

**The molecular basis of *Plasmodium falciparum* merozoite invasion
inhibition via anti-PfCyRPA antibodies**



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the requirements for the degree of
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Robert J. Ragotte

St. Edmund Hall

University of Oxford

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Abstract

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Robert J. Ragotte

Plasmodium falciparum, the pathogen responsible for the deadliest form of malaria, continues to exact an immense human toll, disproportionality shouldered by countries in sub-Saharan Africa. Significant progress has been made this century in reducing global malaria mortality and morbidity, but these gains remain fragile. The development of a malaria vaccine that protects against clinical disease would be an invaluable addition to the malaria control toolkit, but has remained elusive to date. As a result, the search for new, more potent vaccine antigens continues. Emerging from these efforts was the identification of the PfRH5:PfCyRPA:PfRipr complex (RCR), which is essential for merozoite invasion into the host erythrocyte. Here, a new panel of anti-PfCyRPA antibodies are evaluated for their ability to block merozoite invasion, including two new mAbs that are the most potently inhibitory anti-PfCyRPA mAbs described to-date. Structural studies revealed a single critical face of PfCyRPA that elicits neutralizing antibodies, where all four of the newly described inhibitory mAbs bound, opening the door for further studies on synthetic PfCyRPA immunogens that can focus the immune response on this vulnerable region of PfCyRPA. Finally, studies of synergy and antagonism between pairs of anti-PfCyRPA mAbs revealed that non-competing inhibitory anti-PfCyRPA mAbs potently synergize with each other. A novel mechanism of synergy to explain this observation was described, whereby lateral interactions between adjacent antibody Fab arms stabilize and enhance antibody affinity for PfCyRPA. Together, these data can inform the design of the next generation of malaria therapeutics.

To Granny and Papa,

for their unwavering support of education

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Acronyms and Abbreviations

- Ab:** antibody
- ABC:** dimethylamine-borane complex
- AMA1:** apical membrane antigen 1
- AU:** arbitrary units
- BCR:** B-cell receptor
- bnAb:** broadly neutralizing antibody
- C-half:** C-terminal half of a protein
- CD:** cluster of differentiation
- CDR:** complementarity determining region
- CeRTOS:** cell traversal protein for ookinetes
- CHMI:** controlled human malaria infection
- CSA:** chondroitin sulphate A
- CR1:** complement receptor 1
- CSP:** circumsporozoite protein
- CSS:** cysteine-rich small secreted protein
- CV:** column volumes
- DMEM:** Dulbecco's modified eagle medium
- EBA:** erythrocyte binding antigen (140/175/181)
- EBL-1:** erythrocyte binding antigen 1
- EC₅₀:** effective concentration (50% maximum effect)
- EC₈₀:** effective concentration (80% maximum effect)
- EG:** ethylene glycol
- EGF:** epidermal growth factor
- EPCR:** endothelial protein C receptor

fHBP: factor H binding protein

Fab: fragment antigen binding

FPLC: fast protein liquid chromatography

Fc: fragment crystallizable

GIA: grow inhibitory activity

GPA: glycophorin A

GPB: glycophorin B

GPI: glycosylphosphatidylinositol

HAART: highly active anti-retroviral therapy

HEK293: human embryonic kidney cell 293

HIV: human immunodeficiency virus

ICAM-1: intercellular adhesion molecule 1

IgG: immunoglobulin G

iRBC: Infected red blood cell

LB: luria broth

mAb: monoclonal antibody

MHC: major histocompatibility complex

MW: molecular weight

MPD: 2-methyl-2,4-pentanediol

MSP: merozoite surface protein

N-half: N-terminal half (of a protein)

NMR: nuclear magnetic resonance

PBS: phosphate buffered saline

PCR: polymerase chain reaction

PEG: polyethylene glycol

PfAMA1: *Plasmodium falciparum* apical membrane antigen 1

PfCyRPA: *Plasmodium falciparum* cysteine-rich protective antigen

PfEMP1: *Plasmodium falciparum* erythrocyte membrane protein 1

PfGARP: *Plasmodium falciparum* glutamic acid rich protein

PfRH1: *Plasmodium falciparum* reticulocyte binding protein homolog 1

PfRH2a: *Plasmodium falciparum* reticulocyte binding protein homolog 2a

PfRH2b: *Plasmodium falciparum* reticulocyte binding protein homolog 2b

PfRH4: *Plasmodium falciparum* reticulocyte binding protein homolog 4

PfRH5: *Plasmodium falciparum* reticulocyte binding protein homolog 5

PfRipr: *Plasmodium falciparum* RH5-interacting protein

PfRON2: *Plasmodium falciparum* rhoptry neck protein 2

PLP1: perforin-like protein 1

PTRAMP: thrombospondin-related apical membrane protein

PMR: parasite multiplication rate

RBC: red blood cell

RIFIN: repetitive interspersed families of polypeptides

RU: response units

SEC: size exclusion chromatography

SDS-PAGE: sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNP: single nucleotide polymorphism

SPECT1: sporozoite protein essential for cell traversal 1

SPR: surface plasmon resonance

STEVOR: subtelomeric variant open reading frame

TBS: tris buffered saline

TFF: tangential flow filtration

VLP: virus-like particle

VSA: variable surface antigen

Chapter 1: Introduction

Authorship Statement

Parts of this introduction relating to the biology of the PfRH5-PfCyRPA-PfRipr complex, Figures 1.1, 1.2, 1.3; Table 1.1 and 1.2; text section 1.2 and excerpts from text sections 1.1.4, 1.1.5, 1.3.1 and 1.3.2 have been reproduced from a review article I wrote with my supervisors Prof. Simon Draper and Prof. Matthew Higgins. In accordance with the guidelines from the Nuffield Department of Medicine and University of Oxford available on Canvas,

‘Prior publication of material arising from research undertaken while studying is fully acceptable... [So long as] you actually authored/produced the material (e.g. text or figures) to be reused with no more input/advice than would be considered appropriate or acceptable for a thesis... You should fully and clearly acknowledge that parts of the thesis have previously been published, and indicate which parts.’

I wrote the reproduced sections in full with input from Profs. Draper and Higgins.

Ragotte RJ, Higgins MK, Draper SJ. The RH5-CyRPA-Ripr complex as a malaria vaccine target.

Trends in Parasitology **36**, 545-559 (2020).

1.1 *Malaria*

1.1.1 *History and clinical presentation*

As early as the 5th century BCE, there are documented descriptions of intermittent fevers from India to Greece to China, consistent with what would now be described as malaria¹. Indeed, the history of human civilization is closely intertwined with the history of malaria, having changed the course of wars and empires, reshaped the human genome, and brought into existence the noble gin and tonic.

The etymology of malaria, coming from the Italian meaning ‘bad air’, belies its vector-borne transmission through the *Anopheles* mosquito. It was not until the identification of the parasite in the blood of febrile soldiers that Charles Louis Alphonse Laveran established the microbial origins of malaria.

Despite the intervening millennia, humans have not escaped this disease that plagued our Neolithic ancestors. Although significant progress has been made in reducing the global burden of malaria, particularly in the past twenty years, in 2018 there were still 405,000 deaths attributed to malaria². Progress towards malaria elimination has not been evenly distributed with some countries still shouldering disproportionately high malaria burdens, particularly in sub-Saharan Africa, where three countries - Nigeria, the Democratic Republic of Congo and Uganda - account for more than 40% of global malaria cases². As well, the ongoing COVID-19 pandemic threatens to derail this precarious progress, with some predicting a doubling of malaria mortality, through the interruption of critical supply chains and the diversion of resources to address COVID-19³.

There are six species of *Plasmodium* that cause malaria in humans: *P. falciparum*, *P. vivax*, *P. knowlesi*, *P. ovale curtisii*, *P. ovale wallikeri* and *P. malariae*. Of these, *P. vivax* is the most widespread¹, but *P. falciparum* is responsible for the vast majority of mortality². The term 'malaria' refers to the collection of symptoms associated with infection by one of these pathogens. The focus of the research described here is *P. falciparum*. Any mentions of 'malaria' will be in reference to the disease caused specifically by *P. falciparum* infection, unless otherwise specified.

The clinical presentation of [falciparum] malaria begins 9-14 days after initial infection, once the parasite has progressed to the blood-stage of infection (**Figure 1.1**). Symptoms often include intermittent fevers, alongside malaise, myalgia, bouts of sweating, chills, nausea, headaches and/or gastrointestinal involvement. If left untreated, serious cases can result in anaemia, renal failure, pulmonary oedema and, in its most severe forms, progress to cerebral malaria, the most lethal form of the disease. Malaria poses the greatest risk to infants and young children, as 67% of malaria deaths occur in children under 5 years of age². Severe malaria is thought to result from a combination of excessive inflammation in response to infection as well as sequestration of parasites in the microvasculature^{4,5}. Malaria also poses substantial risk to pregnant women via the sequestration of parasites in the placenta where it can lead to low birthweight or stillbirth⁶.

1.1.2 *P. falciparum* life cycle

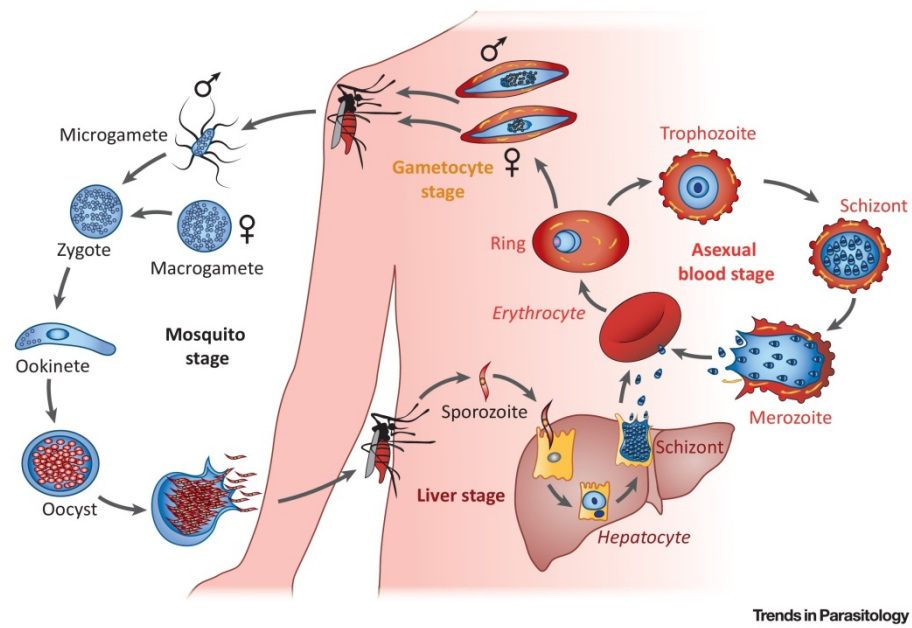


Figure 1.1 *P. falciparum* life cycle. Reproduced from Maier AG *et al.* 2019 with permission from Elsevier⁷.

The life cycle (**Figure 1.1**) begins when 20-200 sporozoites are injected into the dermis through the bite of an *Anopheles* mosquito⁸. These sporozoites then enter the blood vessels where they can migrate to the liver and commence hepatocyte invasion, which involves transversal of epithelial cells, fibroblasts and/or Kupffer cells before reaching the target hepatocyte⁹. This process is poorly understood, but transversal relies on a number of protein families including perforin-like protein 1 (PLP1), sporozoite protein essential for cell traversal 1 (SPECT1) and cell traversal protein for ookinetes and sporozoites (CeTOS)⁹. These proteins are thought to form pores in host cell membranes, allowing the parasite to pass through without being destroyed by the resident macrophages (Kupffer cells). The invasion of hepatocytes by *P. falciparum* is mediated, at least in part, by the host receptor cluster of differentiation 81 (CD81)^{10,11}.

Despite the complexity of this event, within minutes of injection, the sporozoite will have successfully invaded hepatocytes, where they are able to replicate before releasing merozoites into the blood-stream seven days later. Once in the blood, merozoites invade red blood cells through a complex and highly orchestrated invasion event described in **Section 1.1.3**. Over the course of 48 hours, parasites divide by schizogony, progressing from a single ring-stage parasite, to schizonts, releasing 16-32 new merozoites upon egress from the erythrocyte. It is during the blood-stage of infection that the clinical symptoms of malaria occur.

