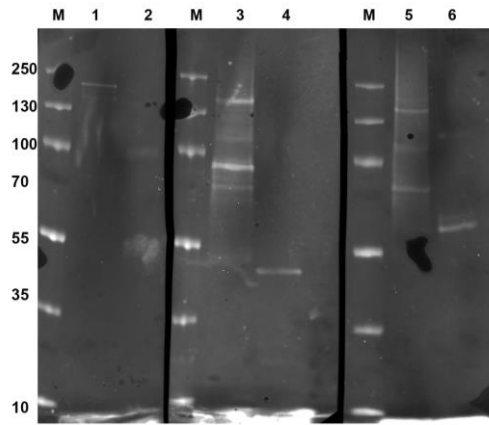


Figure 5.7: Western blot of purified MSP-multihybrid affinity-purified on the mAb 12.2 column. Western blots were probed with mAb 12.3 (anti-MSP-2A, lanes 1 and 2), sera against MSP-2B (lanes 3 and 4) and mAb12.2 (lanes 5 and 6). M= molecular weight markers; lanes 1,3 and 5; MSP-multihybrid; lane 2, MSP-2A; lane 4, MSP-2B; lane 6, MSP-1 hybrid).

## 2.4 Assays of antigenicity

The antigenicity of the MSP-multihybrid antigen was tested by ELISA for recognition by human sera from naturally exposed Africans, in parallel with recognition of MSP-1 Block 2 antigens and the MSP-1 hybrid. African sera reactive with at least one serotype of MSP-1 Block 2, MSP-2A or MSP-2B were used to probe the antigenic structure of the MSP-multihybrid. European sera from malaria naïve individuals were used as negative controls. Using our existing ELISA protocols, 50ng of the purified MSP-multihybrid antigen was coated onto wells of 96 well plates and assays performed as described Chapter 2, Section 19.

The MSP-multihybrid was recognised on Western Blots and dot blots, by sera that from mice immunised with either the MSP-1 hybrid or MSP-2 antigens. A pool of sera from adults from Brefet, Gambia, known to recognise the MSP-1-hybrid, both types of MSP-2, and all five types of MSP-1 Block 2, reacted with the MSP-multihybrid in Western blots. A panel of sera, from Gabonese adults, known to react with both MSP-1 Block 2 antigens and/or MSP-2, were also tested against the MSP-multihybrid. As shown in Figure 5.8, the antibodies from these selected sera recognised the MSP-multihybrid. Positive correlations were observed between antibody reactivity to MSP-multihybrid and other antigens, including the MSP-1 hybrid, MSP-1 Block 2 antigens, and both serotypes of MSP-2. In

general, the reactivity of these sera with the MSP-multihybrid was as least as high as to other antigens.

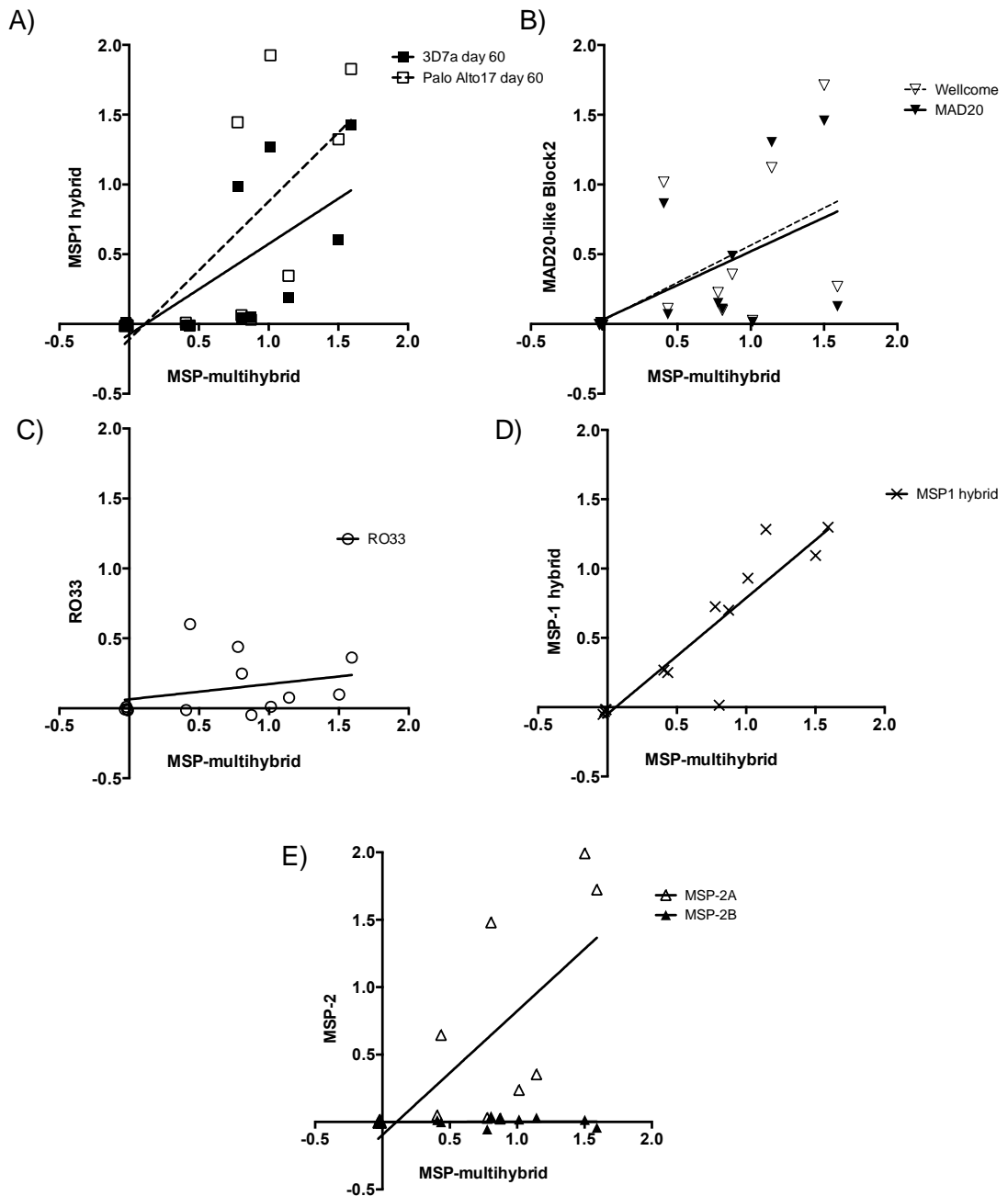


Figure 5.8: Reactivity of Gabonese adult sera with the MSP-multihybrid. ELISA reactivity to MSP-multihybrid, Block 2 antigens derived from MSP-1, the MSP-1 hybrid and full-length MSP-2A and MSP-2B antigens were measured by ELISA. Reactivity of each serum sample against MSP-1 and MSP-2 antigens (Y axis) is plotted against reactivity with the MSP-multihybrid (X axis).

