B cell responses to recombinant CIDRα domains of the *Plasmodium falciparum* erythrocyte membrane protein 1

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Thesis submitted to the University of Liverpool for the degree of

Doctor of Philosophy

April 2019

Abstract

Malaria remains one of the major infectious diseases with high mortality and morbidity particularly in children under the age of 5 years. Mature forms of asexual erythrocytic stages of the parasite adhere to receptors expressed on endothelial cells resulting in the blockage of capillaries and local organ damage. Among all variable surface antigens (VSA) expressed on the surface of infected erythrocytes, the Plasmodium falciparum erythrocyte membrane protein 1 (*Pf*EMP1) is the major modulator of the cytoadhesion. Structurally, *Pf*EMP1 molecule consists of tandem repeats of several duffy binding-like (DBL) and cysteine-rich interdomain region (CIDR) domains that have different cytoadhesive preferential. Most importantly, an EPCR binding subsets of CIDR domain namely α1.1 and α1.4 have been associated with cerebral and severe malaria. However, naturally acquired immunity to these subsets exists and are associated with protection against severe malaria.

In the present study, we produced 12 recombinant CIDRα domains and used it to characterise 29 plasma sample obtained from recruited individuals from the southern region in the Kingdom of Saudi Arabia. Consequently, two donors were selected for single cell isolation of CIDRα1-specific B cells using a tetramerised recombinant CIDRα1.1 and CIDRα1.8 antigens. Following FACS based single cell sorting of isolated CIDRα1-specific B cells, cDNA and amplification of immunoglobulin genes were carried out using single step RT-PCR. Subsequently, paired immunoglobulin heavy and light chains genes were co-transfected into

HEK293 cell line for production of mAbs. Out of 65 mAb produced, nine mAbs demonstrated a variable degree of cross-reactivity against a panel of recombinant CIDRα antigens. However, only two mAbs were able to show a neutralisation potency of up to 67% when tested using a static-based multiplex assay. Further testing for flow adhesion assay as well as epitope mapping is required to conclude the competency of isolated mAbs.

Acknowledgments

First of all, thanks to ALLAH, the Lord and Cherisher, for the all the blessing He bestowed upon me and for willing me to finish my PhD project. Indeed, without His help and will, nothing is accomplished.

I would like to express my most gratitude to my academic supervisor, Dr. Britta Urban for the opportunity to work within her group and agreeing to supervise my PhD project. My sincere thanks for her open door policy, kind flexibility, continuous guidance and encouragement throughout the course of my research program. My appreciation is also extended to my second supervisor Prof. Alister Craig for his support and advice. Many thanks to my PAP members Prof. Richard Pleas and Dr. Qibo Zhang for their support and advice. Also, I would like to thank my dear friend Mr. Hisham Alharbi for his help with laboratory work, scientific support and encouragement throughout the research period. I also would like to extend my thanks to fellow Saudi students for their help and encouragement.

In addition, I would like to acknowledge specific contributions from the following:

- Dr. Thomas Lavstsen and Dr. Louise Turner from the University of Copenhagen for carrying out the Multiplex characterisation for donor plasma samples and isolated mAbs as well as performing the neutralisation assay.
- Dr. Aymen Madkhali, Dr. Mohammed Alzahrani, Mr. Yahia Khawaji and Mr. Abdo Alfaify for their help with donors recruitment and samples processing.

Finally, and most importantly, I am grateful to my parents to whom I owe everything. Thanks could never be enough. My greatest gratitude and love to my wife, for her understanding and encouragement during the long period of my research work. Thanks to my dear children who have patiently tolerated my absent-mindedness.

TABLE OF CONTENTS

List of Figures

List of Tables

Abbreviation

ABC	Activated B cells
APC	Activated protein c
ASC	Antibodies secreting cells
BBMX	BugBuster Master Mix
BC	B cells
BCR	B cell receptor
BME	Beta Mercapto ethanol
BSA	Bovine serum albumin
CIDR	Cysteine rich Interdomain Region
CM	Cerebral malaria
DBL	Duffy binding like
DCs	Domain cassettes
DTT	Dithiothreitol
DVCU	Disease and Vector Control Unit
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein c receptor
FACS	Florescent activated cell sorting
HRP	Horseradish peroxidase
IB	Inclusion bodies
ICAM1	Intercellular adhesion molecule 1
iRBCs	Infected red blood cells
LPS	Lipopolysaccharide
mAb	Monoclonal antibody
MBC	Memory B cell
MFI	Median fluorescence intensity
MOI	Multiplicity of Infection
NSwBC	Non-switched B cells
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffer Saline
PC	Protein C
PEI	Polyethyleneimine
PfEMP1	Plasmodium falciparum erythrocyte membrane protein 1
PTM	Post translational modifications
RT-PCR	Reverse transcription polymerase chain reaction
SDS-Page	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SM	Severe malaria
SwBC	Switched B cells
TOI	Time of Infectivity
TT	Tetanus toxoid
VSA	Variable surface antigens
WHO	World health organisation

Chapter 1. Introduction

Plasmodium falciparum parasite remains the leading causative agent of malaria disease and is responsible for huge mortality and morbidity particularly in younger children. Recently the World Health Organisation (WHO) has estimated 214 million worldwide cases of malaria infection resulting in 438 thousand deaths mostly in children under the age of 5 years(1). Additional disease burden is increasingly realised; in 2014 three-quarters of the countries monitoring insecticide resistance in malaria vectors reported increased resistance. Furthermore, antimalarial drug resistance is confirmed and has now been reported for artemisinin in Southeast Asian countries(2).

1.1 Malaria Life Cycle

Malaria disease has a complex life cycle (Figure1.1) involving both human and mosquito hosts. Transmission is most commonly acquired through a nocturnal bite of infected female Anopheles mosquitoes by inoculating its parasitised saliva into the bloodstream of victims(3). The injected Plasmodium sporozoite stage is transitory, but within an hour, liver cells would be infected with the first stage sporozoites that further evolve to a second stage hypnozoite before evolving to the tertiary stage schizont. Following the completion of the clinically silent liver stage, hepatocyte originating merozoites enter the bloodstream and start invading red blood cells, initiating the blood stage replication. Consequently, the rapidly repeated cycles of invasion, replication and shedding from infected red

blood cells (iRBCs) results in an exponential growth of the parasite population hence the manifestation of symptoms(4).

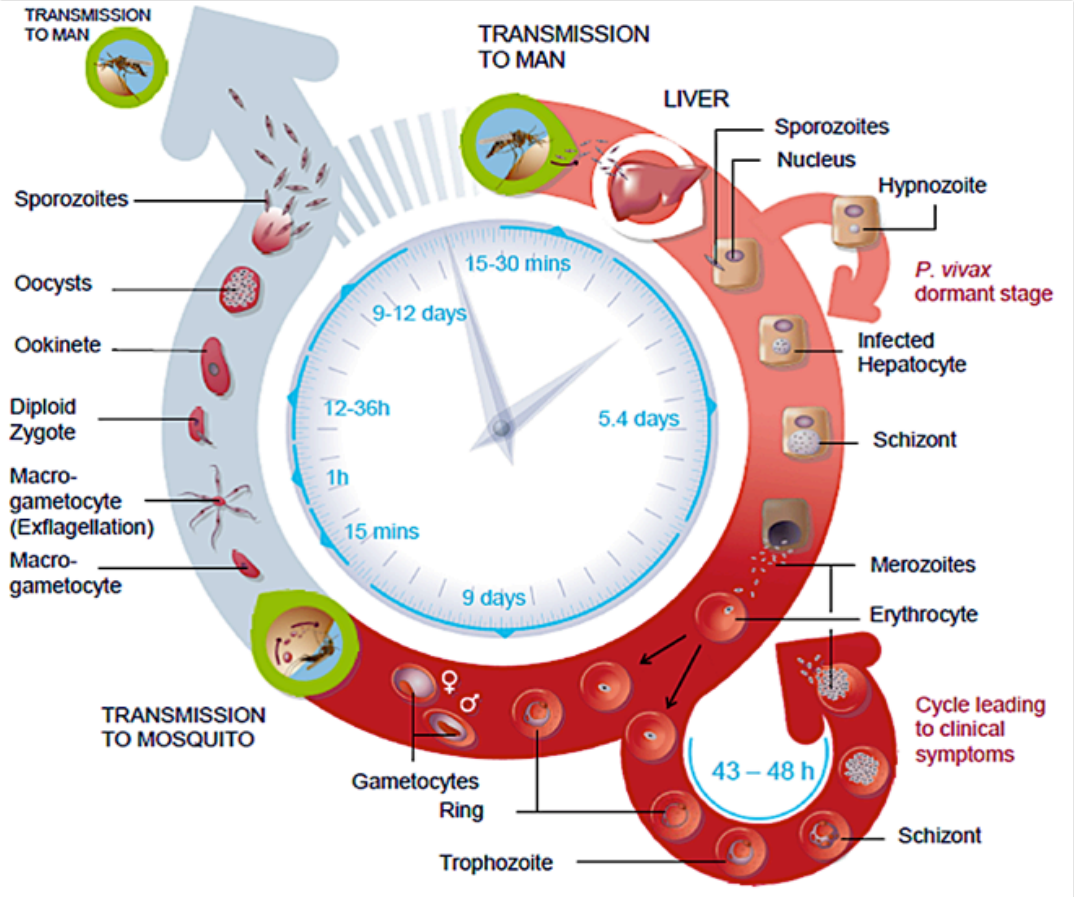

Figure 1. 1 Malaria Parasite Life Cycle.

The parasitic infection initiates when mosquitoes feed on human blood injecting the parasitic sporozoites into the skin. Injected sporozoites start to undergo mitotic replication after it migrates and infect hepatic cells in less than an hour time initiating its first asymptomatic replication phase. Following the completion of the clinically silent liver stage, hepatocyte originating merozoites enter the bloodstream and start invading red blood cells initiating the blood stage replication. Consequently, the rapidly repeated cycles of invasion, replication and shedding from infected red blood cells (iRBCs) results in exponential growth of the parasite population hence the manifestation of symptom. Figure from *Plasmodium falciparum* erythrocyte membrane protein 1 diversity in seven genomes - divide and conquer by Rask *et al.,* 2010 (65).

1.2 Malaria pathogenicity

Globally, most infections with malaria are clinically silent, reflecting the ability of the adaptive immune system to prevent disease progression. However, in a non-immune individual, malaria infections are more clinically apparent with a range of overlapping symptoms that appear between seven- and fifteen-days post-infection. Furthermore, a minority of these cases may progress to a severe life-threatening infection, which is characterised by the presence of impaired consciousness, coma, anaemia, breathing difficulty and multi-organ failure. These clinical features of severe malaria (SM) are thought to happen due to the combinatorial effect of a high parasite burden and the sequestration of iRBCs in the microvasculature(5). Indeed, the sequestration of iRBCs results in obstruction of the microvasculature bed that leads to metabolic disturbance and induction of inflammatory response thus contributing to disease severity(6). Cerebral malaria (CM) is the most severe form of SM infection, which is clinically characterised by fever, headache, seizure and progressively coma. Moreover, secondary bacterial infection may be observed in malaria cases presented with shock, and accounts for the majority of late deaths. The mortality rate in CM is very high, and even the surviving patients may sustain long-term neurocognitive complication. It is estimated that 11% and 5% of CM in children and adults respectively are discharged with gross neurological deficits and about 25% of these cases may sustain long-term impairments such as cognition, motor function or behaviour impairments and epilepsy. Thus, other than causing thousands of deaths, cerebral

malaria is now considered a leading cause of neurocognitive disability in malaria endemic area(7).

1.3 Plasmodium falciparum Erythrocyte Membrane Protein 1 (*Pf*EMP1)

Although cumulative studies have been done on the complex interaction between the *P.falciparum* malaria and Human host, the mechanism of immunity is still unclear. However, it is clear that the *P.falciparum* expresses a highly polymorphic antigenic surface structure on (iRBCs) collectively known as variable surface antigen (VSA), and these antigens are the primary targets of the immune system and mediators of disease pathogenicity(8,9). *P.falciparum* encodes several multigene families with apparent surface expression on iRBCs, these includes var, stevor, rif and pfmc-2tm genes coding for variable *Pf*EMP1 (P. falciparum erythrocyte membrane protein 1), rifin (repetitive interspersed family), stevor (subtelomeric variable open reading frame) and pfmc-2tm (P. falciparum Maurer's clefts 2 transmembrane) respectively(10). The functions and roles of the small VSA families rifin, stevor and pfmc-2tm is not well characterised, however recent studies have shown that stevor mediate parasite invasion and that both stevor and rifin contribute to sequestration of RBCs (rosetting)(11,12). *Pf*EMP1 is a large VSA encoded by a multigene family of 60 var genes per haploid parasite genome containing extracellular regions consisting of tandem repeat of duffy binding-like (DBL), cysteine-rich interdomain regions (CIDR) domains; transmembrane region and cytoplasmic tail(13). Based on phylogenetic analysis across multiple known P. falciparum var genomes, CIDR and DBL domains can be subclassified

into DBLα-ζ and CIDRα-δ with a further subdivision into 147 subtypes (e.g. CIDRα1.1)(14). Although var repertoires are extremely divergent, the majority of *Pf*EMP1 can be classified based on upstream promoter, transcription direction and chromosome location of var gene (Figure 1.2A) into three major groups (A, B and C). Group A and B are present in subtelomeric chromosome regions and transcribed in opposite direction while Group C is found in central chromosome regions. Additionally, Group B can be subclassified into B, having the most frequent domain structure and a chimeric B/A with a more complex domain structure like those found in group A(15). Finally, subsequent sequence comparisons have led to the identification of 23 conserved tandem arrangements of two or more domains of particular subclasses termed as Domain cassettes (DCs)(16). Different subgroups of *Pf*EMP1 variants have been associated with differences in adhesion profiles and disease syndromes. Most of these variants bind to CD36 expressed on endothelial cells, as well as antigen-presenting cells(17). However, small subsets of *Pf*EMP1 variants bind to endothelial protein C receptor (EPCR). Adhesion to CD36 has been mapped to the CIDRα2-6 domain of nearly all group B and C *Pf*EMP1 variants while adhesion to EPCR mapped to subset of CIDRα1 from groups A and B/A. Furthermore, group B and C *Pf*EMP1 variants tend to be associated with asymptomatic malaria whereas subset of group A (DC13) and B/A (DC8) have been implicated in SM (Figure 1.2B)(18). The DC8 consists of the first four domains (DBLα2-CIDRα1.1-DBLβ12-DBLγ4/6) while DC13 consists of the first two domains (DBLα1.7-CIDRα1.4). *Pf*EMP1 clearly contributes to disease pathogenicity by facilitating cytoadhesion and

sequestration of iRBCs through the utilisation of host receptors(16). Several studies demonstrated that the ability to switch between var gene expressions enable *Pf*EMP1 variant to bind different receptors including ICAM1, CD36, complement receptor 1, CSA, blood group A antigen, PECAM1, IgM, IgG and endothelial protein C receptor (EPCR)(17–27).

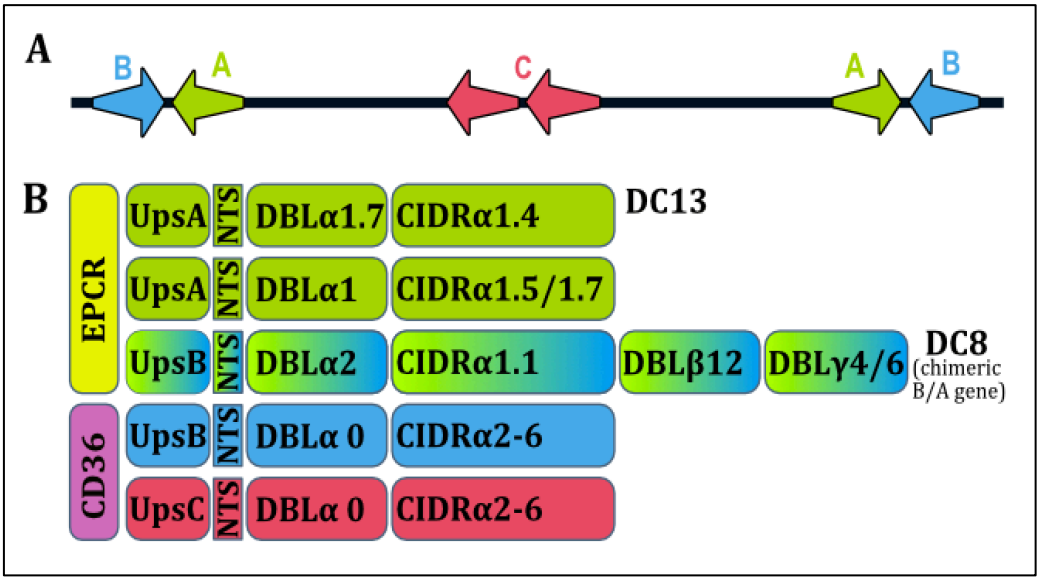

Figure 1. 2 Var gene organisation and protein domain architectures.

(A) Chromosomal organisation of var genes. Group B and A var genes are located in the subtelomeric regions and transcribed in opposite orientations. Group C are typically found in central chromosome clusters. (B) Head structure of CD36/EPCR binder. DC8 is chimeric gene between group B and group A var gene.

1.4 The scavenger receptor CD36

CD36 is a member of scavenger receptor proteins with a binding capability to many ligands including oxidised low-density lipoprotein, oxidised phospholipids, long-chain fatty acids, collagen, thrombospondin and *Pf*EMP1. CD36 is widely expressed on endothelial and epithelial cells, platelets, macrophages, monocytes, erythrocyte precursors and differentiated adipocytes. Depending on expressing cells, this type II membrane protein is physiologically involved in several key processes including lipid metabolism, glucose metabolism, thrombostasis, angiogenesis, phagocytosis of effete cells and innate immunity(28–34). Almost all *P. falciparum* isolates derived from malaria patients bind to CD36, approximately 50 of the average repertoire of var genes per parasite genome are capable of binding to region 139-184 amino acid of CD36 receptor via the N-terminal CIDR domain, though involvement of other regions has not been excluded(3). However, the role of CD36 in malaria pathogenesis remains controversial, studies that evaluate its role as a mediator of severe disease reported negative correlation in African isolates while studies on Asian isolates reported conflicting results(35–42). Moreover, studies investigating the effect of CD36 polymorphism on disease outcome also shown disagreeable results(43–46). In contrast, new evidence from studies investigating the macrophage CD36 has shown its role as a mediator of innate recognition and phagocytic clearance of multiple types of infectious pathogens including *P.falciparum* iRBCs(47–49). It is reasonable to assume that this paradoxical role of CD36 is attributed to its differential cell distribution, however, because the majority

of *P.falciparum* wild isolates uses CD36 as a facilitator for vascular adhesion and sequestration, it is safe to assume that it generally offers a survival advantage to the parasite.

1.5 Endothelial Protein C Receptor (EPCR)

Although the majority of *P.falciparum* wild isolates bind to CD36, in most cases even non-immunes do not progress to SM and sequestration occurs mainly in tissues were CD36 abundantly expressed(35–37). These findings together with the current notion that CD36 is only expressed at a low level in cerebral vessels and is not induced by inflammatory cytokines, suggest the involvement of other receptors in SM(50,51). Indeed, autopsy of fatal *P.falciparum* malaria cases shown elevated expression of multiple receptors in cerebral vessels including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin, whereas CD36 level was not altered(52). To determine host receptors to *P.falciparum* strains associated with SM, including isolates expressing DC8 and DC13, Turner et al. screened full-length recombinant DCs against several plasma proteins and reported the ability of CIDRα1.1 domain from DC8 and DC13 to bind EPCR. Moreover, they reported that DC8 expressing isolates are capable of binding to EPCR in multiple vasculature bed including cerebral, cardiac, pulmonary and bone marrow endothelium(18). EPCR is a transmembrane type 1 molecule expressed variably on most endothelial cell lines as well as other cell types including vascular smooth muscle cells, neutrophils, monocytes, eosinophils, keratinocytes, hippocampal neurons, cardiomyocytes, and placental

trophoblasts. EPCR play a central role in coagulation and immune regulation by exerting its function as a cell surface conductor of cytoprotective coagulation factor signalling(53). EPCR recruit and present Protein C (PC) to thrombin-thrombomodulin (TM) complex facilitating PC activation via the N-terminal cleavage by thrombin. Once formed, Activated Protein C (APC) associates with platelets or endothelium Protein S and proteolytically inactivates cofactors Va and VIIIa employing its anticoagulant activity. Also, EPCR-APC complex exerts multiple cytoprotective effects including modulation of gene expression profile, antiapoptotic activity, anti-inflammatory activities, and preservation of endothelial barrier function. While still attached to EPCR, APC exerts its cytoprotective activities via proteolytic activation of the protease-activated receptor 1 (PAR1), which result in down-regulation of proinflammatory and proapoptotic pathways and up-regulation of anti-inflammatory and antiapoptotic pathways(54). Remarkably, imbalances of each of these APC cytoprotective activities have been implicated in CM pathogenesis. Moxon et al. reported that the normally low levels EPCR in brain microvessels are further downregulated in CM(55). Moreover, TM expression in subcutaneous microvessels, localised to iRBCs sequestration was markedly reduced. The down-regulation in EPCR and TM expression were found to be associated with noted increased levels of soluble EPCR and TM in the cerebrospinal fluid. Collectively, these findings suggest that EPCR and TM are shed from the surface of cerebral endothelial cells (Figure 1.3) resulting in a proinflammatory and pro-coagulant effects and loss of blood-brain barrier(56).

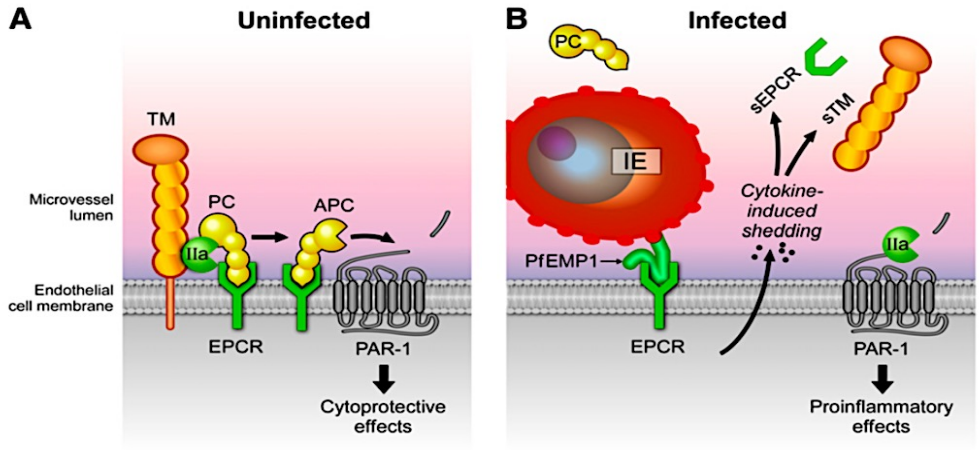

Figure 1. 3 Effects of P. falciparum infection on the protein C system.

(A) A normal functional protein C system. (B) IEs bind to EPCR and promote Endothelial cells (ECs) activation. Activated ECs release proinflammatory cytokines, thus, induce shedding of TM and EPCR from the cell surface and release of the ectodomain soluble TM and soluble EPCR (sEPCR) in the circulation. In addition, IEs binding to EPCR inhibits the function of the receptor. Collectively, these changes severely impair the ability of the endothelium to generate APC, setting up a vicious cycle of procoagulant and proinflammatory reactions leading to further endothelial dysfunction augmented by proinflammatory PAR-1 signalling by thrombin. Figure from Plasmodium falciparum picks on EPCR by *Aird et al*,. 2014 (55)

1.6 Naturally acquired immunity

Individuals with *Pf*EMP1-specific antibodies had a significantly reduced risk of developing symptomatic malaria, whereas antibodies to other VSA were not associated with protective immunity. In addition knockdowns of var gene thus lack of *Pf*EMP1 on the surface of iRBCs resulted in nearly complete loss of antibody recognition and cytoadhesive properties, suggesting the role of *Pf*EMP1 as a mediator of disease pathogenicity and a major target of naturally acquired immunity(57). Serological analysis of *Pf*EMP1 from clinical isolates obtained from different geographical regions revealed the existence of cross-reactivity between isolated epitopes suggesting presence of some degree of multilevel structural conservation(58,59). Though the utilisation of cross-reactive antibody with sufficient extent to reduce adhesion of clinically relevant *Pf*EMP1 could be the key to the development of a successful vaccine, the practicality of isolating such antibody from polyclonal plasma is not feasible. However, the better utilisation of memory B cells will present a better chance in obtaining high specific cross-reactive antibodies and access to the history of an individual's exposure to pathogens(60). Advances in the isolation and molecular analysis of single, antigen-specific memory B cells now allows detailed characterisation and library construction of the B cell receptor (BCR) diversification, V(J)D gene and isotypes usage in addition to the production of recombinant monoclonal antibodies for functional studies(61). Interestingly, memory B cells specific for malarial antigens can be detected in the peripheral circulation even when antibody levels have declined, and

they might maintain protection against recurrent episodes(62). Additionally, a cross-sectional study analysing memory B cell responses showed that about 50% of cross-reactive memory B cells recognise two full-length *Pf*EMP1 derived from laboratory-adapted lines(63).

1.7 Single B-cell technologies

The 1st generation of antibodies developed for therapeutic applications were of mouse origin, as both myeloma and B cells were sourced from mice. OKT3 is the first artificial mouse-originated antibody approved for treatment to reduce the damaging effects of T cell-mediated antigraft reactions(64). Nevertheless, the clinical efficacy of OKT3 was limited by its immunogenicity in humans, amongst other problems, arose due to its origin(65). However, the 2nd generation of therapeutic antibodies were human-mouse chimeric antibodies in which the mouse IgG Fc part was replaced with those of human. Infliximab is a chimeric antibody against the tumour necrosis factor (TNF) which is used in inflammatory diseases therapy such as Crohn's disease and rheumatoid arthritis(66).

Further advancement in the field resulted in the production of fully humanized antibodies in animals, either by grafting the complementarity determining region (CDR)3 into the human IgG framework or through the use of transgenic animals with human Ig genes(67). In parallel with the use of transgenic animals, additional technologies have been developed to directly generate fully human antibodies. Two examples of such technologies are Phage display and single-cell isolation(68)(65). Phage display method utilises a library of bacteriophages that have been

engineered to present antibody fragments on their surface, which are screened for binding to target antigen to identify the antibody fragments of interest(68). The phage display technology has an advantage over animal-based methods as it represents a naturally occurring repertoire. However, the phage display method is labour intensive and antibodies produced through this method are frequently unstable due to artificial pairing of heavy and light chains(66). Conversely, the single B-cell technology provide the ability to produce functionally mature human mAbs possessing naturally paired heavy and light chain that may be difficult to reproduce in vitro. The first step in single B-cell isolation is the identification of antigen-positive B cells by fluorescence-activated cell sorting, cell-based microarray chip, antigen-coated magnetic bead systems, micro-engraving systems(69–71). Isolated B cells could be used directly for RNA extraction or immortalized through the Epstein-Barr virus (EBV) transformation or even expanded in vitro with CD40L and cytokines cocktails including interleukin (IL)-2, IL-4, IL-10, and IL-21(72,73). Encoded heavy and light chains gene sequences are then obtained through reverse transcription-polymerase chain reaction (RT-PCR). Following successful amplification of immunoglobulin genes, co-transfection of both heavy and light chain immunoglobulin genes in HEK293 cells for later production of antigen-specific monoclonal antibodies(60). The main limitation of this technology is the availability of target antigen as well as access to semi-immune donors. Though, if a particular antigen of interest is known, it can be used to identify a suitable donor by screening a large pool of samples. Once identified, the same antigen can be used to probe memory B cells specific to target antigen in

donors PBMC sample. However, isolation of antigen-specific memory B cells Is technically challenging due to their very low frequency in the circulation which can be as low as 0.003% of total B cells population(74). Successful isolation by flow cytometry consequently would require a BCR-ligand that brightly labels these rare cells based on their antigen specificity. Overcoming such an obstacle can be easily achieved through the use of antigen tetramer for memory B cell labelling. The general consensus is that a tetrameric form of a given antigen could offer several advantages compared to its monomeric form. Indeed, fully structured IgG antibodies bind with greater avidity to their target antigen when compared to its partial monomeric Fab fragment. Furthermore, this increase is even several folds greater for pentameric IgM antibodies due to their ten combined Fab fragments(75). Additionally, a tetramerized antigen can be constructed with brightly labelled streptavidin molecules which alleviates the need for chemical modification to add fluorophore and subsequently preserving binding epitopes(76).

1.8 Project aim and Objectives

Aim

The aims of this project are to design and test a platform of techniques for production and characterization of antigen specific mAb from semi immune individuals with previous history of *P.falciparum* malaria infections.

Objectives

1) Production of selected recombinant *Pf*EMP1 CIDRα domains of clinical importance.
2) Collection of blood samples from healthy donors with historical exposure to *P.falciparum* malaria infection.
3) Screening donor samples to identify highest reactive sera with good recognition of at least three recombinant *Pf*EMP1 CIDRα1 domains.
4) Single cell sorting of CIDRα1-specific memory B cells (MBC)
5) Amplification and cloning of immunoglobulin genes from isolated CIDRα1-specific MBC
6) Sequence analysis of variable region of immunoglobulin sequences.
7) Production of recombinant monoclonal antibodies (mAb).
8) mAb characterisation and neutralisation potency testing

Chapter 2. Material & Methods

2.1 Recombinant *Pf*EMP1 domains

2.1.1 Codon optimization and gene synthesis

CIDRα sequences downloaded from global var gene data base available: http://www.vardb.org/vardb/. Aligned and distance matrix created based on sequence identity. 13 Selected representative CIDRα for blocks of similar CIDRα were optimized for expression in bacteria or insect cell using the software Geneious and synthesised by Eurofins (Table 2.1).