Gametocyte production commences alongside continued asexual reproduction, with the former taking up to 12 days, as opposed to 2 days for asexual reproduction¹². It is these gametocytes that are then taken up by mosquitoes during a blood meal. Within the mosquito the parasites undergo their sexual stage of reproduction within the midgut and basal lamina¹³.

Upon completion of the sexual stage of reproduction, the parasites migrate to the mosquito salivary glands where they can prepare for the infection of a new host.

1.1.3 *Erythrocyte invasion*

From when the merozoite egresses from the infected erythrocyte until entry into a new erythrocyte, the parasite is directly exposed to the host immune system where it can be targeted by antibodies, rather than sequestered within host cells. Thus, it is in the parasite's interest for invasion to occur rapidly, minimizing exposure to the host immune response¹⁴. The result is a highly orchestrated invasion event, allowing merozoites to invade within thirty seconds of being released¹⁵.

Cell entry occurs in three key steps: initial attachment, tight junction formation, followed by active invasion. Merozoite surface proteins (MSPs) could mediate initial attachments, although the evidence for this is conflicting as MSP1 was initially reported to be involved in this process, but it is now thought MSP1 is involved in egress^{16,17}. At present, it is not clear which ligand receptor interactions are important for initial attachment.

Tight attachment is mediated through a number of redundant host parasite interactions including erythrocyte binding antigen 175 (EBA-175) binding to glycophorin A (GPA), EBA-140 binding to glycophorin C (GPC), erythrocyte binding ligand 1 (EBL1) binding to glycophorin B (GPB), and *P. falciparum* reticulocyte binding protein homolog 4 (PfRH4) binding to complement receptor 1 (CR1), as well as EBA181, PfRH1, PfRH2a, and PfRH2b binding to yet-to-be-identified receptors¹⁷. Tight attachment is coupled to membrane deformation where microscopy shows the erythrocyte membrane 'buckling'¹⁷. Though the overall function of these families of proteins is essential, these parasite genes can be individually disrupted and invasion is still able to proceed^{17,18}. This is thought to be one

mechanism of immune evasion whereby redundancy allows for individual interactions to be blocked without arresting invasion entirely¹⁴. *P. falciparum* reticulocyte binding protein homolog 5 (PfRH5), a member of the same family of proteins as the other PfRH proteins, is an exception to this. PfRH5 forms a tertiary complex with two other essential invasion proteins, cysteine-rich protective antigen (PfCyRPA) and RH5-interacting protein (PfRipr), known as the RCR complex¹⁴. This complex binds to basigin via the tip of PfRH5¹⁹, which is a non-redundant interaction essential for invasion²⁰.

PfRH5 is thought to bind to basigin immediately following membrane deformation²¹. A series of experiments, using both inducible knockout parasite lines as well as antibodies targeting members of the RCR complex, show that the RCR complex acts downstream of membrane deformation^{21,22}. PfRH5-basigin engagement is also coupled with a spike in calcium concentration within the erythrocyte that is correlated with echinocytosis^{17,21}. The source of the calcium, whether emanating from the merozoite, from the media, or caused by blocking calcium export from the erythrocyte, remains unclear²¹. A recently published model suggested that PfRipr and PfRH5 form a pore in the erythrocyte membrane which allows calcium to move from the merozoite into the erythrocyte²³. However, this was largely speculative as the recombinant complex on its own did not induce an intra-erythrocytic calcium spike²³. Separately, one study showed that PfRH5 on its own could induce a calcium concentration spike within the erythrocyte²⁴. More work is needed to understand the relationship between the PfRH5-basigin interaction and the intra-erythrocytic calcium spike it may or may not induce.

Following PfRH5-basigin engagement, tight junction formation occurs. This is mediated by apical membrane protein 1 (AMA1) binding to rhoptry neck protein 2 (RON2), which is part of the RON complex, a group of proteins secreted from the merozoite rhoptries

which then insert into the erythrocyte membrane^{25,26}. After attachment of AMA1 to RON2, the merozoite is propelled into the erythrocyte via an actin-myosin motor²⁷, while simultaneously secreting the contents of apical organelles to generate a parasitophorous vacuole, where asexual replication can commence.

1.1.4 Mechanisms of immune evasion

There is no single reason why the establishment of blood-stage immunity requires repeated infection. Rather, it is likely the result of many complementary and highly evolved immune evasion tactics employed by the parasite. The aforementioned problems of both diversity and redundancy are perhaps evolutionary responses to host immune pressure, but there are numerous other ways through which *P. falciparum* evades the host immune response.

The first, and perhaps most important, is the choice of host cell. Erythrocytes do not express major histocompatibility complex class I molecules (MHCI), and thus are not subject to CD8⁺ T cell-mediated killing. This is in contrast to *P. vivax* which infects reticulocytes, which retain some protein translation machinery and express MHCI, thereby enabling cytotoxic T-cells to kill infected erythrocytes²⁸. This is an essential strategy of immune evasion as infected cells go largely undetected by traditional mechanisms of adaptive immune surveillance.

Infected red blood cells (iRBCs) express parasite proteins known as variable surface antigens (VSA) on their surface from the erythrocyte membrane protein (PfEMP1), repetitive interspersed families of polypeptides (RIFIN) and subtelomeric variant open reading frame (STEVAR) families of proteins: *var*, *rif* and *stevor* gene families, respectively²⁹. VSAs are involved in a number of immune escape mechanisms including adherence to the vascular endothelium to prevent splenic clearance²⁹ and by acting as immune inhibitory receptors^{30,31}.

Cytoadherence to the endothelium is mediated by PfEMP1 binding to CD36³², endothelial protein C receptor (EPCR)³³, chondroitin sulfate A (CSA)⁶, and intercellular adhesion molecule-1 (ICAM1)³⁴, which leads to vascular congestion and contributes to the most severe manifestations of malaria^{33,35}.

In addition to this, VSAs bind uninfected erythrocytes in a process known as rosetting³⁶. Though the precise purpose of this remains unknown, it is thought that this could be a mechanism of immune escape whereby the uninfected erythrocytes shield iRBCs from immune recognition³⁷. Rosetting contributes to congestion of the microvasculature, as iRBCs bind uninfected RBCs and form clusters of erythrocytes^{37,38}. This is in addition to the blood flow occlusion that occurs as a result of cytoadherence to the endothelium³⁸. iRBCs have also been shown to bind non-immune IgM via the Fc³⁹. The purpose of this is thought to be either mediating rosette formation^{39,40} or a mechanism to escape immune detection⁴¹.

The speed of parasite invasion and the parasite's complex multi-host, multi-organ life cycle contribute to immune escape, regardless of whether it was immune pressure that originally resulted in the evolution of these systems. Parasite invasion into erythrocytes is usually completed in approximately thirty seconds¹⁵. The brevity of this extracellular foray appears to limit the efficacy of invasion inhibitory antibodies⁴². The parasite's complex life cycle also means that the expression of most vaccine antigens of interest is restricted to a single stage of the parasite's life cycle. For example, when considering liver-stage vaccination, this means that immunity is relatively dichotomous, since any parasite infiltration into the liver will result in an uncontrolled infection upon emergence into the blood, as there is no immunity against the blood-stage of infection to limit parasite growth. A partially effective vaccine may result in a delay in time to diagnosis due to lower liver-to-blood inoculum, but

overall parasite expansion during the blood-stage of infection will be equivalent to unvaccinated individuals⁴³.

1.1.5 *Naturally acquired immunity and the assay of growth inhibition activity*

Despite numerous strategies that parasites use to evade the host immune response, individuals living in endemic areas still develop immunity against clinical malaria. A seminal experiment performed by Cohen, McGregor and Carrington showed that the passive transfer of antibodies from adults living in malaria endemic areas to susceptible children protected against disease⁴⁴. However, while it is clear that immunity against *P. falciparum* infection eventually develops, this process is slow and requires repeated exposure over many years^{45,46}. Naturally acquired immunity seems to be predominantly mediated through immune responses to the blood-stage of infection, with limited evidence to show that immune responses to the parasite in the skin or liver confer immunity^{4,47}.

The mechanisms underlying naturally acquired malaria immunity are yet to be fully understood. During the first stage of naturally acquired immunity, individuals become immune from severe disease, which is thought to be mediated in part by a switch from an inflammatory to a more tolerogenic immune response⁴⁸, as inflammation (coupled with sequestration of parasites in the microvasculature) is thought to be a cause of severe disease. This is characterized by a switch from a pro-inflammatory tumour necrosis factor α (TNF α) to an anti-inflammatory IL-10 and TGF- β response^{4,49,50}. Immune responses that directly target the parasite or iRBC directly can be thought of as having two distinct arms, anti-disease and anti-parasitaemia, which both develop in parallel⁵¹. The former protects primarily against the most severe manifestations of the disease via immune responses targeting VSAs^{52,53}. The

latter reduces the parasite burden in the infected individual, but how this is achieved remains controversial.

Antibody effector functions, namely complement deposition and opsonization, have been reported to contribute to controlling parasite growth among naturally immune individuals^{54,55}. In addition to this, recent studies have outlined a role of natural killer cells in killing iRBCs to control parasite growth⁵⁶. This helps to explain the puzzling observation that despite a growing body of evidence that antibody growth inhibition activity (GIA) correlates with immunity after vaccination, it is not strongly associated with naturally acquired immunity among adults living in endemic areas^{57,58}. The GIA assay is primarily designed to measure antibody-mediated neutralization, which suggests that antibody-mediated invasion inhibition is not the primary mechanism of naturally acquired immunity.

The GIA assay has been an important tool to evaluate the antibody response induced through vaccination. The assay begins with a synchronized culture of ring-stage parasites maturing into schizonts, which then rupture and the merozoites are allowed to reinvade fresh, uninfected erythrocytes, in the presence or absence of an antibody or small molecule of interest. In the assay's standardized form, the impact of the antibody on invasion is then quantified by measuring lactate dehydrogenase activity in the treated samples compared to the untreated parasites that invade in media alone. In some forms of this assay, a two-cycle approach is used where invasion is quantified at the end of two cycles of growth. This approach usually gives higher values of inhibition, preventing direct comparison to the one-cycle GIA assay⁵⁹. The GIA reference centre at the National Institutes of Health uses a one-cycle GIA assay, which is also the approach used in the experiments in this thesis⁶⁰.

As mentioned above, the presence/absence of GIA activity does not predict naturally acquired immunity. However, it has been shown to be a useful correlate of immune protection in studies that examined vaccine induced immunity⁶¹⁻⁶³. Furthermore, it has been shown that the passive transfer of inhibitory monoclonal antibodies lacking antibody effector functions can protect against infection⁶⁴. Thus, although their role in naturally acquired immunity may be limited, inhibitory antibodies can confer an 'unnatural' form of immunity¹⁴.

1.2 RH5-CyRPA-Ripr Complex

1.2.1 *Plasmodium falciparum* reticulocyte binding protein homolog 5 (PfRH5)

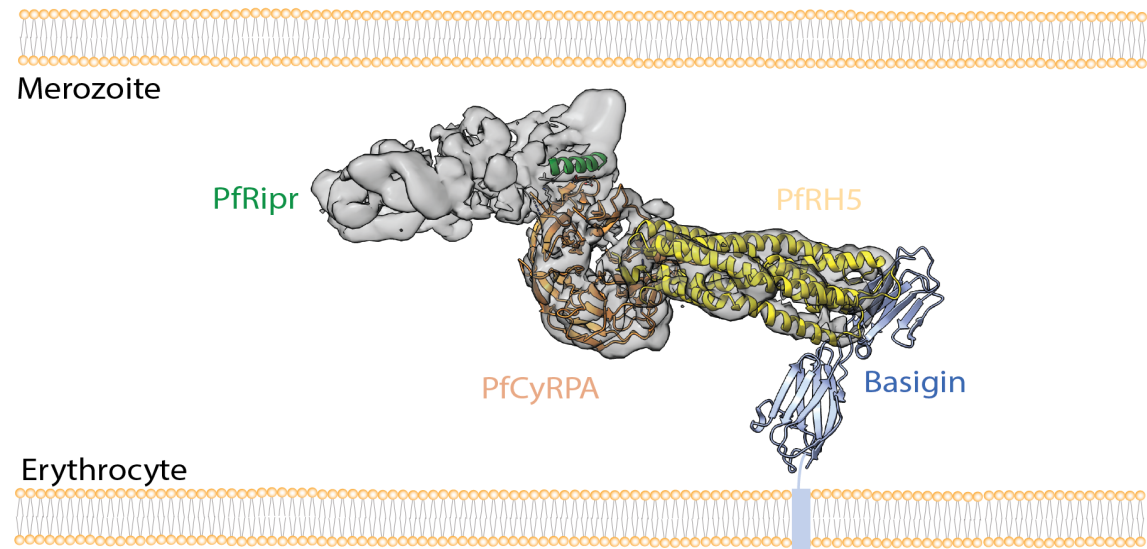


Figure 1.2 RCR complex bound to basigin reproduced from Ragotte *et al.* 2020. Grey density is from the cryo-EM structure of the RCR complex (EMD-9192, PDB: 6MPV) which has been superimposed on the crystal structure of PfRH5 bound to basigin (PDB: 4U0Q).