### 3. Discussion

The MSP-multihybrid was designed as a vaccine that would elicit antibody responses by immunization against multiple parasite antigens to overcome the problems associated with parasite antigenic diversity. This single polypeptide antigen incorporating most of the allelic and antigenic diversity of both MSP-1 and MSP-2 was designed to overcome the problems of administration of mono-allelic vaccines, which have been shown to be ineffective or incapable of inducing a protective response against heterologous parasite strains (Ogutu et al. 2009; Flück et al. 2004; Genton et al. 2002). Although the MSP-multihybrid was expected to be relatively easy to produce, there were a number of problems and setbacks in producing, expressing and purifying this protein.

#### 3.1 Difficulties in gene cloning and protein production

The PCR technique is inexpensive, easy to manipulate and rapid. However, some genes can be difficult to amplify. The MSP-multihybrid gene is about 2.5 kb long and sequences of this length may exhaust the PCR reaction. As a result, the PCR product may be truncated. In addition, the MSP-multihybrid contains domains within the two MSP-2 components that are repeated. When two types of MSP-2 templates were present in the PCR reactions, they can potentially hamper the specificity of binding of the primers as they can both serve as alternative primer sites. Therefore, there is a chance that more than one product may be produced. This problem was overcome by separately joining each type of the MSP-2 to the MSP-1 hybrid. Restriction enzymes were then used to cleave the relevant sites within MSP-1 hybrid and the two PCR fragments were sequentially joined by ligation reactions.

This expression system gave very low amounts of MSP-multihybrid protein, which was insufficient for intensive downstream studies. Many possible reasons have been proposed to account for the low production of this protein. The DNA sequence of the MSP-multihybrid contains low-complexity regions and repetitive sequences. This kind of DNA sequence is more common in eukaryotes than in bacteria and the protein produced in *E. coli* may be particularly sensitive to proteolytic degradation. The more low-complexity regions there are in the gene, the less likelihood that the protein will be expressed well in a bacterial host (M. R. Dyson et al. 2004). Nevertheless, the MSP-1 hybrid, which contains similar repetitive sequences was expressed with much higher yields using similar expression and purification protocols. This suggested that the difficulty of MSP-multihybrid expression may be not only due to the structure of the protein but also its size. This protein is more than twice the size of the MSP-1 hybrid and to make the complete protein, *E.coli* needs to transcribe and translate a relatively long heterologous mRNA which has not been optimised for stability in bacterial systems. Experiments outlined in this chapter showed that extending

the expression period helped to improve total cell mass. However, the yield of MSP-multihybrid was not significantly improved. Like most malaria genes, the MSP-multihybrid is a relatively AT rich gene, apart from the codon optimised MSP-1 hybrid component. The *E.coli* host may not have enough tRNA resources for the production of this foreign protein, thus resulting in low expression and/or truncated products. Expression media were also important for growth of *E.coli*, serving as an energy source, and therefore important for protein yields. Experiments reported in this chapter showed that in different media, *E.coli* grew at different rates. However, none of media used showed any significant capacity for improving the expression yield of this protein.

To improve protein expression, the DNA sequence of this protein may have to be codon-optimised to make it more compatible with the *E.coli* expression host. A codon adjustment strategy, known as codon harmonization, may be another interesting way of improving heterologous protein expression (Angov et al. 2008). Other kinds of bacterial host and expression media could be used to find the best conditions for MSP-multihybrid expression. Using a different expression host, such as yeast, could be an alternative method to improve expression efficiency of this synthetic parasite protein hybrid, although yeast expression systems are prone to hyperglycosylation and post-translational degradation of heterologously expressed proteins. Since this protein is expected to be used in immunological studies and further developed as a vaccine candidate, contaminants from yeast, or even bacterial products (such as LPS) can trigger the immune system (Medzhitov 2001). These unintended immune responses may cause difficulty in the interpretation of the vaccine performance or could reduce the efficacy of any vaccine against malaria parasites. Purification methods have to be carefully designed to eliminate yeast or bacterial proteins from the protein product as much as possible.

In the purification of the MSP-1 hybrid, heat treatment served as an efficient purification step that eliminated the majority of host bacterial protein contaminants. It was expected that this purification step could be used in a similar way for this protein purification, because the MSP-multihybrid was also predicted to be an IUP. Since the expression protocols used provided very low protein yield, this caused difficulty in measuring protein concentration and detecting the MSP-multihybrid protein. Thus, this purification step has been neither properly validated for efficiency nor has it been intensively tested. It would be interesting to re-test the heat treatment as a purification step if expression of the MSP-multihybrid could be improved. Further development of this purification method could be made, by adjusting the salt concentrations in the buffers and testing a range of heating temperatures to improve the yield of partially purified MSP-multihybrid. Further

purification steps, such as anion exchange chromatography should be re-investigated as more scalable methods than using antibody-affinity columns such as the 12.2 mAb column used here.

Assuming that this protein would perform in a similar way to the MSP-1 hybrid on SDS-PAGE, it was predicted that it would migrate more slowly than a globular protein of the same molecular weight. Based on the predicted size of the MSP-multihybrid of 81kDa, this protein is likely to migrate like a protein of twice its size (around 160kDa or higher). The evidence from SDS-PAGE and Western blotting indicates a variety of sizes that are potentially the MSP-multihybrid. Based on the bands visualised by silver stained SDS-PAGE and the reactivity of the bands on Western Blots, the ~200 kDa band is likely to be the full-length product of the MSP-multihybrid gene, while the ~130kDa and ~100 kDa bands are assumed to be degradation products which lack some part of the MSP-2A region, as reactivity with these bands was not visible with anti-MSP-2A sera.

### **3.2 Properties**

From the amino acid sequence, the MSP-multihybrid should have a molecular weight of 81 kDa and is likely to be an IUP. However, to show that the protein is intrinsically disordered, the protein should be characterised by circular dichroism and dynamic light scattering as the MSP-1 hybrid was, to obtain more conclusive data on the structure of this protein.