Table 2. 1 *Pf*EMP1 CIDRα domains included in study

ID	Domain-Strain	Note	Length	Size
K01	CIDRα1.1_IT4var20	Reference genomes	263 aa	30.7 kDa
K02	CIDRα1.1_PX0201-C.g34	Clinical isolate	264 aa	30.7 kDa
K03	CIDRα1.1_PX0205-C.g37	Clinical isolate	263 aa	30.6 kDa
K04	CIDRα1.1_PX0209-C.g28	Clinical isolate	263 aa	30.6 kDa
K05	CIDRα1.1_PX9997-C.g4296	Clinical isolate	263 aa	30.5 kDa
K06	CIDRα1.4_HB3var03	Reference genomes	252 aa	29.5 kDa
K07	CIDRα1.6_PFD1235w	Clinical isolate	250 aa	29.2 kDa
K08	CIDRα1.6_PX0208-C.g61	Clinical isolate	261 aa	30.6 kDa
K09	CIDRα2.0_PX0009-C.g9	Clinical isolate	235 aa	27 kDa
K10	CIDRα2.0_PX0202-C.g70	Clinical isolate	257 aa	29.8 kDa
K11	CIDRα2.1_PX0202-C.g26	Clinical isolate	250 aa	29.6 kDa
K12	CIDRα2.1_PX0205-C.g24	Clinical isolate	251 aa	29 kDa
K13	CIDRα3.5_IT4var15	Reference genomes	263 aa	30.7 kDa

2.1.2 Infusion cloning primers design

All primers were designed in Geneious software and synthesised by Eurofins UK. Generally, primers were designed based on Infusion cloning requirements. The 5' end of each primer contain at least 15 bases that are homologous to the expression vector while the 3' end contains at least 18 bases that are homologous to CIDR genes. Additionally, the GC content were 40-60% and melting temperature (Tm) for each primer within 58-65°C with no more than 4°C difference between primer pairs. Primers were also checked for complementarity within each primer and between pairs to avoid primer dimers. Two forward and one reverse primers were designed for each recombinant CIDR and expression vector (full length domain and short length).

2.1.3 Preparation of expression vectors

Expression of recombinant CIDR domains in *E.coli* or insect cell were performed using modified pTriEx-2 vectors pOPINF & pOPING (Addgene). The *E.Coli* compatible pOPINF vector contains an N-terminal His-tag and a 3C protease cleavable site whereas the insect cell compatible pOPING vector contains an N-terminal secretion leader and C-terminal His-tag. Linearization of vectors pOPINF and pOPING were carried out using endonucleases KpnI-HindIII and KpnI-PmeI respectively (New England biolabs). Briefly, double digest was performed on pOPINF and pOPING vectors using KpnI-HindIII and KpnI-PmeI endonucleases sets respectively for 1 hours at 37^{o}C. This is followed by 1% agarose gel purification and quantification using nanodrop.

2.1.4 Infusion cloning and sequence verification

*Pf*EMP1 CIDR domains sequences were amplified using the designed primers and gel purified using GeneJet purification kit following manufacturer protocol (Thermo). Ligation of amplified *Pf*EMP1 CIDR domains sequences to its respected linearized expression vector were performed using the infusion cloning kit according to manufacturer instruction (Clonetech, Takara). All infusion cloning product were transformed in to *E.coli* Steller competent cells (Clonetech, Takara) then cultured and purified using Qiaprep spin MiniPrep kit (Qiagen) followed by sequence verification using Smartseq kit (Eurofins).

2.1.5 Preparation of competent cell

There are several methods for preparation of competent cells, however the calcium chloride method is the most widely adapted and is the one described here. In this method about 20 µl glycerol stock of an *E. coli* strain was allowed to thaw at RT then added to 80 ml of SOC media and incubated at 37^{o}C for 1 hr. This is then used to inoculate a 10 ml LB starter culture with added ampicillin antibiotic and incubate overnight at 37^{o}C shaking at 200 rpm. The 10 ml starter culture was then used to inoculate 1 litre of LB media with added antibiotics and incubated at 37^{o}C shaking at 300 rpm until an OD600 of 0.4 is reached. This is followed by cooling culture until it reached 4^{o}C then split in to four fractions of 250 ml and centrifuged at 3200 RCF for 7 min at 4^{o}C, then resuspended in 50 ml/bottle of sterile cold 100 mM MgCl2 and combine all fractions in to one bottle. After another

centrifugation step as above, the resulting cell pellet was resuspended in 200 ml of sterile cold 100 mM CaCl2 and incubated on ice for 30 min. After additional centrifugation, the cell pellet was resuspended in 50 ml of sterile cold 85 mM CaCl2, 15% glycerol before last centrifugation as before and then resuspended in 4 ml of sterile cold 85 mM CaCl2, 15% glycerol to yield the final competent cell suspension. The competent cell suspension aliquoted as 100 ul fraction and stored in -80^{o}C.

2.1.6 Expression of recombinant *Pf*EMP1 domains in *E.coli*

BL21(DE3)pLysS, Rosetta2(DE3)pLysS and Rosetta-gami2(DE3)pLysS competent cells were transformed with recombinant pOPINF plasmids containing CIDRα1.1_IT4var20 (K01B or K01C) or CIDRα1.1_PX0201-C.g34 (K02B or K02C) as inserts. The transformed bacteria were selected on LB agar plates containing strain specific antibiotics and ampicillin (100 µg/ml). A single colony of the transformed bacteria was inoculated in 10 ml LB medium with added antibiotics and incubated overnight at 37°C. Aliquots of 5 ml from each culture were used to inoculate 500 ml LB medium with added antibiotics and incubated at 37°C while shaking at 225 rpm until OD A_{600} reached 0.7, then IPTG was added to a final concentration of 1 mM to induce the expression. Subsequently, cultures were incubated at 37°C for 6 hours and harvested afterwards as 50 ml aliquots by centrifugation at 4000 RCF for 15 minutes, then stored at -20°C. Aliquots of 1 ml were taken before induction and similarly after 2, 4 and 6 hours of induction to analyse expression by SDS-PAGE.

2.1.7 Cell Lysis and purification of IB

Bacterial cell pellets were thawed and suspended in BugBuster Master Mix (BBMX) and rotated for 30 minutes at room temperature then centrifuged at 4000 RCF for 35 min at 4°C. Obtained cell pellets were resuspended again in BBMX and vortexed to obtain homogenous mixture followed by addition of equal amount of diluted BBMX and centrifuged at 32000 RCF for 30 min at 4°C. Washing of cell pellets with diluted BBMX was repeated for an additional 3 times followed by centrifugation at 32000 RCF for 35 minutes to obtain purified recombinant proteins however in the form of inclusion bodies (IB).

2.1.8 Protein purification and refolding

Purified IB pellet were suspended in 5 ml denature binding buffer (DB) (50 mM Phosphate buffer pH 8, 8 M Urea, 300 mM NaCl, 10 mM Imidazol) and rotated for 4 hours at room temperature, then added to 1 ml washed and equilibrated Ni-NTA resin and incubated for 30 minutes. The denatured-resin mixture was transferred to equilibrated column and washed with 8 washing buffers (50 mM Phosphate buffer pH 8, 300 mM NaCl, 20 mM Imidazol and decreasing concentration 7-0 mM Urea) each wash with 15 columns volume followed by elution buffer (50 mM Phosphate buffer pH 8, 300 mM NaCl, 300 mM Imidazol and 5% Glycerol) with 3 ml of elution buffer. The collected fractions were then analysed by SDS-PAGE and suitable elution fraction pooled together and dialyzed overnight against 2 L of PBS pH 8.0. After dialysis, proteins concentration was determined using

Pierce BCA Protein Assay Kit (Thermo) and endotoxin level were assayed using Pierce® LAL Chromogenic Endotoxin Quantitation Kit (Thermo).

2.1.9 Production of recombinant Baculovirus

Production of recombinant Baculovirus was carried out in 6 well plate that been seeded with $5*10^5$/well of Sf9 Insect cells (Invitrogen) which was grown in serum-free medium suspension. The seeded plate was left at RT for 1 hour to allow Sf9 cells to adhere while preparing transfection master mix (200µl SF900II serum-free medium, 100ng Flashback virus DNA, recombinant pOPING transfer vector and 4µl Fugene HD transfection reagent). Transfection master mix was added after discarding 1ml of media from seeded well and left to incubate for 6 days at 27°C. At the end of incubation period, supernatant which contains the recombinant baculovirus was collected into a separate tube labelled as P0 and stored at -80°C. In the case of the positive control well, recovered supernatant was replaced with fresh media that contains 2% W/V X-gal and incubated for 2 hours to check for blue colour development, before storage at -80°C.

2.1.10 Proteins expression in insect cells

The first step in expression involved amplification of recombinant baculovirus P0 stock. This was achieved by inoculating $1*10^6$ Sf9 cells in T75 flask (Star lab) with 120µl P0 virus stock and left to incubate at 27°C for 6 days. Following the incubation period, supernatant was harvested and labelled as P1 and stored at 4°C. This P1 stock was used to optimize protein expression by testing 3 different transfection concentrations (0.5, 2.5 and 5µl) for multiple incubation periods (3,5,6 and 7 days). Supernatant

was harvested and stored at -80°C after using small aliquots for western blots.

2.1.11 SDS-Page analysis for expressed recombinant *PF*EMP1 CIDR proteins

Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used for initial confirmation of recombinant *Pf*EMP1 CIDR proteins expression. Proteins were diluted at 1:4 ratio in RunBlue sample buffer (Expedeon) then heat denatured at 95°C for 5 minutes after adding dithiothreitol solution (DTT) or Beta-Mercaptoethanol (BME) reducing agent at a ratio of 1:20 relative to total sample volume (Thermo Scientific). RunBlue 4-12% polyacrylamide (Expedeon) was washed with distilled water and assembled in electrophoresis chambers then filled with RunBlue SDS buffer (Expedeon). Denatured protein samples were loaded at 15µl into wells of acrylamide gel as well as protein ladder. The chamber was connected to a power supply and an electrical current of 200 volts was run for 35 minutes. Following electrophoresis, gels were stained with Instant Blue stain (Expedeon) or used in western blotting.

2.1.12 Western blot analysis for expressed recombinant *PF*EMP1 CIDR proteins

Western blot assay was performed to confirm the expression of recombinant *Pf*EMP1 CIDR through detection of tagged poly histidine motif. SDS-PAGE gels were performed as described before then transferred to nitrocellulose membrane (Biorad) in transfer buffer at 120V for one hour. To block non-specific binding, the membrane was incubated in PBSTM

blocking solution (PBS, 0.1% Tween® 20 and 5% non-fat milk) for 2 hours, followed by 3x washes in PBST (PBS, 0.1% Tween® 20). The membrane was then probed with HRP-conjugated anti-polyhistidine monoclonal antibodies (Sigma) for 1 hour followed by 3x washes in PBST. Visualisation of proteins was achieved by developing HRP conjugated Abs with TMB (Biolegend) substrate for 10-20 minutes and stopped by washing membrane with dH2O.

2.2 Blood sample collection & screening

2.2.1 Ethical application and approval

Ethical application forms were prepared and submitted to the LSTM research ethics committee and the in country ethical approval granted based on LSTM approval as part of the joint program between LSTM and the Saudi Arabian ministry of health (Figure 2.1).

2.2.2 Sample collection

The donor's samples collected for study were obtained from Jazan region at the southern border of the Kingdom of Saudi Arabia. Malaria caused by *P. falciparum* is endemic in this region with peaks in transmission between December – February and most of the cases are imported from Yemen and Eritrea. All donors were identified by the Disease and Vector Control Unit (DVCU) in Jazan region and recruited by the principle investigator (PI). Identified suitable participants were invited to participate in the study by the PI and upon informed consent, three 5 ml lithium heparinized venous blood

samples were collected by member of the DVCU and transferred within 6 hours of collection to the research centre in Jazan University.

2.2.3 PBMCs isolation

PBMCs (peripheral blood mononuclear cells) were processed within a maximum of 8 hours from collection. Briefly, donor blood samples were pooled in to 50 ml falcon and centrifuged at 1000 x g for 10 minutes at room temperature (RT). After centrifugation, plasma was collected and aliquoted in 1 ml tubes then stored at -80°C. Blood was resuspended with PBS in 2x the volume of plasma removed and transferred to Leucosep tube (Greiner) with added 15 ml Lymphoprep (AXIS-SHIELD) and centrifuged at 900 x g for 13 minutes at RT. PBMCs were collected in to a 50 ml tube containing 25 ml of cold-PBS pH 7.5 and topped up to 45 ml with cold-PBS supplemented with 2% FBS and centrifuged at 300 x g for 10 minutes at 4°C with centrifuge deceleration breaks off. After centrifugation, the supernatant was decanted and the pellet resuspended in 10 ml cold PBS, and PBMCs counted and assessed by analysing 10 μl of sample using a Hemacytometer. Depending on PBMCs cell count, the required quantity of cell freezing solution was prepared (90% FBS: 10% DMSO), and the 10 ml PBMCs-PBS mixture centrifuged at 440 x g for 10 minutes at 4°C followed by discarding the supernatant. PBMCs were quickly but gently resuspend in freezing solution and aliquoted at $1*10^7$/ml. Aliquots were stored in -80°C using cool cell container for controlled freezing for the first 24 hours, then transferred and stored at -80°C. All samples were shipped to LSTM within one month of collection in dry ice then stored in liquid nitrogen cryofreezer.

2.2.4 Enzyme-linked immunosorbent assay (ELISA)

Indirect ELISA was used to measure the presence of antibodies from plasma of immune malaria individuals against recombinant CIDR domains. Recombinant *Pf*EMP1 CIDR proteins were diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and 100 μl were used to coat Nunc-maxisorp micrometre plates (Thermo/Fisher) at 1μg/ml, this is then incubated at 4°C overnight. Any uncoated material was then discarded and the plates washed 5 times with PBST (0.05% Tween-20 PBS). Plates were then blocked with 200 μl blocking buffer (3%BSA, 0.05%Tween PBS) and incubated for 2 hours at room temperature. Wells were washed in PBST 5 times before the addition of 100μl from donor plasma samples or negative control that been diluted at 1:100 in 1%BSA PBS. Plates were incubated for additional 2 hours at room temperature then washed 5 times in PBST before the addition of horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc that had been diluted 1:1000 in 1%BSA PBS, then incubated for 1 hour at room temperature. Plates were then washed as before and then developed using 100 μl of TMB substrate per well and plates were allowed to develop in the dark. After 20 minutes, the reactions were stopped with 1M Sulphuric acid and measured in duplicate at 450nm.

2.3 Single B cell isolation

2.3.1 Thawing of PBMCs

PBMCs vials were transferred from liquid nitrogen storage in dry ice and briefly thawed in 37^{o}C until a small visible frozen pellet remained. Once nearly thawed, vials were disinfected using 70% ethanol and processed by adding 1 ml prewarmed cMedium (10% FBS, 1% Pen Strep, 1% L-Glutamine Iscove's modified Dulbecco's Medium) dropwise and quickly transferred to 14 ml falcon. Wash by adding 9 ml cMedium drop-wise, while gently swirling the tube. The cell suspension was centrifuged at 400 x g for 11 minutes at room temperature. Supernatant decanted and pellet resuspended in 10 ml cMedium containing 500U Benzonase and incubated for 10 minutes at room temperature. The cell suspension was filtered through 0.2 μm filter and centrifuged using same previous setting, supernatant decanted then cell pellet resuspended in 10 ml 2%BSA PBS and incubated in Ice for analysis. 10 μl of cell suspension were used to perform cell count using trypan Blue staining.

2.3.2 Biotinylation of recombinant CIDR domains and Tetanus Toxoid

Biotinylations were performed using EZ-Link Sulfo-NHS-Biotinylation kit (Thermo Scientific) according to manufacturer protocol. Briefly, recombinant *Pf*EMP1 CIDR proteins were buffer exchanged in to 0.1M PBS (pH7.2). The 10mM Sulfo-NHS-Biotin solution (Thermo) was prepared at 4.4 mg/mL in ultrapure water. The Biotinylation reaction was carried out using a 20-fold molar excess of biotin solution to label recombinant *Pf*EMP1

CIDR proteins. After vortexing for 5 min, the mixture was incubated overnight at 4°C. The supplied desalting columns were used next day to remove excess unbound biotin molecules.

2.3.3 HABA Assay

The measurement of biotin incorporation to the recombinant proteins were carried out using Avidin/HABA reagents (Thermo Scientific) following the recommended manufacturer protocol. Briefly, 96 well plate (Starlab) were coated with prepared Avidin/HABA mixture and measured at OD_{500nm} for base line reading. Subsequently, repeat measurement at OD_{500nm} were performed after addition of biotinylated recombinant *Pf*EMP1 CIDR proteins. Obtained measurements were used to calculate the moles of biotin incorporation to each mole of recombinant *Pf*EMP1 CIDR protein.

2.3.4 Tetramerization of recombinant CIDR domains

Biotinylated recombinant *Pf*EMP1 CIDR proteins monomers were incubated at a molar ratio of 4:1 with fluorescently labelled streptavidin BV421 (Biolegend). The addition of labelled streptavidin to the biotinylated CIDR proteins monomers were performed in ten stepwise gradients, each followed by an incubation of 10 min on ice (100 minutes). Prior PBMCs staining, a centrifugation of prepared tetramer for 10 minutes at 32000 RCF were performed to ensure removal of tetramer aggregates.

2.3.5 Probing PBMCs with recombinant CIDR domains

Frozen PBMC were thawed quickly and resuspended in RPMI complete medium then pelleted and resuspended again in 2%BSA PBS. Cells were

then incubated on ice with a titered amount of conjugated or tetramerized recombinant CIDR in 100μl total reaction volume for at least 30 minutes. Following 2x washes in 3ml 2%BSA PBS, cells were labelled for 30 min on ice with a mixture of mouse anti-human MAbs; CD3-APC-Cy7, clone SK7; CD19-BV711, clone HIP19; CD27-PerCP-CY5.5, clone O323; IgD-APC, clone IA6-2; CD20-BV605, clone 2H7; CD38-FITC, clone HB7; CD71-PE-Cy7, clone CY1G4; Anti-His tag, clone J095G46 (Biolegend). CD21-PB, clone LT21; CD138-AF700, clone BA38 (ExBio). Subsequently, labelled cells were pelleted and washed again in serum free PBS before labelling with LIVE/DEAD Fixable Aqua Stain 367⁄526 nm (Life Technologies) for 30 minutes on ice. Following 2x washes in 2%BSA PBS, cells analysed on a FACS LSRII equipped with Diva v7.0 and CS&T v2.0 software (BD Biosciences). Photomultiplier tube voltages and area scaling factors were optimized, and compensation spill over values were determined, using UltraComp eBeads (eBioscience). FCS 3.1 data files were collected for analysis of B cell populations and staining efficiency. Data analysis and transformation were performed using FlowJo v10 (Treestar).

2.3.6 Single cell sorting of CIDR-specific B cells

Cryopreserved PBMC ($10*10^6$ cells) were thawed following PBMC thawing protocol followed by B cells enrichment using Easysep B cell enrichment negative selection kit (Stemcell Tech). Enriched cells were resuspended in sterile PBS supplemented with 1% FBS and incubated for 20 minutes with streptavidin-BV421 labelled tetramerised recombinant CIDR protein. Subsequently cells were washed and stained for another 20 minutes with

CD3-APC-Cy7, clone SK7; CD19-BV711, clone HIP19; CD27-PerCP-CY5.5, clone O323; IgD-APC, clone IA6-2 (Biolegend). This is followed by additional cells wash and staining for 15 minutes with LIVE/DEAD Fixable Aqua Stain 367⁄526 nm (Life Technologies). Finally, cells were washed and resuspended in 2 ml cold RPMI media supplemented with 10% FBS. CD3-/CD19+/CD27+/Tetramer+ cells were sorted with a BD FACS aria in single cell mode into a 96-well plate containing 1M Tris lysing buffer supplemented with Rnasin.

2.4 Production & characterization of the recombinant monoclonal antibodies

2.4.1 Single cell RT-PCR & Ig amplification

Single cell cDNA was synthesised by Reverse Transcription PCR (RT-PCR) from single B cell lysate using One Step RT-PCR kit (Qiagen). Immunoglobulin (Ig) heavy (Igh) and light chains (Igk or Igλ) variable region genes were amplified from cDNA by multiplex nested PCR on separate rounds. The 1st nested round uses primers mix consisted of 5' end forward primers specific for V region and 3' end reverse primer that target the constant region. The amplified products from this round were purified and sent for Sanger sequencing to determine the V(D)J gene usage and Ig type. Paired Ig-variable region gene sequences were identified and selected for recombinant antibody production by 2nd round nested PCR with primers containing restriction sites which enable direct cloning into respective mammalian expression vectors. The RT-PCR amplification program used

for production of cDNA was carried out with 40 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1.5 min before a final extension step at 72°C for 1 min. Slight modifications were added to the setup during the nested RT-PCR, initial denaturation at 95°C for 15 min before a 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 1 min, and extension at 72°C for 1.5 min before a final extension step at 72°C for 10 min. Table 2.2 and Table 2.3 show full list of primers that were used for RT-PCR and nested PCR respectively.

2.4.2 Cloning of Immunoglobulin genes

Cloning of the 2nd round PCR products was carried out by restriction enzyme digestion and ligation into the mammalian expression vectors containing respective human Ig-constant regions. Restriction digestion of PCR products and vectors were carried out with AgeI, SalI, BsiW and XhoI restriction enzymes (New England Biolabs) at 37°C for 4 hours. The T4 DNA ligase (New England Biolabs) was used in the ligation process at 16°C overnight after purifying digested products from 1% agarose gel. This is followed by transforming recombinant expression vectors into Steller competent cells and selected on Ampicillin agar plates. Min-prep plasmid preparations (Qiagen miniprep kit) were performed using 3 different colonies from each transformation followed by colony PCR to confirm presence of Ig gene. Colonies with confirmed insert sequences were used for maxiprep then stored frozen as glycerol stock at -80°C for future use.

2.4.3 Monoclonal antibodies production and purification

HEK293A cells (Invitrogen) were cultured in 100 mm dish (StarLab) under standard conditions in DMEM medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 µg/ml streptomycin, 100 U/ml penicillin (all from Invitrogen). Co-transfections of Ig-heavy chain and matching Ig-light chain expression vectors (2.5µg each vector) were performed using linear polyethyleneimine (PEI) as a transfection reagent in DMEM medium supplemented with 1% Nutridoma-SP (Sigma). Briefly 1:2 concentration ratio of plasmids:PEI were mixed in 500 µl 150 mM NaCl for 10 min at RT, and subsequently added dropwise to 90% confluent cells in a total volume of 25 ml medium for 7 days. Following incubation period, supernatant was collected, and dot blot was used to confirm the production of mAbs. Collected supernatant was purified using Amintra protein G resin (Expedeon) and 500µl spin column (Biorad) following manufacturer protocol.

2.4.4 Indirect ELISA for monoclonal antibodies characterization

Recombinant CIDR domains (K01-K10, K12 and K13) were diluted in 0.05 M carbonate/bicarbonate buffer (pH 9.6) and 100 µl were coated down at 1µg/ml on a Nunc-maxisorp micrometre plates (Thermo/Fisher) then incubated at 4°C overnight. Any uncoated material was then discarded and washed 5 times with PBST (0.05% Tween-20 PBS), plates were blocked with 200 µl blocking buffer (3%BSA, 0.05%Tween PBS) and incubated for 2 hours at room temperature. Wells were washed in PBST 5 times before

the addition of mAbs that been diluted at 1:100 in 1%BSA PBS. Plates were incubated for additional 2 hours at room temperature then washed 5 times in PBST before the addition of horseradish peroxidase (HRP)-conjugated goat anti-human IgG-Fc that has been diluted 1:1000 in 1%BSA PBS then incubated for 1 hour at room temperature. Plates were then washed as before and then developed using 100 µl of TMB substrate per well and plates were allowed to develop in the dark. After 20 minutes, the reactions were stopped with 1M Sulphuric acid and measured in duplicate at 450nm.

Table 2. 2 RT-PCR primers used for amplification of immunoglobulin heavy and light genes from isolated CIDR-specific B cells

RT-PCR Forward primers	
5'LVH1	ACAGGTGCCCACTCCCAGGTGCAG
5'LVH3	AAGGTGTCCAGTGTGARGTGCAG
5'LVH4/6	CCCAGATGGGTCCTGTCCCAGGTGCAG
5'LVH5	CAAGGAGTCTGTTCCGAGGTGCAG
5'LVK1/2	ATGAGGSTCCCYGCTCAGCTGCTGG
5'LVK3	CTCTTCCTCCTGCTACTCTGGCTCCCAG
5'LVK4	ATTTCTCTGTTGCTCTGGATCTCTG
5'LVL1	GGTCCTGGGCCCAGTCTGTGCTG
5'LVL2	GGTCCTGGGCCCAGTCTGCCCTG
5'LVL3	GCTCTGTGACCTCCTATGAGCTG
5'LVL4/5	GGTCTCTCTCSCAGCYTGTGCTG
5'LVL6	GTTCTTGGGCCAATTTTATGCTG
5'LVL7	GGTCCAATTCYCAGGCTGTGGTG
5'LVL8	GAGTGGATTCTCAGACTGTGGTG
RT-PCR Reverse primers	
3'CgCH1	GGAAGGTGTGCACGCCGCTGGTC
3'CK543-566	GTTTCTCGTAGTCTGCTTTGCTCA
3'CL	CACCAGTGTGGCCTTGTTGGCTTG
3' Cu CH outer	GGAAGGAAGTCCTGTGCGAGGC

Table 2. 3 Nested PCR primers used for amplification of immunoglobulin heavy and light genes.

5'AgeIVH1/5/7	CTGCAACCGGTGTACATTCCGAGGTGCAGCTGGTGCAG
5'AgeIVH3	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGGTGGAG
5'AgeIVH4	CTGCAACCGGTGTACATTCCCAGGTGCAGCTGCAGGAG
5'AgeIVH3-23	CTGCAACCGGTGTACATTCTGAGGTGCAGCTGTTGGAG
5'AgeIVH4-34	CTGCAACCGGTGTACATTCCCAGGTGCAGCTACAGCAGTG
5'AgeIVH1–18	CTGCAACCGGTGTACATTCCCAGGTTCAGCTGGTGCAG
5'AgeIVH1–24	CTGCAACCGGTGTACATTCCCAGGTCCAGCTGGTACAG
5'AgeIVH3–9/30/33	CTGCAACCGGTGTACATTCTGAAGTGCAGCTGGTGGAG
5'AgeIVH6–1	CTGCAACCGGTGTACATTCCCAGGTACAGCTGCAGCAG
5′AgeIVH4–39	CTGCAACCGGTGTACATTCCCAGCTGCAGCTGCAGGAG
5′AgeIVH3–33	CTGCAACCGGTGTACATTCTCAGGTGCAGCTGGTGGAG
3'SalIJH1/2/4/5	TGCGAAGTCGACGCTGAGGAGACGGTGACCAG
3'SalIJH3	TGCGAAGTCGACGCTGAAGAGACGGTGACCATTG
3'SalIJH6	TGCGAAGTCGACGCTGAGGAGACGGTGACCGTG
5'PanVK	ATGACCCAGWCTCCABYCWCCCTG
3'CK494-516	GTGCTGTCCTTGCTGTCCTGCT
5'AgeIVK1-5	CTGCAACCGGTGTACATTCTGACATCCAGATGACCCAGTC
5'AgeIVK1-9/1-13	TTGTGCTGCAACCGGTGTACATTCAGACATCCAGTTGACCCAGTCT
5'AgeIVK1D-43/1-8	CTGCAACCGGTGTACATTGTGCCATCCGGATGACCCAGTC
5'AgeIVK2-24	CTGCAACCGGTGTACATGGGGATATTGTGATGACCCAGAC
5'AgeIVK2-28/2-30	CTGCAACCGGTGTACATGGGGATATTGTGATGACTCAGTC
5'AgeIVK3-11/3D-11	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACACAGTC
5'AgeIVK3-15/3D-15	CTGCAACCGGTGTACATTCAGAAATAGTGATGACGCAGTC
5'AgeIVK3-20/3D-20	TTGTGCTGCAACCGGTGTACATTCAGAAATTGTGTTGACGCAGTCT
5'AgeIVK4-1	CTGCAACCGGTGTACATTCGGACATCGTGATGACCCAGTC
3'BsiWIJK1/2/4	GCCACCGTACGTTTGATYTCCACCTTGGTC
3'BsiWIJK3	GCCACCGTACGTTTGATATCCACTTTGGTC
3'BsiWIJK5	GCCACCGTACGTTTAATCTCCAGTCGTGTC
3'BsiWIJK150/3	GCCACCGTACGTCTGATTTCCACCTTGGTC
5'AgeIVL1	CTGCTACCGGTTCCTGGGCCCAGTCTGTGCTGACKCAG
5'AgeIVL2	CTGCTACCGGTTCCTGGGCCCAGTCTGCCCTGACTCAG
5'AgeIVL3	CTGCTACCGGTTCTGTGACCTCCTATGAGCTGACWCAG
5'AgeIVL4/5	CTGCTACCGGTTCTCTCTCSCAGCYTGTGCTGACTCA
5'AgeIVL6	CTGCTACCGGTTCTTGGGCCAATTTTATGCTGACTCAG
5'AgeIVL7/8	CTGCTACCGGTTCCAATTCYCAGRCTGTGGTGACYCAG
3'XhoICL	CTCCTCACTCGAGGGYGGGAACAGAGTG
5'Absense	GCTTCGTTAGAACGCGGCTAC
Ig internal	GTTCGGGGAAGTAGTCCTTGAC
Cu CH1	GGGAATTCTCACAGGAGACGA

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Wednesday, 02 December 2015

Dear Mr Khashab

Re. Research Protocol (15.041RS) Evaluation of cross--reactive B-cells responses to recombinant CIDR domains of the Plasmodium falciparum erythrocyte membrane protein 1

Thank you for your email of 28 November 2015 providing the evidence that in-country ethical approval is not required for this project. I can confirm that the protocol now has formal ethical approval from the LSTM Research Ethics Committee.