Table 1.1 Single nucleotide polymorphisms in the RCR complex with minor allele frequency >5%. Reproduced from Ragotte *et al.* 2020.

Protein	Mutation	Minor allele frequency (%)	Citation
PfRH5	H148D	18.41%	65
	Y147H	17.39%	
	S197Y	16.36%	
	C203Y	13.50%	
	I410M	9.40%	
PfRipr	Y985N	39.67%	
	V190A	13.11%	
	I1039M	10.83%	
	A755G	9.13%	
	H511R	7.97%	
	L673V	6.93%	
PfCyRPA	R339S	14.84%	

Table 1.2 Neutralizing monoclonal antibodies targeting the RCR complex. Reproduced from Ragotte *et al.* 2020.

Antibody	Target	Isotype	Species	Concentration (mg/ml)	GI(%)	Cross Strain Neutralizing	Binding Details	Citation
BS1.2	PfRH5	IgG	Mouse	0.5 0.25 0.125	35 30 25	Yes	N/A	66
2AC7		IgG1	Mouse	0.5 0.25 0.125	80 75 70	Yes	Conformational epitope	67
9AD4		IgG2a	Mouse	0.5 0.25 0.125	70 60 55	Yes	Linear epitope beginning at Y346, basigin binding adjacent	
QA5		IgG1	Mouse	0.5 0.25 0.125	60 50 45	Yes	Linear epitope beginning at Y194	
6BF10		IgG	Mouse	0.5 0.25 0.125	30 30 30	Yes	Conformational epitope	
QA1		IgG	Mouse	0.5	35	Yes	Conformational epitope, basigin blocking	
R5.004		IgG1	Human	0.5 0.25 0.01	90 80 30	Yes	Conformational epitope, basigin blocking	68
R5.008		IgG1	Human	0.5 0.25 0.01	80 75 20	Yes	Conformational epitope, basigin blocking	
R5.013		IgG1	Human	0.5 0.25 0.01	65 50 10	Yes	Conformational epitope, basigin blocking	
R5.016		IgG1	Human	0.5 0.25 0.01	95 85 50	Yes	Conformational epitope, adjacent to basigin binding site	
R5.017		IgG1	Human	0.5 0.25 0.01	90 80 30	Yes	Conformational epitope, basigin blocking	
R5.018		IgG1	Human	0.5 0.25 0.01	50 35 5	Yes	Conformational epitope, basigin blocking	
R5.019		IgG1	Human	0.5 0.25 0.01	90 80 20	Yes	Conformational epitope, basigin blocking	
5G6	PfRipr	IgG	Mouse	0.5 0.25 0.125	35* 25* 15*	Unknown	Conformational epitope	69
1G12		IgG	Mouse	0.5 0.25 0.125	50* 35* 25*	Unknown	Conformational epitope	
c02	PfCyRPA	IgG2b	Mouse	0.5 0.25 0.125	50* 40* 30*	Yes	Conformational epitope between amino acids 26-181	70
c04		IgG3	Mouse	0.5 0.25 0.125	50* 40 30	Yes	Conformational epitope between amino acids 26-181	
c06		IgGa	Mouse	0.25 0.125	50* 40*	Yes	Conformational epitope between amino acids 26-181	

c08		IgG1	Mouse	0.5 0.25 0.125	30* 20* 15*	Yes	Conformational epitope between amino acids 26-181	
c09		IgG3	Mouse	0.5 0.25 0.125	50* 40* 30*	Yes	Conformational epitope between amino acids 26-181	
c10		IgG1	Mouse	0.5 0.25 0.125	45* 35* 25*	Yes	Conformational epitope between amino acids 26-352	
c12		IgG2a	Mouse	0.5 0.25 0.125	50* 40* 30*	Yes	Conformational epitope on blades 1-2	
SB1.6		IgG	Mouse	0.5 0.25 0.125	65* 50* 35*	Unknown	Conformational epitope between amino acids 74-251	71
SB2.1		IgG	Mouse	0.5 0.25 0.125	50* 40* 30*	Unknown	Conformational epitope between amino acids 74-251	
SB2.3		IgG	Mouse	0.5 0.25 0.125	50* 40* 30*	Unknown	Conformational epitope between amino acids 74-251	
SB3.3		IgG	Mouse	0.5 0.25 0.125	40* 30* 20*	Unknown	Conformational epitope between amino acids 74-251	
5B12		IgG	Mouse	2	70*	Unknown	Conformational epitope	72
3D1		IgG	Mouse	2	70*	Unknown	Conformational epitope	
8A7		IgG1	Mouse	2	70*	Unknown	Conformational epitope on blades 2-3	

*Based on only two-cycle growth inhibition while all others are based on one-cycle

1.2.1.1 Structure and function

PfRH5 is currently the most advanced next-generation blood-stage vaccine candidate antigen. It was identified as a member of the PfRH family both through a homology-based search⁷³, and through linkage analysis⁷⁴. PfRH5 is a 63 kDa protein expressed during the mature schizont stages, localized to the rhoptries^{75,76}. It is processed and cleaved to a 45 kDa form, which is subsequently shed into parasite culture supernatant^{75,76}. The structure of PfRH5 reveals a kite-like architecture formed from the coming-together of two three-helical bundles^{19,77}. This same fold was seen in the *P. vivax* reticulocyte-binding protein 2a (RBP2a) invasion ligand, demonstrating the potential for versatile binding properties⁷⁸.

PfRH5 lacks a transmembrane domain or glycosylphosphatidylinositol (GPI) anchor. Initially, cysteine-rich protective antigen (PfCyRPA) was thought to anchor it to the merozoite surface⁵⁹; however, it has since been shown that PfCyRPA also lacks a GPI anchor^{21,79}. P113, a GPI-anchored protein, was thereafter shown to bind the PfRH5 N-terminus, suggesting a possible role in invasion⁷⁹. This is complicated by the observation that P113 is also known to associate with the PTEX translocon, meaning any role alongside the RCR surface complex would be an additional, seemingly unrelated, biological function⁸⁰. Further investigation into this interaction through hydrogen-deuterium exchange mass spectrometry confirmed P113 binding to the PfRH5 N-terminus, but also showed that antibodies that block this interaction do not block invasion⁸¹.

PfRH5 is indispensable for human erythrocyte invasion²⁰. As such, it is refractory to genetic deletion^{74,76}. Intriguingly, PfRH5 orthologues are found in other species belonging to the *Laverania* subgenus, which includes parasites that infect chimpanzees and gorillas, but they are not found in the other species of *Plasmodium* that infect humans, indicating a unique

role in *P. falciparum* invasion of human erythrocytes^{74,82,83}. Genomic studies identified the transfer of an 8kb stretch of DNA encoding both PfRH5 and PfCyRPA from the gorilla parasite *P. alderi* to an ancestor of *P. falciparum* as a critical event in the emergence of *P. falciparum* as a lethal human parasite^{84,85}, while polymorphisms in *PfRH5* play a critical role in defining species-specificity of host cell receptor binding, thereby determining infectivity^{82,86–89}.

One model of invasion suggests that upon binding to erythrocyte basigin, PfRH5 inserts into the membrane, alongside PfRipr, where it unfolds, potentially forming a pore, as mentioned previously²³. However, there is little evidence for this as the structural and biochemical properties of PfRH5 are not consistent with membrane insertion or unfolding; structures of PfRH5 in complex with a variety of antibodies and erythrocyte basigin, in a variety of crystallization conditions, are essentially identical, suggesting a robust and stable protein fold^{19,68,77}.

1.2.1.2 Antibody responses to *PfRH5*

In malaria, cross-strain neutralizing vaccine targets were thought to be subject to considerable natural immune pressure, thus, typically, such targets showed wide antigenic diversity; however, PfRH5 appears to be the first identified exception to this dogma⁹⁰. Indeed, vaccine-induced antibodies that mediate parasite neutralization *in vitro* were effective across all *PfRH5* SNPs tested, including the five most common^{68,91–93} (**Table 1.1**).

In light of these vaccine data, interest arose in naturally acquired anti-PfRH5 responses. Following natural infection, as opposed to vaccination, anti-PfRH5 seropositivity was associated with reduced parasitaemia in a cohort of children from Papua New Guinea, while a study in Malian children drew an association between anti-PfRH5 seropositivity and protection from febrile malaria^{69,94,95}. Although there appears to be an association between

naturally acquired anti-PfRH5 antibodies and protection from clinical *P. falciparum* malaria, PfRH5 also appears to lack the inherent immunogenicity observed with many other well-described *P. falciparum* merozoite proteins in the context of natural exposure. Indeed, when antibody responses in previously infected Kenyan adults were examined, researchers found markedly reduced antibody levels to PfRH5 compared to PfAMA1 and nine other blood-stage vaccine targets⁹⁰. This immuno-epidemiological observation has now been consistently observed across numerous studies, with a number including large panels or arrays of malaria antigens^{94,96–98}.

The reasons why PfRH5 is poorly immunogenic in natural malaria infection remain unclear, although these probably relate to low antigen abundance, with PfRH5 protein lost in a sea of many other highly abundant immuno-dominant (decoy?) targets, such as MSP1. Early concerns about issues of human tolerance to PfRH5 due to self-similarity have floundered in light of data confirming strong vaccine-induced immune responses against PfRH5 following small animal, *Aotus* monkey and now human vaccination^{62,90,93}.

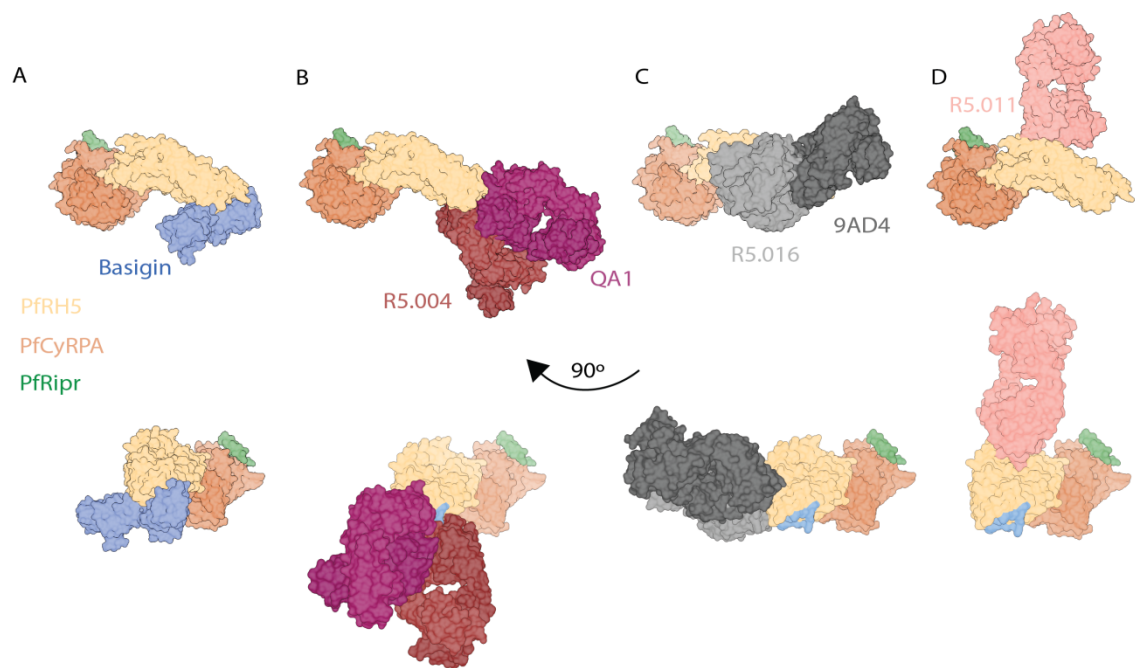


Figure 1.3 PfRH5 bound to basigin, inhibitory or non-inhibitory antibodies. **A.** RCR bound to basigin. **B.** RCR bound to basigin blocking inhibitory antibody R5.004 and QA1. **C.** RCR bound to basigin proximal inhibitory mAbs R5.016 and 9AD4. **D.** RCR bound to non-inhibitory (dilatatory) mAb R5.011.