Antigenically the MSP-multihybrid contains epitopes recognised by antibodies in pooled and individual sera from two separate populations naturally exposed to malaria. Further analysis showed that all subunit antigens within this molecule are recognised by these selected sera, and by antibodies to all three MSP components raised by immunisation in mice. Positive correlations between serum recognition to this MSP-multihybrid and MSP-1 Block 2 based antigens was shown. Similar trends were also observed when this antigen was tested against both MSP-2 serotype antigens. However, compared to the other antigens, these selected sera reacted weakly with the RO33 Block 2 and the MSP-2B antigen. This may be due to the low allele frequency of the RO33 Block 2 serotype in the studied population and, therefore, these individuals were infected with RO33serotype parasites less often. This may also be the same reason why these sera recognised MSP-2B antigen less frequently. Testing of more serum samples from different endemic regions might provide more conclusive data on the reactivity of the MSP-multihybrid with endemic sera.



### 3.3 Future plans

To make a further progress with this vaccine antigen, MSP-multihybrid expression will need to be improved to allow further investigations of this protein. For the expression step, a combination of alternative *E. coli* strains, especially those that are designed for proteins with low complexity regions, for example protease-deficient strains or strains secreting the protein into the periplasm could be tested. By improving the expression media used, cell mass could be increased, which would increase protein yield. Heat treatment has proved to be a very efficient way to partially purify IUPs, such as the MSP-1 hybrid. This purification step should be re-tested on cell lysates of the MSP-multihybrid, once expression levels have been improved. If this is successful in partially purifying the antigen, further purification by anion exchange chromatography may be used as needed.

Immunogenicity testing of the MSP-multihybrid in mice and rabbits should provide important data to assess the potential of this protein as a vaccine candidate. Once (or perhaps if) sufficient purified antigen is available, testing of the antigen in combination with the more promising adjuvants used with the MSP-1 hybrid would be possible. Again, if more purified protein was available, it would be of benefit to test the antigen for its reactivity with a more extensive set of endemic sera, to confirm the antigenic integrity of the purified product. Sera raised by immunization of animals could also then be tested in functional antibody assays such as GIA, ADCI or the recently described antibody-dependent respiratory burst assay, which is reported to correlate with protective immunity (Joos et al. 2010)

## Chapter 6: IUP identification methods

### 1. Introduction

Developing novel vaccines for malaria is challenging because the malaria parasite has a complex life cycle and expresses many different proteins during its different life cycle stages. Although there are at least five thousand candidate malaria parasite proteins that could be or are being researched, not many antigens have proved successful as malaria vaccine candidates. This leads us to the question of whether the critical targets for producing a protective vaccine are still undiscovered. If that is the case, then what are these targets, and how can we identify them?

Despite the completion of the *P. falciparum* genome, most of the functions of the predicted proteins encoded and their interactions with other parasite or host proteins are still largely unknown. One of the reasons for this is the fact that the malaria parasite genome is enriched with many low complexity regions (Aravind et al. 2003; Pizzi & Frontali 2001; Brocchieri 2001). These regions cause difficulties in identifying homologues of plasmodium proteins (Feng et al. 2006). The genes encoding *P. falciparum* proteins are also hard to express in heterologous systems (Mehlin et al. 2006). One well-known feature of low complexity protein sequences is tandem repeat regions, and these are abundant in the surface antigens of malaria parasites (Pizzi & Frontali 2001). Low complexity regions are closely linked to IUPs (Mohan et al. 2008), which are abundant in pathogenic eukaryotes (Mohan et al. 2008; Feng et al. 2006; Vucetic et al. 2003).

IUPs are abundant in the malaria parasite. Up to 70% of *P. falciparum* proteins contain long disordered regions (Vucetic et al. 2003), and IUPs are predicted to take up a large proportion of the malaria parasite's proteome (Feng et al. 2006). Our knowledge of these types of proteins, however, is still very limited and opinions as to their functions in malaria are very controversial. Some studies of the tandem repeats in proteins have hypothesized that they may function as a smoke-screen (Reeder & G. V. Brown 1996) possibly involved in an immune evasion strategies (Ferreira et al. 2003). Since IUPs generally contain arrays of short tandem repetitive sequences it has been suggested that IUPs may not be suitable as vaccine candidates (Pizzi & Frontali 2001).

However, many antigen candidates of the malaria parasite, such as MSP-1, MSP-2, MSP-3 CSP, AMA-1 and GLURP contain disordered regions or have been described as having IUP regions (Feng et al. 2006; Pizzi & Frontali 2001; Yang et al. 2007; Brocchieri 2001; Nair et al. 2002; Burgess et al. 2005). Some of these repeats are very immunogenic

and the antigens that contain them have shown potential as protective immunogens. It is therefore possible that not all malaria parasite IUPs are functionless and/or used in an immunological screen strategy, but may play important roles for parasite survival. IUPs are thought to have a role in binding to different protein partners (Feng et al. 2006; Turoverov et al. 2010), and may provide conformational plasticity for regions that require interaction to multiple partners (Mehlin et al. 2006; Singh et al. 2006a). For example, this plasticity may enable the surface antigens of the malaria parasite to interact with a broad variety of host ligands (Pizzi & Frontali 2001; Dunker et al. 2002). Alternatively, they may serve as linkers between two functional domains, controlling distance and timing of these interactions (Mohan et al. 2008; Dunker et al. 2002). Earlier in this thesis, other IUP vaccine candidates, which are derived from the surface proteins of malaria parasites, were able to elicit specific antibodies with functional anti-parasite activity *in vitro*. In addition, the evidence that naturally exposed African children with antibodies to the IUP regions of two of these antigens (MSP-1 Block 2 and MSP-2) are less likely to suffer from clinical malaria episodes argues that this type of protein target may be a promising source of new vaccine antigens. In the light of these data, it may be profitable to identify more IUPs from the blood-stages of malaria that may elicit protective immune responses, and that may potentially be made into useful anti-malaria vaccines.

## 1.1 IUP identification

There are two main ways to identify IUPs. Firstly, the IUP can be identified using *in silico* prediction tools. The unique characteristics of IUPs, such as their low content of bulky hydrophobic amino acids, the presence of low complexity sequences, and a high proportion of polar and charged amino acids, are used as the basis of algorithms for prediction of IUP tendencies. However, for greater accuracy, combinations of the algorithms and computational programs available for IUP prediction are recommended (Mohan et al. 2008; Ferron et al. 2006).