The approval is for a fixed period of three years and will therefore expire on 01 December 2018. The committee may suspend or withdraw ethical approval at any time if appropriate.

Approval is conditional upon:

- Continued adherence to all in-country ethical requirements.
- Notification of all amendments to the protocol for approval before implementation.
- Notification of when the project actually starts.
- Provision of an annual update to the Committee.
  Failure to do so could result in suspension of the study without further notice.
- Reporting of new information relevant to patient safety to the Committee
- Provision of Data Monitoring Committee reports (if applicable) to the Committee

Failure to comply with these requirements is a breach of the LSTM Research Code of Conduct and will result in withdrawal of approval and may lead to disciplinary action. The Committee would also like to receive copies of the final report once the study is completed. Please quote your Ethics Reference number with all correspondence.

Yours sincerely

Dr Angela Obasi
Chair
LSTM Research Ethics Committee

Figure 2. 1 Ethical approval letter

Chapter 3. Production of recombinant Plasmodium falciparum Erythrocyte Membrane Protein 1 CIDRα domains

3.1 Introduction

Cytoadhesion and sequestration of Plasmodium falciparum infected red blood cells iRBCs the microvasculature is the main virulence determinant for the disease pathological complications. The binding phenomena of iRBCs are mediated by interactions between the P. falciparum erythrocyte membrane protein 1 (*Pf*EMP1) family and several ligands on the surface of endothelial cells lining the microvasculature(3). *Pf*EMP1 is a large variable surface antigen (VSA) encoded by a multigene family of 60 var genes per haploid parasite genome.

Structurally, the extracellular regions of *Pf*EMP1 consist of tandem repeat of duffy-binding-like (DBL), cysteine-rich interdomain regions (CIDR) domains; transmembrane region and a cytoplasmic tail. Based on phylogenetic analysis across multiple known P. falciparum var genomes, CIDR and DBL domains can be subclassified into DBLα-ζ and CIDRα-δ with a further subdivision into 147 subtypes(14).

A large body of immunoepidemiologic evidence has linked specific CIDR domains expression with disease severity(77). Indeed, recent studies on *Pf*EMP1 gene expression in severe malaria cases have identified a subset of *Pf*EMP1 proteins carrying a specific CIDRα1 domain that mediates adhesion to endothelium by coupling to endothelial protein C receptor (EPCR)(78).

These observations make CIDRα1 domains a main target to be included in the development of vaccines and therapeutics, which target the reduction of *Pf*EMP1 mediated pathology. Hence, the current chapter aims to express recombinant *Pf*EMP1 domains, which are of clinical importance including groupB-DC8 (CIDRα1.1), groupA-DC13 (CIDRα1.4), groupB (CIDRα2) and group-B/C (CIDRα3). A total of 13 CIDRα domains will be produced; 5 of which are from IT4 or HB3 parasite lines, the remaining 8 are novel domains which show 99% identity between clinical isolates from Malawi, Kenya, Ghana and The Gambia (Chris Newbold and Thomas Otto, unpublished data).

3.2 Results

3.2.1 Production of pOPINF-CIDRα and pOPING-CIDRα constructs

The CIDRα domains (Table 3.1) were amplified from synthetic genes through the use of two primers set with varying domain boundaries that introduces flanking homologues region and restriction sites enabling sub-cloning into prokaryotic (pOPINF) and eukaryotic (pOPING) system compatible vectors. The pOPINF vector contains a hexa-histidine motif in the N-terminus while the pOPING vector contains a secretion motif in the N-terminus and a hexa-histidine motif in the C-terminus. This was done to compare recombinant proteins expression in prokaryotic and eukaryotic systems to evaluate the best system to use for large scale expression. Despite the successful amplification of all CIDRα variants genes (Figure 3.1) and (Figure 3.2), only two recombinant pOPING-CIDRα were successfully sequenced and verified while almost all recombinant pOPINF-CIDRα sequence verified except K11 protein. Repeated sequencing of pOPING-CIDRα also failed, therefore I decided to use the *E.coli* expression system for all CIDRα domains expression while test only the two sequenced pOPING-CIDRα domains in Baculovirus expression system.

Table 3. 1 Synthetic CIDRα domains from PFEMP1 variants

ID	Domain-Strain	Note
K01	CIDRα1.1_IT4var20	P. falciparum reference genomes
K02	CIDRα1.1_PX0201-C.g34	Clinical isolate
K03	CIDRα1.1_PX0205-C.g37	Clinical isolate
K04	CIDRα1.1_PX0209-C.g28	Clinical isolate
K05	CIDRα1.1_PX9997-C.g4296	Clinical isolate
K06	CIDRα1.4_HB3var03	P. falciparum reference genomes
K07	CIDRα1.6_PFD1235w	Clinical isolate
K08	CIDRα1.6_PX0208-C.g61	Clinical isolate
K09	CIDRα2.0_PX0009-C.g9	Clinical isolate
K10	CIDRα2.0_PX0202-C.g70	Clinical isolate
K11	CIDRα2.1_PX0202-C.g26	Clinical isolate
K12	CIDRα2.1_PX0205-C.g24	Clinical isolate
K13	CIDRα3.5_IT4var15	P. falciparum reference genomes

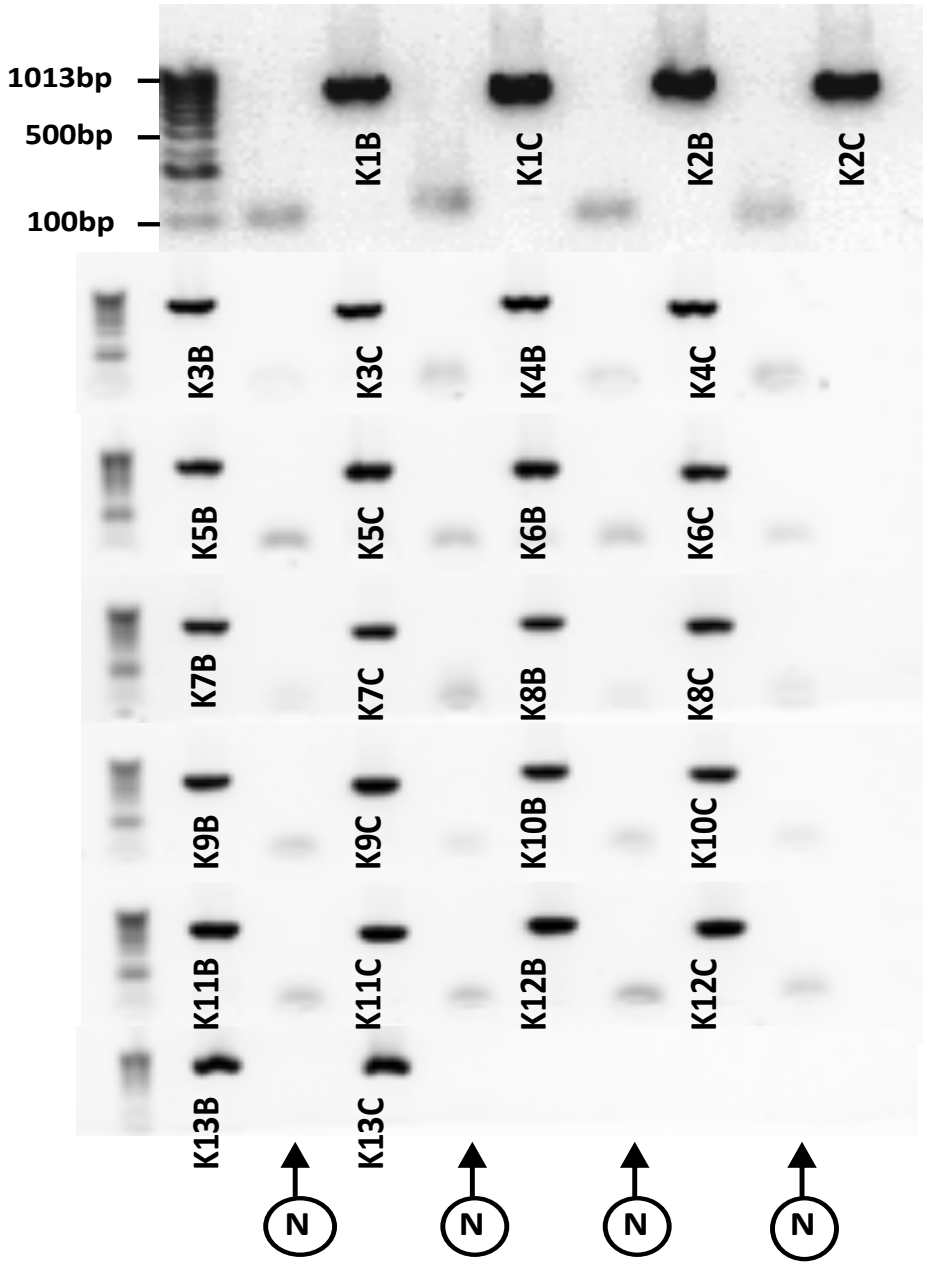


Figure 3. 1 Agarose gel analysis of amplified CIDR genes compatible with pOPINF vector.

Shown are amplicons representing different domain boundaries (B and C). Negative controls are indicated by N.

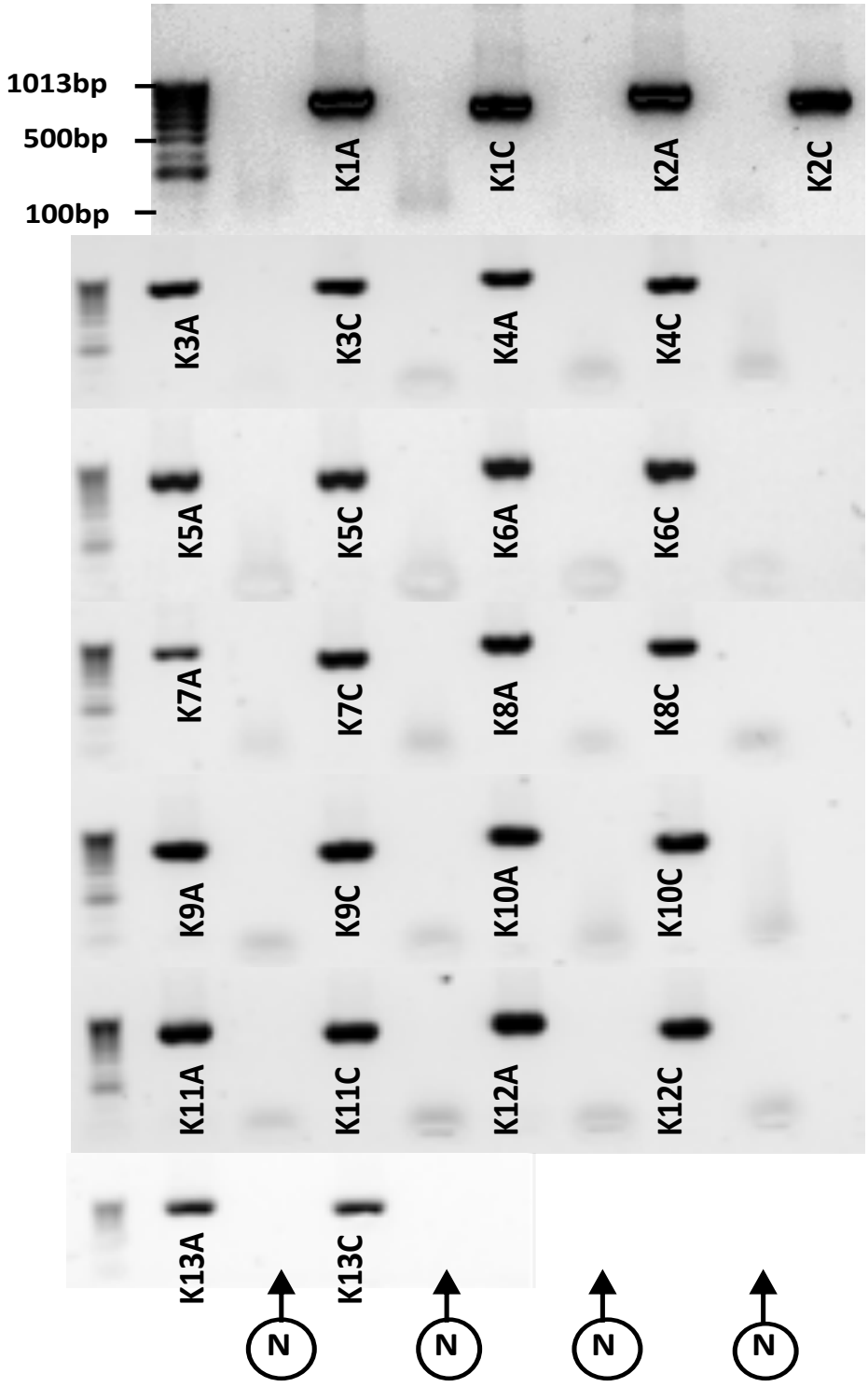


Figure 3. 2 Agarose gel analysis of amplified CIDR genes compatible with pOPING vector.

Shown are amplicons representing different domain boundaries (A and C). Negative controls are indicated by N.

3.2.2 Recombinant CIDRα domains expression

3.2.2.1 Proteins expression in *E.coli*

Expression of malaria proteins in prokaryotic system specially membrane type such as *Pf*EMP1 proves to be challenging and require careful planning. Therefore, we decided to use the pOPINF vector which is a modified T7 expression vectors carrying a chromosomal copy of the T7 RNA polymerase gene under the control of the lacUV5 promoter. The use of pOPINF vector with an expression strain that contains plasmid encoding T7 lysozyme, a natural inhibitor of T7 RNA polymerase, will suppress basal expression of our proteins thus stabilizing growth and viability. Consequently competent cell strains BL21(DE3)pLysS, Rosetta2(DE3)pLysS and Rosetta-gami2(DE3)pLysS were chosen for optimization of the protein expression protocol. The BL21 strain was included as a gold standard in bacterial protein expression, the Rosetta2 strains enhances the expression of eukaryotic proteins that contain codons rarely used in *E.coli* by supplying tRNA for 7 rare codons. The Rosetta-gami2 strain have mutations in both the thioredoxin reductase (trxB) and glutathione reductase (gor) genes, which greatly enhance disulphide bond formation in the cytoplasm combined with the advantage of Rosetta2 strain alleviating codon bias and enhancing disulphide bond formation(79). The preparation of all strains were performed using the calcium chloride methods under standardized condition, however, the preparation of *E.coli* Rosetta-gami2(DE3)pLysS competent cells was not successful and excluded. The BL21(DE3)pLysS and Rosetta2(DE3)pLysS competent cells showed moderate competency, thus were used for initial proteins

expression trial. Two CIDRα genes K01 (CIDRα1.1_IT4var20) and K02 (CIDRα1.1_PX0201-C.g34) were selected for the test expression trial. Transformed cells were grown at 37°C in LB media to an ODA_{600} of 1.6, then induced with 1.5 mM IPTG for 6 hours. SDS-PAGE analysis of protein expression showed very weak expression in *E.coli* BL21(DE3)pLysS strain as seen by comparing the uninduced sample against 2, 4 and 6 hours post-induction samples (Figure 3.3). Contrary, induction carried out in *E.coli* Rosetta2(DE3)pLysS strain under the same condition successfully showed expression of all recombinant proteins variants (Figure 3.4). Subsequently a large-scale expression was tested for same proteins in Rosetta2 strains with an induction time of 3 hours and 2 litres of LB culture. Analysis of large-scale cultures revealed a satisfactory level of recombinant proteins expression, however, all proteins were expressed in the form of inclusion bodies (IB) which require further processing and refolding steps during the purification step (Figure3.5).

Further optimization of expression protocol was performed in an attempt to produce recombinant proteins in its soluble forms. Thus, additional recombinant plasmids encoding K04 and K07 were used to transform *E.coli* Rosetta2(DE3)pLysS strain and induced at variable ODA_{600} for 3 hours. Cultures transformed with recombinant K04 failed to show any sign of protein expression (Figure 3.6 A) while those transformed with recombinant K07 successfully expressed the recombinant K07 protein at all tested ODs, however, to a variable degree. Further analysis of culture fractions revealed the presence of recombinant K07 in the pellet as an IB (Figure 3.6 B & C).

In a final attempt to express recombinant proteins in its soluble forms, two additional *E.coli* strains (Shuffle LysS and Shuffle express) were transformed with all recombinant plasmids. Colonies thought to contain recombinant genes were cultured and induced at 1.9 ODA_{600} for 3 hours at 37^{o}C. Apart from the K11 plasmid, all other proteins expressed at satisfactory level, however, in the form of IB. Further optimization to the protocol were not favoured due to time constraints and all subsequent large-scale expressions were performed using either Rosetta2 or Shuffle *E.coli* strains under last optimized condition. Successful expression of all domains was confirmed by Western blot analysis by probing with HRP conjugated anti-histidine antibody (Figure 3.6 D & E).

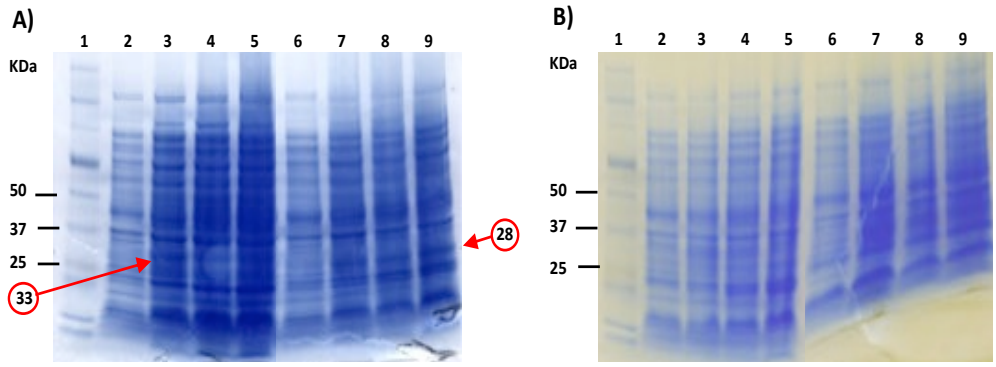


Figure 3. 3 Analysis of K01 (CIDRα1.1_IT4var20) and K02 (CIDRα1.1_PX0201-C.g34) protein expression.

(A) and (B) SDS-page analysis for K01 and K02 expression respectively in *E.coli* BL21 (DE3)pLysS. (Lane 1, prestained protein ladder; Lane 2-5, uninduced, 2hours, 4hours and 6hours induction time respectively for long protein sequence; Lane 6-9, uninduced, 2hours, 4hours and 6hours induction time respectively for the short protein sequence).

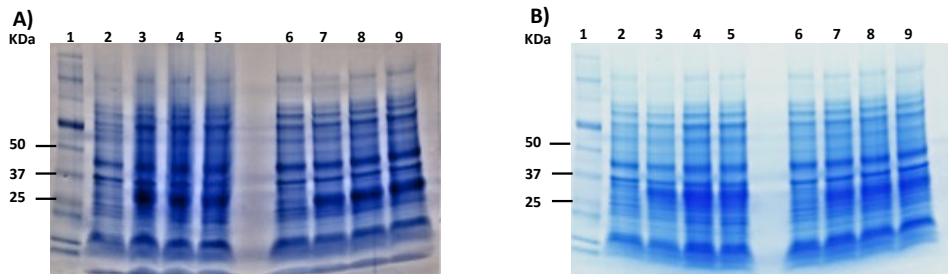


Figure 3. 4 Analysis of K01 (CIDRα1.1_IT4var20) and K02 (CIDRα1.1_PX0201-C.g34) protein expression.

(A) and (B) SDS-page analysis for K01 and K02 expression respectively in *E.coli* Rosetta2 (DE3) pLysS. (Lane 1, prestained protein ladder; Lane 2-5, uninduced, 2hours, 4hours and 6hours induction time respectively for long protein sequence; Lane 6-9, uninduced, 2hours, 4hours and 6hours induction time respectively for the short protein sequence).

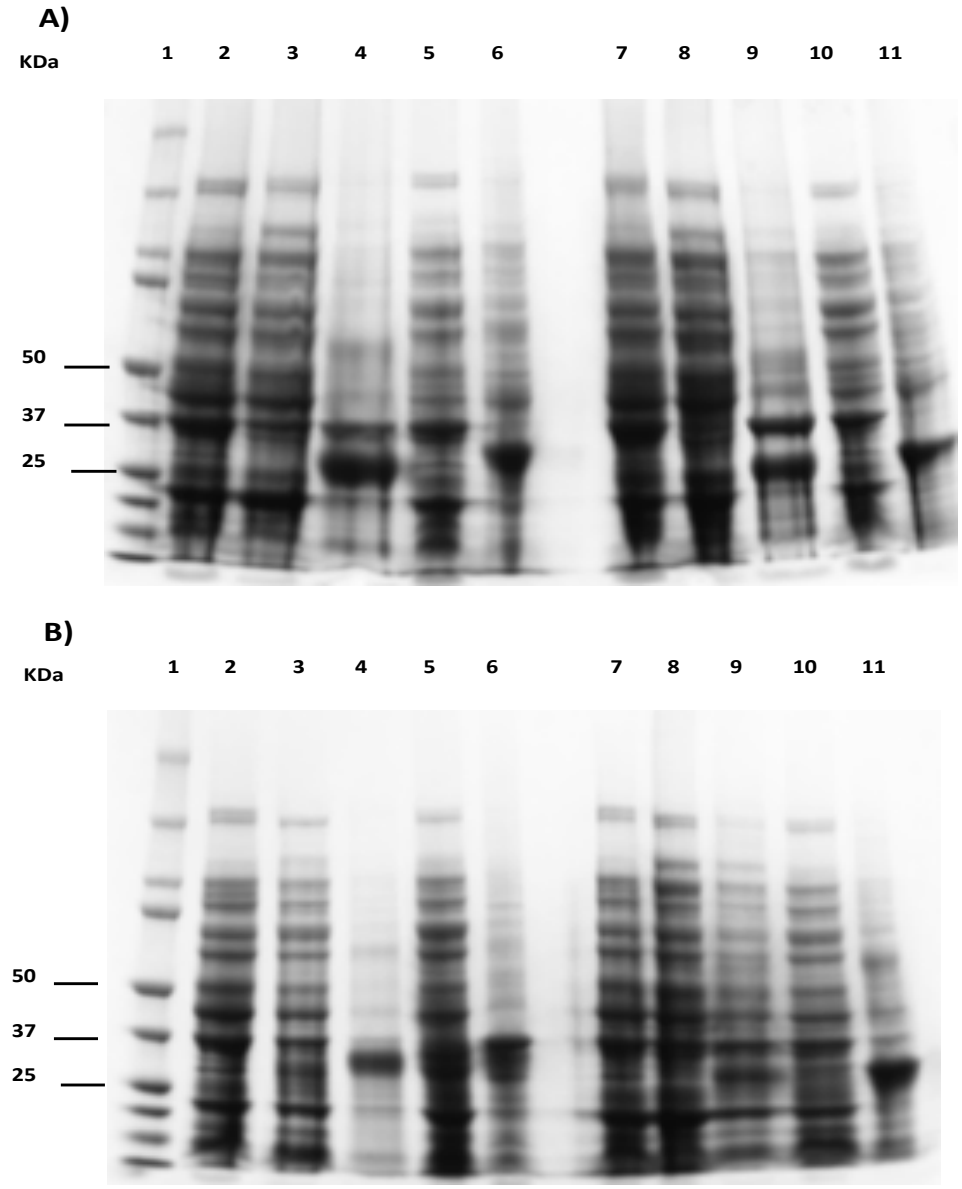


Figure 3. 5 Analysis of K01 (CIDRα1.1_IT4var20) and K02 (CIDRα1.1_PX0201-C.g34) protein expression.

(A) and (B) SDS-page analysis for cell lysate fraction separated into supernatant and pellet of both test and large scale proteins expression in *E.coli* Rosetta (DE3) pLysS of K01 and K02 respectively (Lane 1, prestained protein ladder; Lane 2-6, uninduced, scale up supernatant, scale up pellet, test expression supernatant and test expression pellet respectively for long protein sequence; Lane 7-11, uninduced, scale up supernatant, scale up pellet, test expression supernatant and test expression pellet respectively for the short protein sequence).

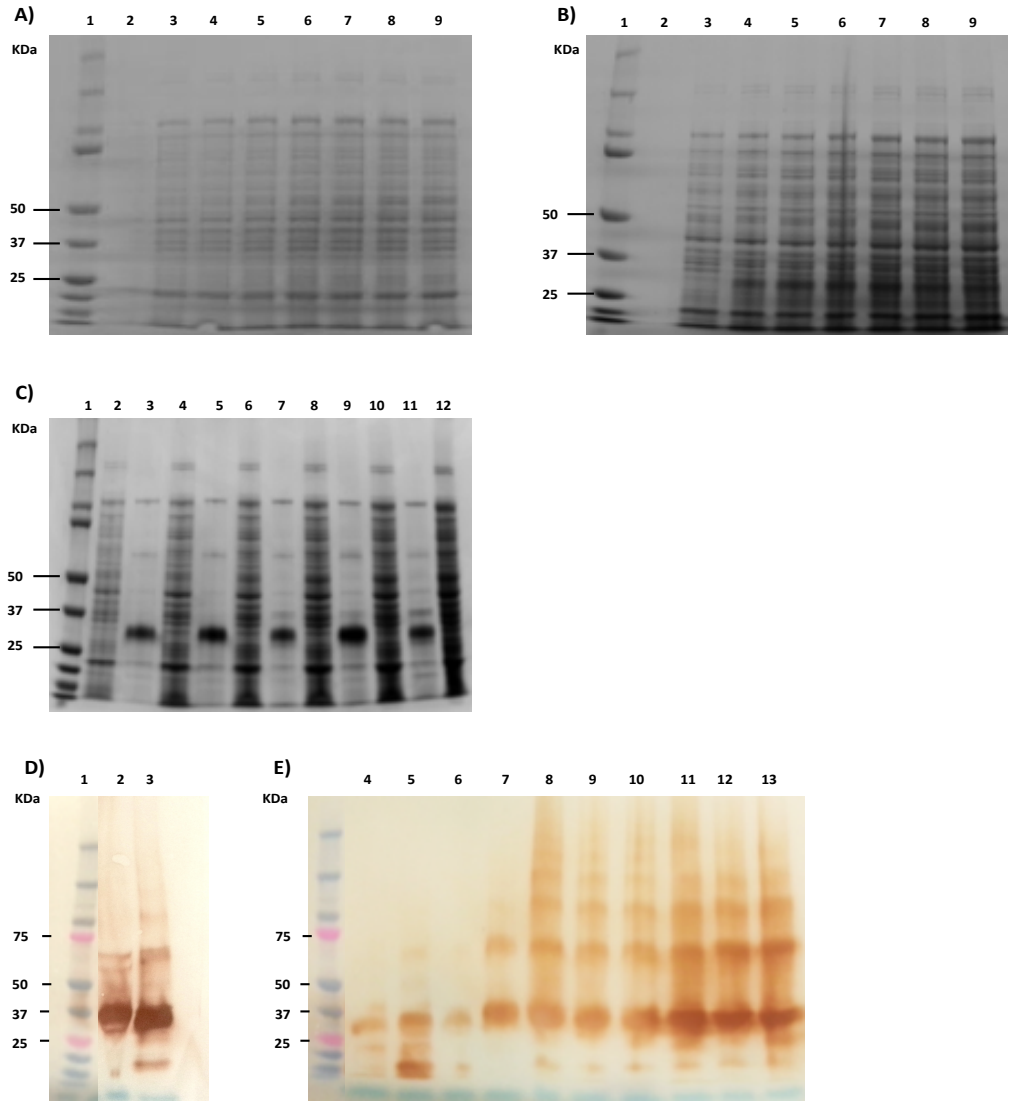


Figure 3. 6 Production of recombinant CIDRα in *E.coli*.

(A) and (B) SDS-Page for expression of recombinant proteins K04 (CIDRα1.1_PX0209-C.g28) and K07 (CIDRα1.6_PFD1235w) respectively (Lane 1, prestained protein marker; Lane 2, Uninduced sample; Lane 3-9, protein induction at OD600 of 0.4, 0.6, 0.8, 1.0, 1.6, 1.9 and 2.1 respectively); (C) SDS-Page analysis for expression of recombinant K07B fractions at various OD600 (Lane1, prestained protein marker; Lane2, uninduced sample; Lane3-4, pellet and supernatant fractions respectively at OD600 0.6; Lane5-6, pellet and supernatant fractions respectively at OD600 1.0; Lane7-8, pellet and supernatant fractions respectively at OD600 1.6; Lane9-10, pellet and supernatant fractions respectively at OD600 1.9; Lane11-12, pellet and supernatant fractions respectively at OD600 2.1); (D) and (E) Western blots for all successfully expressed recombinant proteins (Lane1, prestained protein marker; Lane 2-13, K01B, K02B, K03C, K04B, K05B, K06B, K07B, K08B, K09B, K10B, K12B AND K13B respectively).

3.2.2.2 Protein expression in insect cells

Two CIDRα genes, K01 and K02, were selected for the test expression trial utilizing a Baculovirus expression vector. Production of recombinant Baculovirus was achieved through co-transfection of pOPING recombinant vectors and Baculovirus vector in SF9 insect cells. Cultures were incubated at 27°C for 6 days before harvesting and subsequently amplifying the recombinant Baculovirus for another 6 days. Successively amplified recombinant baculovirus DNA were used in the test expression using three variable concentrations at 27°C for total of 7 day. SDS-page analysis for fractions collected at day 4, 5, 6 and 7 shows no clear evidence of proteins expression (Figure 3.7 A-D), however, when analysed by western blot, low expression can be observed (Figure 3.8). Further analysis of western blot suggests that 5 μl of P1 recombinant virus are the optimal transfection concentration when incubated for 5 days. Although expression of recombinant proteins in baculovirus were successful, I decided to stop further optimization and focus on producing the recombinant proteins in *E.coli* system.