At present, anti-PfRH5 monoclonal antibodies are the most potent among those targeting each of the three antigens within the RCR complex (**Table 1.2**), as assessed by the standardized *in vitro* assay of GIA. Notably this assay was shown to strongly associate with protection outcome in vaccinated *Aotus* monkeys and was then confirmed as a mechanistic correlate (i.e. causative of protection) following mAb passive transfer^{62,64}, with similar results also obtained in humanized mice. The most potent reported anti-PfRH5 mAb, R5.016, has a one-cycle GIA EC₅₀ at 9.6µg/ml, matching or surpassing many of the anti-PfRH5 polyclonal sera that have been tested (**Table 1.2**)^{67,68,91,92,99}.

The primary mechanism through which inhibitory antibodies to PfRH5 are thought to work is by disrupting the PfRH5-basigin interaction^{19,68}. This is likely how anti-PfRH5 mAbs R5.004, R5.008, R5.013, R5.017, R5.019 and QA1 (which clearly block basigin in SPR experiments and/or based on their crystal structures) inhibit parasite invasion (**Figure 1.3B**)^{67,68}. However, 2AC7 and R5.016, the two most potent anti-PfRH5 mAbs, and 9AD4, inhibit invasion seemingly without blocking the PfRH5:basigin interaction *in vitro*, despite binding close to the basigin-binding site (**Figure 1.3C**)^{67,68}. It was suggested that 2AC7 works by disrupting the PfRH5-PfCyRPA interaction⁶⁹. However, this is inconsistent with data showing 2AC7 competes for binding with R5.016, on the opposite side of PfRH5 from where PfCyRPA interacts⁶⁸. Further, the few published anti-PfRH5 mAbs that block PfCyRPA binding are non-inhibitory⁶⁸. It is clear that the most potent neutralizing anti-PfRH5 antibodies all bind towards the tip of the PfRH5 kite close to the basigin binding site (**Figure 1.3A-C**), making this a promising candidate for structure-guided immunogen design to increase relative concentrations of neutralizing (**Figure 1.3B,C**) to non-neutralizing antibodies (**Figure 1.3D**)¹⁰⁰.

1.2.2 *PfCyRPA*

1.2.2.1 *Structure and Function*

PfCyRPA is a 43 kDa protein that was identified as a blood-stage vaccine candidate antigen through reverse vaccinology based on a predicted N-terminal secretion peptide, upregulation during the blood-stage of infection, and a putative role in erythrocyte invasion owing to proximity to other invasion genes such as *PfRH5* within the parasite genome⁷⁰. Transcriptional analyses also identified PFD1130w, the gene ID of *cyrpa*, as part of the *P. falciparum* invasion pathway¹⁰¹.

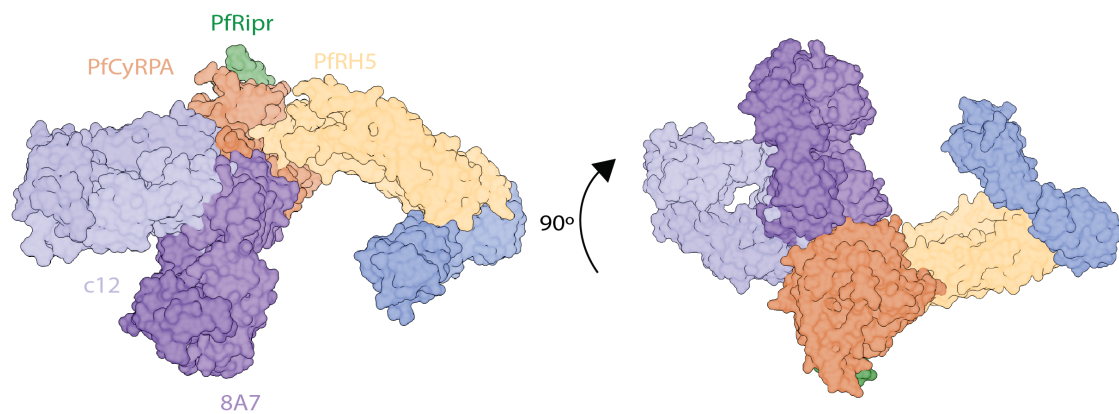


Figure 1.4 *PfCyRPA* bound to inhibitory antibodies 8A7 (PDB:5TIH) and c12 (5EZO). Figure reproduced from Ragotte *et al.* 2020.

Subsequently, and notably after it had already been independently identified as a vaccine candidate, PfCyRPA was coimmunoprecipitated with PfrH5 and PfrRipr^{59,70,92}. Akin to PfrH5 and PfrRipr, PfCyRPA is highly conserved with only a single SNP above 5% prevalence (**Table 1.1**), essential for invasion as conditional knockdown causes the loss of invasion activity²¹, and has poor sero-reactivity from natural exposure^{70,97}. PfCyRPA localizes to the parasite micronemes, as is the case with PfrRipr, but different from PfrH5^{21,59}. Two crystal structures of PfCyRPA have been solved, revealing a six-bladed β -propeller with five disulphide bonds (four intra-sheet, one inter-sheet)^{66,72}. PfCyRPA has some similarity to known sialidases; but lacks critical catalytic residues⁷². Further ELISA-based analyses, now confirmed by the cryo-EM structure²³, show that PfCyRPA acts as a bridging protein, which links PfrH5 and PfrRipr⁷⁹. No further role of the protein beyond this has been reported.

1.2.2.2 *Antibody responses to PfCyRPA*

Like PfrH5 and PfrRipr, PfCyRPA is a weakly immunogenic target during natural infection^{70,97}. However, studies of monoclonal and polyclonal antibodies have shown that PfCyRPA is also susceptible to cross-strain neutralizing antibodies^{70,102,103}. Deyer *et al.* (2012) showed that of their panel of nine mAbs (derived from mice immunized with full-length soluble PfCyRPA protein produced in mammalian HEK293 cells), seven were cross-strain inhibitory, with the best achieving GIA of 58% at 1 mg/ml against 3D7 clone parasites (**Table 1.2**). All seven of the inhibitory antibodies bound a conformational epitope between amino acids 26 and 181⁷⁰.

Analysis of a different panel of five mouse-derived antibodies identified three inhibitory anti-PfCyRPA mAbs⁷². The mechanism for the most potent of these, 8A7, was reported to be disruption of the PfCyRPA-PfrH5 interface^{69,72}. This is in contrast to data on

eight anti-PfRH5 mAbs, spanning three epitope regions, which all disrupted the same PfCyRPA-PfRH5 interaction *in vitro* and yet had negligible GIA against *P. falciparum*⁶⁸. Reconciling these observations will require further study. Interestingly, two additional anti-PfCyRPA mAb clones from the same study, 5B12 and 3D1, competed for binding with 8A7 on PfCyRPA and yet did not disrupt RCR complex formation *in vitro*; instead they stably formed a quaternary complex⁶⁹. The crystal structure of 8A7 Fab bound to PfCyRPA has also been solved⁷². It is not immediately clear how it would block the PfRH5:PfCyRPA interface, as superimposition of 8A7:PfCyRPA onto the PfRH5:PfCyRPA cryo-EM structure does not indicate they would compete for binding (**Figure 1.4**).

These data would thus suggest there is another mechanism of action, beyond disruption of RCR complex formation, which allows for anti-PfCyRPA antibodies to block parasite invasion. The authors of one study noted that inhibitory anti-PfCyRPA antibodies that bind the RCR complex point toward the erythrocyte membrane⁶⁹. From there, it was speculated that there could be steric interference between the bulky antibody Fc constant domain and the membrane, preventing the RCR complex from binding to erythrocyte basigin²³.

Monoclonal antibodies targeting PfCyRPA have also been tested in animal challenge models showing some efficacy. In a NOD-*scid* IL2Ry^{null} mouse (NSG) model grafted with human erythrocytes, there was a marked decrease (90%) in parasitaemia after *P. falciparum* challenge when pre-treated with 2.5 mg of monoclonal antibody SB1.6 or c12 (**Table 1.2**)⁷¹. A follow-up study showed that rabbit immunization with PfCyRPA integrated into influenza virosomes elicits neutralizing antibodies that slow parasite growth in the NSG mouse model¹⁰⁴.

At the time of writing, there have been no human vaccine trials using PfRipr- or PfCyRPA-based immunogens. We can expect that candidate vaccines will begin to appear in animal studies and then human trials in the coming years given the strong scientific rationale for exploring such vaccines and the promising preclinical studies so far.

1.2.3 *PfRipr*

1.2.3.1 *Structure and Function of PfRipr*

PfRipr is an approximately 120 kDa protein, first identified when gel filtration of what was presumed to be ion-exchange purified PfRH5 showed a 150-200 kDa compound, instead of the expected 45 kDa size of the processed fragment⁷⁵. Mass spectrometry revealed the presence of another protein, complexed with PfRH5, corresponding to hypothetical protein, PFC1045c, later renamed PfRipr⁷⁵. This interaction was confirmed by immunoprecipitation. Independently, PFC1045c was identified as a member of the *P. falciparum* 'invadome', a collection of proteins that are hypothesized to be involved in invasion¹⁰¹.

PfRipr is composed of putatively unstructured regions, 10 epidermal growth factor-like (EGF) domains and has 87 cysteines. The full-length 120 kDa protein is processed into two fragments of similar size, an N-terminal fragment (including EGF domains 1 and 2) and a C-terminal fragment (including EGF domains 3-10)⁷⁵. Whether cleavage is critical to the function of PfRipr is not clear. Like PfRH5, recombinant expression of this large and complex antigen in its entirety, proved difficult; however, the first reported success to provide reasonable quantities was again using the *Drosophila* S2 stable cell line platform. This led to a recent 7Å resolution cryo-electron microscopy (cryo-EM) structure of the RCR complex (**Figure 1.2**), revealing that PfRipr interacts with PfCyRPA through blade six of the PfCyRPA β-propeller²³. However, the moderate resolution of this structure precluded building of an atomic model of

PfRipr, and indeed, the electron density attributed to PfRipr is sufficient to include only around half of the molecule. There is still significant work to do to understand the structure of PfRipr and how it contributes to invasion.

Interestingly, PfRH5 and PfRipr localize to different compartments during the schizont stage of the *P. falciparum* life cycle: PfRipr in the micronemes and PfRH5 in the rhoptries⁵⁹. Despite this, parasites with conditional knockouts of PfRipr have a similar invasion phenotype to parasites that try to invade in the presence of anti-PfRH5 antibodies; these parasites induce membrane deformation, but cannot complete invasion²¹.

1.2.3.2 *Antibody Responses to PfRipr*

Much like PfRH5, PfRipr is highly conserved, essential for invasion, capable of inducing cross-strain neutralizing antibodies in assays of GIA, but is not a target of naturally acquired immunity^{94,96}. There are only six non-synonymous SNPs with a minor allele frequency above 5% in *RIPR* (**Table 1.1**). In line with this, polyclonal antibodies raised against EGF domains 6-8 (amino acids 791-900) of PfRipr are cross-strain neutralizing^{69,75}. In one report, GIA assays using the FCR3 laboratory-adapted strain and the 3D7 clone of *P. falciparum* showed nearly complete versus 50% growth inhibition at 3mg/ml total rabbit IgG, respectively. In a separate study, polyclonal anti-PfRipr total rabbit IgG was produced using a fragment of PfRipr spanning amino acids 279-995 and then tested for GIA with homologous (3D7 clone) and heterologous (FVO strain) parasites, both of which were successfully inhibited by the purified polyclonal IgG¹⁰⁵.