IUPs can also be identified using experimental protein chemistry assays, based on their unique properties, such as lack of 3D structure and lower electron density compared to folded proteins. Biophysical techniques such as NMR, analytical ultracentrifugation, circular dichroism, dynamic light scattering or X-ray crystallography can be used to distinguish IUP profiles from the profiles of folded proteins (Feng et al. 2006; Dunker et al. 2001; Vucetic et al. 2003). These techniques, (once conditions are optimised), are very efficient, reliable and suitable for small samples. However, each of these techniques has different individual requirements and may not be suitable for use with every kind of protein. For example, high protein concentrations are required for NMR and protein samples for circular dichroism need

to be in soluble form and in a limited range of buffer conditions (Vucetic et al. 2003; Kelly & Price 2000). IUPs of malaria parasites appear to be an important focus for further development as vaccine candidates or as drug targets. However, ways of identifying parasite IUPs are limited. Since the proteomic profile of the malaria parasite is yet to be completed, there is still a need for improvement of the IUP prediction algorithms. Also there are certain limitations in the available biophysical techniques for identification of IUPs from other structured proteins in the malaria cellular extracts.

A study by Csizmok in 2005 showed that IUPs can be distinguished from globular proteins by an unconventional two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) technique (Feng et al. 2006; Csizmok et al. 2006). This technique is based on the differences in biochemical properties between IUPs and globular, structured proteins. Firstly, IUPs can be selectively isolated from total cell protein extracts by heating, because IUPs are generally heat-stable, whereas many globular proteins are irreversibly denatured by heat treatment. This step is optional, as some IUPs may be associated with globular proteins and could therefore be lost by heat treatment. Heat-treated protein extracts, including IUPs and the remaining heat-stable globular proteins, are then resolved by native gel electrophoresis, separating the products according to their charge/mass ratio. A second dimension electrophoretic process then separates these proteins further under denaturing conditions. To do this, lanes from the native gel are cut from the gel, stained briefly and then fixed sideways along the top of a denaturing gel containing 8M Urea. The rationale for this is that generally, the structure of IUPs in a chemical denaturing environment, such as 8M Urea, is the same as under native non-denaturing conditions. Therefore, IUPs should theoretically migrate in both dimensions at the same speed. This results in the deposition of the IUPs on a diagonal line when resolved in the 2<sup>nd</sup> dimension, while globular proteins, which are unfolded in the denaturing environment of the second dimension, will run at a slower rate and accumulate in the area above the diagonal (Reeder & Brown 1996; Csizmok et al. 2006).

This novel method has been shown to be helpful in identifying new IUPs. It can be used to identify IUPs from a pool of unclassified proteins when run with known control samples of both globular proteins and IUPs. This separation of unstructured from structured proteins can be very effective when used in combination with mass spectrometric analysis. When compared with other predictors of unstructured protein such as PONDR (Xue et al. 2010), and techniques such as CD analysis, and gel filtration chromatography, results from the 2D gel technique were generally in agreement with other applications (Feng et al. 2006)

In this part of the thesis the aim was to modify and develop a 2D gel IUP identification method as reported by Csizmok *et al.* (Csizmok et al. 2006). The protocol was

reported to work with protein extracts from several eukaryotic species (Pizzi & Frontali 2001; Csizmok et al. 2006), but had not been used with protein extracts from malaria parasites. It was envisaged that a validated protocol from this study would be established as an alternative method for identification of the IUPs from malaria parasites, supporting other methods of characterisation and being introduced as a novel tool for vaccine antigen discovery.

## **2. Materials and results**

### **2.1 Establishing a working protocol using *E. coli* protein extracts**

To validate the pilot 2D gel protocol, protein extracts were prepared from a litre of the *E.coli* BL21 (DE3) pLysS culture. A single colony of cells was inoculated into a flask containing LB medium and grown overnight. The culture was harvested by centrifugation (Chapter 2, Section 8) and the pellet was washed several times in PBS. Cells were ruptured in 1/20 original volume of 25mM Tris pH8 by freeze-thawing. 1Units mL<sup>-1</sup> DNase (Benzonase; Novagen, UK) was added, incubated for 20 minutes, and then insoluble material removed by centrifugation (13,000 xg for 10 minutes) Cell lysates were either heated at 80 °C for 10 minutes, and clarified by centrifugation (13,000 xg for 10 minutes) or left untreated. Soluble lysates were collected and stored at -20°C until use. The protocol for the 2D acrylamide gel was derived from Csizmok *et. al.* (Csizmok et al. 2006). Native polyacrylamide gels were made by the protocol given in Materials and Methods (Section 13), but without SDS. In these experiments, two configurations of polyacrylamide gel concentrations were used; a) 3% acrylamide stacking gel and 8% resolving gel, or b) 4.5% and 12% respectively). Two resolving buffer conditions were also used; either 1.5M Tris pH8.8, or 0.5M CAPS pH10.5. The 1<sup>st</sup> dimension sample was resolved on 0.7mm thickness gels in a small format (8x6 cm Mini Protean II from Bio-Rad). The 1<sup>st</sup> dimension native gel was briefly stained with Coomassie blue dye, then soaked in 8M Urea solution (8M Urea in 1M Tris pH6.6) for 45 minutes and lanes containing protein bands were cut into ~1cm wide strips. The 2<sup>nd</sup> dimension 8M Urea gel, which formed the denaturing step of the protocol used only the resolving gel without a stacking gel. The gel strip from the first dimension was placed between 1-mm spacers to allow the 1<sup>st</sup> dimension native gel strip, (which was swollen after incubation in 8M Urea buffer), to be fitted onto the second dimension gel. Both gel dimensions were run at the same voltage (90-120 V), using the same running buffer as described in Materials and Methods, Section 13, but without SDS, until the dye-front

reached around 5-10 mm from the bottom edge. Gels were then stained with silver nitrate stain and used for further analysis.

Both percentages of native gel (3% gel and 8% resolving gel, and 4.5% and 12% respectively) were successfully used in separating bacterial proteins. These 'Diagonal gels' showed different protein patterns between the heated protein extracts and the untreated protein samples. On both gels, some of proteins resolved on the diagonal between the two dimensions, indicative of IUPs, while other proteins ran above diagonal, as expected of globular, structured proteins (Figure 6.1).