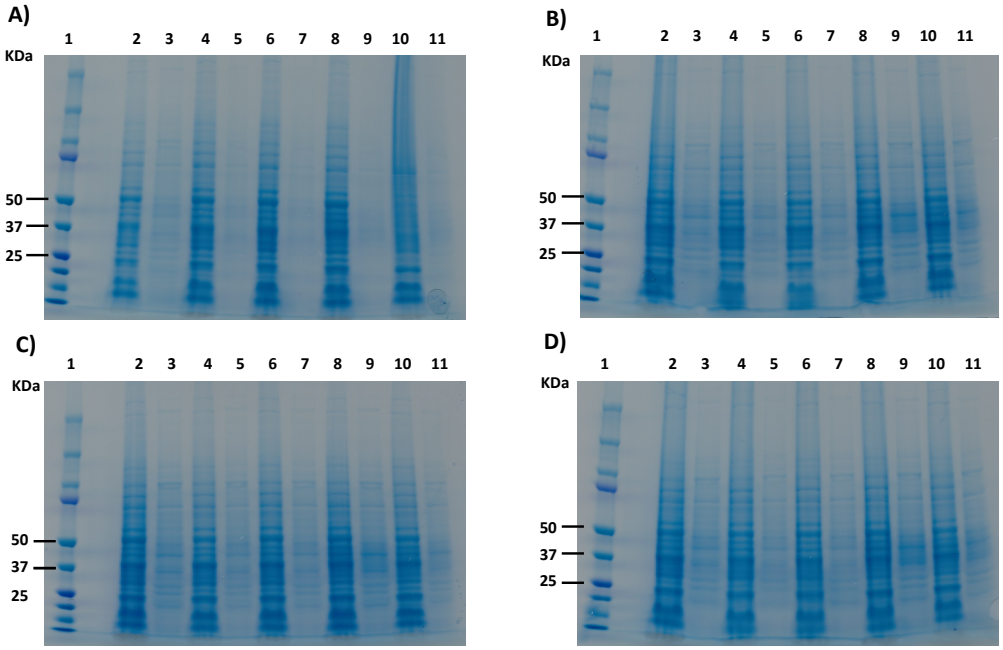


Figure 3. 7 Production of recombinant CIDRα K01 and K02 in insect cell.

(A) to (D) SDS-Page analysis of recombinant CIDRα K01 and K02 expression from fractions collected at day 4, 5, 6 and 7 respectively (Lane 1, prestained protein marker; Lane 2-3, cell pellet and supernatant respectively for K01 infection @ 0.5ul/ml; Lane 4-5, cell pellet and supernatant respectively for K01 infection @ 2.5ul/ml; Lane 6-7, cell pellet and supernatant respectively for K01 infection @ 5.0ul/ml; Lane 8-9, cell pellet and supernatant respectively for K02 infection @ 5.0ul/ml; Lane 10-11, cell pellet and supernatant respectively for K02 infection @ 2.5ul/ml).

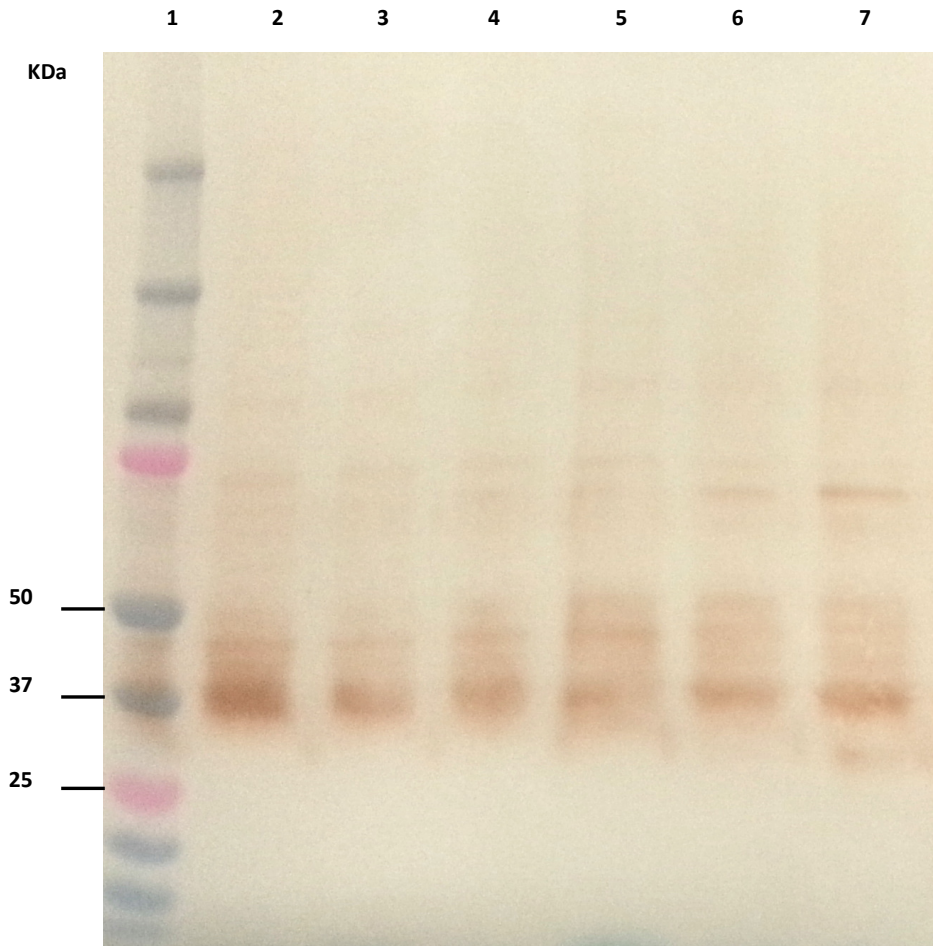


Figure 3. 8 Production of recombinant CIDRα K01 and K02 proteins in insect cells.

Immunoblots for day 5 using anti-His tag antibody (Lane 1, prestained protein marker; Lane 2-4, recombinant K01 @ 5.0, 2.5 and 0.5 μl/ml infection respectively; Lane 5-7, recombinant K02 @ 0.5, 2.5 and 5.0 μl/ml infection respectively).

3.2.3 Purification and refolding of recombinant CIDRα domains

Cell pellet fractions from the protein expression experiments were lysed and washed with BugBuster master mix detergent, then loaded on a column containing anti-Histidine resin. Several mixtures of buffers with variable additives and concentrations were used during the optimization of purification procedure, however, phosphate buffer was found to be the optimal buffer in the process. All purification experiments were performed at room temperature with eight washing buffers containing decreasing concentrations of Urea ranging from 8M to null thus facilitating the correct refolding of recombinant proteins while washing out contaminating proteins. After the last washing step, proteins were eluted with phosphate buffers in three steps containing increasing concentrations of imidazole ranging from 300mM to 500mM. Analysis of the wash and eluted fractions using SDS-PAGE shows a clear band in the eluted fractions with correct size for almost all expressed recombinant proteins (Figure 3.9). Following purification experiment, suitable eluted fractions were pooled and either dialyzed (3 KDa) or buffer exchanged (10 KDa) to remove imidazole from preparation, and then quantified using BCA quantitation kit (Table 3.2).

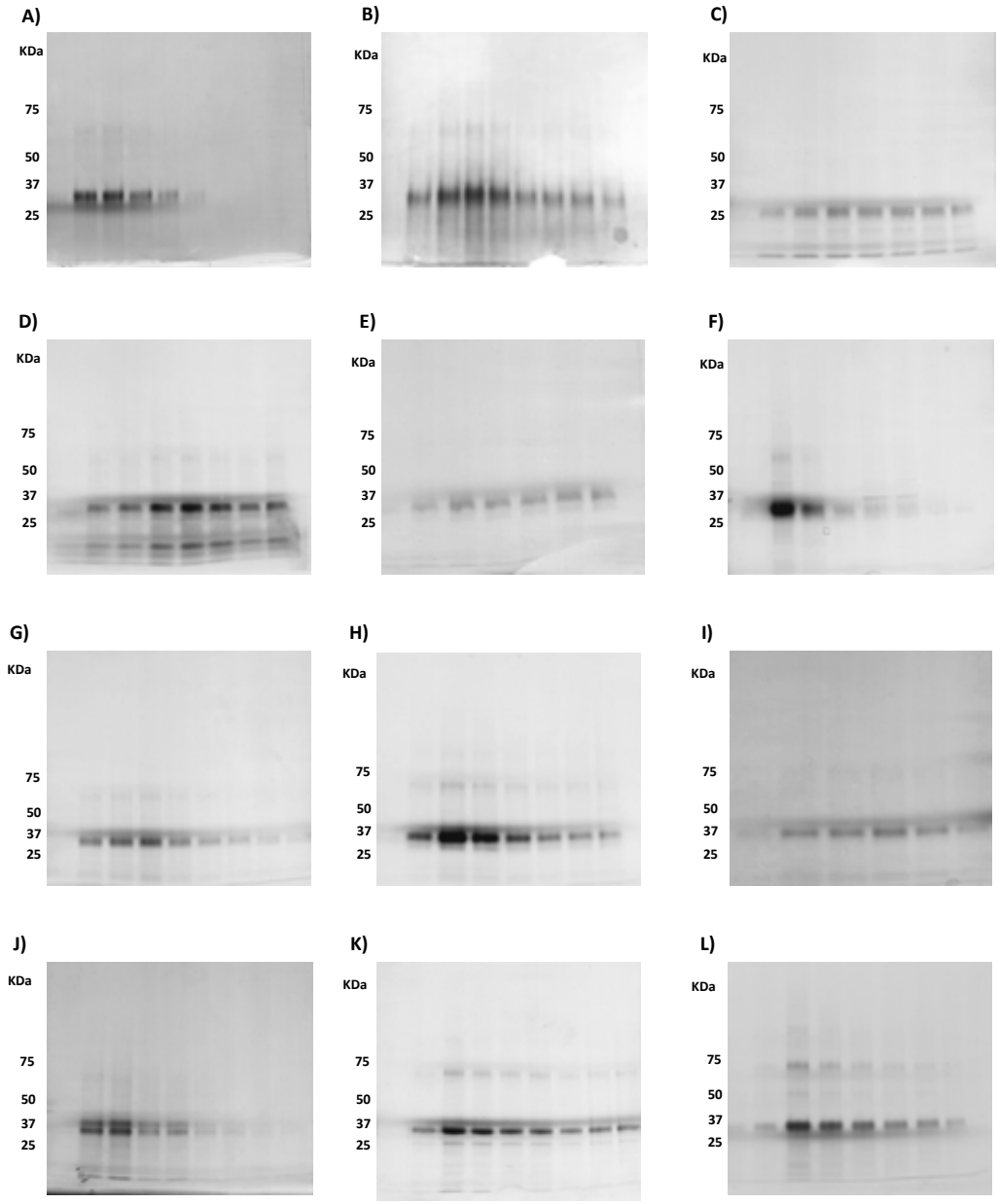


Figure 3. 9 On column recombinant CIDRα proteins refolding and purification.
(A) to (L) are elution fractions of K01B, K02B, K03C, K04B, K05B, K06B, K07B, K08B, K09B, K10B, K12B and K13B respectively. All lanes represent elution fractions.

Table 3. 2 Successfully expressed and purified recombinant CIDR proteins.

Protein	PI	Buffer	Purification	Domain	Quantity
K01B	7.5	Phosphate PH8.0	Dialysis	CIDRα1.1_IT4var20	8.85 mg
K02B	5.8	Phosphate PH8.0	Dialysis	CIDRα1.1_PX0201-C.g34	3.05 mg
K03C	6.5	Phosphate PH8.0	Dialysis	CIDRα1.1_PX0205-C.g37	0.34 mg
K04B	6.8	Phosphate PH8.0	Dialysis	CIDRα1.1_PX0209-C.g28	2.83 mg
K05B	7	Phosphate PH8.0	Dialysis	CIDRα1.1_PX9997-C.g4296	0.39 mg
K06B	6.5	Phosphate PH8.0	Dialysis	CIDRα1.4_HB3var03	1.04 mg
K07B	6.8	Phosphate PH8.0	Buffer Exchange	CIDRα1.6_PFD1235w	0.87 mg
K08B	6.5	Phosphate PH8.0	Dialysis	CIDRα1.6_PX0208-C.g61	2.97 mg
K09B	8.2	Phosphate PH6.0	Buffer Exchange	CIDRα2_PX0009-C.g9	0.19 mg
K10B	6.9	Phosphate PH8.0	Buffer Exchange	CIDRα2_PX0202-C.g70	0.31 mg
K12B	6.4	Phosphate PH8.0	Buffer Exchange	CIDRα2_PX0205-C.g24	1.13 mg
K13B	6.3	Phosphate PH8.0	Buffer Exchange	CIDRα3.5_IT4var15	0.97 mg

3.3 Discussion

*Pf*EMP1 is a VSA encoded by a multigene family of 60 var genes per haploid parasite genome. The extracellular regions of *Pf*EMP1 which consist of tandem repeat of DBL and CIDR domains are the mediator of malaria pathogenicity. A large body of evidence has linked specific CIDR domains expression with disease severity(77). The aim of this chapter was to produce different recombinant CIDRα of clinical importance to use later for screening of antibodies in plasma of malaria-immune individuals.

*Pf*EMP1 is considered one of the most difficult to express malaria proteins in heterologous systems due to their genetic codon usage and richness of A/T in its mRNA template(80). To overcome such obstacles, we decided to run test expression trials in both insect cell (eukaryotic systems) and *E. coli* (prokaryotic system) before deciding which system to use for large scale expression. Additionally, we decided to produce and test two construct of each CIDRα genes with variable domain boundaries; full domain sequence and a shorter sequence missing amino acids sequence encoding the first few cystine residue. However, no noticeable difference was noted during the test trial expression, so we decided to proceed with full domain boundary sequence in large scale proteins expression.

The test expression trial in insect cell were carried out using baculovirus Flashbac vector (Oxford Technology). Two CIDRα genes; K01 and K02 were cloned into Flashbac vector via homologues recombination between flanking sequences when co-transfected in to SF9 insect cells. Following the recombination and amplification process, it was essential to determine the optimum Multiplicity of infection (MOI) and Time of infection (TOI) for

protein expression. Ideally, optimising MOI and TOI for each construct require testing under 9 different conditions which obviously too many reactions, so we minimize the MOI optimisation to include only 0.5µl, 2.5µl and 5µl P1 virus, and cut down the TOI to 4 days. Although, successful production for both recombinant CIDRα proteins was confirmed, no significant differences were noted between all tested MOI or TOI. This finding was unexpected and suggests that further optimisation to MOI using higher amount of P1 virus is needed before commencing with large scale expression. However, due to the need for further optimisation and high cost consumables, we chose to stop testing this system and focus on the prokaryotic system using *E.coli.*

When it comes to proteins expression, the *E. coli* expression system is considered the gold standard due to its flexibility, low costs and ability to produces large amounts of recombinant proteins in short amount of time. However, in certain cases efficient expression of heterologous proteins in *E. coli* is diminished because it lacks certain tRNAs codon that are present in the organisms from which the heterologous protein is derived. The lack of these tRNA codons may result in termination of the translation process due to drop off of the ribosomal unit from the mRNA(80). The presence of high A/T content in *Plasmodium* genome may explain the relative difficulty in expressing *Pf*EMP1 within a heterogenous system such as *E.coli* without first optimising its codons. This may explain the failure of BL21 strains to express any recombinant CIDRα during test expression trial. In contrast, expression carried out in Rosetta2 strains succeeded with all tested genes. This is likely due to the ability of Rosetta2 strains in enhancing expression

of eukaryotic proteins by supplying tRNA for 7 rare codons. Although we were successful in expressing twelve out of thirteen recombinant CIDRα proteins at satisfactory level, we did not anticipated it to be in the form of IB. A possible explanation for this result is the lack of proper post translational modifications (PTM) in *E. coli* which may prove to be essential for correct protein folding, stability and activity. The formation of disulphide bonds is one of the most important PTM for mammalian proteins. These covalent bonds formed in the endoplasmic reticulum of mammalian cell through oxidation of thiol group between two cysteine residue and function as stabilizer of tertiary and quaternary structure of many mammalian proteins(81). In contrast, the reducing environment of *E. coli* cytoplasm is not permissible for catalysing the formation disulphide bonds due to presence of numerous reductases such as glutaredoxin and thioredoxin. Although disulphide bonds may form transiently, they are quickly reduced and becomes unstable before finally forming inactive IB(82).

In reviewing the literature, many protein refolding methodologies exist. However, most rely on denaturing the insoluble protein through the use of strong reducing agents such as guanidinium hydrochloride or urea. Subsequently, denatured proteins gradually refolded in a set of intermediate refolding buffers before finally eluted or dialyzed against the final buffer(83). However, the major drawback to protein refolding methodologies involves the formation of an incorrectly folded protein or aggregation of folding intermediates(84). Therefore, it was necessary to develop a protocol that minimizes the probability of such problems from happening during the refolding process. The on-column gradient protein

purification-refolding that we adopted facilitate proper protein refolding through serial washing with sets of buffers containing a decreasing gradient of a denaturing agent. Furthermore, the on-column immobilization of refolded protein reduces contact with other proteins chains thus aggregation is minimized.

Chapter 4. Acquisition of antibodies targeting *Pf*EMP1 CIDRα domains in malaria exposed adults

4.1 Introduction

In malaria endemic regions where transmission is high and stable, older individuals acquire natural clinical immunity as a result of repeated exposure to malaria infections (85). The age at which such immunity develops depends primarily on the intensity of malaria transmission within area of residence (86,87). However, immunity to severe malaria infections is acquired early in life, possibly 1 to 2 febrile infection may be sufficient to confer immunity against severe and life threatening disease (88). Several studies have provided evidence that naturally acquired antibodies mainly target the *P. falciparum* erythrocyte membrane protein 1 (*Pf*EMP1) member of clonally related variable surface antigen expressed on surface of infected red blood cells(77,89,90). Each parasite genome carries 60 *var* genes that encode single *Pf*EMP1 at a time providing large repertoire of types that mediates adhesion to several host receptors such as endothelial protein C receptor (EPCR) and CD36 (18). Structurally, the extracellular regions of *Pf*EMP1 consist of tandem repeat of duffy-binding-like (DBL), cysteine-rich interdomain regions (CIDR) domains which can be classified as CIDR α, β, γ, and δ (91). Studies of *var* gene transcription and *Pf*EMP1 expression demonstrated strong association between severe malaria in children and EPCR binding *Pf*EMP1 variants. Additionally, these studies suggest that severe malaria pathogenicity stem as a result of *Pf*EMP1 binding to EPCR, impairing its functional role as a cytoprotective, anticoagulative, and anti-

inflammatory(16,92–94). Adhesion of *Pf*EMP1 variants to EPCR host receptor is mainly mediated by specific subtypes of N-terminal CIDRα domains namely CIDRα1.1 or CIDRα1.4 to CIDRα1.8 (95).

The aim of this chapter is to screen plasma samples from 29 participants with current or previous history of malaria infection from Jazan region, Saudi Arabia. Enzyme-linked immunosorbent assays (ELISAs) and multiplex Luminex assay will be used to measure IgG antibodies level against several EPCR-binding and CD36-binding CIDRα regions of *Pf*EMP1. Results from both assays will be used to identify at least 2 samples with highest proportion of response and magnitude against the EPCR-binding CIDRα domains. Additionally, Spearman's rank correlation will used to compare results from both assays.

4.2 Results

4.2.1 Study recruits

Ethical approval for blood sample collection was granted by LSTM Research Ethics Committee on the 2nd of December 2015. Study recruitment was carried out in Jazan city region, at the southern border of the Kingdom of Saudi Arabia. Unfortunately, reliable and generally accessible area-specific information is not available even though transmission appears to have declined in recent years. Sample collection started after arrangements with the Disease and Vector Control Unit in Jazan region (DVCU) and authorizations from the ministry of Health and the Ministry of Higher Education in the Kingdom of Saudi Arabia. Blood samples were collected from 29 adult males aged between 18 and 46 years (Table 4.1). Almost all donors were immigrants from Yemen or Eritrea (15 and 9 donors respectively) except for 5 donors from Saudi Arabia. Historical samples were collected from individuals with previous history of malaria (HXX) in the last three years, while acute samples (AXX) collected within 7 days from treatment initiation.

4.2.2 Sample processing

For the isolation of PBMCs and plasma from participants, blood samples were collected in lithium heparin tubes (20-30 ml) and processed within a maximum of 8 hours from collection time. Plasma and PBMCs fractions were isolated through the use of Leucosep tubes (Greiner) and Lymphoprep (Axis-Shield). Following separation, all fractions were stored

frozen at -80C using controlled temperature gradient storage containers for 1 month. After completion of sample collection and processing, arrangements were made with a third party company to ship all processed samples on dry ice, and delivery was made within 3 days of sample shipment. All samples were then stored in liquid nitrogen cryostorage.

Table 4. 1 Characteristic of recruited donors

I.D	Age (years)	Gender	Nationality	Episodes Count (n)
H01	46	Male	Saudi Arabia	1
H02	26	Male	Saudi Arabia	1
H03	19	Male	Saudi Arabia	1
H04	18	Male	Saudi Arabia	1
H05	28	Male	YEMEN	1
H06	27	Male	ERITREA	3
H07	24	Male	YEMEN	2
H08	37	Male	Saudi Arabia	3
H09	22	Male	YEMEN	1
H10	33	Male	YEMEN	1
H11	27	Male	YEMEN	3
H12	35	Male	YEMEN	1
A13	19	Male	ERITREA	2
A14	28	Male	ERITREA	3
A15	21	Male	ERITREA	2
A16	25	Male	YEMEN	2
H17	29	Male	YEMEN	1
H18	34	Male	YEMEN	1
H19	43	Male	YEMEN	3
H20	20	Male	YEMEN	1
A21	18	Male	YEMEN	1
A22	35	Male	YEMEN	4
A23	38	Male	YEMEN	2
H24	30	Male	YEMEN	1
H25	22	Male	ERITREA	2
A26	33	Male	ERITREA	2
A27	30	Male	ERITREA	2
A28	34	Male	ERITREA	1
H29	18	Male	ERITREA	1

4.2.3 Indirect ELISA for screening reactivity of human plasma against a panel of twelve recombinant CIDRα domains

Human plasma samples used in the present study were collected from twenty-nine male adults living in Jazan region, Saudi Arabia. In order to identify suitable donors with good serological recognition of at least 6 recombinant *Pf*EMP1 domains, plasma samples were tested for reactivity with a panel of twelve recombinant CIDRα proteins using indirect ELISA. The calculated mean reactivity against the twelve recombinant proteins are shown in Table 4.2 and Figure 4.1. A high proportion of donors exhibited a broad range of immune responses against the recombinant CIDRα proteins above the cut-off value calculated as 2SD above the Mean Negative control. Plasma sample H25 and H06 showed the highest levels of reactivity (2.05±0.72 OD_{450nm}) and (1.84±0.66 OD_{450nm}) respectively against all twelve CIDRα proteins. On the other hand, CIDRα proteins responses (Figure 4.2) were variable even within domains that belong to the same class, the highest immune response noted for the CIDRα1.6a_PFD1235w (1.64±0.58 OD_{450nm}). Thus, each adult is likely to have a repertoire of antibodies to different CIDRα antigens, probably due to repeated exposure to malaria infection.

Table 4. 2 ELISA Plasma IgG reactivity to 12 CIDRα domains

ID	Mean OD_{450}	SD	LCL	UCL	Domains (n=12)
H01	1.41	0.40	1.16	1.67	12
H02	1.61	0.62	1.22	2.00	12
H03	0.80	0.54	0.46	1.14	9
H04	0.69	0.51	0.37	1.01	5
H05	1.51	0.70	1.06	1.95	12
H06	1.84	0.66	1.41	2.26	12
H07	0.92	0.31	0.72	1.12	11
H08	0.61	0.36	0.38	0.84	5
H09	0.74	0.22	0.60	0.89	10
H10	0.76	0.36	0.53	0.99	11
H11	1.05	0.51	0.73	1.38	11
H12	0.69	0.24	0.54	0.84	9
A13	0.83	0.45	0.54	1.12	8
A14	1.11	0.44	0.84	1.39	10
A15	1.36	0.75	0.88	1.84	10
A16	1.25	0.84	0.72	1.78	9
H17	1.50	0.52	1.17	1.83	12
H18	1.26	0.40	1.00	1.51	12
H19	0.76	0.50	0.44	1.08	6
H20	1.61	0.51	1.29	1.94	12
A21	1.06	0.44	0.78	1.34	11
A22	0.88	0.47	0.58	1.18	7
A23	1.24	0.60	0.86	1.63	12
H24	0.95	0.25	0.79	1.11	12
H25	2.05	0.72	1.59	2.50	12
A26	0.62	0.23	0.47	0.77	7
A27	1.27	0.58	0.90	1.64	12
A28	1.56	0.72	1.11	2.01	12
H29	1.02	0.25	0.86	1.19	11

SD(Standard deviation), LCL(Low confidence limit), UCL(Upper confidence limit), Domains(Number of reactive recombinant CIDRα antigens)

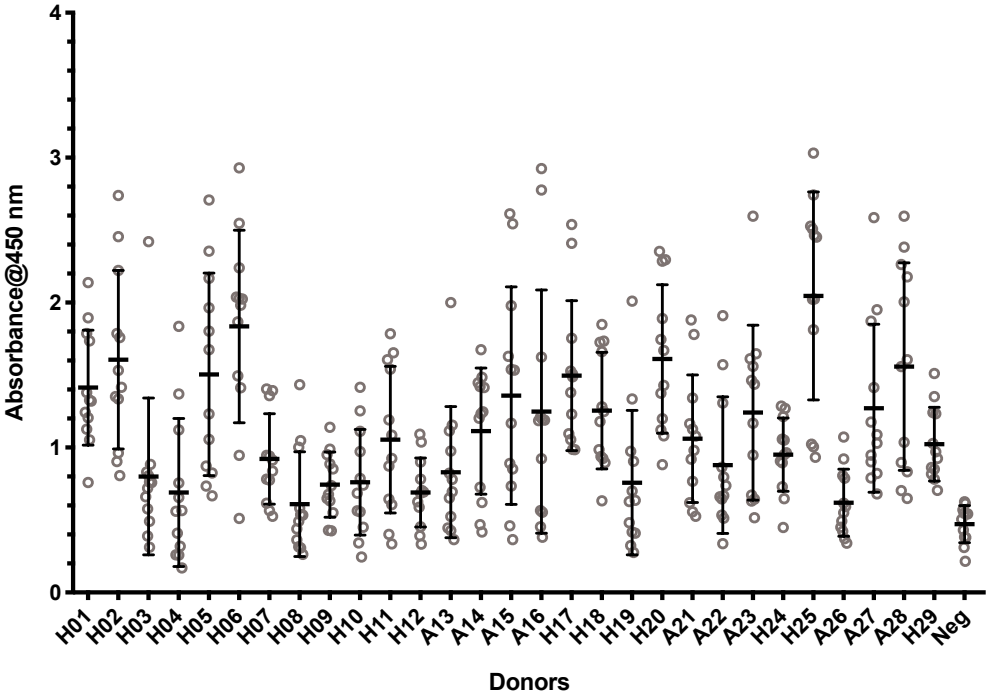


Figure 4. 1 ELISA assay for characterisation of donors plasma samples.

Indirect ELISA was performed to detect reactivity of plasma samples collected from 29 individuals with current or previous history of malaria infection against twelve recombinant CIDRα proteins. Malaria naive European sample was included as a negative control. Each dot represents a reactivity against single CIDRα protein. Results were considered positive if the mean absorbance value was more than the cut-off value calculated as 2SDs above the mean of negative control absorbance values.

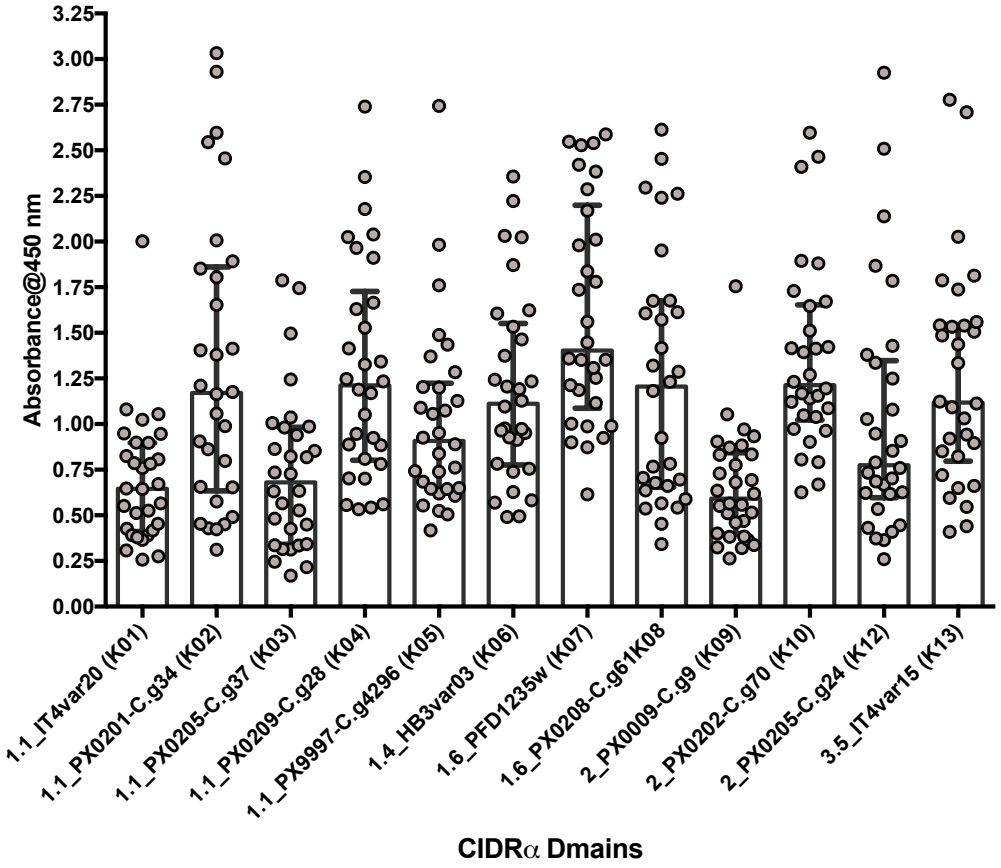


Figure 4. 2 Serological reactivity against different CIDRα domains from PfEMP1 proteins in human plasma.