Against the entirety of the large PfRipr molecule, only two neutralizing mAbs have been mapped so far, targeting EGF-7⁶⁹. The most potent mAb, 1G12, has an EC₅₀ of 0.35mg/ml, approximately ten-fold less potent than the best PfRH5 mAbs (**Table 1.2**)¹⁰⁶.

Notably, the only region of PfRipr known so far to be targeted by neutralizing mAbs (EGF-7) appears to be located away from the PfCyRPA:PfRipr interface, which is towards the N-terminal half of the protein. Accordingly, the mechanism behind neutralization for anti-PfRipr antibodies does not appear to be related to RCR complex formation and remains to be elucidated¹⁰⁶.

1.2.4 Targeting the RCR complex

Owing to the different subcellular locations of PFRH5 versus PfCyRPA and PfRipr, the RCR complex likely only forms during invasion when the components are secreted from either the rhoptries or micronemes, at which point the complex forms on the surface of the invading merozoite^{21,59}. It was also recently shown in the rhesus malaria *P. knowlesi* that PkRipr forms a complex with two other proteins: cysteine-rich small secreted protein (PkCSS) and thrombospondin-related apical membrane protein (PkPTRAMP)⁸³. *P. knowlesi* lacks an orthologue of PFRH5 altogether; and, despite being present, PkCyRPA does not, surprisingly, interact directly with PkRipr, although both are still essential for invasion⁸³. This then raises the question of whether the potentially different ancestral roles of Ripr and CyRPA are retained in *P. falciparum* alongside their established function within the RCR complex mediating the PFRH5-basigin interaction. Moreover, it is not currently known what the functions of PkCSS or PkPTRAMP are in *P. falciparum*, and whether they still interact with PfRipr. Encouragingly, however, for vaccine development against the other human malaria parasite species, the authors also reported strong GIA when using antisera raised against the C-terminal EGF domain region of PkRipr⁸³.

A critical question for future vaccine development is the merits of targeting the wider set of RCR complex antigens, and to what extent this could lead to much higher levels of

vaccine efficacy than targeting a single component alone. Indeed, spreading immune pressure over at least three essential antigens, rather than just one, could be viewed as a distinct advantage. Studies are in their early stages, however, there has been some work examining the growth inhibitory activity of mixtures of antibodies that target the three RCR complex components. Evidence suggests that antibody mixtures to PfCyRPA, PfRH5 and/or PfRipr typically act additively, and in some cases synergistically, when combined in both *in vitro* and *in vivo* experiments^{59,66,69,102,103,107}. These differences potentially relate to the antibody clones that may dominate different polyclonal responses following immunization of different species with different vaccine formulations. In any case, either scenario (additivity and/or synergy) should be highly beneficial to vaccine efficacy outcome, assuming a mixture of vaccine antigens maintains the same quantitative levels of immunogenicity and does not lead to significant immune interference.

1.2.5 Other interacting partners

In addition to P113, which has already been discussed, RhopH3 and Cyclophilin B (CypB) have also been reported as potential binding partners, that may in turn associate with PfRH5-basigin¹⁰⁸. Whether or not RhopH3 is essential for invasion has yet to be determined, as some parasites expressing a truncated RhopH3 can still invade erythrocytes¹⁰⁹. The importance of cyclophilin B was recently called into question as a study on CypB knockout erythroblasts showed these cells could still support *P. falciparum* invasion¹¹⁰. RAP2 and basigin were also found to interact, but this was thought to be independent of the PfRH5-basigin complex¹¹¹.

1.3 *Malaria vaccines*

1.3.1 *Pre-erythrocytic malaria vaccines*

To date, the cornerstones of malaria eradication efforts have been vector control through expanded access to insecticide-impregnated bed nets and indoor residual spraying, alongside enhanced availability of anti-malarial medications to treat infected patients. Undoubtedly, these tools will continue to be essential to controlling and reducing the burden of malaria globally. However, modelling studies suggest that in high transmission settings, these tools alone will not be enough to eradicate the disease¹¹². To do so new tools will be required. The path to malaria eradication is further complicated by the possibility of existing tools falling into obsolescence as a result of the development of resistance: mosquitoes to pyrethroid insecticides¹¹³ and parasites to artemisinin combination therapy¹¹⁴. Fortunately, the pipeline of new anti-malarial drugs and insecticides remains healthy.

The only malaria vaccine to complete a phase III clinical trials is RTS,S from GlaxoSmithKline, which is currently undergoing pilot implementation in three countries: Ghana, Malawi and Kenya. RTS,S is a VLP-based vaccine, made of a circumsporozoite protein (CSP) fusion to hepatitis B surface antigen formulated in AS01 adjuvant. This vaccine is thought to work by inducing antibodies that target the CSP on the surface of the sporozoite^{115,116}. However, the exact mechanism underlying RTS,S induced immunity remains unclear. Anti-CSP antibodies are thought to cause precipitation of CSP off the surface of the sporozoite, thereby limiting motility^{117,118}. However, more recent studies have suggested that effector functions of antibodies, notably those related to neutrophil phagocytosis, are also critical to immunity¹¹⁹. RTS,S includes the NANP repeat region of CSP as well as the C-terminal

domain. It is the antibodies that target the NANP region that are thought to mediate immunity, with antibodies targeting the C-terminal domain being largely non-functional¹²⁰.

Studies of anti-CSP mAbs induced through whole sporozoite vaccination suggests that a critical protective epitope at the junction of the N-terminal domain and NANP repeats, may be missing from RTS,S^{121,122}. This may pave the way for a second generation CSP-based vaccine. Recently, however, the importance of this junctional epitope was called into question when it was shown that the strongest predictor of anti-CSP mAb efficacy was overall affinity for NANP repeats, which then often resulted in cross-reactivity with the junctional epitope¹²³. Antibodies that only bound exclusively to the junction were not more protective than those that bound NANP repeats¹²³. This suggests that in fact, RTS,S may have all of the necessary epitopes to elicit a potent antibody response, and thus vaccine formulation and dosing to optimize for antibody affinity remain more critical to vaccine performance than adding additional CSP epitopes.

R21, like RTS,S, is a VLP that fuses a portion of CSP to hepatitis B surface protein¹²⁴. The primary difference is that in RTS,S, conjugated and unconjugated hepatitis B surface antigen is mixed in a ratio of 1:4. R21, conversely, is composed of exclusively conjugated hepatitis B¹²⁴. The advantages of this approach remain largely unproven, but it has been speculated that it may be more immunogenic or allow dose sparing, which would be relevant for a vaccine that could be needed in the hundreds of millions of doses per annum^{43,124}.

Radiation attenuated sporozoites have proven to be effective in small studies in malaria unexposed individuals with high levels of protection against controlled human malaria infection (CHMI)¹²⁵. Efficacy was lower when tested in individuals living in endemic areas, and efficacy against heterologous strains was reduced compared to homologous

challenge, suggesting that breadth of protection may be a problem for this vaccine^{43,126}. Finally, the immunogenicity of this vaccine for individuals living in endemic areas appears significantly reduced¹²⁷. Four weeks post-vaccination, volunteers in the United States had ten-fold higher responses than Equatorial Guinean adults, five-fold higher in Malian adults and three-fold higher than Tanzanian adults¹²⁷. Weak antibody responses appeared to be associated with high levels of malaria exposure, which suggests that this approach will be least effective in high burden areas¹²⁷.

Another sporozoite-based approach to malaria prophylaxis is sporozoites under chloroquine drug cover whereby unattenuated live sporozoites are delivered intravenously and allowed to infect the liver in the presence of chloroquine, which will only kill the blood-stage parasites. The parasites can complete their liver-stage life cycle before emerging into the blood where they are killed by the chloroquine. This approach has shown a high degree of durable protection in CHMI studies¹²⁸. However, the shortcoming of this approach is that it seems to elicit strain-specific immunity¹²⁹. Further, the operational complexities of delivering live sporozoites with the additional requirement of taking a drug to prevent malaria infection belie the necessary mass scalability of any malaria vaccine.

Viral vectored vaccines that produce malaria antigen *in situ* have been explored. The most successful of these encodes multiple epitope string (composed of LSA1, LSA3, EXP1, STARP, CSP and TRAP) and thrombospondin related protein (ME-TRAP) has shown some, albeit modest (21%), efficacy following CHMI when delivered through a viral vector¹³⁰. It went on to show some efficacy in a field trial in Kenya (67% protection during 8-week follow-up)¹³¹, but then failed to confer any protection in a phase 2b field trial in Senegal¹³². Other viral vectored antigens including LSA1 and LSAP2 showed promise in preclinical studies, but results from initial efficacy studies are still awaited¹³³.

1.3.2 Erythrocytic (blood-stage) malaria vaccines

Vaccines that target the parasite during the blood-stage of its life cycle would not be required to provide sterile protection, as is the case with pre-erythrocytic vaccines. Rather the goal of these interventions would be to reduce parasite replication to a level that prevents the development of clinical symptoms and allows the immune system to clear parasites⁴³, as is observed in *Aotus* monkeys after vaccination with PfRH5⁶².

AMA1 and MSP1 have been explored extensively as vaccine targets in both protein-in-adjuvant formulations as well as viral vectored vaccines^{63,134–137}. Interest has waned in recent years with efforts consistently being hindered by antigenic diversity¹³⁸, though some interest in a multi-allele AMA1 vaccine persists^{139,140}. Still, AMA1 polyclonal sera has an EC₅₀ of approximately 100µg/ml antigen- (and strain-) specific IgG in GIA assays, before accounting for the challenges of antigenic diversity⁴³. Recently, it was found that vaccinating with AMA1 in complex with a fragment of its natural ligand RON2 (RON2L) resulted in a higher quality antibody response¹⁴¹. However, this was only tested in strain-specific GIA and homologous challenge; since AMA1 is polymorphic it is likely this approach will only induce strain-specific immunity^{141,142}.

The identification of the RCR complex as a blood-stage malaria vaccine target has addressed the problem of antigenic diversity, as all three antigens are highly conserved, as described in **Section 1.2**. As PfRH5 was the first member of the RCR complex to be identified as a promising vaccine candidate, vaccine development for this protein is the most advanced. A study in *A. nancymae* showed that both a protein-in-adjuvant formulation, as well as chimpanzee adenovirus-poxvirus prime-boost vectored vaccines, effectively prevented malaria after a heterologous challenge⁶². The protein-in-adjuvant formulation resulted in

sterile protection in 3/6 animals, with the other 3/6 self-curing after challenge, while never exceeding 10,000 parasites/ μL ⁶². All six animals in the control group required treatment by day 14 post-challenge⁶².

Given these promising results, first-generation PfRH5-based vaccines quickly progressed to human trials with the first results being reported in 2017⁹³. In this Phase Ia clinical trial, PfRH5 was delivered through intramuscular injection of recombinant chimpanzee adenovirus serotype 63 (ChAd63) with or without a modified vaccinia virus Ankara (MVA) poxvirus boost eight weeks later⁹³. Both viruses are replication-defective in humans, but express the full-length PfRH5 antigen *in situ* from virally-infected cells. The results from this study showed a favourable safety profile and the induction of anti-PfRH5 antibodies of approximately 10 $\mu\text{g}/\text{ml}$ at peak, far exceeding the aforementioned levels acquired through natural malaria infection⁹³. The vaccine-induced anti-PfRH5 polyclonal antibodies exhibited cross-strain functional GIA *in vitro* and inhibited key interactions within the RCR complex as well as between PfRH5 and basigin (as assessed using ELISA-based assays)⁹³. A Phase Ib field trial in Tanzania of the viral vectored PfRH5 vaccine is ongoing, with a dose-escalation and age-de-escalation study design (Clinicaltrials.gov NCT03435874).