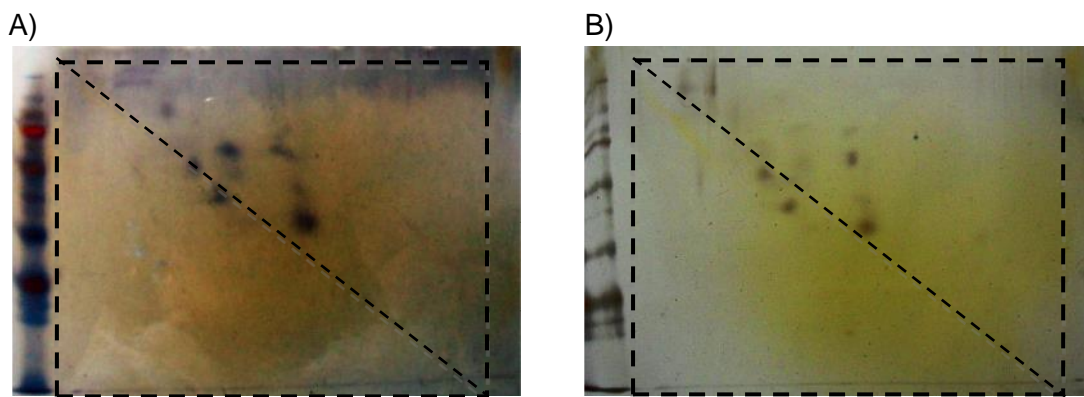


Figure 6.1: Diagonal gels of bacterial protein extracts, with either A) no heat treatment or B) lysates heat treated at 70 °C for 20 minutes. Proteins were resolved on both gels using 3% acrylamide stacking gel and 8% resolving gel for the 1<sup>st</sup> dimension and a homogeneous 8% acrylamide 8M Urea gel for the 2<sup>nd</sup> dimension. Note that the protein marker on the left was used as a staining control and as an indicator that the protein sample had reached the bottom of the gel, but did not act as a reliable marker for molecular weight estimations.

## 2.2 Parasite sample preparation

Parasite cultures for this experiment were prepared as described earlier in Materials and Methods (Section 21). Schizont rich cultures were harvested by centrifugation at 830 xg for 5 minutes and washed three times in PBS. Initially, a 0.15% saponin solution was used to lyse both uninfected and infected erythrocytes. Cell culture pellets were re-suspended in the saponin solution and incubated at room temperature for 5 minutes. An equal volume of

PBS was added to the mixture and parasites released from erythrocytes were pelleted by centrifugation at 830 xg for 5 minutes. The parasite pellet was washed several times in PBS and stored at -20°C until use. Parasite pellets were re-suspended in 0.15% saponin solution and incubated on ice for 5 minutes. Insoluble material was then removed by centrifugation at 13,000 xg for 5 minutes at 4 °C. The supernatant was stored at -20°C until use.

The saponin extracted parasite samples resolved on native PAGE gels were stained with silver nitrate stain, but resolution of individual protein bands was very poor, due to a strong gel background and smearing (Figure 6.3 lane 1). The saponin treated samples were then heated at 80°C for 10 minutes, and clarified by centrifugation at 13,000 xg 5 minutes at 4°C. Heat-treated samples have lower levels of protein on the silver nitrate stained native gels and individual protein bands were readily visible, although smearing was still visible (Figure 6.3 lane 2).

As problems were encountered with the first protein extraction method, an alternative method was tried. Erythrocyte membranes from parasite cultures were broken under mild conditions by freeze-thawing on ice. The parasite pellet was collected and washed with PBS and frozen until use. Using this parasite pellet, different methods were then used to extract parasite proteins.

Further freeze-thawing cycles were performed. Parasite pellets were re-suspended in PBS containing protease inhibitors (Roche). Cycles of freezing at -20°C and thawing on ice were performed three times. Cell debris was separated from the soluble protein by centrifugation at 13,000 xg 5 minutes at 4°C and stored at -20°C until use.

Detergent or chemical treatments were used to disrupt parasite cell lysates. Parasite pellets were treated with either a) 1% NP40, b) 0.5% Triton X100, c) 0.5% NaCl in PBS or d) 0.1M NaCO<sub>3</sub> pH 11.0 and incubated for 10 minutes on ice. Soluble proteins were then harvested from the supernatant after clarification by centrifugation at 13,000 xg 5 minutes at 4°C. During all extractions protease inhibitors were used at the concentrations recommended by the manufacturer (cOmplete Tablets, Roche, UK) and, depending on the experiment, DNase (1Units mL<sup>-1</sup> Benzonase, Novagen, UK) was added. Clarified supernatants from DNase treated lysates were collected and samples were resolved on native gels to assess quantities of proteins in each extraction, and the quality of resolution of these proteins on native PAGE gels. The most efficient extraction method was selected for sample preparation and further investigation by 2D gel electrophoresis.

Heat treatment was also used with some of the protein extract samples. Soluble protein lysates were heat-treated at 80°C for 10 minutes. Samples were then chilled on ice

for a further 10 minutes and denatured proteins were removed by centrifugation at 13,000 xg for 5 minutes at 4°C.

Using the freeze-thawing method, erythrocyte membranes are gently disrupted and the parasite pellets can be collected. Thereafter, several techniques can be used to produce a suitable parasite protein extract. The most successful treatment was freeze-thaw lysis which produced soluble proteins harvested from the parasite pellet that could be detected as discrete bands on silver stained native gels (Figure 6.3, lane 3). This sample provided clearer protein bands and lower background on the gel than samples prepared by saponin lysis (Figure 6.3, lane 1). Heat treatment was performed on this protein sample, but soluble proteins from the heating step were at very low concentrations and protein bands were barely visible on silver stained gels (Figure 6.3, lane 4).