Indirect ELISA was performed to detect serological reactivity of plasma samples taken from 29 individuals and a European naïve sample used as a negative control against 12 recombinant CIDRα proteins. Each bar represents mean absorbance@450 OD with 95%CL.

4.2.4 Luminex based screening for reactivity of human plasma against a panel of forty-nine recombinant CIDRα domains

To confirm our results, we asked our collaborator (Thomas Lavstsen, Copenhagen University) to screen all 29 donor's plasma with a wider range of CIDRα proteins. Thus, plasma samples were analysed by Luminex against a panel of 48 CIDRα proteins. The calculated mean reactivity against tested recombinant proteins are shown in Table 4.3 and Figure 4.3. Almost all plasma samples exhibited a broad range of immune responses against the recombinant CIDRα proteins above the calculated cut-off (2SD Mean Negative control), with A13 and H20 showing the highest levels of reactivity (7538±3374 MFI) and (6839±2231 MFI) respectively. On the other hand, CIDRα proteins responses (Figure 4.4 A) were variable even within domains that belong to the same class. However, the highest two recognised domains identified were those belonging to the EPCR binding class; CIDRα1.1_IGHvar19 (4779±3018 MFI) and CIDRa1.4 1974-5 (3693±2019 MFI). Additionally, the magnitude of seroreactivity was also compared between grouped domains belonging to the same subclass (Figure 4.4 B). Thus, each adult is likely to have a repertoire of antibodies to different CIDRα antigens, probably due to repeated exposure to malaria infection.

Table 4. 3 Luminex assay for plasma IgG reactivity to 48 CIDRα domains

ID	Mean MFI	SD	LCL	UCL	Domain
H01	1503	1546	1059	1947	40
H02	2141	1084	1830	2453	46
H03	1234	2030	650.7	1817	26
H04	325.7	567.5	162.6	488.7	4
H05	2900	1677	2418	3382	49
H06	5119	1993	4547	5691	49
H07	4696	2017	4116	5275	48
H08	634.2	673.1	440.9	827.6	7
H09	2259	879	2006	2511	45
H10	1130	601.5	957.4	1303	37
H11	2074	1828	1549	2600	45
H12	1830	1274	1464	2196	45
A13	7538	3374	6569	8507	49
A14	3127	2072	2531	3722	48
A15	3425	1322	3045	3805	48
A16	1921	1423	1513	2330	42
H17	3556	1723	3061	4051	49
H18	2120	1282	1752	2488	47
H19	1009	830.3	771	1248	29
H20	6839	2231	6198	7480	47
A21	1049	790.5	822.1	1276	31
A22	1281	1026	986.6	1576	39
A23	1520	591.5	1350	1690	46
H24	671.4	267.7	594.5	748.3	24
H25	2308	959.4	2033	2584	46
A26	1056	1203	710.4	1402	24
A27	2910	995.9	2624	3196	48
A28	3468	1604	3007	3928	48
H29	1433	1602	973	1893	39

MFI(Mean fluorescence intensity), SD(Standard deviation), LCL(Low confidence limit), UCL(Upper confidence limit), Domains(Number of reactive recombinant CIDRα antigens)

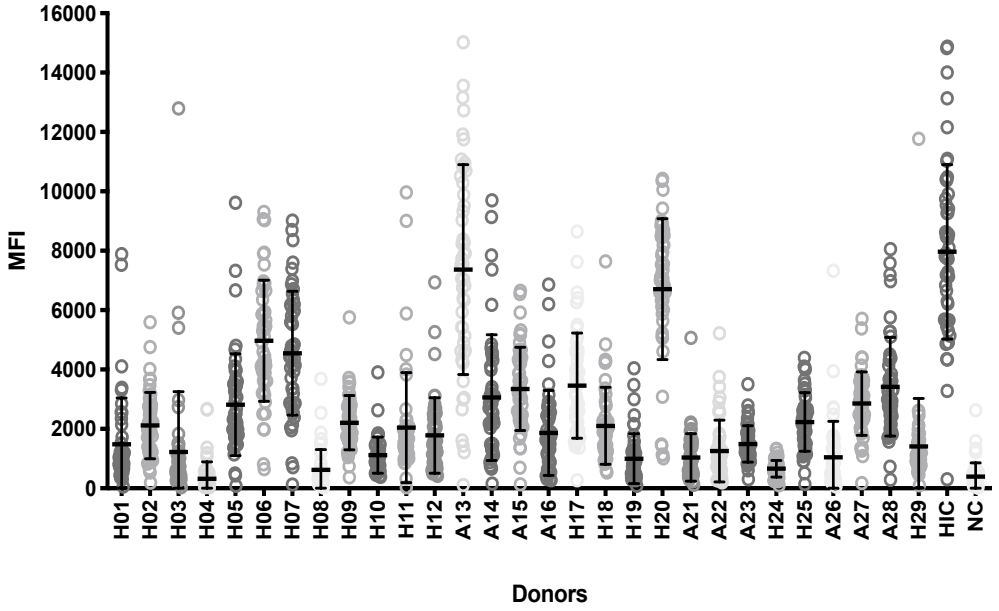


Figure 4. 3 Multiplex assay for characterisation of donors plasma samples

Luminex based assay was performed to detect reactivity of plasma samples collected from 29 individuals with current or previous history of malaria infection against 48 recombinant CIDRα proteins. Malaria naive European sample was included as a negative control (NC) as well as a hyperimmune immune pool as positive control (HIC). Each dot represents a reactivity against single CIDRα domain. Results were considered positive if the median fluorescence intensity (MFI) was more than the cut-off value calculated as 2SDs above the mean of negative control values.

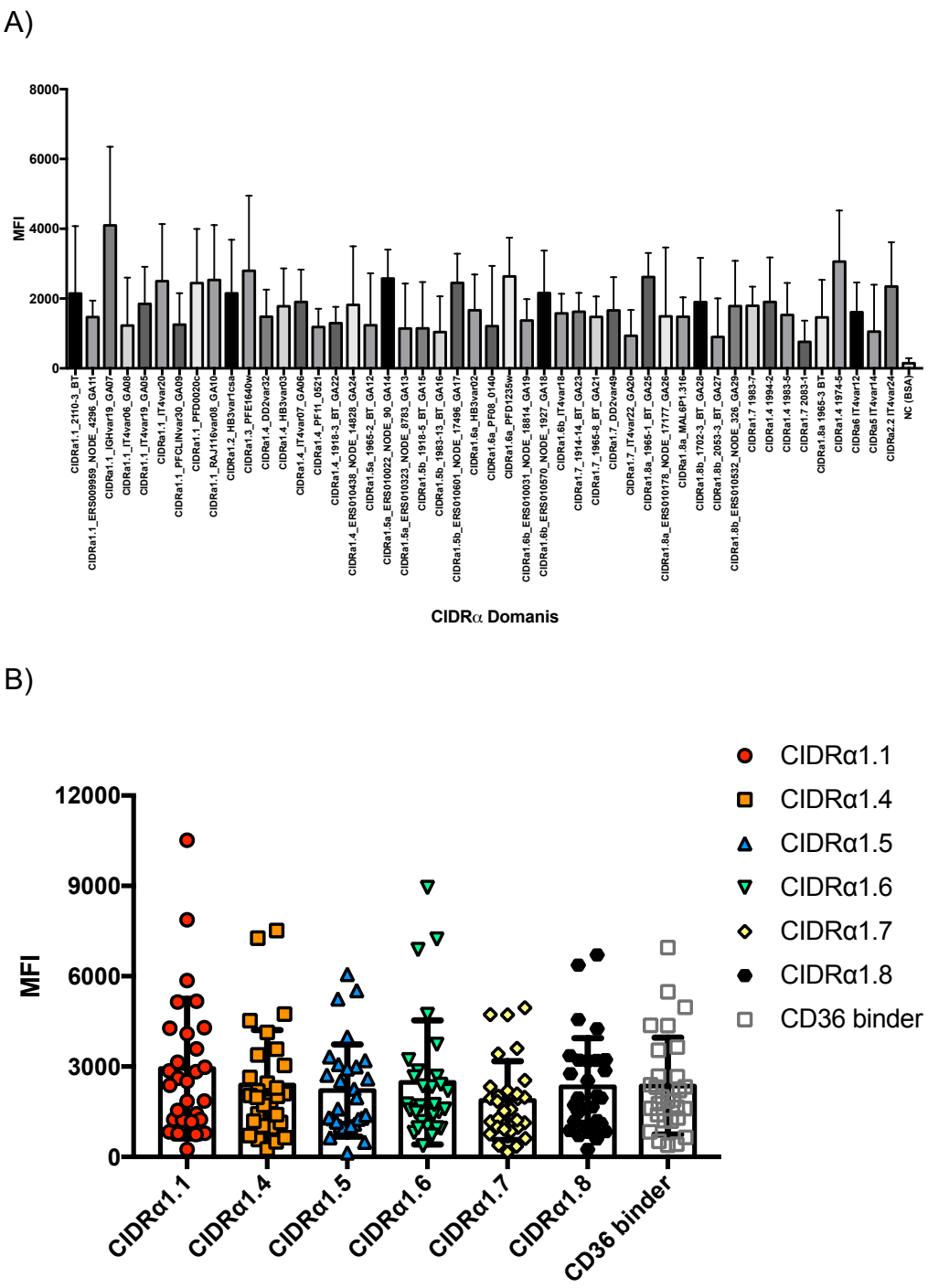


Figure 4. 4 Levels of immunoglobulin G (IgG) against different cysteine-rich interdomain region (CIDR) domain types.

A) Serological reactivity of 29 plasma samples against 48 CIDRα domains (Column bar: median with 95%CL). B) Serological reactivity of 29 plasma samples against 5 different CIDRα domains groups (Scatter plot: median with 25th and 75th percentiles). Reported results were those obtained as mean reactivity of duplicate reactions.

4.2.5 Relationship between ELISA and Luminex assays

The results obtained from measuring antibody seroreactivity to recombinant CIDRα1.1IT4var20 and CIDRα1.6PFD1235w in 29 plasma samples by Luminex were compared to those obtained by ELISA. The correlation between data points for each sample generated by Luminex relative to the data points generated by ELISA was evaluated by Spearman's rank correlation coefficients (Rho) with two tailed p-values <0.05 considered significant. Data obtained from Luminex based assay were performed using 1:80 dilution factor whereas ELISA assay was performed after 1:100 dilution of plasma samples. Analysis of data from both recombinant CIDR revealed statistically significant correlation between ELISA and Luminex determinations, although weak. The correlation scatter plots of seroreactivity to recombinant CIDRα1.1IT4var20 and CIDRα1.6PFD1235w for the entire sample set (n = 29) by ELISA and Luminex are depicted in Figure 4.5, panels A and B, respectively. The Spearman rank Rho for CIDRα1.1IT4var20 was 0.536, (P=0.0027), indicative of weak but acceptable correlation between the two assays. However, the correlation between both assays is extremely poor when using CIDRα1.6PFD1235w Rho 0.372, (P=0.0466).

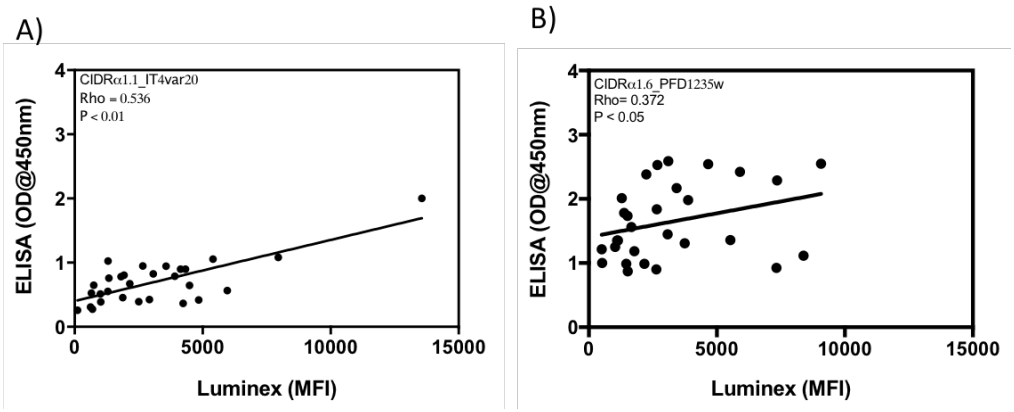


Figure 4. 5 Relationship of the Luminex based assay and ELISA.

Two recombinant CIDRα1.1IT4var20 and CIDRα1.6PFD1235w were analysed by ELISA and Luminex assay using 29 plasma samples from individuals with current or previous history of malaria infection. The results for Luminex based assay were expressed as median fluorescent intensity (MFI) and Absorbance @OD 450 for ELISA. The Spearman's rank correlation coefficients (Rho) for CIDRα1.1IT4var20 and CIDRα1.6PFD1235w were 0.536 and 0.372 respectively, ($P < 0.05$).

4.2.6 Identification of suitable donors for the single cell isolation of memory B cells

Single cell isolation will be performed on samples with no concurrent infection (Historical) that demonstrated both highest and widest reactivity against all CIDRα proteins in both assays. Summary of the highest 3 plasma samples with no concurrent infection from both assays is shown in Table 4.4. The highest serological reactivity based on Luminex data was noted for plasma sample H20 (6839±2231), H06 (5119±1993) and H07 (4696±2017). Comparably, the highest serological reactivity based on ELISA data was noted for plasma sample H25 (2.05±0.72), H06 (1.84±0.66) and H20 (1.61±0.51). Therefore, by combining both data, PBMCs samples from the highest donors H06 and H20 will be used to isolate CIDRα specific memory B cells. The serological reactivity of H06, H20 and naïve European negative control against 12 and 49 recombinant CIDRα proteins assayed by ELISA and Luminex respectively are depicted in Figure 4.6.

Table 4. 4 Summary of highly reactive plasma samples from both assays

	ID	Mean OD_{450}	SD	LCL	UCL	Domains (n=12)
Elisa	H20	1.61	0.51	1.29	1.94	12
	H06	1.84	0.66	1.41	2.26	12
	H25	2.05	0.72	1.59	2.5	12
	ID	**Mean MFI**	**SD**	**LCL**	**UCL**	**Domains (n=49)**
Luminex	H07	4696	2017	4116	5275	48
	H06	5119	1993	4547	5691	49
	H20	6839	2231	6198	7480	47

OD(Optical density), MFI(Mean fluorescence intensity), SD(Standard deviation), LCL(Low confidence limit), UCL(Upper confidence limit), Domains(Number of reactive recombinant CIDRα antigens)

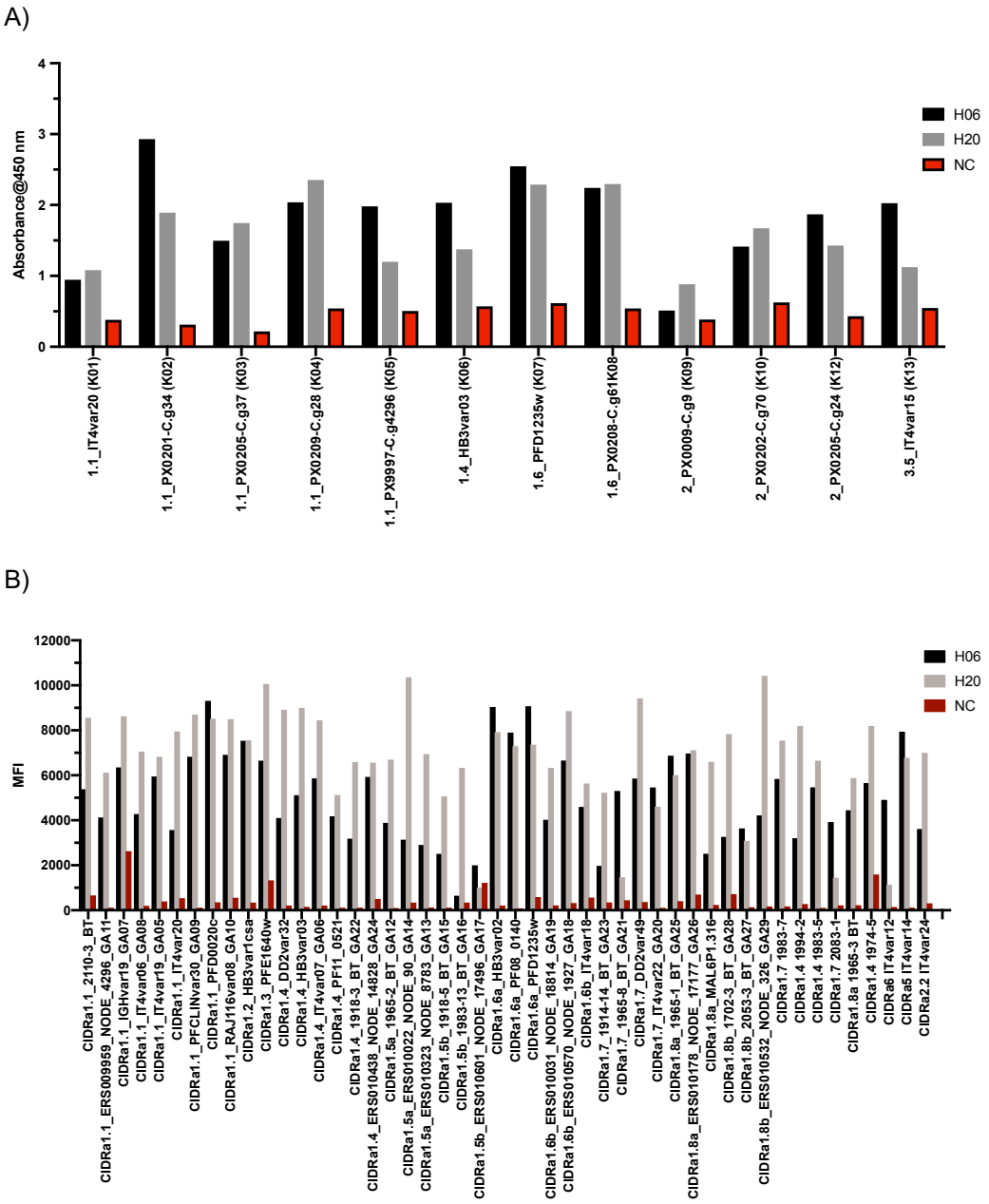


Figure 4. 6 Antibody reactivity to recombinant CIDRα proteins in human plasma.

A) 12 recombinant CIDRα proteins were analysed using ELISA technique against H06, H20 and naïve European negative control. B) 48 recombinant CIDRα proteins were analysed using Luminex based assay against H06, H20 and naïve European negative control. Reported results were those obtained as mean reactivity of duplicate reactions

4.3 Discussion

Regardless of the many ongoing prevention and intervention programs, malaria remains the most life-threatening disease in young children, especially in areas with high transmission rates. A strong body of evidence suggests that naturally acquired antibodies targeting VSAs on iRBCs, such as *Pf*EMP1, are associated with protection against severe malaria disease(8,87,90). Moreover, disease severity and outcomes during malaria episode are largely dependent on the binding phenotype of the blood stage parasite. Severe malaria is thought to be caused by *Pf*EMP1 binding to the EPCR receptor and impairing its functional roles as a cytoprotective, anticoagulative, and anti-inflammatory mediator(93). Adhesion of *Pf*EMP1 variants to EPCR host receptor is mainly mediated by specific subtypes of N-terminal CIDRα domains namely α1.1 or α1.4 to α1.8 (95). Immunological profiling of plasmas for the presence of reactive antibodies targeting VSAs is a commonly used tool for evaluating acquired immunity and identifying potential candidates for vaccine development.

In this chapter, we describe the use of multiplex and ELISA assays for characterizing the antibody response of 29 plasma samples obtained from adult donors living in Jazan, southern region, Saudi Arabia. We chose to focus our studies on EPCR binding *Pf*EMP1 phenotypes due to its strong association with reduced risk of severe malaria making it a possible vaccine candidate. Therefore, we evaluated the serological reactivity of these samples against 12 and 48 recombinant CIDRα domains from *Pf*EMP1 using ELISA and multiplex assay respectively. By doing so, we were able

to identify at least 2 semi-immune adults exhibiting high and wide serological recognition across multiple recombinant CIDRα1 domains.

The results obtained from ELISA experiment clearly show that all recombinant CIDRα domains were recognized by most donors' sera with inter-variable levels of recognition indicating presence of cross-reactive population of serum IgG in the tested sera. Additionally, 15 out of 29 donor sera (52%) that we tested were able to react with all 12 recombinant CIDRα domains while the remaining 14 samples were able to recognise at least 5 recombinant CIDRα domains. There are two possible explanations for such higher proportion of cross-reactivity against all tested recombinant CIDRα domains. In accordance with previous conclusions by Newbold (1992) and Gamain (2001), it could be due to acquisition of IgG antibodies of multiple specificities against the tested recombinant CIDRα domains (96,97). However, it may also be that the existing IgG antibodies in sera recognises conserved epitopes across tested recombinant CIDRα domains, which is in agreement with previous suggestions that antibodies recognise conserved epitopes in VSAs (98).

Consistent with ELISA experiment, results from Multiplex assay demonstrate even higher rate of recognition for recombinant CIDRα domains confirming presence of cross-reactive population of serum IgG in tested sera. Moreover, 15 donor sera (52%) were able to react with all 48 recombinant CIDRα domains, while 13 sera were able to recognise at least 38 recombinant CIDRα domains, and only 11 CIDRα domains were recognised by the remaining sera sample. Also, acquisition of anti-CIDRα1.1 IgG antibodies were significantly higher than CD36 binding

CIDRα domains (P<0.01 ANOVA, Bonferroni corrected) which is consistent with a previous study looking at IgG acquisition to EPCR-binding CIDR domains in Tanzanian children. They demonstrated that EPCR binding CIDRα domains are acquired early in life and most likely before other CIDR domains. However, sustained exposure to malarial antigen is a key factor for maintaining the level of acquired antibody against disease. They concluded that CIDRα1.1, α1.4 to α1.8 domains are not only associated with severe malaria, but also that on individual's first *P. falciparum* infections in endemic area are likely to be caused by parasites expressing *Pf*EMP1 encoding EPCR-binding domains(19).

Taken together, results from both experiments show an agreement for historical donors H20 and H06 as having the highest reactive sera that were able recognise almost all tested recombinant CIDRα domains. To further test agreement between both methods, results obtained from measuring antibody reactivity to recombinant CIDRα1.1_IT4var20 1.1_ and CIDRα1.6_PFD1235w domains by the multiplex assay were compared to those obtained by ELISA assay. Spearman's correlation coefficients (Rho) were 0.53 and 0.37 (P<0.05) for CIDRα1.1_IT4var20 1.1_ and CIDRα1.6_PFD1235w respectively reflecting positive but poor correlation between data. Close examination of correlation curve from both domains indicate consistent low signal in ELISA assay compared to the multiplex assay, which may be attributed to several factors. Generally, such variation may possibly be caused by the differences in the plasma dilution factor, the amount of protein for coating, buffers, the anti IgG concentration and the detection reagents. For example, the multiplex assays were performed by

our collaborators from Copenhagen university (Lavstsen and Turner) with 15μg CIDR/well and plasma dilution at 1:80 fold, while we ran the ELISA assay using 0.05μg CIDR/well and plasma diluted at 1:100 fold. In a similar study, Cham et al (2008) compared data obtained from measuring the seroreactivity of 80 plasma samples against 4 recombinant *Pf*EMP1 proteins by ELISA and bead-based multiplex. They reported a strong correlation between both methods, although, marked difference between methods values were noted for few samples (low ELISA value/high bead-based multiplex assay values or vice versa) which is in agreement with our findings(99). However, they concluded that the bead based multiplex assay is faster, more sensitive and less labour intensive compared to ELISA. Even so, both studies suffer from some degree of variation, yet it is debatable whether true comparisons can be made due to differences in methods setup and sample size. Nonetheless we took samples H06 and H20 forward for single cell isolation, based on their breadth of binding to tested recombinant CIDRα1 protein antigens.

Chapter 5. Single cell sorting of CIDR-specific B cells

5.1 Introduction

The precise characterization and isolation of human antigen-specific memory B cells (MBC) is often challenging due to their low frequencies in peripheral blood. It is estimated that tetanus toxoid (TT)-specific MBC constitute about 0.003% of total B cells (BC) in adults that were vaccinated during their childhood (100). Furthermore, signal generated by FACS (florescent activated cell sorting) analysis utilising labelled antigens monomer often are insufficiently bright and tend to overlap with unlabelled cells. To overcome such obstacles, most studies have relied on the in vitro expansion and conversion of MBC into plasmablasts using different combination of cytokines including IL-2, IL4, IL-6, IL-15, IL-10 and IL-21(101–105). However, other studies have considered the utilization of antigen tetramers to increase the avidity of B cell receptor (BCR) labelling, thereby improving identification of antigen-specific MBC (74).

In this chapter, we aimed to isolate CIDR-specific MBC cells from PBMCs samples collected from participants with previous history of malaria infection. Two PBMC samples H06 and H20 were chosen for single MBC isolation, based on their breadth of binding to tested recombinant CIDRα1 protein antigens (see section 4.2.6 page 71). We chose to test the most widely used techniques to isolate CIDR-specific MBC. This involve testing fluorescently labelled antigen monomer, BC culture and fluorescently labelled antigen tetramer.

Through the use of two tetramerised recombinant CIDR (K02-CIDRα1.1_PX0201-C.g34 and K08-CIDRα1.6_PX0208-C.g61) we were able to isolate 395 CIDR-specific MBC from two donor PBMC samples.

5.2 Results

5.2.1 Defining B cell subsets after immunization

To directly detect and isolate antigen-specific MBC cells we designed several multicolour flow cytometry panels to better characterize and distinguish BC populations. The aim of this experiment was to construct a FACS panel that can effectively characterize seven BC populations from PBMCs samples from healthy donors. During sample acquisition, we used a gating strategy (Figure 5.1) to measure the relative proportion of class switched B cells (Sw BC) ($CD3^-CD19^+IgD^-CD27^+$), MBC ($CD3^-CD19^+IgD^-CD27^+CD38^-CD20^+$), activated B cells (ABC) ($CD3^-CD19^+IgD^-CD27^+CD71^{hi}CD20^+CD38^-$), antibody secreting cells (ASC) ($CD3^-CD19^+IgD^-CD27^+CD71^{hi}CD20^-CD38^+$), naive B cells ($CD3^-CD19^+IgD^+CD27^-$), non-switched B cells (NSw BC) ($CD3^-CD19^+IgD^+CD27^-$) and atypical B cells ($CD3^-CD19^+IgD^-CD27^-$). As is the case with ASC, all antigen specific B cells should be actively proliferating upon encountering its antigen, thus we used CD71 as a surface marker to label proliferating B cells, allowing us to clearly distinguish ASC and ABC within Sw B cells pool in addition to CD38 and CD27 markers.

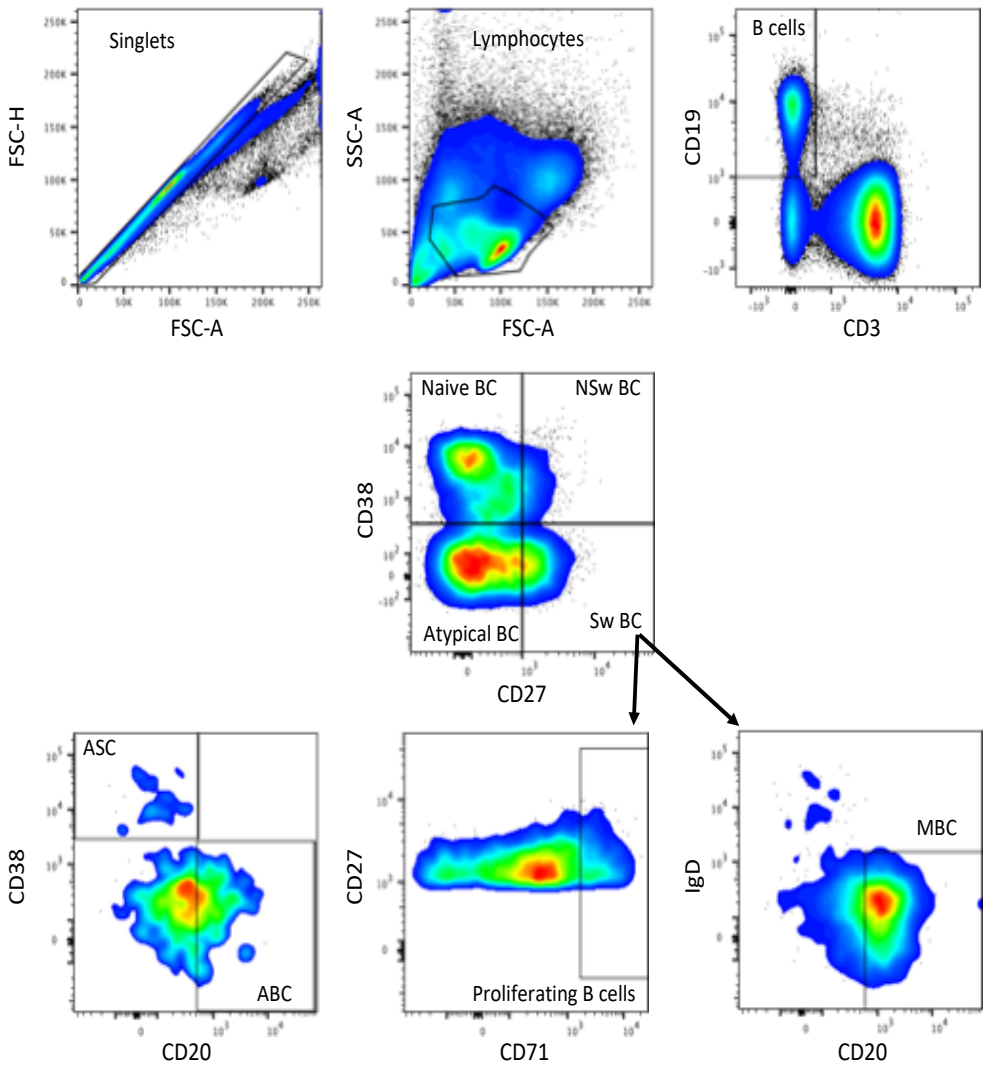


Figure 5. 1 Gating strategy for the BC repertoire analysis of ABC, ASC and MBC clonal lineages.