A second-generation full-length PfRH5 protein vaccine was designed, using a *Drosophila* S2 stable cell line system for expression and C-tag purification technology^{143,144}. This protein, termed RH5.1, was subsequently manufactured to good manufacturing practice (GMP), and has been through a Phase I/IIa clinical trial in healthy UK adults formulated in GSK's AS01_B adjuvant (Clinicaltrials.gov NCT02927145), including a blood-stage *P. falciparum* CHMI study to assess vaccine efficacy. Although peer-reviewed publication of these results is awaited, these data are likely to lay important foundations on which future iterations of vaccines targeting the wider RCR complex will build.

As vaccine design is often an iterative process, development of future iterations of PfRH5 vaccines has continued as these earlier vaccine formulations make their way through clinical evaluation. This includes the successful heat stabilization of PfRH5, a potentially important characteristic for a vaccine component, which also enabled inexpensive and scalable expression in *E. coli*¹⁴⁵.

There are a number of other strategies that are currently being explored to try to address the persistent issue of needing high antibody quantity and potency. Antibody quantity may increase simply by moving the vaccine from studies in adults living in the United States and Europe to the target population, which is African infants, as has been observed previously¹⁴⁶. Other possibilities to improve quantity include more potent adjuvants, different vaccine formulations such as the use of VLPs, or different dosing regimens. Delayed fractional dosing, which is a vaccination regime where the final dose is administered in a much lower quantity and after a six-month delay^{147,148}, has been shown to boost peak antibody concentrations. To improve antibody potency, the main approach explored in this area to-date is the use of structure-guided vaccinology (**Section 1.3.5**), whereby the immune response is focused on regions of the antigen that are known to induce potent neutralizing antibodies¹⁰⁰.

1.3.3 Anti-disease vaccines

One subset of blood-stage vaccines is a category that has become known as anti-disease vaccines¹⁴⁹. Here, instead of preventing parasite invasion into the erythrocyte to limit parasite growth, antibody responses target the infected erythrocyte to prevent the most severe forms of disease. This approach was borne out of the observation that immunity

against severe malaria is rapidly conferred, unlike clinical immunity which requires repeated infection over many years.

Recently, glutamic acid-rich protein (PfGARP) was identified as a promising vaccine candidate¹⁵⁰. Anti-PfGARP antibodies were associated with protection from severe disease and this is thought to be mediated by antibody-induced programmed cell death in iRBCs¹⁵⁰. In a non-human primate study of PfGARP, animals vaccinated with mRNA expressing PfGARP showed reduced parasitaemia and clinical disease after heterologous challenge¹⁵⁰.

Another approach proffered is to elicit anti-VSA antibodies to block binding to their ligands thereby preventing adherence to the endothelium and/or rosette formation, which are mechanisms associated with manifestations of severe disease¹⁵¹. Such a vaccine could be clinically useful if it prevents the sequestration of iRBCs in the microvasculature, but it would have to overcome the immense sequence diversity between different VSAs¹⁵². Each of these multigene families are large and diverse, with the PfEMP-1 family having over 60 members¹⁵³ which are able to recombine, allowing the parasite to cycle between superficially different genes, evading antibodies that target these variable parts of the protein, while still retaining key binding properties¹⁵².

Initial results of vaccines targeting VSAs have proven disappointing. In an *Aotus* study that used a single domain of PfEMP1 protein MCvar1 in a protein-in-adjuvant formulation using MF59, an oil-in-water emulsion, animals were protected against severe disease in homologous challenge, but were not protected in heterologous challenge¹⁵⁴. Early results in clinical trials using the only known PfEMP-1 protein that is associated with pregnancy-associated malaria, VAR2CSA¹⁵⁵, confirmed that antigen diversity will pose a significant challenge¹⁵⁶.

1.3.4 Transmission Blocking Vaccines

The final category of malaria vaccines does not protect against disease at all, but rather limits the ability of one infected individual to transmit the parasite. As such, they are not particularly relevant to the work presented here, but will be described for completeness. So-called transmission blocking vaccines target antigens that are necessary for sporozoite development within the mosquito. During a blood meal, the mosquito takes up the sexual-stage parasites, along with antibodies targeting critical antigen(s) that then prevent parasite development thereby limiting onward transmission. Though there is no proven efficacy of this approach in large-scale field trials, it is often cited as a critical component of a multi-antigen multi-stage vaccine¹⁵⁷. The leading transmission blocking antigens are Pfs25, Pfs48/45 and Pfs230, all of which are still in early stages of clinical testing⁴³.

1.3.5 Structure-guided vaccinology

High antibody concentrations required for protection against malaria pose a challenge to the development of vaccines against all stages of parasite development. One approach to eliciting higher titre neutralizing antibodies is to focus the immune response on critical inhibitory regions of a given antigen through structure guided vaccinology.

Structure-guided vaccinology is an emerging field of biochemistry where bespoke synthetic immunogens are rationally designed to optimize the immune response elicited. There are three main applications of this approach¹⁵⁸. The first is germline immunisation, which is of particular interest for diseases where the types of antibody needed are rare, or poorly induced by the pathogen of interest. In the context of HIV, the challenge is that the known (hyper-mutated) broadly neutralizing antibodies (bnAb) do not bind to the HIV envelope protein in their germline state¹⁵⁹. Thus, a synthetic germline targeting immunogen

can be used to induce the expansion and maturation of this population of B cells that are capable of maturing into bnAb producing cells¹⁵⁹.

The second application is for the design of immunogens that can induce strain-transcending immunity when the pathogen is highly diverse¹⁵⁸. Chimeric antigens that incorporate different structural features from different pathogen strains can be designed with the goal of eliciting a humoral response that overcomes the antigen's diversity to provide strain-transcendent protection. This was used in the design of a synthetic version of factor H binding protein (fHBP), a highly diverse vaccine target from *Neisseria meningitidis*^{158,160}.

The final application is the induction of epitope-specific responses. This is the most relevant for PfRH5-PfCyRPA-PfRipr vaccines, where the challenge is neither antigen diversity (as all three complex members are highly conserved), nor induction of rare antibodies (as neutralizing antibodies against all three components can be routinely isolated from vaccinated individuals). Rather, the challenge is ensuring that neutralizing antibodies are sufficiently abundant in the polyclonal sera. This could be achieved by increasing total antibody concentration while keeping the fraction of neutralizing antibodies constant, or keeping the antibody concentration constant and increase the proportion of neutralizing antibodies. Realistically, both could be necessary.

If a large and complex antigen only has one important epitope (or region) where all of the neutralizing antibodies bind, then ideally, all antibodies elicited from vaccination would target this single epitope/region. This is the principle behind epitope-focused vaccine design. It has been applied to numerous infectious diseases including, but not limited to, HIV, influenzas A and B, chlamydia and lyme disease¹⁵⁸.

Epitope focused vaccine design can be done by truncating the original antigen, expressing instead only a single domain or region of the protein. Alternatively, if it is a highly specific epitope that is of interest, the epitope can be grafted onto a protein scaffold, maintaining the three-dimensional shape of the epitope of interest¹⁶¹.

For the PfRH5-PfCyRPA-PfRipr complex, this approach is being used for the redesign of the PfRH5 antigen using the structural data that mapped out the important neutralizing epitopes to the tip of the PfRH5 kite (**Figure 1.3**)^{19,68}. However at present, the Fab:antigen structure has only been solved for two weakly neutralizing anti-PfCyRPA antibodies and no PfRipr antibodies, although a weakly neutralizing anti-PfRipr antibody has been mapped to EGF-like domain 7. As a result, more structural information is needed, particularly with potently neutralizing antibodies, for PfCyRPA and PfRipr in order to move forward with epitope focused vaccine design for these antigens.

1.4 *Antibody synergy*

Two mAbs are said to act synergistically when the measured activity of the combination of the mAbs is greater than the predicted activity of the combination if each mAb acted entirely independently. That is to say, synergy implies that the action of two mAbs are not independent, but rather the presence of one mAb enhances the activity of another. This is not only relevant to mAbs, but indeed to all drugs. Exactly how the 'predicted activity' is defined remains an area of intense debate that is explored in depth in Chapter 5 of this thesis.

The use of drug combinations to enhance efficacy or reduce toxicity is not a new concept¹⁶². In the context of cancer, drug synergy has been investigated extensively, often with the goal of combining drugs with different toxicity profiles to enhance tumour-killing without a coordinated increase in toxicity¹⁶³. For infectious diseases, the possibility of synergy has often been considered when designing antibody cocktails whereby the combination could be more potent than any individual mAb delivered at the same concentration¹⁶⁴.

The mechanisms underlying synergy between two drugs vary widely, and often depend on whether the drugs target the same pathway, related pathways or separate pathways. A comprehensive review on synergy mechanisms was published a decade ago, but remains a good reminder of the creative ways that drugs can cooperate to enhance efficacy¹⁶⁵.

Canonically, synergy is often explained by allosteric effects where one antibody can lock a protein in a conformation that enhances the activity of a second antibody. This is a recognized mechanism of antibody synergy to block pathogen invasion¹⁶⁶. Conversely, one antibody could inhibit the binding of another allosterically by locking an antigen in an

unfavourable conformation. Alternatively, if two antibodies target redundant mechanisms within the same pathway, inhibiting one of these arms of the pathway may not stop invasion, but when both arms of the pathway are inhibited then the entire pathway is blocked. This is particularly relevant in malaria where redundant invasion pathways complicate vaccine design¹⁶⁷.

Antibody combinations are of interest in the context of malaria vaccines and mAb therapies because at present, even the best antibodies available still require a very high concentration of antibody to block parasite entry, meaning these can be extremely difficult to achieve through human vaccination, let alone maintain for durable immunity. However, if different combinations of antibodies, either administered directly or induced through vaccination, could work together 'synergistically', this would reduce the amount of circulating antibody required to achieve protection. Consequently, understanding how antibodies targeting specific epitopes can combine to enhance or hinder antibody-mediated inhibition can inform the design of vaccines that aim to elicit higher potency polyclonal responses.

1.5 *Thesis aims*

The goal of the work described in this Thesis is to further characterize the regions of PfCyRPA that are able to induce potent monoclonal antibody clones and then define these structurally, in order to establish foundational knowledge for future structure-guided vaccinology targeting this antigen. Then, I aimed to understand whether different neutralizing antibodies both targeting PfCyRPA can cooperate to enhance GIA.

- a. Chapter II describes the materials and methods used throughout this Thesis.
- b. Chapter III describes the characterization of a panel of anti-PfCyRPA antibodies in terms of their GIA, binding characteristics and ability to disrupt the RCR complex.
- c. Chapter IV describes the structural characterization of the most potentially inhibitory anti-PfCyRPA clones bound to PfCyRPA.
- d. Chapter V describes the assessment of synergy between different anti-PfCyRPA antibody clones and the mechanism underlying the observed synergy.
- e. Chapter VI brings the newly established insight from this thesis into context within the broader body of literature.

Chapter 2: Materials and Methods

2.1 Key methods overview

2.1.1 Assay of growth inhibition activity

The assay of growth inhibition activity (GIA), and its associated advantages and disadvantages, were introduced in the previous Chapter. It relies on the measurement of parasite invasion and subsequent growth over one 48-hour life cycle in the presence of a recombinant antibody or serum compared to media alone. This allows for the degree to which invasion (and/or growth) is inhibited by the antibody/serum to be determined. Throughout this Thesis, it was used extensively to assess ‘antibody potency’ and ‘synergy’ on the basis of how well an antibody or a combination of antibodies block parasite invasion in the GIA assay.

The maintenance of *P. falciparum* in culture, first reported in 1976¹⁶⁸, was foundational work enabling a greater understanding of parasite biology. Initial cultivation was done using HEPES-buffered RPMI supplemented with sodium bicarbonate and human serum. Since this initial cultivation success, *P. falciparum* culture media now routinely includes hypoxanthine as a purine source to improve parasite yields¹⁶⁹.

GIA assays rely on tightly synchronized cultures of parasites so that all invasion events occur at approximately the same time. This means that plate set-up can be coordinated with the life cycle allowing inhibitory compounds to be added during the trophozoite stage, immediately before the invasion events that will be measured. Here, treatment with 5% sorbitol was used to synchronize the cultures, which selectively lyses erythrocytes that are infected with parasites in the trophozoite and schizont stages due to their increased

permeability^{170,171}. Other methods, such as magnetic separation based on hemozoin, can be used to pull out stage-specific infected erythrocytes (schizonts, in the case of magnetic separation).