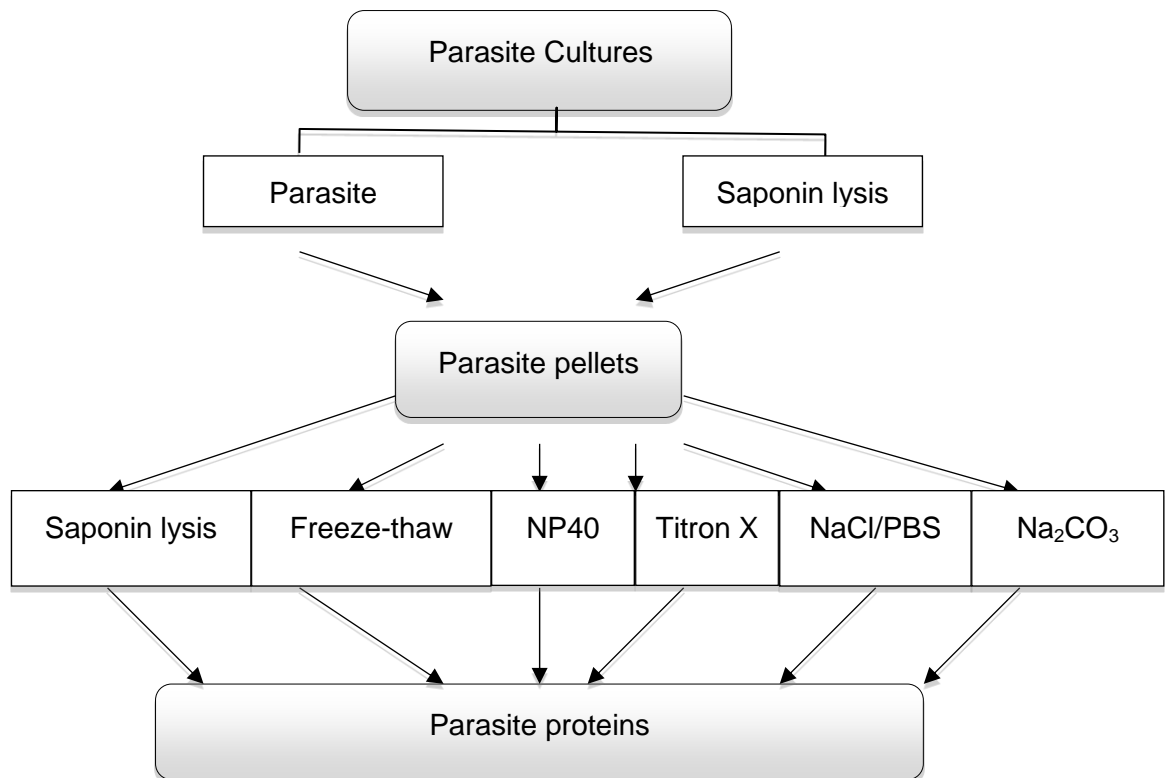


Figure 6.2: Flow chart showing the protein extraction methods used to prepare parasite proteins from malaria parasite cultures.



Soluble proteins were also extracted from the parasite pellet using the detergent NP40. This detergent is not powerful enough to break nuclear membranes on mammalian cells, but can break their cytoplasmic membranes. As such, it can be used to obtain the cytoplasmic contents of a cellular culture. Using NP40, the length of incubation time period correlated with the amount of proteins extracted (Figure 6.3, lanes 5-6). However, other chemical extraction methods, including high salt treatment (0.5% NaCl in PBS) and high pH treatment (0.1M Sodium Carbonate pH11) gave very low concentrations of protein extract (data not shown).

Due to the limited time scale of this part of my project, the samples from NP40 extraction were not further treated by the heating method. Only the protein samples from the freeze-thawing method were further tested on 2 dimension gels (Figure 6.4).

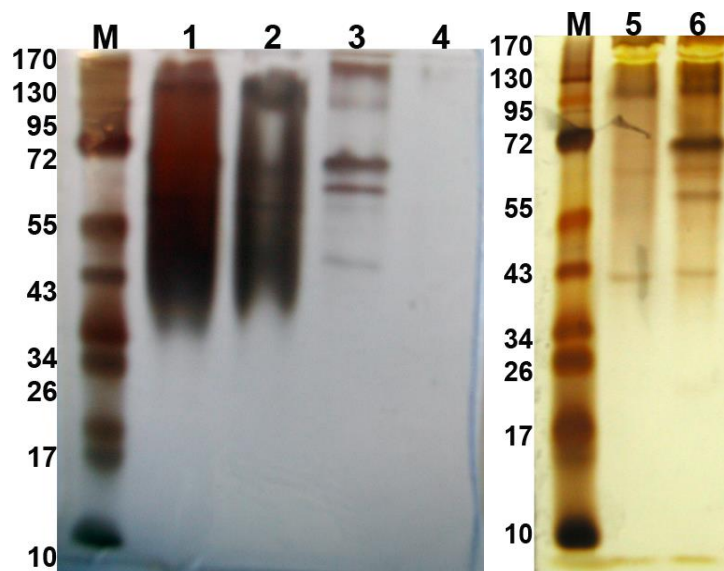


Figure 6.3: Parasite protein samples from different extraction methods resolved on native PAGE. Proteins from parasite pellets were prepared either by saponin lysis (lane 1) or by freeze-thawing method (lane 3). These two samples were heated and the clarified solutions are shown in lane 2 (saponin) and lane 4 (freeze-thawing). Subsequently, parasite pellets prepared by freeze-thawing were incubated with 1% NP40 for 10 minutes (lane 5) or 20 minutes (lane 6). (M= molecular weight markers in kDa)

### 2.3 Native/denaturing 2D gels of parasite protein extracts

Unlike the bacterial protein samples, protein extracts from malaria cultures contained some contaminants from red blood cells, which appeared to interfere with the gel staining dyes and give background on the 2D gels. It was also noticed that these protein samples migrated relatively slowly on the 1<sup>st</sup> dimension native gel. Because of this slow migration, lower percentages of native gel (3% stacking gel and 8% resolving gel) and the 2<sup>nd</sup> dimension 8M Urea gel (8% acrylamide ) were used in order to allow proteins to migrate further. To test whether higher pH in the gel might improve this migration by increasing the negative charge on the proteins, a running buffer of 0.5M CAPS pH10.5 was used as an alternative to the 1.5M Tris pH8.8 in the standard protocol. However, it did not improve resolution of the protein dots on the 2D gel (data not shown). The velocity of protein migration, which is directly proportional to the voltage across the terminals in the PAGE tank, was another factor that affected the resolution of these diagonal gels. I was able to show that using a lower voltage could improve the resolution of the 2D gel.

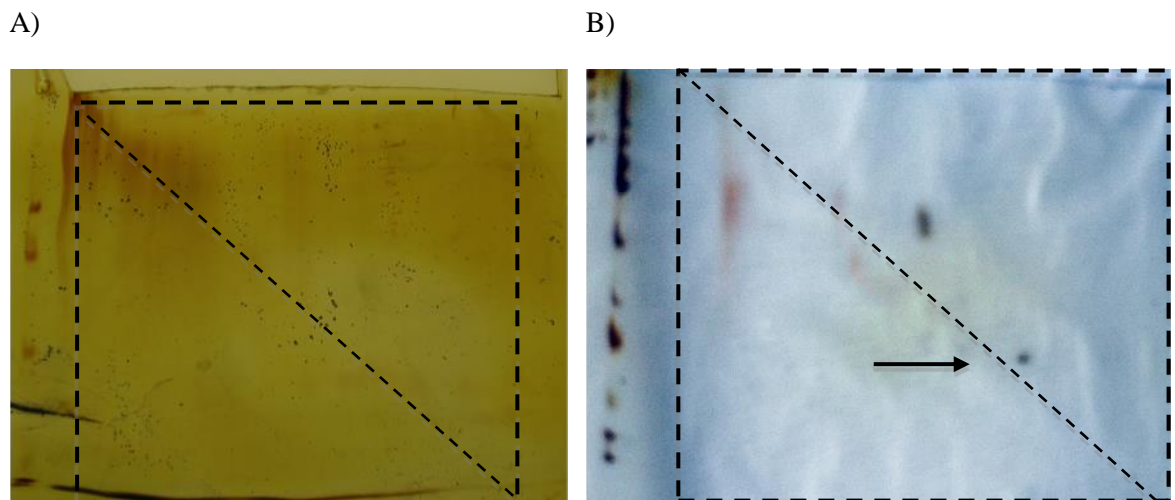


Figure 6.4: Representative native/denaturing 2D gel of parasite proteins extracted by the freeze-thawing method, and then A) heat treated or B) without heat treatment afterward. A small number of proteins were observed to migrate at equal velocities in both dimensions, and thus migrate close to the diagonal. One prominent spot was observed close to the diagonal in the lower right hand part of the gel (arrowed).