PBMCs were first gated to exclude doublets by comparing the FSC area and height measurements. Single cells were gated by forward (FSC) and side scatter (SSC), extending the normal lymphocyte gate beyond the standard limits to account for the increased size and granularity of plasmablasts.

5.2.2 Titration of Histidine-tagged recombinant CIDR

The general aim of this chapter is to isolate CIDR-specific memory B cells from PBMC samples of donors exhibiting high and wide response against multiple CIDR. This can be achieved by measuring cell binding to a hexa-histidine tagged recombinant CIDR followed by a fluorescently tagged anti-histidine conjugate. However, due to absence of a suitable control PBMC sample with validated CIDR-specific memory B cells levels, it was challenging to effectively evaluate and titrate our recombinant CIDR proteins. As an initial step, we decided to use 4 Histidine-tagged recombinant CIDR to label a PBMC sample obtained from a healthy donor with no history of malaria infection, two of which were an EPCR binder (K01 and K02) while the remaining two were CD36 binders (K12 and K13). The plan was to label the control sample with different concentrations of His-tagged CIDR proteins followed by cell staining with CD3, CD14, CD19 and anti-histidine fluorochromes to evaluate non-specific binding. Subsequently, FACS acquisition of 50,000 cells was performed before using a gating strategy to first exclude doublets, then gate on B cells (CD3-/CD14-/CD19+) and monocytes (CD3-/CD19-/CD14+) then finally gating on anti-histidine positive subset in both B cells and monocytes populations (Figure 5.2).

In term of B cell labelling, non or low background noise was detected even at higher concentrations when staining with K02, K12 and K13 CIDRs, however, cell labelling with K01 protein generated increased level of non-specific binding. In contrast, 90% of monocytes population labelled with K13 CIDR at optimum concentration as expected due to binding to CD36,

while only 0.12 and 0.22% labelled with K02 and K12 respectively. Results from the initial experiment suggest that 1μg/ml of Histidine-tagged CIDR was the optimum staining concentration as it produced least background noise in case of negative staining (B cells with the 4 tested CIDR) or positive staining (Monocytes with K13 CIDR). Consequently, follow-up experiment was performed to test remaining histidine-tagged CIDR proteins (K03-K10) at 1μg/ml and 5μg/ml concentration which again confirm that 1μg/ml was the optimum concentration needed to stain $2.5x10^5$ Cells (Table 5.1-5.2).

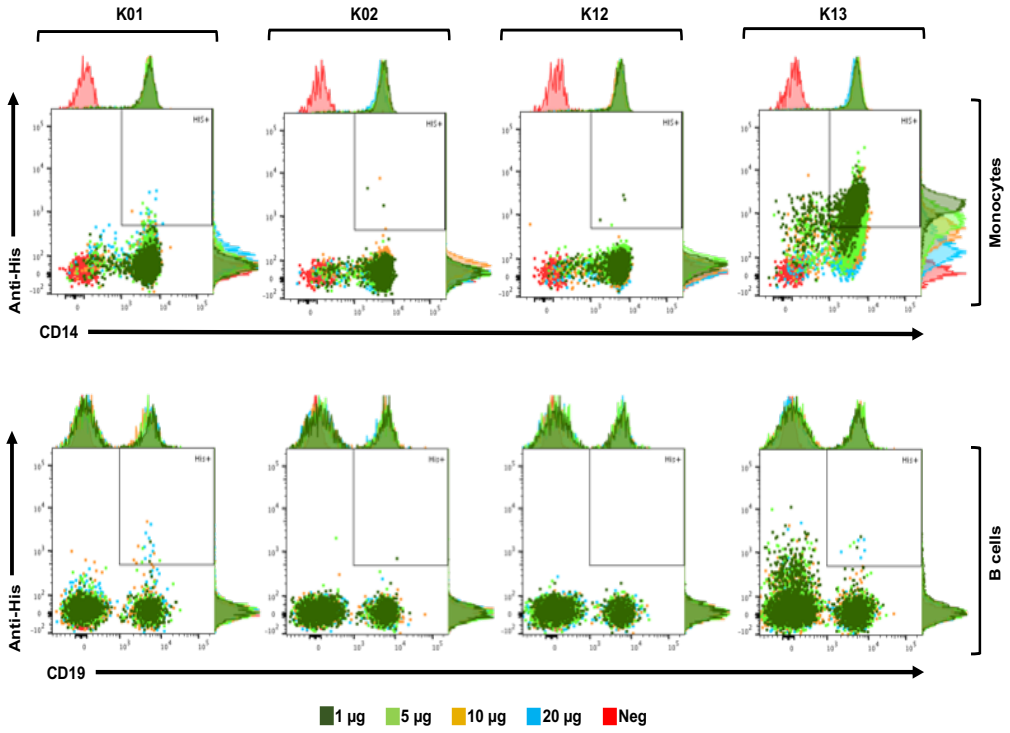


Figure 5. 2 Titration of recombinant histidine-tagged CIDR proteins.
Four recombinant histidine-tagged CIDR proteins were used to stain PBMC sample from malaria naïve donor. Overlay of dot plots showing five different concentrations with adjunct histogram. Upper panel showing frequency of stained monocytes while lower panel showing frequency of stained B cells.

Table 5. 1 Frequency of stained B cells (malaria naïve donor) at different concentrations of 11 recombinant histidine-tagged CIDR proteins.

	0.5ug/ml	1ug/ml	5ug/ml	10ug/ml	20ug/ml
K01	---	0.160%	0.270%	0.390%	0.630%
K02	---	0.078%	0.000%	0.000%	0.000%
K03	---	0.037%	0.076%	---	---
K04	---	0.038%	0.037%	---	---
K05	---	0.000%	0.110%	---	---
K06	---	0.076%	0.190%	---	---
K07	---	0.000%	0.000%	---	---
K08	---	0.077%	0.037%	---	---
K09	---	0.000%	0.000%	---	---
K10	---	0.000%	0.064%	---	---
K12	---	0.000%	0.000%	0.000%	0.000%
K13	0.180%	0.073%	0.074%	0.000%	0.190%

Table 5. 2 Frequency of stained monocytes (malaria naïve donor) at different concentrations of 11 recombinant histidine-tagged CIDR proteins.

	0.5ug/ml	1ug/ml	5ug/ml	10ug/ml	20ug/ml
K01	---	0.000%	0.940%	0.440%	1.050%
K02	---	0.120%	0.000%	0.150%	0.000%
K03	---	0.220%	0.280%	---	---
K04	---	2.540%	2.660%	---	---
K05	---	0.220%	0.480%	---	---
K06	---	2.070%	1.710%	---	---
K07	---	3.710%	3.890%	---	---
K08	---	0.072%	0.067%	---	---
K09	---	0.066%	0.380%	---	---
K10	---	0.640%	0.310%	---	---
K12	---	0.180%	0.056%	0.000%	0.000%
K13	86.300%	90.400%	63.200%	38.300%	11.800%

5.2.3 Surface labelling of malaria naïve PBMC samples with recombinant histidine-tagged CIDR, CD36 and EPCR

Results from previous titration experiments suggests presence of some degree of binding to B cells, especially when using K01 protein to label PBMC samples. Such binding may be caused by contaminants from recombinant CIDR preparation or due to dead cells in PBMCs preparation or even that the naïve donor was unaware of previous malaria infection during his childhood. Thus, we decided to address such issues in this experiment by screening all recombinant CIDR proteins against 4 naïve malaria donors with addition of more markers such as Live/Dead fluorochrome and B cell subset markers (CD27 and IgD). Additionally, we constructed two more panels to FACS screen PBMC samples from the 4 recruited naïve donors for surface expression of EPCR and CD36 cell markers on B cell subsets.

Results from this experiment (Table 5.3) confirm our previous findings in that K01-CIDR protein bind B cell specifically and not due to dead cells, but perhaps due to bacterial contaminants carried over during protein production. Interestingly, most binding was confined to IgD+ subtypes (Naïve and NSw BC) which is also true even with CIDR exhibited lower binding to BC such as K08. However, even though our aim was to isolate MBC, which is a switched type BC meaning we still able use K01 to try isolating MBC, we preferred using recombinant K02-CIDR due to its low background staining, and it was among those that demonstrated highest reactivity among CIDRα1.1 domain proteins. Most intriguingly, data from EPCR and CD36 experiment indicate that no evidence of EPCR expression

could be detected within screened B cell subsets which was expected, however, low expression of CD36 marker was detected on BC (Table 5.4).

Table 5. 3 Median percentage of anti-6Histidine (CIDR+) from 4 naïve malaria control

	Mono	BC	Sw BC	NSw BC	Naïve	Atypical
K01	0.59%	1.95%	0.38%	2.29%	2.40%	0.76%
K02	0.13%	0.12%	0.17%	0.51%	0.09%	0.10%
K03	0.46%	0.30%	0.18%	0.60%	0.25%	0.18%
K04	0.25%	0.13%	0.13%	0.38%	0.11%	0.16%
K05	0.38%	0.15%	0.17%	0.26%	0.13%	0.18%
K06	1.01%	0.37%	0.52%	0.92%	0.35%	0.29%
K07	0.47%	0.22%	0.36%	0.39%	0.14%	0.15%
K08	0.11%	0.61%	0.36%	0.79%	0.71%	0.22%
K09	0.02%	0.05%	0.13%	0.03%	0.04%	0.07%
K10	0.02%	0.04%	0.11%	0.11%	0.02%	0.01%
K12	0.16%	0.14%	0.24%	0.20%	0.08%	0.09%
K13	74.70%	0.47%	0.39%	0.36%	0.48%	0.48%
Blank	0.02%	0.10%	0.16%	0.21%	0.05%	0.05%

Table 5. 4 Median percentage of CD36 marker expression.

	Mono	BC	Naïve	NSw BC	Sw BC	Atypical
Blank	0.00%	0.02%	0.00%	0.04%	0.04%	0.00%
CD36+	99.35%	5.36%	4.83%	4.54%	5.18%	7.14%

Abbreviation: BC, B cells; Sw BC, Switched B cells; NSw BC, Non-Switched B cells.

5.2.4 B cell sorting and Culturing conditions

The isolation of encoded antibodies from memory B cells of semi immuned individuals can be very useful in vaccine development. However, the precise identification and isolation of antigen specific memory B cells is technically challenging due to their low frequencies in the periphery and access to sample donors. Therefore, we planned to establish B cells culture to enrich for antigen specific MBC. Thus, two donor samples H03 and H04 that exhibited medium reactivity against screened CIDR were taken into culture using two different approaches. Donor sample H03 was first activated by Histidine-tagged recombinant K07 CIDR for 1 day before further 3 days culture with added IL2, IL4 and IL21 cytokines cocktail. A different approach was used with a sample of donor H04, it was first enriched for CD19+ cells using magnetic cell sorting technique followed by activation with Histidine-tagged recombinant K07-CIDR and homologues APC (antigen presenting cells) obtained from the depleted fraction during the enrichment process. Samples were left in culture for 3 days before refreshing with IL2, IL4 and IL21 cytokines cocktail and further incubation for an additional 3 days.

Results obtained from H03 culture experiments indicated a 23 fold increase in BC labelling following activation, however most of the expansion was in the non-Sw BC compartment (Figure 5.3). Furthermore, a better resolution in Sw BC labelling was noted in the pre-activation fraction compared to activated compartment which indicate that most of the increased labelling was non-specific (Table 5.5). In addition, H04 sample culture indicated huge cell death, so we decided to repeat this experiment while addressing

the issues arising from the first two experiments. Thus, donor A21 PBMC sample was taken in to culture using same approach as H03 culture while only changing activation antigen to K02 and K03 as well as re-using both CIDR antigen in post activation staining before FACS analysis. Though, A21 labelling efficiency improved within total BC population when re-staining with CIDR used for activation process, almost all of the histidine positive BC belonged to the naïve BC subset (Figure 5.4). Further optimisation of culture method was needed in order to properly label CIDR-specific MBC, however, we preferred to drop this technique and proceed with a different approach.

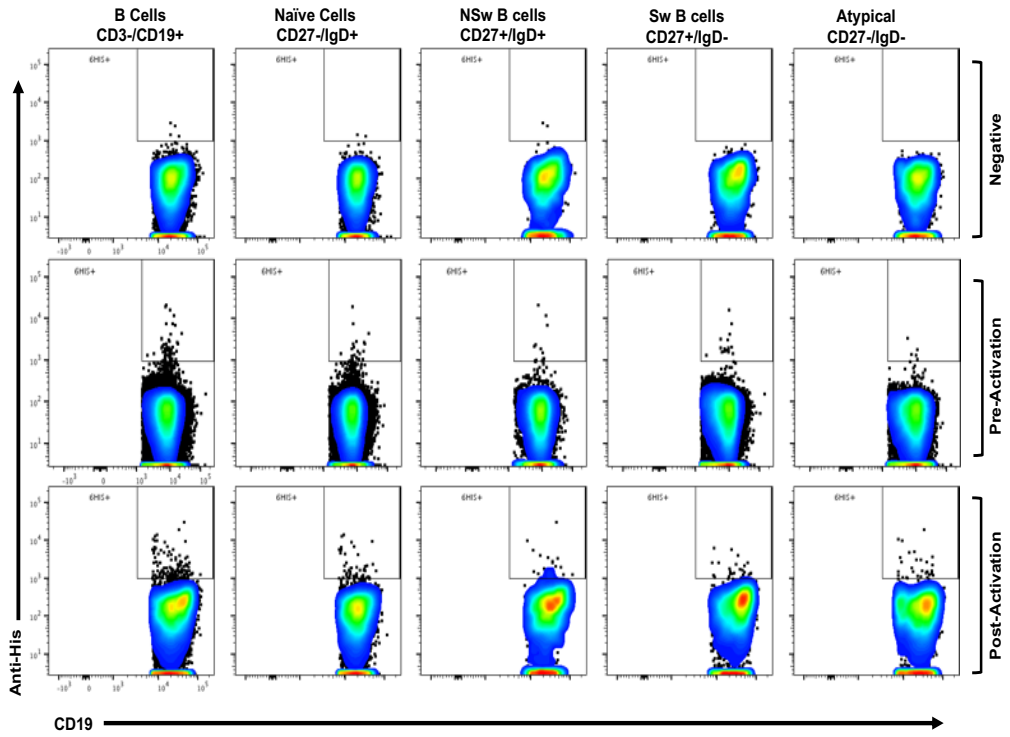


Figure 5. 3 H03 PBMC activation and culture.

Donor H03 PBMC sample activated by Histidine-tagged recombinant K07-CIDR and cultured for 3 days. Dot plot graph showing frequency of B cells subsets before and after activation/culture.

Table 5. 5 H03 PBMC activation and culture. Frequency of B cells subsets before and after activation/culture.as well as fold increase in labelling.

	CD3-/CD19+	CD27-/IgD+	CD27+/IgD+	CD27+/IgD-	CD27-/IgD-
Neg	0.004%	0.003%	0.000%	0.000%	0.001%
Pre-activation	0.019%	0.008%	0.003%	0.006%	0.002%
Post-activation	0.440%	0.170%	0.073%	0.095%	0.100%
Fold increase	23.000%	21.000%	24.000%	16.000%	50.000%

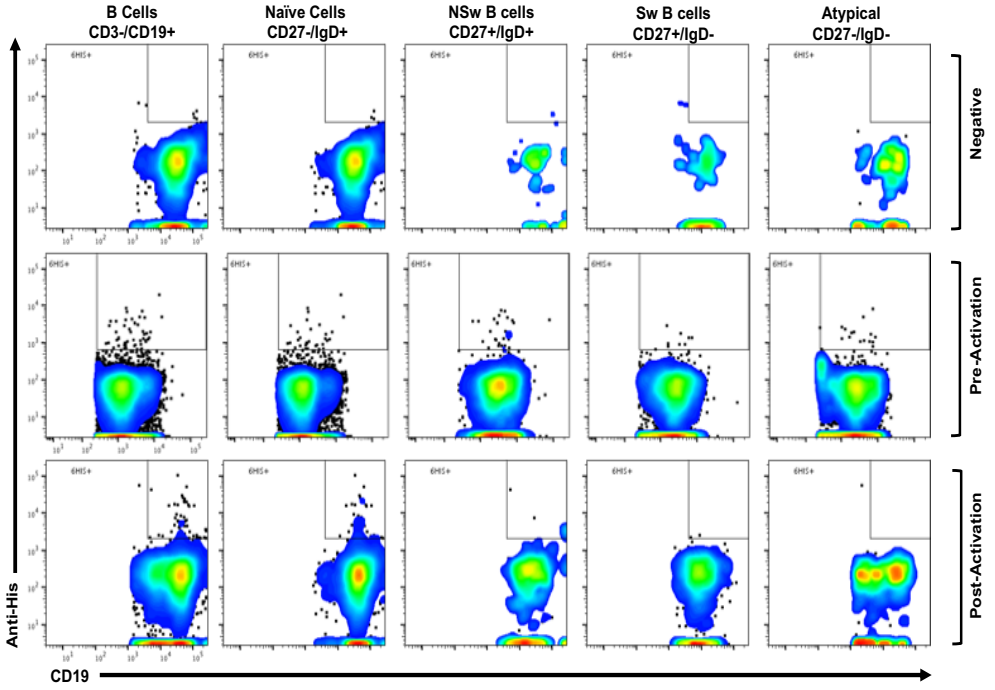


Figure 5. 4 A21 PBMC activation and culture.

Donor A21 PBMC sample activated with a mixture of Histidine-tagged recombinant K02-CIDR/K03-CIDR proteins and cultured for 3 days. Dot plot graph showing frequency of B cells subsets before and after activation/culture.

5.2.5 Tetramer based detection of antigen-specific B cells

The goal of this chapter was to characterise and isolate CIDR-specific MBC using FACS technique, however, such cells are hard to identify due to their very low frequencies in peripheral blood or PBMC samples. Results from our previous experiment prove the difficulty in identifying these subsets and distinguishing them from background noise. We reason that their characterisation and isolation by FACS requires a ligand for the BCR which brightly labels these cells on the basis of their antigen specificity. The histidine tagged recombinant CIDR used in previous experiment were all antigen monomers conjugated to phycoerythrin bright fluorochrome, however this approach failed to effectively characterise CIDR-specific MBC subsets. Therefore, the experiments aim was to produce antigen tetramer conjugated to brighter fluorochrome allowing better labelling of antigen-specific MBC with minimal background noise. Thus, all recombinant CIDR proteins as well as a commercial recombinant Tetanus toxoid were biotinylated and assessed for level of biotin incorporation in preparation for making tetramerised form. Subsequently, FACS screening of all biotinylated protein monomers was conducted on a malaria naïve PBMC sample which revealed comparable results to those obtained using Histidine-tagged protein monomers (Table 5.6). Follow up experiments were performed on same negative PBMC sample, however, this time we aimed to titrate the tetramerised K13 and TT proteins prepared at 1:4 molar ratio. By utilising data obtained from K13 and TT antigens tetramers experiment, we were able to determine optimum concentration for both

antigen tetramer as well as streptavidin fluorochrome used to stain labelled cells while maintaining low background noise.

Next, we used TT antigen tetramer to label PBMC samples taken from healthy donor who recently received a booster TT immunization (Figure 5.5). Interestingly, using tetramerised form of TT antigen, we were able to clearly identify TT-specific Sw BC in PBMC sample while maintaining reduced background noise (Table 5.7).

Table 5. 6 FACS analysis for biotinylated protein monomers.

	CD3-/CD19-	BC CD3-/CD19+	Naïve CD27-, IgD+	NSw BC CD27+, IgD+	Sw BC CD27+, IgD-	Atypical CD27-, IgD-
K01	0.058%	0.500%	0.120%	0.110%	0.130%	0.130%
K02	0.044%	0.130%	0.050%	0.033%	0.017%	0.033%
K03	0.021%	0.036%	0.022%	0.007%	0.007%	0.000%
K04	0.088%	0.370%	0.090%	0.083%	0.120%	0.069%
K05	0.024%	0.440%	0.110%	0.086%	0.093%	0.160%
K06	6.720%	0.910%	0.140%	0.220%	0.280%	0.270%
K07	0.034%	0.260%	0.030%	0.090%	0.100%	0.037%
K08	0.100%	0.950%	0.210%	0.110%	0.230%	0.400%
K09	0.027%	0.061%	0.007%	0.027%	0.020%	0.007%
K10	0.051%	0.130%	0.034%	0.041%	0.034%	0.021%
K12	0.027%	0.053%	0.023%	0.023%	0.000%	0.008%
k13	84.200%	0.730%	0.270%	0.140%	0.110%	0.200%
TT	0.017%	0.062%	0.017%	0.017%	0.026%	0.002%
neg	0.033%	0.013%	0.000%	0.007%	0.000%	0.007%

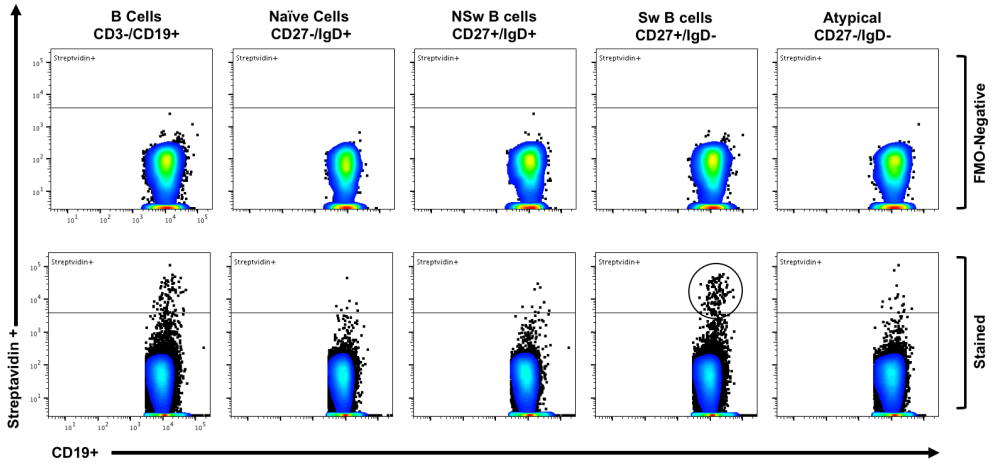


Figure 5. 5 Detection of tetanus toxoid (TT) specific B cells.

Frequency of B cells labelling after staining with TT antigen tetramer.

Table 5. 7 Frequency of B cells labelling after staining with TT antigen tetramer.

	BC	Naïve CD27-/IgD+	NSw BC CD27+, IgD+	Sw BC Q3: CD27+, IgD-	Atypical CD27-, IgD-
FMO-Neg	0.0000%	0.0000%	0.0000%	0.0000%	0.0000%
Stained	0.0370%	0.0019%	0.0021%	0.0300%	0.0040%

5.2.6 CIDR-specific single B cell isolation

Following our success in characterising TT-specific BC in the previous experiment, we decided to commence with CIDR-specific single MBC isolation from previously identified malaria immuned donors. Two experiments were performed separately to sort CIDR-specific MBC in single cell mode from two different donors. We used K02-CIDR tetramer to label PBMC sample from donor H06, while donor H20 PBMC was first enriched for BC using MACS negative selection cocktail then labelling it with two tetramers (K02 and K08). The sorting strategy was to gate on live single cells that lack expression of CD3 marker and are positive for CD19, CD27 and CIDR tetramer (Figure 5.6). Sorting was performed in single cell mode using FACS aria machine and sorted cells were collected into 96 well plates containing RNA lysing buffer in preparation for subsequent cDNA synthesis.

Total of 395 CIDR-specific BC were obtained from both donors, however most of acquired cells (276 cells) were from H06 donors while only 119 cells were isolated from donor H20. Possible reason for low acquisition from donor H20 was because we placed the gate for CIDR-specific BC to at least 1 log brighter than the negative control for most of acquisition duration before reducing it to ½ log toward the end of acquisition.

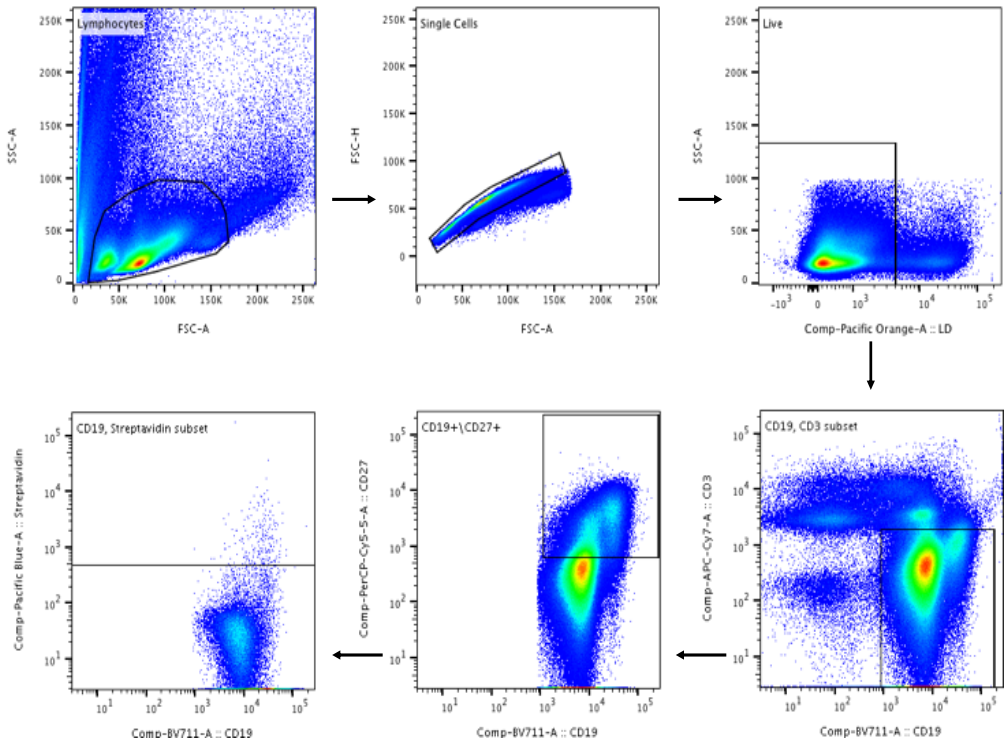


Figure 5. 6 Gating strategy for sorting CIDR-specific B cells.

PBMCs were first gated by forward (FSC) and side scatter (SSC), extending the normal lymphocyte gate beyond the standard limits to account for the increased size and granularity of plasmablasts. Doublets were excluded by comparing the FSC area and height measurements which follow gating on live cells then CD3-/CD19+ cells. Sorting was performed by gating to CD3-/CD19+/CD27+/CIDR tetramer+.

5.3 Discussion

The goal of this chapter was to establish a protocol for characterization and isolation of CIDR-specific memory B cells. However, due to their very low frequencies in peripheral blood, a successful isolation by flow cytometry consequently would require a BCR-ligand that brightly labels these rare cells on the basis of their antigen specificity. Thus, we decided to label these cells using a histidine-tagged recombinant CIDR that demonstrated high reactivity against plasma obtained from the same donor, then use a phycoerythrin conjugated anti-histidine fluorochrome (Anti-Histidine/PE) to identify and isolate these rare events. However, despite several attempts to titrate the histidine-tagged recombinant CIDR and the Anti-histidine/PE, high background was detected which makes it hard to distinguish such very rare events. This became even more apparent when we screened all recombinant CIDR against 4 different malaria naïve donors. This could be caused by E.coli protein contaminants present with recombinant CIDR preparation carried over from expression and purification process, especially those that are co-purified using His-tag affinity column, or that these proteins may indeed bind to their target receptors; EPCR or CD36 expressed selectively on lymphocytes (106). Won et al. (2007) reported upregulation of CD36 expression on IgM+ plasma cells but not class switched plasma cells in response to T-dependent and T-independent Ag challenge (107). However, Feistritzer et al. (2006) reported EPCR expression only on CD3+ lymphocytes which may participate in the inhibitory effects of PC and activated PC on cell migration (108). Indeed, data obtained from our FACS analysis of naïve PBMC samples indicated

CD36 expression on all CD19+ B cells subsets but not EPCR. However, it became clear that such an approach is not suitable to effectively isolate antigen-specific MBC, so we decided to establish B cells culture hoping to expand and maintain supply of these rare cells.

During natural infection, activation of resting B cells requires three stimulatory factors, two of which are antigen specific. The earliest signal derived from the induction of phosphoinositide 3-kinase (PI3K) activity which initiate a cascade of signals driving progression of cell cycle from resting phase to DNA synthesis phase, thereby promoting cell proliferation (109). However, differentiation to plasmablasts and mature plasma cells is derived mainly by cytokines regulating expression of B-cell lymphoma 6 protein (BCL-6) and B-lymphocyte induced maturation protein 1 (BLIMP-1). The transcription factor BLIMP-1 downregulates BCL-6 expression which consequently upregulates the expression of differentiation factor X-box binding protein 1 (XBP1) (110–113).