Once synchronized as ring-stage infected erythrocytes, the parasites are left to develop into trophozoites before cells are moved into 96-well plates where the invasion inhibitory compounds of interest are added. Then, when these erythrocytes rupture, they are able to reinvade uninfected erythrocytes and continue their life cycle. In order to measure the number of invasion events, many methods have been developed using microscopy, flow cytometry and various biochemical viability assays¹⁷². A distinction is sometimes made between growth inhibition activity and invasion inhibition activity. Biochemical viability assays provide measurements of growth inhibition, as they allow for the quantification of parasite intracellular viability, not only invasion¹⁷². Here, I relied on measurements of parasite lactate dehydrogenase activity to measure the number of viable parasites 48 hours after invasion. pLDH is useful in this respect as it is an enzyme of the glycolytic pathway and its ability to consume 3-acetylpyridine adenine dinucleotide (APAD) is taken as a measurement of the parasite's metabolic state¹⁷³. Since pLDH is distinct from host LDH, the APAD reaction occurs much more slowly with human LDH, thus enabling viable parasite quantification¹⁷⁴. A % GIA can then be determined on the basis of the quantification of viable parasites in the test sample relative to the control sample.

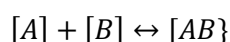
2.1.2 Surface plasmon resonance

Surface plasmon resonance (SPR) is a biophysical technique that can be used to detect interactions between an immobilized ligand and a free moving analyte. It is commonly used to assess ligand-receptor, protein-carbohydrate, and protein-DNA interactions, among

others¹⁷⁵. Here, it was applied to measure kinetics and affinity of antibody-antigen interactions, as well as to conduct antibody competition assays.

The principle of SPR is that changes in the angle of reflection of the minimum intensity of a wave of polarized light is proportional to the refractive index of the material, which changes with the mass concentration immobilized on a sensor¹⁷⁵. A chip coated with a thin metal film, in this case gold, resonates with the wave of polarized light at a particular incident angle resulting in the absorption of the light at that angle, which appears as a band of minimum intensity that can be measured by a detector¹⁷⁵. As the mass concentration on the chip increases, the resonance angle shifts proportionally to quantity of immobilized mass. In the context of biomolecular interactions, this shift in resonance angle allows for the calculation of binding parameters including kinetics and affinity based on the rates of mass accumulation, and thus shift in the SPR angle, during the association phase when analyte is injected and the rate of dissociation after binding has stopped.

Antibody binding kinetics describes how fast an antibody can bind to its target (k_a) and how quickly it falls off again (k_d) (**Equation 2.1**). Together these values describe the affinity (K_D) (**Equation 2.2**). More precisely, k_a describes the number of complex formation events per unit time, which is dependent on both the concentration of ligand and analyte ($M^{-1} s^{-1}$); k_d describes the fraction of complexes that decay per unit time and is measured in s^{-1} .



Equation 2.1 Complex kinetics are defined by the rate of association (forward reaction measured in $M^{-1} s^{-1}$) and dissociation (reverse reaction measured in s^{-1})

$$K_D = \frac{[A][B]}{[AB]} = \frac{k_d}{k_a}$$

Equation 2.2 Affinity constant is defined in terms of the rate of complex dissociation over association.

When the dissociation of a complex is too fast (this is machine dependent, but this work uses the Biacore X100, which has an upper-limit of $0.1 s^{-1}$), affinity cannot be calculated based on binding kinetics as shown above. Instead, overall affinity can be determined based on steady-state measurements. At steady state, the rate of complex decay is equal to the rate of complex formation (**Equation 2.3, 2.4**).

$$\frac{d[AB]}{dt} = k_a[A][B] - k_d[AB]$$

Equation 2.3 Change in complex concentration is the difference between complex formation and decay.

$$0 = k_a[A][B] - k_d[AB]$$

$$k_a[A][B] = k_d[AB]$$

Equation 2.4 At steady-state, the rate of complex formation is equal to the rate of decay.

Using a dilution curve of analyte of a known concentration, the affinity can be calculated (**Equation 2.5**). Running this experiment in a dilution curve beginning at 10x the K_D allows for the R_{max} to be estimated, which describes the measured response when all analyte binding sites are occupied. For a given dilution curve, R is the response measured at steady-state for one concentration of analyte.

$$k_a[C][R_{max} - R] = k_d[R]$$

$$K_D = \frac{C \cdot R_{max} - C \cdot R}{R}$$

$$K_D = \frac{C \cdot R_{max}}{R} - C$$

Equation 2.5 Determination of the affinity constant based on Rmax, R and analyte concentration.

When the analyte concentration is equal to the K_D , the R is equal to 50% of the R_{max} (**Equation 2.6**).

$$1 = \frac{R_{max}}{R} - 1$$

$$R = \frac{R_{max}}{2}$$

Equation 2.6 R is Rmax/2 when analyte concentration is equal to the affinity constant.

Although steady-state measurements allow for the calculation of the overall affinity of an antibody-antigen interaction, this readout lacks critical kinetic data. Due to the fact that affinity is relative to both association and disassociation rate, an antibody with very slow association and very slow dissociation will show the same affinity as an antibody with rapid association and rapid dissociation. In the case of anti-PfPRH5 antibodies, greater inhibition of parasite invasion was associated with faster association rather than slower dissociation. As such, the k_a was better correlated to % GIA compared to the K_D ⁶⁸. This relationship has also been noted for neutralization of vesicular stomatitis virus¹⁷⁶. For this reason, the kinetic data provides additional information regarding the underlying biological processes and, where possible, should be collected instead of steady-state affinity data.

2.1.3 X-ray crystallography

Growing Crystals

The principle of X-ray crystallography, as applied to understanding the structure of proteins, is that diffraction data from X-rays diffracted from an ordered array of protein molecules can be measured to obtain information about the intensity of reflections across a given plane. This, when combined with phase information (either experimentally derived or determined through molecular replacement) can be used to provide a map of electron density in the crystal from which a protein model can be built.

Protein crystallization remains an empirical process. Crystallization occurs when a protein sample is mixed with a crystallization condition that contains a precipitant. This drop of protein-crystallization condition is left in the presence of a reservoir of the crystallization condition. Slowly, the concentration of water in the protein sample and the reservoir equilibrate, but due to the lower precipitant concentration in the reservoir, the result is that water is removed from the protein sample ideally resulting in nucleation and crystal growth. Typically, the process of crystallization requires the screening of hundreds of conditions, which vary in precipitant, pH and salt content, in a 'broad screen' and then conditions that yield crystals can be further optimized. This optimization usually involves slight changes to pH, salt concentration or precipitant concentration, but can also include an 'additive screen', which includes a variety of chemicals (amino acids, chelating agents, detergents, carbohydrates etc.) that can alter the conditions of crystallization and yield better diffraction data. For single crystal experiments, crystals between 100-500 μ M across all three dimensions are typically used. Smaller crystals can be used particularly when using serial crystallography approaches, but these remain outside the scope of the work described here.

After crystallization, crystals are harvested which involves ‘fishing’ the crystal through a nylon loop. Since most crystallography is carried out under cryogenic conditions, crystals must be cryoprotected before harvesting, as they are then plunged into liquid nitrogen to enable vitrification. Cryogenic temperatures limit beam damage by reducing the spread of free radicals generated through high energy X-rays through the crystals¹⁷⁷.

Data collection

Data collection was carried out on synchrotrons with wavelengths equal to approximately 1Å at 100K. To collect data, a crystal-in-a-loop must be mounted on a goniometer and centred on the beam. Three images at 0°, 45° and 90° were taken initially to determine approximate diffraction resolution and index the data using the pipeline available through the Diamond Light Source platform (MOSFLM¹⁷⁸ and EDNA¹⁷⁹). These programs use a Fast Fourier Transformation (FFT) approach to rapidly index the data¹⁷⁸. The process of indexing and space group determination is based solely on spot presence or absence during this process, which can give rise to pseudosymmetry, which can only be deconvoluted by looking at intensities. Still, based on this initial indexing process, a data collection strategy that is defined by a starting angle and degrees of rotation required is suggested to give the highest likelihood of a complete dataset with the fewest degrees of rotation.

A full dataset is collected through small angle rotations on the goniometer. After dataset collection, the unit cell dimensions and space group for the full dataset must be determined, which here was done using Pointless¹⁸⁰. After indexing reflections were scaled and merged. Scaling relies on the known symmetry of the crystal to adjust differences in reflection intensity caused by systematic changes in the experimental conditions (e.g. exposed area of crystal, beam intensity) between images¹⁸¹. Intensities can be scaled to

account for these differences and then merged to establish an average intensity for a given reflection. The number of times a given reflection is measured is referred to as the multiplicity.

The data quality control throughout this process is essential and there are a number of key metrics that are assessed. First, an $I/\sigma I$ cut-off is often used. This is the signal to noise ratio that is allowed during the spot finding process. Higher resolution reflections have weaker intensity and thus, the $I/\sigma I$ cut-off impacts the resolution cut-off. A cut-off of approximately 2 is typical. Alternatively, and perhaps more reliably, a metric known as $CC_{1/2}$ (correlation coefficient) can be used. Here, reflections are split into subsets x and y to determine a correlation coefficient between the two groups based on the Pearson correlation coefficient formula¹⁸² (**Equation 2.7**). As variation in the intensity measurements increases, the $CC_{1/2}$ decreases. A typical $CC_{1/2}$ cut-off is between 0.3 and 0.5.

$$CC_{1/2} = \frac{\sum(x - \bar{x})(y - \bar{y})}{\sqrt{\sum(x - \bar{x})^2 \sum(y - \bar{y})^2}}$$

Equation 2.7 $CC_{1/2}$ equation where x and y are two halves of a set of intensity measurements for the same reflection.

The R_{merge} is a measure of data consistency and is the variation in intensity across multiple measurements of the same reflection (**Equation 2.8**). This measurement typically increases with multiplicity as it does not account for the added confidence of the average intensity with increasing multiplicity. As a result, a similar measurement, the R_{pim} , is now widely used which incorporates an additional term ($\sqrt{\frac{1}{N-1}}$) where N is the number of measurements of a given reflection, to address this.

$$R_{merge} = \frac{\sum_{hkl} \sum_{i=1}^n |I_{(hkl)i} - \bar{I}_{(hkl)}|}{\sum_{hkl} \sum_{i=1}^n I_{(hkl)i}}$$

Equation 2.8 Calculation of the R_{merge} where R_{merge} is calculated from the difference of each intensity measurement from the average intensity of that measurement.

Determining phases from diffraction data

X-ray diffraction from crystalline structures yields reflections when Bragg's law is satisfied (**Equation 2.9**). That is, when the distance between planes is equal to an integral multiple of the wavelength. When this condition is satisfied, the waves interfere constructively and produce a reflection that can be detected. Otherwise, the waves will be out of phase and interfere destructively, and no reflection is observed.

$$2d_{hkl} \sin \theta = n\lambda$$

Equation 2.9 Bragg's law describes the conditions that produce diffraction where $2d_{hkl} \sin \theta$ is equal to the difference in distance between two waves interfering constructively, n is any integer and λ is the wavelength.

Each diffraction spot, called a reflection, is based on the electron density across a single set of planes described by their miller indices: h , k and l . A diffraction pattern is defined by the unit cell dimensions of a given lattice while the intensity of the spots is based on the structure of the protein. Each detected reflection is the sum of contributions from each individual atom in the unit cell. This is known as the structure factor which can be expressed as follows:

$$F_{hkl} = \sum_{j=1}^n f_j e^{2\pi i(hx_j + ky_j + lz_j)}$$

Equation 2.10 The structure factor (F) equation describing the contributions of atom j at position x , y , z in the unit cell to reflection h , k , l where f is the amplitude.¹⁸³

In order to obtain an electron density map, the goal of a crystallography experiment, structure factors can be used to obtain electron density data through a Fourier transform as shown in **Equation 2.11**.

$$\rho_{(x,y,z)} = \frac{1}{V} \sum_h \sum_k \sum_l |F_{hkl}| e^{-2\pi i(hx+ky+lz-\phi_{hkl})}$$

Equation 2.11 Electron density equation describing the electron density as a Fourier sum of structure factors where V is the unit cell volume, F is the amplitude (proportional to $\sqrt{I_{hkl}}$), and where ϕ is the phase of the reflection¹⁸³.