### 3. Discussion

#### 3.1 Protein sample preparation

These experiments confirmed that the 2D native/denaturing gel technique could be performed in the laboratory, using soluble proteins from bacteria. However, this protocol is not yet optimised for malaria IUP identification. The main problems encountered were that 1) protein samples from parasite cultures were not sufficiently concentrated to detect IUPs, especially in heat-treated samples and 2) samples contained some red blood cell contaminants, which interfered with the migration of proteins in both the native and denaturing dimensions. The protein extract that was prepared for this experiment turned out to be too dilute to visualize proteins well on the 2D gel. As a consequence, it was hard to compare the proteins from each extraction process qualitatively and quantitatively. By examining earlier studies by others, using conventional 2D gels (isoelectric focusing/SDS-PAGE gels) for *P. falciparum* protein identification, approximately 30 mL of parasite culture at 8% parasitaemia, 5% haematocrit was used to reliably detect parasite proteins in that system (Smit et al. 2010). Therefore, it seems that this technique requires larger amounts of soluble protein than I expected, and that a much greater amount of starting material was needed in my experiments than I initially estimated.

The optimisation of lysis buffers and protein extraction methods is however equally important. Optimised protocols should not only maximise solubility of parasite proteins and therefore allow more proteins to be detected, but may also play an important role in eliminating the contaminants that interfere with protein migration, cause non-specific background staining, and allow further assays on gels such as mass spectrometry. Ideally, the protein lysate used should to give high-resolution spots and have low streaking effects on the 2D gel. In this study, different detergents were tested for their ability to extract different cohorts of proteins. The freeze-thawing method appeared to be the gentlest technique for disrupting the erythrocyte membrane and the parasite membrane. Saponin treatment was also able to disrupt these two membranes as well as the parasitophorous vacuolar membrane and should therefore release more proteins from the parasite. However, saponin seems to interfere with protein migration on PAGE and cause smearing effects. NP40 was also used for protein extraction, producing a soluble protein extract that could be visualised on native PAGE. Other extraction methods (high salt, high pH) seemed to give protein samples of very low concentration.

Haemoglobin from red blood cells has been shown in other studies to interfere with assays such as conventional 2D PAGE, as well as mass spectrometry (Alvarez Llamas et al. 2009). It is likely that haemoglobin carried over from the cell lysis step may have affected

the resolution of these 2D gels. This suggests that future protein preparation steps should be designed to exclude haemoglobin as much as possible. This should improve the clarity and reduce background staining of the gel and ensure that any IUPs identified can be further analysed by mass spectrometry. Other purification steps, such as anion exchange chromatography, can remove haemoglobin (Sun & Palmer 2008), and may be used to eliminate haemoglobin from the sample, while allowing collection of other proteins from the parasite extract. Nevertheless, this protocol has to be tested with the malaria infected red blood cell sample to be validated before it can be used. The work by Smit, *et. al* in 2010 suggests that using the lysis buffer of Nirmalan *et al* (Nirmalan et al. 2004) yielded parasite protein extracts suitable for use with conventional 2D isoelectric focusing/SDS-PAGE. In that study, it was reported that a lower percentage of saponin, shorter sonication time, and an increase in washing steps reduced haemoglobin contaminants significantly and improved resolution on 2D gels (Smit et al. 2010). This protocol could be tested for preparation of samples to be run on these native/denaturing diagonal gels.

The protein extraction methods used in this study should be repeated using more parasite material, to establish which method(s) are most suitable for malaria parasite proteins. These different methods may extract different groups of proteins, which may localize at different parts of either the erythrocyte or parasite structures. Sequential protein extraction, using different combinations of these methods may also be useful. Each protein sample from each step of the extraction procedure could be tested separately on different gels. By separating whole cell proteins extracts into subsets by these fractionation methods, gel resolution may be improved by decreasing the number of individual spots on each gel. This should greatly facilitate downstream analysis such as mass spectrometry for final identification of IUPs in the parasite proteome.

Heat treatment was expected to remove most heat-sensitive globular proteins, precipitate DNA/protein complexes and to leave the sample with a higher proportion of IUPs. Heating certainly improved the resolution of the 1<sup>st</sup> dimension native gel step, giving more defined protein bands. However, in the samples used with their low concentration of protein it was difficult to detect protein spots on the 2D PAGE gels. Again, more concentrated samples would be needed to validate this technique in future experiments. Although IUPs are heat stable, they may be very sensitive to proteases (Dunker et al. 2001; Wright & Dyson 1999). Protease inhibitors should therefore be used through the extraction process.

### 3.2 Limitations of the diagonal gel assay

The native gel PAGE method used for the 1<sup>st</sup> dimension of this diagonal gel method has poor resolving power. This was reported by Csizmok *et al*, who stated that the resolution of the native/denatured 2D system is not as high as that of conventional isoelectric focusing/SDS-PAGE 2D gels. However, they also suggested that this can be overcome by using non-charged, mild detergents in protein extraction steps (Csizmok *et al*. 2006). The same paper by Csizmok *et al* also concluded that the buffer system also affected the outcome of the assay. Since roughly half of IUPs would have a net positive charge in the buffers used in this system, i.e. that they have a basic isoelectric point, they would not resolve on these gels when standard conditions are used (Csizmok *et al*. 2006). In our experiments an alternative buffer with a higher pH was used. However, it did not seem to improve the detection of IUP spots, but since these gels were tested with low concentration samples, these experiments should be repeated.

The limitations and sensitivity of the gel staining methods used are also to be considered. Ideally, a sensitive, reproducible, and easy staining method is required and crucially, it should not interfere with further analysis, such as mass spectrometry. Coomassie Blue, which is used in the original paper by Csizmok *et al.*, is based on direct or indirect binding of aromatic and/or basic amino acids and cysteine. It has been shown that this method underestimates the concentration of IUPs, which have low levels of these types of amino acid residues (Szollosi *et al*. 2007). Thus, the less abundant IUPs may not be able to be visualized by this method. Using a more sensitive staining dye, such as silver nitrate staining, was suggested. However, since different proteins stain differently according to their unique physical and chemical properties, several different staining methods should be tested and the results should be compared.