In our experiments, we included cytokines IL2, IL4 due to their role in activating PI3K which drive resting MBC into a proliferative phase (109). Furthermore, IL21 was also added to the cytokine mixture for its ability to induce the expression of BLIMP 1 and XBP1 thereby promoting differentiation in to plasmablasts (114). However, data from all culture experiments failed to show any noticeable expansion in CIDR-specific MBC compartment. Further optimisation of the B cell culture method was required to identify the cause of high cell death as well as inability of cultured cells to differentiate in to plasmablasts, however we decided to test a new approach for the isolation process before commencing with culture

optimisation. We aimed to design and test a tetrameric form of our recombinant CIDR, since the signal generated by monomeric molecule is typically not bright and tends to overlap with the unlabelled cell population. Through the use of tetramerized TT antigen, we were able to identify TT-specific MBC cells from PBMC sample obtained after TT immunization. Following our success with characterising TT-specific MBC, we made a tetramerised K02-CIDR antigen and used it to isolate CIDR-specific BC from donor H06 and H20 PBMC samples. We were able to isolate 276 BC from donor H06 and 119 BC from donor H20. The reason for the low yields from donor H20 PBMC sample was due to placing the sorting gate to 1 log brighter than the negative control compared to ½ log during H06 acquisition, thereby reducing probability of acquiring non-specific BC.

The general consensus is that a tetrameric form of a given antigen could offer several advantages compared to its monomeric form. Indeed, fully structured IgG antibodies bind with greater avidity to their target antigen when compared to its partial monomeric Fab fragment, furthermore this increase is even several fold greater for pentameric IgM antibodies due to their 10 combined Fab fragments (76). Additionally, tetramerized antigen can be constructed with brightly labelled streptavidin molecules which alleviates the need for chemical modification to add fluorophore and subsequently preserving binding epitopes. This principle was first adopted by Altman and colleague labelling T cells-TCR with peptide-major histocompatibility complex tetramers, before becoming widely used in the field of single cell characterization (115). Since then, several researchers have been using this approach to isolate antigen-specific B cells, Cox and

colleagues were able to produce dengue neutralizing antibodies from single cell sorted memory B cell using a tetramerised dengue envelope protein (74). This technique thereby permits efficient isolation of these rare cells for subsequent production of human monoclonal antibodies.

Chapter 6. Production and characterisation of CIDRα1-specific mAb

6.1 Introduction

Individuals with *Pf*EMP1-specific antibodies had a significantly reduced risk of developing symptomatic malaria, whereas antibodies to other VSA were not associated with protective immunity(116). Furthermore, a knockdowns of var genes which diminished *Pf*EMP1 expression on the surface of iRBCs resulted in nearly complete loss of antibody recognition and cytoadhesive properties suggesting the role of *Pf*EMP1 as mediator of disease pathogenicity and major target of naturally acquired immunity (57). Serological analysis of *Pf*EMP1 from clinical isolates obtained from different geographical regions revealed the existence of cross-reactivity between isolated epitopes suggesting presence of some degree of multilevel structural conservation (58,59). Though the utilization of cross-reactive antibody with sufficient extent to reduce adhesion of clinically relevant *Pf*EMP1 could be the key to the development of successful vaccine, the practicality of isolating such antibody from polyclonal plasma is not feasible. However the better utilization of memory B cells will present a better chance in obtaining highly specific cross-reactive antibodies and access to the history of an individual's exposure to pathogens (60). Advances in the isolation and molecular analysis of single, antigen-specific memory B cells now allows detailed characterization and library construction of the B cell receptor (BCR) diversification, V(J)D gene and isotypes usage, in addition to the production of recombinant monoclonal antibodies for functional

studies (61). Interestingly, memory B cells specific for malarial antigens can be detected in the peripheral circulation even when antibody levels have declined and they might maintain protection against recurrent episodes (62). Additionally, a cross sectional study analysing memory B cell responses showed that about 50% of cross-reactive memory B cells recognize two full-length *Pf*EMP1 derived from laboratory adapted lines (63).

We single sorted a total of 395 CIDRα1-specific B cells from PBMC samples of two healthy donors previously infected with malaria disease and found highly reactive B cells against a panel of multiple *Pf*EMP1 CIDRα antigens. The objectives of this chapter were to first amplify and sequence the immunoglobulin genes encoded by sorted B cells. This was followed by production of mAb through identifying and cloning paired genes in to their respected expression vector before transiently transfecting mammalian HEK293 cells. Consequently, running initial characterisation to select functionally reactive mAbs for subsequent large-scale production and purification before testing their ability to neutralize CIDRα1 antigens.

6.2 Results

6.2.1 CIDR-specific B cell isolation

For the production of Ig-gene repertoire, we FACS sorted a total of 395 individual CIDRα1-specific B cell (CD19+/CD27+/CIDR-tetramer+) from two healthy donors with a previous history of malaria infection within the last two years. Both donors were selected for B cell isolation due to their high and wide reactivity against panel of 48 recombinant CIDRα proteins. As a negative control, no cell was sorted into H1-H12 wells.

6.2.2 Single cell RT-PCR

Variable genes for immunoglobulin heavy (VH) chain and both light chain (Kappa and Lambda) variants were amplified separately by RT-PCR from single cell cDNA generated by amplifying Ig-mRNA transcript using primers that target constant region. The 1st round of PCR amplification was performed with a mixture of forward primers targeting the FR1-region of all identified Ig-V families, and a reverse primer specific for the constant region of IgH, Igκ or Igλ chains. All RT-PCR amplicons were purified and analysed using 1% agarose gel, however, Sanger sequencing was performed only for wells that expressed both heavy and light chains. Out of 395 individual CIDRα-specific B cells sorted, RT-PCR amplification of IgH, Igκ and Igλ genes produced 108, 36 and 45 amplicons respectively, resulting in amplification efficiency of 27% based on obtained IgH amplicons. Sanger sequencing of paired PCR amplicons produced 65, 30 and 40 readable IgH, Igκ and Igλ sequences respectively, resulting in sequencing efficiency of 60%, 83% and 89% respectively.

6.2.3 Repertoire analysis

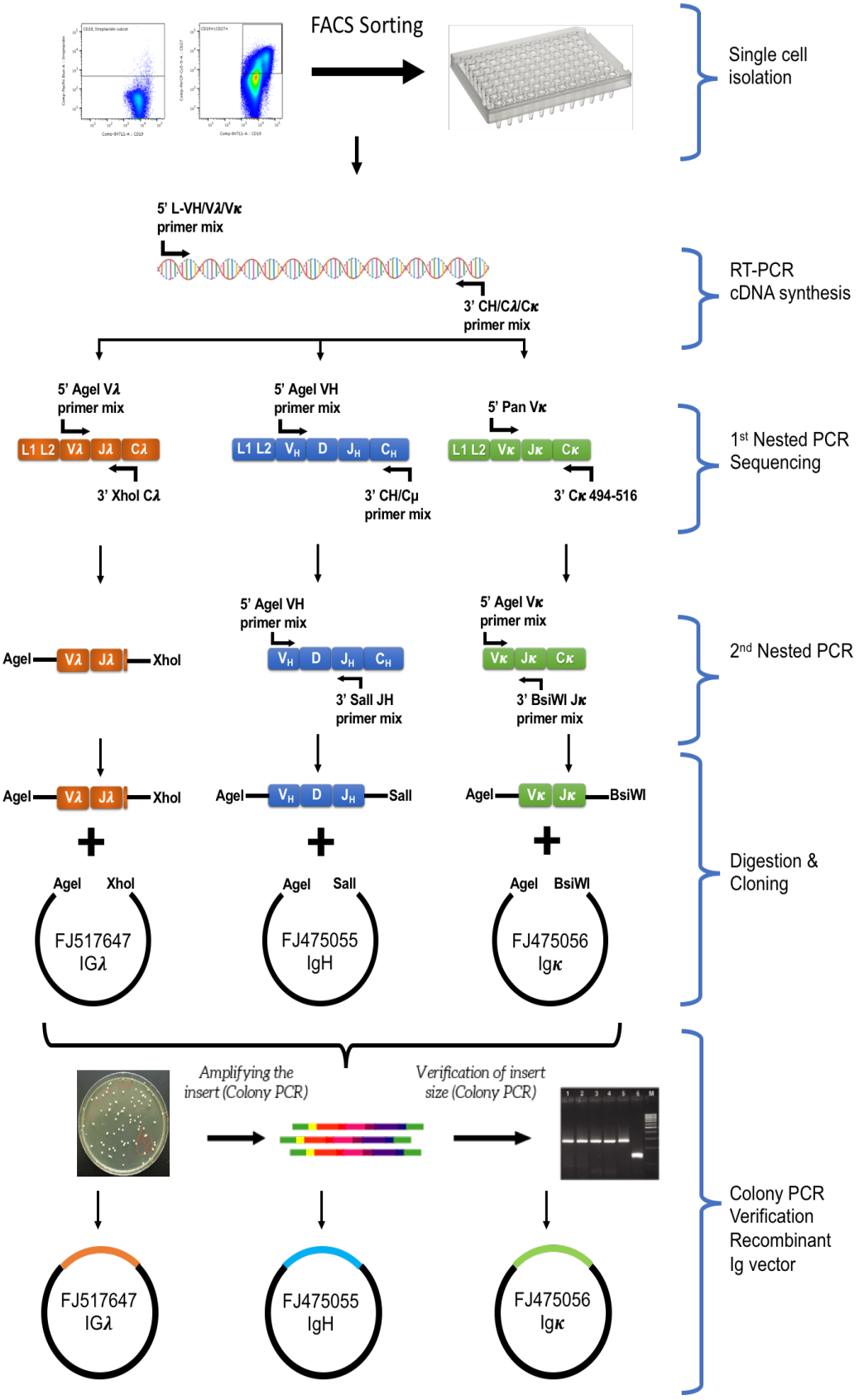
Sequencing data with clearly readable antibodies sequences were analysed for the presence of productive variable domains as well as to identify V(D)J gene arrangement and CDR3 sequence using the International Immunogenetics Information System IMGT/V-QUEST which can be accessed from (http://www.imgt.org/IMGT_vquest/vquest).

Cumulative sequence analysis within isolated cell population revealed a preferential usage of lambda light chain compared to kappa light chain with usage ratio of 0.8 (Kappa:Lambda). Additionally, repertoire analysis of Ig-VH (78), Ig-Vk(36) and Ig-Vλ (45) amplicons revealed mostly Ig heavy chain genes belonging to the VH3 gene family (63%), followed by VH4 (22%), VH1 (10%), VH5 (4%) and VH7 (1%) families (Fig. 1). Likewise, light VK2 gene family were most commonly used (54%), followed by VK1 (20%), VK3 (14%) and VK4 (12%) families. However, light Vλ gene usage were more diversified divided to six families, highest Vλ3 (38%), followed by Vλ2 (22%), Vλ1 (20%), Vλ8 (11%), Vλ7 (4.5%), Vλ4 (4.5%) families.

Productivity of Ig-heavy chain V(D)J genes rearrangement was also checked; a total of eleven sequences were flagged as unproductive or unknown. However, this was due to poor quality of sequencing which resulted in an inability of IMGT blast platform to correctly produce reliable results. Likewise, five sequences from both Kappa and lambda light chain genes were also flagged as either unproductive or unknown. However, all produced amplicons from RT-PCR were included in mAb production regardless of IMGT data.

6.2.4 Production of recombinant mAb

Sequencing of heavy and light chain sequences were performed on amplicons obtained from the 1st round of nested PCR, however, recombinant monoclonal antibody production was performed after 2nd round of nested PCR with primers containing restriction sites compatible with Ig mammalian expression vectors. Subsequently, cloned recombinant vectors were propagated in culture then purified and subjected to colony PCR to confirm the presence of inserts. Pairs of heavy and matching light chain recombinant vectors (323 and 360) were transiently co-transfected using linear Polyethyleneimine (PEI) in to mammalian HEK-293 cells for test production of recombinant monoclonal antibodies. The initial transfection was performed using multiple PEI:Vecors ratio (1:1, 1:2 and 1:3) however, no apparent difference was noted when assessed by dot blot assay, therefore, we decided to use 1:1 ratio of PEI:Vectors for all remaining antibodies productions. Following 6 days culture, dot blot analysis for mAb culture supernatant showed clear mAb production in 70% of cultures. The mAb production strategy and process flow through were summarised in Figure 6.1.


FACS Sorting
Single cell isolation
5' L-VH/Vλ/Vκ primer mix
3' CH/Cλ/Cκ primer mix
RT-PCR cDNA synthesis
5' AgeI Vλ primer mix
L1 L2 Vλ Jλ Cλ
3' XhoI Cλ
5' AgeI VH primer mix
L1 L2 VH D JH CH
3' CH/Cμ primer mix
5' Pan Vκ
L1 L2 Vκ Jκ Cκ
3' Cκ 494-516
1st Nested PCR Sequencing
AgeI Vλ Jλ XhoI
5' AgeI VH primer mix
VH D JH CH
3' SalI JH primer mix
5' AgeI Vκ primer mix
Vκ Jκ Cκ
3' BsiWI Jκ primer mix
2nd Nested PCR
AgeI Vλ Jλ XhoI
AgeI VH D JH SalI
AgeI Vκ Jκ BsiWI
AgeI XhoI
FJ517647 IGλ
AgeI SalI
FJ475055 IgH
AgeI BsiWI
FJ475056 Igκ
Digestion & Cloning
Amplifying the insert (Colony PCR)
Verification of insert size (Colony PCR)
FJ517647 IGλ
FJ475055 IgH
FJ475056 Igκ
Colony PCR Verification Recombinant Ig vector

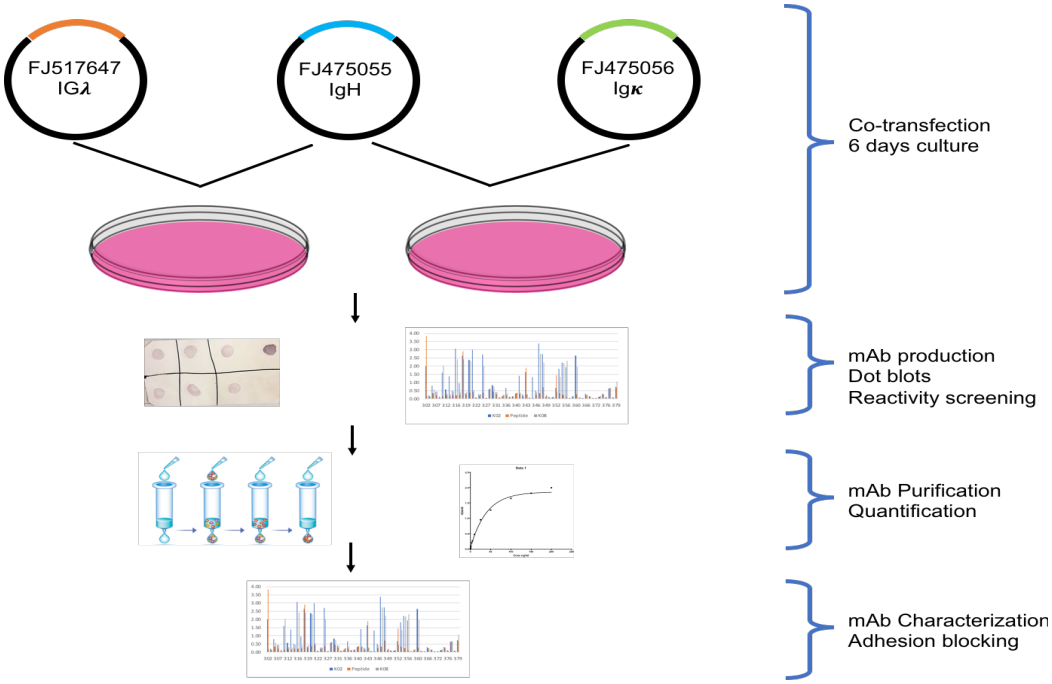


Figure 6. 1 Strategy to generate CIDR-specific recombinant human monoclonal antibodies (mAb).

Ig-heavy and light chain genes were amplified by RT-PCR from single cell cDNA generated by amplification with random hexamers primers. The cDNA generated from single cells were used as template to amplify Ig genes with forward mixed-primers targeting leader region and reverse primers specific for the respective IgH, Igκ or Igλ constant region. This is followed by 1st round nested PCR with either forward primer mixes or single primer specific for gene region FWR1 in the case of IgH and Igλ genes or Igκ gene respectively. Reverse primers used in 1st round nested PCR were either single or mixed for Igκ and Igλ or IgH respectively. All amplicons from 1st round nested PCR were purified and subsequently sequenced. The 2nd nested PCR were performed with V and J genes specific primers containing restriction sites compatible with human monoclonal antibody expression. Both, Ig sequences and respective expression vector were digested with respective set of enzymes then purified and subcloned. Colony PCR were performed on cloned recombinant vectors to verify presence of Ig inserts. Recombinant antibody production was performed with verified paired Ig-heavy and light chain plasmids by co-transfected into 293HEK cells and culturing for 6 days. Dot Blot analyses were performed on fractions from culture supernatant to assess production of mAb before screening all culture supernatant for reactivity to pre-isolation CIDR antigens (K02 and K08) and a synthetic peptide derived from CIDR-K02. Confirmed reactive mAb were selected for next round of screening against 10 recombinant CIDRα antigens. Finally, 10 mAb were selected for large scale production and purification before being characterised and tested for adhesion blocking against a panel of 48 recombinant CIDRα antigens.

6.2.5 Characterization of mAb for neutralization potency

6.2.5.1 Identification of CIDRα-specific mAb

Indirect ELISA assay was used to test aliquots from mAb culture supernatant for reactivity against pre-isolation recombinant CIDRα1 antigen (K02 and K08) as well as a 70 amino acid synthetic peptide representing EPCR-binding region from K02- CIDRα1.1 protein (K02-P) (Figure 6.2). Out of 65 mAb cultures, about 27% (18 mAb), 20% (13 mAb) and 7% (5 mAb) were clearly reactive against recombinant K02-CIDRα1.1, K08-CIDRα1.4 and K02-P respectively. Mixed reactivity was also noted; mAb 318 recognised all three tested CIDR antigens, eleven mAb were reactive to recombinant K02-CIDRα1.1 and K08-CIDRα1.4, while three mAb show reactivity to recombinant K02-CIDRα1.1 and its derivative K02-P. Most of all reactive mAb were isolated from donor H06, with only three reactive mAb from donor H20.

A second characterization against additional eight recombinant CIDRα was performed on selected 32 mAb that showed reactivity against recombinant CIDRα antigens in the previous characterisation (Figure 6.3). Consistent with previous results, mAb 318 was the only reactive antibody to all tested CIDRα antigen including the synthetic peptide. Additionally, a total of seven mAb (311, 316, 320, 325, 347, 348 and 360) were found reactive to all recombinant CIDR antigen but not the synthetic peptide suggestive of either a cross creativity or presence of contaminants. However, fewer reactivity and subsequently more specificity were also noted, eleven mAb were found reactive to six or less recombinant CIDRα antigens. Noteworthy, mAb 302

and 343 were both reactive to the synthetic peptide (K02-P) as well as to an additional four and three recombinant CIDRα antigens, respectively, belonging to CIDRα1.1 subclass. In addition, mAbs 321 and 345 reacted to K02 recombinant CIDR while 352 mAb reacted only to the synthetic peptide K02-P, but not the K02 recombinant CIDRα antigen.

6.2.5.2 Indirect ELISA for characterization of selected CIDR-specific mAb

We used K02 recombinant CIDRα antigen during B cell isolation from donor H06, while using a mixture of K02 and K08 for the isolation process from donor H20. Hence, we selected nine mAbs (302, 313, 316, 318, 321, 343, 345, 352 and 356) that reacted to recombinant K02- CIDRα1.1 or the K02-P peptide as well as the mAb-319 as negative control for subsequent large-scale production, purification and consequently testing of neutralization potency. Dot blot analysis were performed to confirm successful production of all 10 cultures supernatant. Subsequently, protein G beads were used to purify mAbs from culture supernatant, as described in materials and methods. A standard curve, generated using standardised concentrations of mAbs, was used to measure the concentration of purified mAbs, however, observed concentrations varied greatly. The highest concentration noted was 170 μg/ml for mAb-316 while the lowest concentration was 1.5 μg/ml for mAb-345, and an average of 86 μg/ml from 75 ml cultures. As a final confirmation, an indirect ELISA as well as a multiplex assay were used to re-characterise the ten purified mAbs. Multiple concentration of mAbs were used during ELISA based characterization against 14 recombinant CIDRα antigen as well as the synthetic K02-P

peptide and lipopolysaccharide (LPS). mAbs 302, 313, 316, 318, 319, 321, 343 and 352 were all used at serial concentrations 5, 2.5, 1 and 0.5 μg, while mAb-345 and mAb-356 were used at concentrations 1, 0.5 and 0.25 μg due to their low yields (Figure 6.4). Both mAb-352 and mAb-356 did not show reactivity to K02-CIDR or any of the other recombinant CIDR antigens, and were therefore excluded from further testing. The mAb-316 again showed a wide reactivity against all tested recombinant CIDR antigens except the K02-P peptide and LPS antigen, which may very well be a cross reactivity or reactivity against the 6-Histidine tag in the recombinant CIDR antigens. Likewise, mAb-318 also showed high reactivity against all tested antigens including the K02-P peptide and LPS antigen indicative of non-specific reactivity against recombinant CIDR but rather specificity toward LPS contaminants. However, mAb-313 showed some degree of specificity as it reacted to K02, K03 and K13 recombinant CIDR antigens while mAb-345 reacted only against K02 recombinant CIDR. Interestingly, mAb-302 and mAb-343 were the only mAbs that reacted to both K02 and the synthetic K02-P peptide as well as other recombinant CIDR antigens but not the LPS protein. Collectively these results seemed encouraging, however still need further confirmation to rule out reactivity against 6-Histidine tags as well as testing its ability to block adhesion of CIDRα domains to EPCR receptor.

6.2.5.3 Multiplex based assay for characterization of selected CIDRα-specific mAb

We sent the ten mAbs (302, 313, 316, 318, 319, 321, 343, 345, 352 and 356) that were previously characterised by the indirect ELISA to our

colleagues from Copenhagen University (Lavstsen and Turner) for further characterization and adhesion blocking potency against panel of 49 CIDRα antigens as well as a two negative controls (Bundle Histidine tagged protein and BSA protein) (Figure 6.5). In agreement with our findings, mAb-319, mAb-352 and mAb-356 failed to react with any CIDRα domain, however, the mAb-345 which was reactive to recombinant K02-CIDRα (CIDRα1.1) in our ELISA characterization failed to show reactivity toward any of the 49 tested CIDR antigens in the multiplex assay. As we speculated, mAb-316 reacted with all tested CIDRα antigens as well as the Histidine tagged non-CIDRα proteins which proves that it was reacting to the 6-Histidine tags incorporated to our recombinant CIDRα proteins. Otherwise, data relating to mAb-302 was in partial agreement with our data since it showed reactivity against two CIDRα1.1 and one CIDRα1.8a but not CIDRα2. Likewise, mAb-321 were found reactive to three different CIDRα proteins (CIDRα1.1, CIDRα1.5b and CIDRα1.8a) while our data indicate moderate and low reactivity to one CIDRα1.1 (K02) and one CIDRα1.4 (K06) respectively. However, mAb-343 demonstrated more specificity compared to other mAbs as it was able to react to three CIDRα1.1, one CIDRα1.8 and one CIDR1.4. Most interesting, mAb-313 were found specifically reacting against CIDRα1.1 subclass as it was able to react to a total of six CIDRα1.1 proteins demonstrating cross-reactivity across CIDRα1.1 domain subclass.

6.2.5.3 Multiplex based assay for assessment of neutralization potency of selected CIDRα-specific mAb

Total of four mAbs (343, 313, 302 and 321) were used in neutralization assay to measure ability of the tested mAbs for binding inhibition of CIDRα1 antigen to EPCR receptor. The mAb-343 exhibited the highest and widest inhibition profile compared to other tested mAbs, as it was able to inhibit the binding of three recombinant CIDRα1.1 by up to 67%. In addition, it demonstrated cross reactivity beyond CIDRα1.1 as it was able to inhibit binding of one CIDRα1.8 and one CIDRα1.4 by 46% and 47% respectively. The mAb-313 also exhibited high inhibition capability for one CIDRα1.1, however low inhibition were also noted for five additional CIDRα1.1 by up to 23%. The remaining mAb-302 and mAb-321 did not perform as well as the previous two mAbs, however the mAb-302 was able to inhibit the binding of three CIDRα1.1 by up to 27% while mAb-321 was able to inhibit the binding of only one CIDRα1.1 by 20% (Figure 6.6).

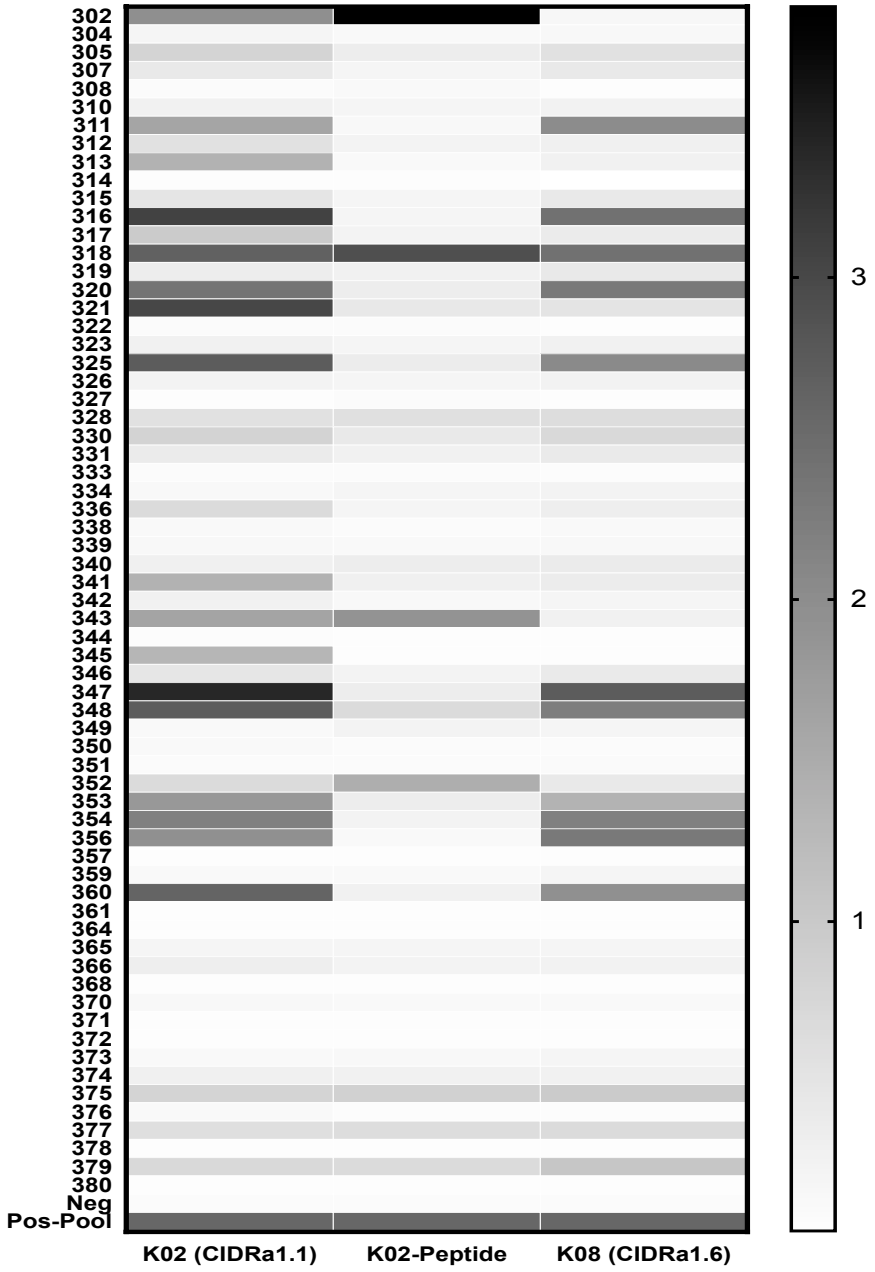


Figure 6. 2 Indirect ELISA assay investigating reactivity of isolated monoclonal antibodies (mAb) against recombinant CIDR antigens.

Fractions from all mAb culture supernatant were screened for reactivity against 3 recombinant CIDR antigen (K02, K08 and K02-P). Signal intensities were quantified as OD450 and are shown on the heat map (white indicates weakest intensity; black, strongest). Non transfected culture supernatant was used as negative control. Reactivity were considered positive if the mean absorbance value was above the cut-off value calculated as 4SD above the mean of negative control. Pooled serum samples from malaria patient were included as positive control.