### 3.3 Future plans

This 2D gel technique was proposed as an alternative method for identifying and confirming proteins as intrinsically unstructured (IUPs). Uniquely, it can be used with proteins from whole cell extracts, which will allow novel IUPs to be discovered from a protein mixture (Csizmok *et al*. 2006). Although these experiments did not prove to be currently suitable for malaria protein extracts, there still a large amount of room for improvement of the protocols of protein extraction and detection. Once these methods are validated for malaria parasite extracts, many novel proteins may be discovered. Newly discovered IUPs can then be identified by mass spectrometry, and investigated for their localization in the parasite, functions and their potential to be developed as vaccine candidates.

## Chapter 7: Discussion

Many malaria antigens under development as vaccine candidates were selected by means of their reactivity with human immune sera. However, due to the nature of the parasite antigens selected (i.e. their relative abundance, immunogenicity, polymorphism, etc.) and our limited knowledge of the mechanisms of acquisition of immunity to malaria, these selection methods are of limited value and empirical. This causes difficulty in comparative studies (Holder & Holder 2009) and there is always the risk that the good vaccines are dropped while poor vaccines are taken into further development stages (Richie & Saul 2002).

Malaria vaccine research has not been focused in any way on IUPs until recently, when many of the existing vaccine candidates were reported to contain unstructured regions (Cowan et al. 2011; Yang et al. 2007; Burgess et al. 2005; Pizarro et al. 2005). With this knowledge, two sides of opinion were formed concerning the roles of malaria parasite IUPs and their relationship with host immunity. The first view proposed that IUPs are functionally important for parasite survival, and therefore may be good targets for protective immune responses. This has been supported by the fact that several of the potential vaccine candidates which are IUPs are targets of antibody responses which appear to be associated with protective immunity or at least reduced incidence of clinically significant malaria infections (Poley et al. 2006; Cavanagh et al. 2004; Roussilhon et al. 2007; Metzger et al. 2003; Conway et al. 2000). In contrast, in the past, others have suggested that IUPs are used as part of the parasite's immune evasion strategies, by eliciting non-protective, cross-reactive antibodies that act as an immunological 'smoke screen' (Kemp et al. 1987). This idea is supported by the fact that IUPs have the ability to take up a variety of conformations, which may hamper the efficiency of antibody recognition and therefore immune responses (Feng et al. 2006).

In this PhD project I have shown that the two studied IUPs, the MSP-1 hybrid and MSP-3.3C were immunogenic when formulated with adjuvants. Antibodies in malaria exposed children specific for the MSP-1 hybrid were associated with protection from clinical malaria episodes in a cohort of Ghanaian children. Antibodies raised to MSP3.3C by immunization in rabbits showed very strong parasite growth inhibitory effects *in vitro*. This encouraging data suggests that these IUPs (and possibly others) are an interesting repertoire of antigens that have the potential to be developed as vaccine candidates. This alternative rationale for antigen selection is supported by the fact that several of the leading vaccine candidates have been shown to possess IUP domains or to be wholly IUP themselves.

IUPS can be distinguished from globular, structured proteins by their unique

biochemical properties. Using the native/denaturing diagonal gel method, I attempted to identify IUPs from parasite protein extracts. Unfortunately, due to problems with the efficiency of the protein extraction procedures, and some underestimation of the volumes of parasite material needed, I was not successful in identifying novel IUPs in parasite extracts. Once this technique has been further developed, this protocol may serve as a simple, novel strategy for proteome-wide IUP screening. Identified protein sequences could then be analysed by bioinformatics, the products expressed in recombinant systems and tested in pre-clinical immunological assays to establish whether these antigens are vaccine candidates.

This PhD project showed that IUPs such as MSP-3.3C, the MSP-1 hybrid and the MSP-multihybrid can be produced either as single recombinant proteins based on one gene or as multi-component vaccine antigens. By designing constructs with synthetic sequences or multiple gene components fused together, this can overcome the problem of antigenic polymorphism, as well as provide multiple targets for immune recognition. Protocols for the production and purification of the MSP-1 hybrid and for MSP-3.3C were easily developed and are both scalable methods amenable to process-scale vaccine production. For larger antigens such as the MSP-multihybrid, further codon optimization of the MSP-2 components may be necessary for optimum yields.

The unique properties of IUPs make these proteins easy to express, handle and purify. The heat stability of the proteins makes production steps simple, and simplifies the purification process. Unlike many proteins expressed in bacteria refolding steps are not required for IUPS, as they lack rigid structure in their native form. Once these IUP antigens have been characterised, Good Laboratory Practice (GLP) procedures should be established in order to guarantee the quality of any vaccine before it can be further tested in non-human primates. The results from these pre-clinical studies will surely be good guidelines for further trials, which require the higher exigencies standard such as Good Manufacturing Practice (GMP). Unlike other vaccines, which can rapidly lose potency due to temperature sensitivity, IUPs such as the MSP-1 hybrid are stable. This unique heat-stable property of IUPs has practical benefits for vaccine delivery, especially for the distribution of malaria vaccines to locations where they are needed without cold chain supply. Older studies of the silk protein fibroin, which has a similar circular dichroism profile to the IUPs in this study (Iizuka & Yang 1966), reported that it was a good stabilizer for labile vaccines, by enhancing thermostability and prolonging the shelf life of the vaccines (Zhang et al. 2012).

This project has shown that surface IUP antigens from malaria parasites are promising malaria vaccine candidates. But how are these IUP antigens processed and presented by the immune system? What could be the molecular activities of antibodies to

IUPs? Why do antibodies to MSP-3.3 seem to be very effective in parasite killing *in vitro*? These questions remain to be resolved and will need to be further investigated by experimentation. However, based on current knowledge, it may be that IUPs adopt stable conformations only for short periods of time when they interact with their partner ligands (Tompa 2002). This 'just in time regulation' of protein structure may benefit parasite survival by narrowing the chance for immune cells or antibodies to recognise discontinuous epitopes within IUPs, which are part of transient conformationally active sites. However, specific antibodies that bind to continuous epitopes on these proteins may also disrupt the 3D structure formation process and impair IUP protein function.

It was believed until recent years that only properly folded proteins could perform physiologically relevant functions; those that failed to develop rigid structures were regarded as the cause of pathology or doomed to be destroyed by internal cellular structures such as the proteasome. Studies of IUPs have reshaped our understanding toward these fundamental aspects of protein functions. Research on IUPs will not only let us understand more about their roles, but will also benefit immunological studies and vaccine development research, especially for pathogens such as malaria parasites, in which IUPs are abundant.



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