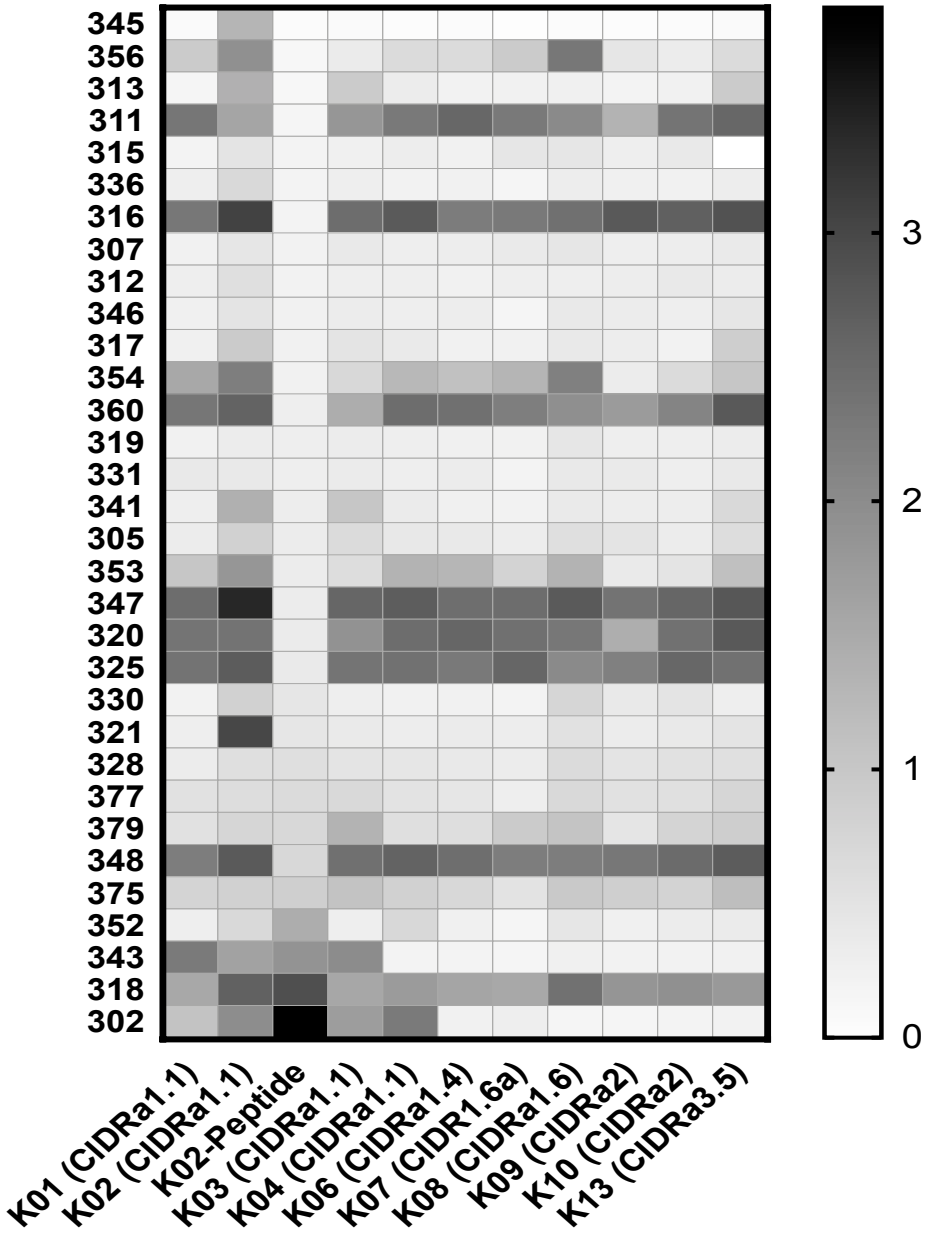


Figure 6. 3 Indirect ELISA assay assessing reactivity of selected mAb culture supernatant against recombinant CIDR antigens.

Heat map showing reactivity of selected monoclonal antibodies (mAb) against 11 recombinant CIDR antigens. Signal intensities were quantified as OD450 and are shown on the heat map (white indicates weakest intensity; black, strongest). Reactivity were considered positive if the mean absorbance value was above 1.0 OD450.

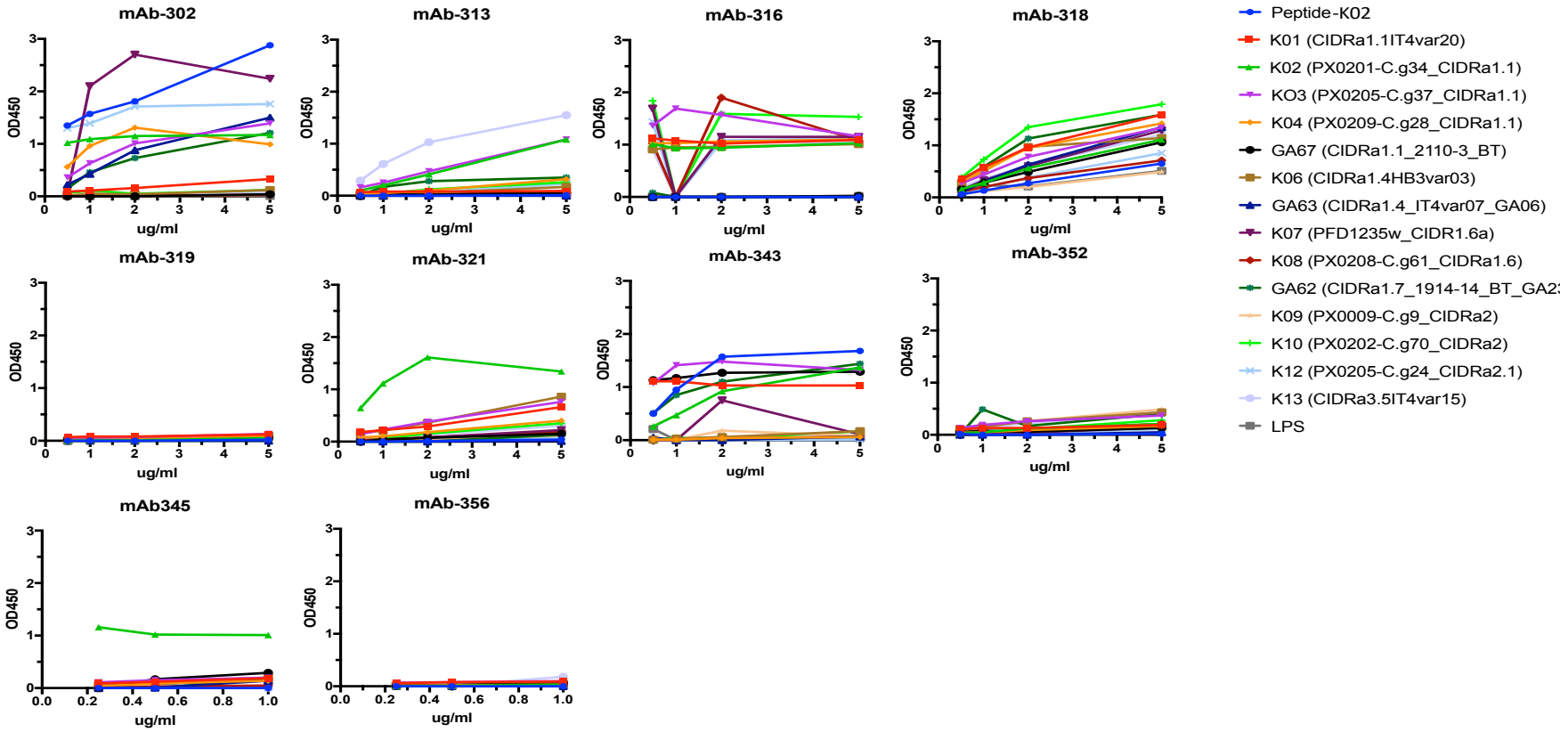


Figure 6. 4 Titration of selected mAbs against panel of CIDRα proteins.

Multiple concentration of mAbs were used in ELISA based characterization against 14 recombinant CIDRα antigen as well as the synthetic K02-P peptide and lipopolysaccharide (LPS). mAbs 302, 313, 316, 318, 319, 321, 343 and 352 were all used at serial concentrations 5, 2.5, 1 and 0.5 μg, while mAb-345 and mAb-356 were used at concentrations 1, 0.5 and 0.25 μg.

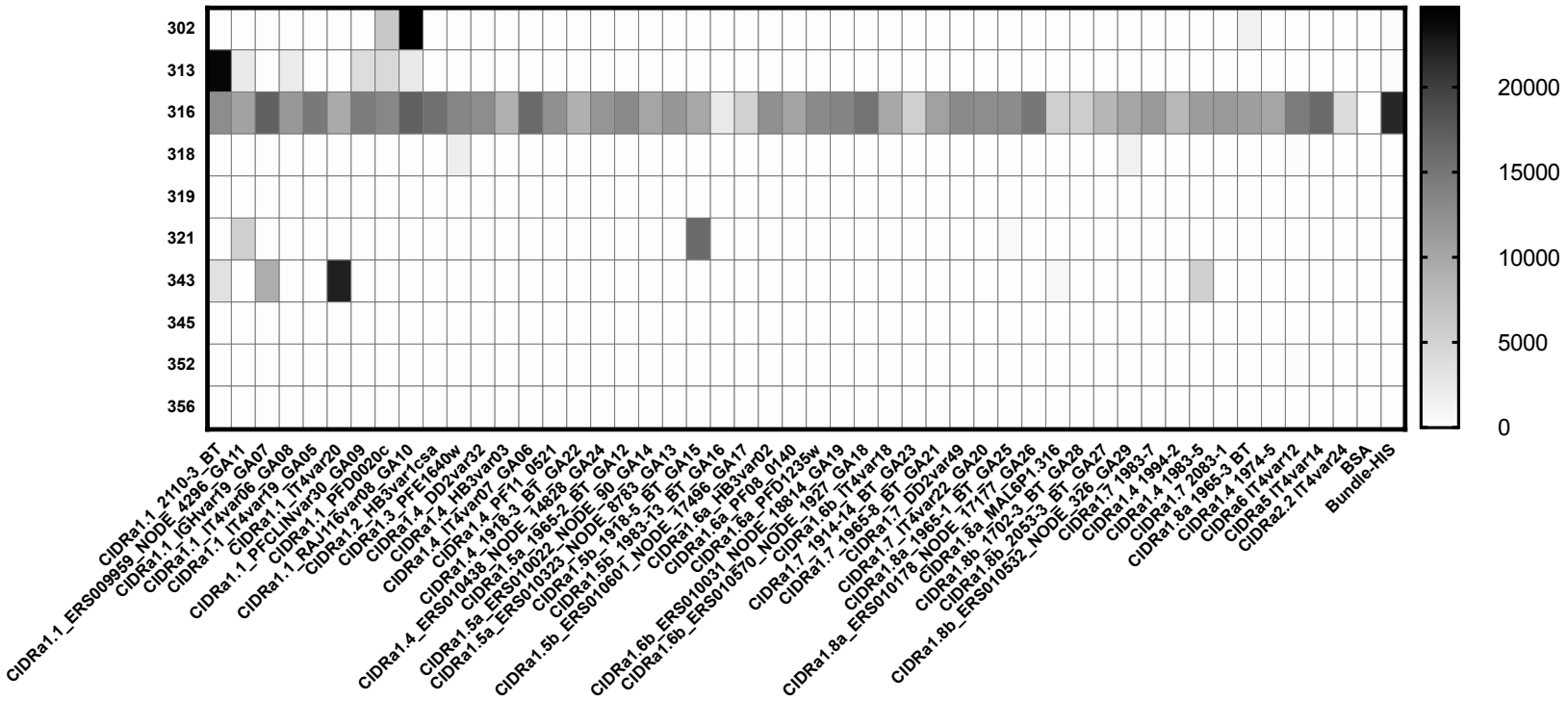


Figure 6. 5 Multiplex assay assessing reactivity of selected purified mAb against panel of recombinant CIDR antigens.

Heat map showing reactivity of selected monoclonal antibodies (mAb) against 49 recombinant CIDR antigens. Signal intensities were quantified as MFI and are shown on the heat map (white indicates weakest intensity; black, strongest).

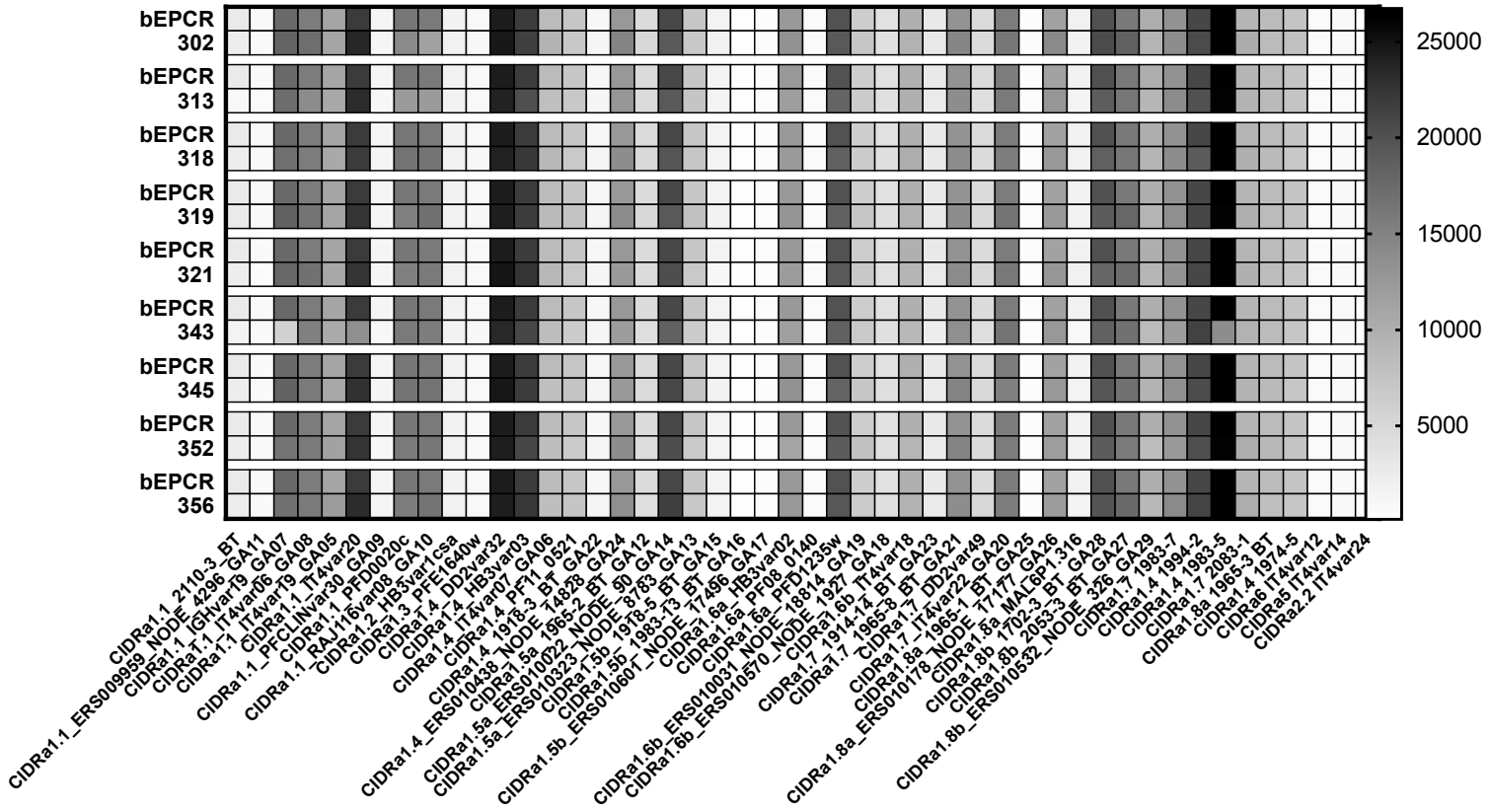


Figure 6. 6 Multiplex assay measuring adhesion inhibition of different CIDRα1 to EPCR receptor under static conditions.

Binding of CIDRα1 proteins to EPCR receptor was measured after pre-incubation with mAb-302, mAb-313, mAb-321 and mAb-343. Signal intensities were quantified as MFI and are shown on the heat map (white indicates weakest intensity; black, strongest).

6.3 Discussion

Plasmodium falciparum (*P.falciparum)* parasite remains the leading causative agent of malaria disease and is responsible for a huge mortality and morbidity particularly in younger children(2). Recently WHO has estimated more than 200 million worldwide cases of malaria infection resulting in above 500 thousand deaths per year, mostly in children under the age of five years(1). Additional disease burden is increasingly realized; three-quarters of the countries monitoring insecticide resistance in malaria vectors reported a continuous increase in resistance. Furthermore, antimalarial drug resistance is confirmed and has now been reported for artemisinin in Southeast Asian countries (2). This calls for the need of a highly efficient malaria vaccine capable of achieving control and subsequently elimination of such global threat. Current strategic goals of the World Health Organization (WHO) are to develop a malaria vaccine with at least 75% efficacy against all clinical isolates with a duration of protection of no less than two years and not requiring more than one booster dose annually (117). Yet, developing a potent and durable vaccine with such criteria is challenging, as this requires careful consideration to identify suitable antigen and epitope capable of inducing long lasting protective immunity. Additionally, induction of memory B cells specific for malaria antigen is a pre-requisite for mounting such durable immunity through the generation of long lived antibody secreting plasma cells (ASC) and subsequently maintaining antibodies level in the periphery (118).

The current chapter focused on producing mAb from B cells isolated previously from two healthy donors with a history of malaria infection. Following B cell isolation, a process conducted as a part of work carried out in previous chapter, RT-PCR amplification of IgH, Igκ and Igλ genes produced 108, 36 and 45 amplicons respectively resulting in amplification efficiency of 27% based on IgH amplification. The efficiency of this step was 3% lower than the minimum reported range of 30-60% PCR amplification efficiency (119). Possible reasons for such low amplification efficiency rate could be failed RT-PCR or low priming efficacy of V genes with included primer set. We adopted the one step RT-PCR method for production and amplification of cDNA from isolated B cells. This PCR technique is not a failsafe and is known for its low sensitivity compared to two step RT-PCR which may result in failed production of cDNA. Furthermore, failed cDNA synthesis may also be due to low mRNA concentration as a result of improper sorting or storage of sorting plate (120). However, the most probable cause would be the gating strategy that we adopted during B cell sorting, which allowed sorting of CD19+/CD27+/Tetramer+ B cells without regards to immunoglobulin type. Such gating would allow sorting of B cells expressing a variety of immunoglobulin types including IgD. We used forward primers that target the L region while the two reverse primers target the constant region of an IgG and IgM variable chains. Subsequently all other immunoglobulin genes would fail to amplify with exception of only IgG and IgM variable genes resulting in low amplification rate. Smith et al. reported a rate of amplification efficiency as high as 70% when using a tight gating strategy by excluding all other immunoglobulin beside IgG, which in

turn improved the priming efficiency of constant region in immunoglobulin gene (121). Following IgH, Igλ and Igκ genes amplification and cloning to their corresponding expression vectors, we were able to confirm the presence of all inserted immunoglobulin genes through the use of colony PCR technique. Subsequently, co-transfection in to HEK293A cells using (PEI) precipitation technique were performed at PEI:DNA ratio of 1:1 which resulted in 70% transfection efficiency rate. Although the transfection rate was in agreement with similar studies, tailored PEI:DNA ratio specific to each failed transfection may result in successful production of mAb. Furthermore, using HEK293T instead of HEK293A cell lines may have had a significant enhancement to transfection efficiency and the amount of produced mAbs (122). This is due to integrated SV40 large T antigen in HEK293T cell line which allow episomal amplification of plasmids containing the SV40 origins of replication. Consequently, increasing recombinant mAb expression through uptake and retaining more plasmid copies inside the cell during production phase (123).

Out of 65 mAb reconstructed, only forty mAb were confirmed positive for production by dot blot assay. However, seven mAb (302, 313, 316, 318, 321, 343 and 345) demonstrated clear reactivity against one or more recombinant CIDRα1.1 or CIDRα1.6 domain antigens. Most interestingly, these seven mAb were all produced from isolated B cells sorted from donor H06 PBMC sample and non from donor H20. Looking back at initial plasma screening, we can see that the indirect ELISA screening identified donor H20 (1.61 ± 0.51 OD_{450}) as 3rd highest reactive after H06 (1.84 ± 0.66 OD_{450}) and H25 (2.05 ± 0.72 OD_{450}) donors. Conversely, plasma screening

data obtained using Luminex assay identified donor H20 (6839 ± 2231 MFI) as highest reactive historical sample, followed by donor H06 (5119 ± 1993 MFI). It is safe to assume that ELISA assay was more accurate compared to the multiplex assay in screening plasma samples and perhaps during the characterization process too. Although the multiplex assay seemed less accurate compared to ELISA assay, it remains a valuable asset since it requires considerably lower amount of test sample. Since we ended up with low mAbs concentrations, we took advantage of such option and performed the neutralisation screening using the multiplex assay. Out of the seven tested mAbs, only mAb-343 showed promising results with its ability to inhibit three recombinant CIDRα1.1 as well as one CIDRα1.4 and CIDRα1.8 domain antigens. Most interestingly, all three domains were of an EPCR binding type while both CIDRα1.1 and CIDRα1.4 domains were of DC8 and DC13, a subtype of *Pf*EMP1 protein known for its association with severe malaria (16).

Chapter 7.

7.1 Reiteration of project aim and objectives

Aim

The aim of this project was to design a platform of methods allowing production of human mAbs in vitro with neutralising potency against *Pf*EMP1-CIDRα1 antigens.

Objectives

1. Production of selected recombinant *Pf*EMP1 CIDRα domains of clinical importance.
2. Collection of blood samples from healthy donors with historical exposure *to P.falciparum* malaria infection.
3. Screening donor samples to identify highest reactive sera with good recognition of at least three recombinant *Pf*EMP1 CIDRα1 domains.
4. Single cell sorting of CIDRα1-specific memory B cells (MBC)
5. Amplification and cloning of immunoglobulin genes from isolated CIDRα1-specific MBC
6. Sequence analysis of variable region of immunoglobulin sequences.
7. Production of recombinant monoclonal antibodies (mAb).
8. Characterisation and neutralisation potency testing for mAb

7.2 Results summary

7.2.1 Chapter 3: Production of recombinant Plasmodium falciparum Erythrocyte Membrane Protein 1 CIDRα domains

In this chapter we present data detailing production of 12 clinically important CIDRα domains from *Pf*EMP1. This involved cloning of CIDRα genes, testing expression in prokaryotic and eukaryotic system and finally producing a purified recombinant CIDRα proteins. The attempts made to express two CIDRα domains in a eukaryotic system were only nominally successful, therefore requiring further adjustment and optimization to transfection protocol. Although proteins expression carried out in *E.coli* yielded insoluble recombinant CIDRα proteins in the form of IB, we managed to refold/purify all expressed proteins in a simple reproducible procedure.

7.2.2 Chapter 4: Acquisition of antibodies targeting *Pf*EMP1 CIDRα domains in malaria exposed adults

In this chapter we present data detailing the recruitment of 10 malaria patients and nineteen healthy individuals with previous history of malaria infection in the southern border region of the Kingdom of Saudi Arabia. Most of the recruited individuals were immigrants from neighbouring countries with moderate to high malaria endemicity profile. Two assays, indirect ELISA and multiplex bead assays were used for the characterisation of donor's plasma samples. Although data obtained from both assays were poorly correlated, we were able to identify two healthy donors that are suitable for isolation of CIDRα-specific B cells from respected PBMC

samples. Additionally, plasma samples from both donors demonstrated high cross reactivity against tested recombinant CIDRα antigens. Donor H06 plasma sample demonstrated full reactivity against all tested recombinant CIDRα antigens from both assays. However, plasma sample from donor H20 reacted to 14 out of 49 tested recombinant CIDRα antigens in multiplex assay while full reactivity was also noted during ELISA assay. Most importantly, both samples demonstrated high cross reactivity against recombinant CIDRα1.1 and CIDRα1.4 antigens that are closely associated with severe and cerebral malaria.

7.2.3 Chapter 5: Single cell isolation of CIDRα1-specific B cell

This chapter described the employment of multiple techniques for the characterization and isolation of antigen-specific memory B cells (MBC). Despite several attempts to use the recombinant CIDRα1 antigen monomer for the characterisation of CIDRα-specific B cells, high background was detected which made it hard to distinguish such very rare events. Subsequently, a different approach was adopted which focused on activating and expanding these rare events in culture, however, this approach also fails due to huge cell death or expansion of non CIDR-specific B cells. Conversely, through the use of tetanus toxoids (TT) antigen tetramer, we were able to characterise these rare TT-specific MBC from PBMC sample of healthy volunteer in both base line and after receiving booster immunization. Thus, we utilised the tetramerization technique to construct a CIDRα1.1 and CIDRα1.8 antigen tetramers and use it for the

isolation process. Consequently, we were able to single sort 276 and 119 CIDRα1-specific B cells sample from H06 and H20, respectively.

7.2.4 Chapter 6: Production and characterisation of CIDRα1-specific mAb

In this chapter we present data detailing the production of monoclonal antibodies (mAb) from the isolated CIDRα1-specific B cells. This involved synthesising cDNA from isolated B cells, which in turn were used as the template for amplification of immunoglobulin genes. Subsequently these genes were sequenced, and selected pairs of heavy and light chain genes were cloned in to immunoglobulin expression vectors before finally transiently transfected in to HEK293A cell line for production of mAb. A total of 40 culture supernatants were confirmed as positive for the production of immunoglobulin by dot blot analysis. However, initial characterisation of culture supernatant identified only 9 cross-reactive mAb, which in turn were selected for upscaled production and purification. This was followed by repeat characterisation for these 9 mAb as well as testing for neutralization potency. Out of all tested mAbs, only mAb-343 showed promising results with its ability to inhibit three recombinant CIDRα1.1 as well as one CIDRα1.4 and CIDRα1.8 domain antigens. Most interestingly, both CIDRα1.1 and CIDRα1.4 domains were part of DC8 and DC13 respectively, a subtype of *Pf*EMP1 protein known for its association with severe malaria and cerebral malaria.

7.3 Concluding remarks

Single B-cell technologies provide the ability to produce functionally matured human mAbs possessing naturally paired heavy and light chain that may be difficult to reproduce in vitro. A significant improvement in this field was introduced through the use of single-cell reverse transcription (RT)-PCR to synthesize the encoded immunoglobulin heavy and light chain gene pairs from isolated memory B cells. Following successful amplification of immunoglobulin genes, co-transfection of both heavy and light chain immunoglobulin genes in HEK293 cells was performed for later production of antigen-specific monoclonal antibodies. The main limitations of this technology are the availability of a suitable target antigen as well as access to semi-immune donors. Indeed, the first limitation in our platform is related to the production of *Pf*EMP1-CIDRα1 antigens in prokaryotic system which resulted in expression as an IB requiring additional step for denaturing and refolding. To overcome such obstacle, we used manual on-column rapid refolding and purification technique which resulted in variable concentrations of purified *Pf*EMP1-CIDRα1 antigens. The variation of protein production concentration is largely attributed to formation of misfolded intermediates during refolding process(84). This could be easily resolved through the use of automated size exclusion chromatography compared to the labour-intensive technique that we used. Expression in a eukaryotic system serves to provide an alternative pathway alleviating the need for the additional refolding step necessary in prokaryotic system. Our trials using the eukaryotic system (insect cells) showed promising results,

however, further optimisation to the transfection protocol was needed before we are able to apply it.

The second limitation which greatly affected the outcome of this project is related to donor's recruitment methodology represented in the lack of pre-screening step for candidates as well as inability to recall donors. Ideally, pre-screening candidates against panel of *Pf*EMP1-CIDRα1 antigens through the use of micro array chip could have greatly enhance our finding as it could have yielded more antigen-specific B cells.

Once a suitable candidate is identified, the same antigen can be re-used to probe memory B cells specific to target antigen in a donors PBMC sample. However, isolation of antigen-specific memory B cells is technically challenging due to their very low frequency in the circulation, which can be as low as 0.003% of total B cells population(124). Successful isolation by flow cytometry consequently would require a BCR-ligand that brightly labels these rare cells based on their antigen specificity. Overcoming such an obstacle can be easily achieved through the use of antigen tetramer for memory B cell labelling. The general consensus is that a tetrameric form of a given antigen could offer several advantages compared to its monomeric form. Indeed, fully structured IgG antibodies bind with greater avidity to their target antigen when compared to its partial monomeric Fab fragment. Furthermore, this increase is even several folds greater for pentameric IgM antibodies due to their ten combined Fab fragments(76). Additionally, a tetramerized antigen can be constructed with brightly labelled streptavidin molecules which alleviates the need for chemical modification to add fluorophore and subsequently preserving binding epitopes.

The aim of this project was to design a platform of methods to isolate human mAbs directly from the peripheral blood of a donor with a naturally acquired immunity to particular pathogen. The resulting platform would thereby provide the required technology to produce mAbs with the ability to restrict the functionality of such pathogen which can be exploited in vaccine development.

Currently, blood stage malaria vaccine have a relatively low efficacy, offering only short- lived protection. Most of the blood stage malaria vaccines in clinical trials uses a single merozoites proteins (AMA1 and MSP1/2/3), however, few other used a combination of VSA providing moderate efficacy that wane quickly(118).

The results presented in this work show that the platform can be used to successfully identify, isolate and reproduce cross-reactive mAbs against variant CIDRα1 proteins of PfEMP1. This cross-reactive CIDRα1-specific mAb, suggests that V(D)J recombination and antibody diversification via somatic hypermutation can result in the recognition of a semi-conserved epitope. Characterization of the epitopes that inhibit interaction between CIDR and its co-receptor EPCR may lead to the engineering of antibodies that are effective against erythrocytes-stage malaria.

Furthermore, our platform can be easily adapted to other emerging or re-emerging infectious diseases such as those caused by RNA viruses. Moreover, isolated mAb with significant cross neutralising activity can be utilised as therapeutic agents against these emerging viruses. This is exemplified by the severe acute respiratory syndrome (SARS) or Middle East respiratory syndrome (MERS). Both infections are caused by

members of corona viruses family which encodes more than 10 proteins including the spike protein (S), membrane protein (M), nucleo protein (N) and the highly conserved highly immunogenic envelope protein (E)(125).

Several studies have successfully isolated hmAbs against both diseases. 80R, S3.1, and m396 are anti-SARS monoclonal antibodies produced either through phage display or EBV immortalization technologies, which exhibited high neutralisation efficiency by targeting the S1 domain of the spike S protein(72,126,127). Similarly, m336 and LCA60, a potent anti-MERS-CoV mAbs targeting the S protein were isolated from human antibody library using phage display method or by using cellclone technology from immortalized B cells derived from recovering MERS patient(128,129).

In conclusion, through the use of these series of methods we were able to produce and characterise mAbs with high degree of purity that can be later analysed to identify its epitope updating current database contributing toward designing an effective vaccine.

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