As indicated by the electron density equation, the phase of a given reflection is required, but it cannot be determined from the reflection data alone, which only contains intensities. There are three primary methods of determining the phase information, also known as ‘solving the phase problem’: isomorphous replacement, anomalous scattering and molecular replacement. Both isomorphous replacement and anomalous scattering rely on the presence of heavy atoms, which can be added through heavy atom soaks, while molecular replacement does not¹⁸³. Instead molecular replacement relies on a highly similar structure having already been solved (>30% sequence identity), which is then used to ‘guess’ the phase information. Here, because PfCyRPA has been solved^{66,72} previously, as have many Fab structures, search models to enable molecular replacement were readily available and thus, this was the approach used.

If the unit cell dimensions are identical, as is often the case if a protein is crystallized in the same way but with a variety of different ligands, then phases can be taken directly from the search model to the unknown structure as a starting point for refinement. However, here, because different Fab fragments were being co-crystallized with PfCyRPA, the unit cell

dimension changed between crystallization conditions meaning this approach was not possible.

Instead, when the unknown structure has crystallized with different unit cell dimensions, the position of the search model relative to the unknown structure's unit cell dimensions must be determined. In this work, Phaser¹⁸⁴ was used to carry out molecular replacement. Phaser relies on a two-step rotation-translation function in order to orient the search model, whereby the position of the search model placement is optimized through a maximum likelihood approach, which is probabilistic, to provide the orientation that is most statistically likely. Maximum likelihood replaced the Patterson methods that were previously used for molecular replacement. A Patterson map contains only amplitudes, but no phase information, as all phases are set to 0. Then, the rotation and translations of the search model that best align the known structure's Patterson map with the unknown structure's map can be used to determine the molecular replacement solution. Both methods proceed in by two-step rotation and translation functions, but maximum likelihood has the advantage that it is better able to address discrepancies between the search model and the unknown structure.

Refinement and Model Building

Models are improved through alternating cycles of real space model building and reciprocal space refinement to improve both the electron density map and the model. As the model is improved, the phase information that determines electron density is also updated, which should lead to improvements in agreement between the model and the electron density map. This introduces the problem of model bias whereby an incorrect model is used to update phase information resulting in the electron density map aligning with the model, regardless of its actual correctness.

For model building in real space, two electron density maps are used, $2F_{\text{obs}} - F_{\text{calc}}$ and $F_{\text{obs}} - F_{\text{calc}}$, which was carried out in Coot¹⁸⁵. The former is the typical map that shows the density contained in the unit cell, while the latter is the 'difference map' which shows where the model and the electron density do not agree. This indicates areas where there is density in the model, but not in the map, or alternatively, where the model may be missing sources of electron density. The difference map guides the construction of missing regions that are not included in the search models, such as Fab CDR loops, which are likely to differ between the search model and unknown structure and thus removed prior to molecular replacement.

After a cycle of model building, refinement was carried out in Buster¹⁸⁶. Buster refinement involves a number of steps, but after molecular replacement, initial refinement cycles begin with a rigid body refinement where each chain of the model is refined as a collective unit. After this, individual atoms are refined based on their x,y,z coordinates and B factors that result in highest agreement between the experimental data and the theorized model. During this process, the models are restrained based on known physical parameters, such as bond lengths and angles. Other restraints typically used ensure that multiple copies of the same protein/protein complex in the asymmetric unit do not deviate substantially (non-crystallographic symmetry restraints). Alternatively, if there is a higher resolution solution to a structure, such as for PfCyRPA which was solved to 2.44Å⁷², external restraints can be used which ensures agreement between the new lower resolution structure and the higher resolution structure.

Ultimately, the quality of a model is assessed based on its agreement with known geometrical restraints (dihedral angles, rotameric conformers, clashes) and the agreement between the model and the experimental data (R_{work} and R_{Free}) (**Equation 2.12**).

$$R = \frac{\sum |F_{obs}| - |F_{calc}|}{|F_{obs}|}$$

Equation 2.12 R_{work} measures the difference between the measured structure factors and those calculated based on the model.

In order to address the problem of over fitting, R_{free} is used which involves the same calculation as above but on a random subset of data that was kept aside during refinement. In the case of overfitting, the R_{work} may improve but the R_{free} should not. Thus, significant deviations (>10%) between these two parameters is indicative of overfitting.

2.2 Chapter III methods

2.2.1 Plasmid preparation

Transformed *Escherichia coli* cultures were grown in 150ml of Miller Luria broth for 18 hours at 37°C in an orbital shaking incubator (225 rpm). After incubation, 100ml of culture was harvested via centrifugation at 6000×g for 15 minutes. DNA extraction was carried out using a Plasmid *Plus* Maxi kit (Qiagen) according to the manufacturer's protocol, except for the elution step when DNA was eluted in water instead of TE buffer. After purification, plasmid DNA was sterile filtered through a 0.22µm spin filter (Sigma-Aldrich) prior to transfection.

2.2.2 HEK Expi293 culture maintenance

Cells were maintained in suspension in Expi293 expression medium (Thermo Fisher Scientific) at 37°C in a shaking incubator at 120 rpm. Cells were split to 0.5×10^6 cells/ml every Monday and Wednesday and to 0.3×10^6 cells/ml on Fridays. Before transfection, cells were split to 1×10^6 cells/ml the day prior to transfection.

2.2.3 Recombinant protein expression in mammalian cells

HEK Expi293 cells (Thermo Fisher Scientific) were transfected following the manufacturer's protocol. Briefly, cells were grown to a density of 2×10^6 cells/ml for the day of transfection. Per 100ml of cells, 100µg of plasmid was diluted in 5ml of opti-mem reduced serum media (Thermo Fischer Scientific) and 267µL of expifectamine (Thermo Fisher Scientific) was diluted in 5ml of opti-mem reduced serum media. After five minutes, the plasmid and expifectamine solutions were mixed and incubated at room temperature for 20

minutes to allow for complex formation. The plasmid/expifectamine solution was then added directly to the cells and incubated for 18 hours at 37°C. After 18 hours, 500µL of enhancer 1 and 5ml of enhancer 2 (Thermo Fisher Scientific) were added to the cells. The cells were incubated for a further three days before harvesting the supernatant via centrifugation at 4°C and 3000×g for 15 minutes.

2.2.4 mAb purification

Four antibody monoclonal plasmids were sent from collaborators at the National Institute for Biological Standards and Controls (Cy.003, Cy.004, Cy.007 and Cy.009) (**Figure 2.1**). Cy.001, Cy.002, Cy.005 and Cy.010 were derived from mouse hybridomas, where variable domains were sequenced (GeneArt, Thermo Fisher Scientific), codon optimized for human expression (GeneArt, Thermo Fisher Scientific) and then cloned into a previously described hIgG1 backbone⁶⁸ to enable transient transfection. The same approach was taken for c12 and 8A7, except in this case, sequences were retrieved from the amino acid sequences associated with their structures deposited on the protein databank^{66,72}. Antibody plasmids were transiently transfected, as described above. The antibodies were purified from the harvested supernatant in two steps using an Äkta pure (GE Healthcare). Harvested supernatants were run over a 5ml protein G column (GE Healthcare) at 5ml/min, followed by a wash step of 20 column volumes (CVs) using TBS (150mM NaCl, 20mM Tris pH 7.4). Antibody was eluted in 1.6ml fractions in glycine (200mM pH 2.0) into Tris buffer (1M, pH 9.0). Next, fractions were pooled and concentrated to 2ml before size exclusion chromatography. Concentrated fractions were purified in TBS using an S200 16/600 column (GE Healthcare). For storage, protein samples were flash frozen in liquid nitrogen in 1ml aliquots.

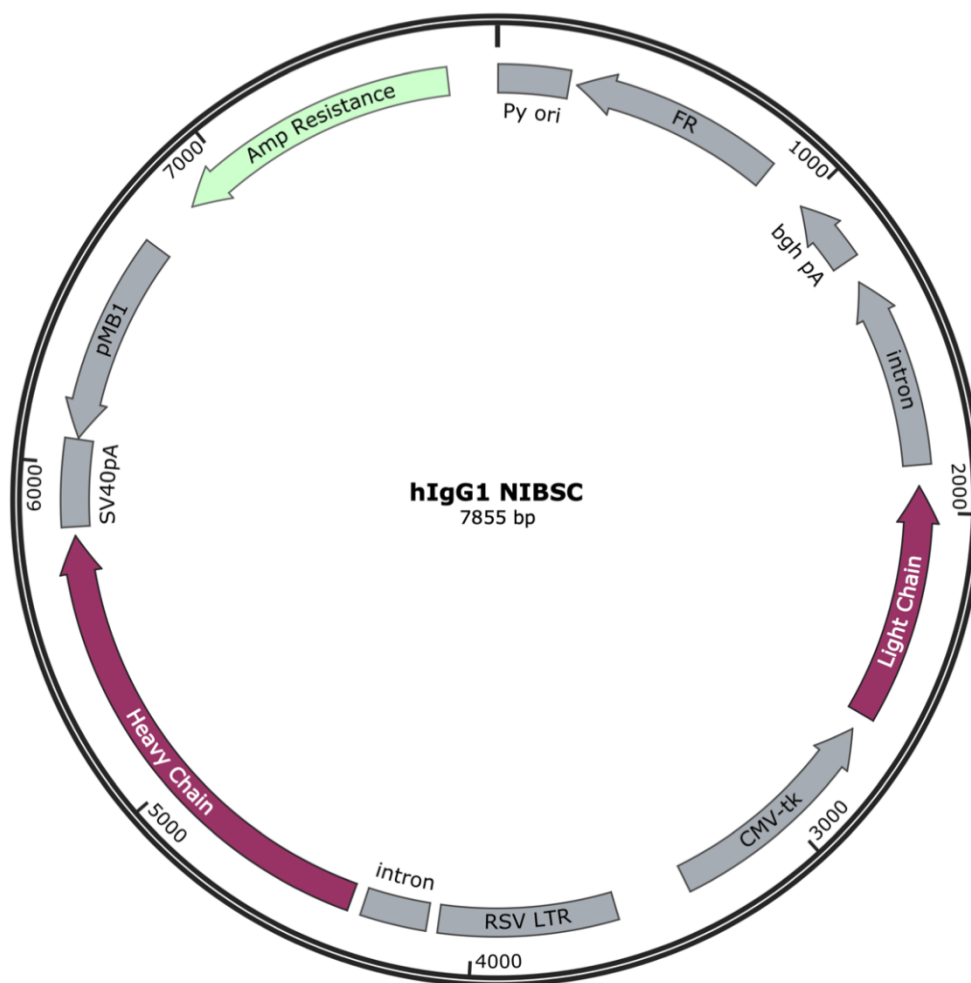


Figure 2.1 hlgG1 expression vector for Cy.003, Cy.004, Cy.007 and Cy.009.

2.2.5 *PfCyRPA expression and purification*

Production of recombinant PfCyRPA from mammalian expression systems has been previously described^{66,68,72}. The PfCyRPA construct (**Figure 2.2**) used was based on the PfCyRPA sequence from the 3D7 strain encompassing amino acids 29 – 362¹⁸⁷. A mammalian secretion peptide, MEFQTQVLMSLLCMSGAAA, was added upstream of residue 29 to enable secretion from a mammalian expression system, along with a four-glycine linker followed by an EPEA ag (C-tag) on the C-terminus. The following mutations were introduced to remove three potential N-linked glycosylation sites: S147A, T324A and T340A. Transfection and supernatant harvest followed the same protocol as described in **2.2.3**. Following supernatant harvest, PfCyRPA was purified in two steps: first using a 10ml column packed with CaptureSelect C-tag affinity resin (Thermo Fisher Scientific), harvested supernatants were run over the column at 5ml/min and then washed for 10CV with TBS; second, PfCyRPA was eluted using 2M MgCl₂ in 1ml fractions. Fractions were pooled and concentrated to 2ml before size exclusion using an S200 16/600 column (GE Healthcare) on an Äkta pure (GE Healthcare) into TBS (150mM NaCl, 20mM Tris pH 7.4). For storage, protein samples were flash frozen in liquid nitrogen in 1ml aliquots.

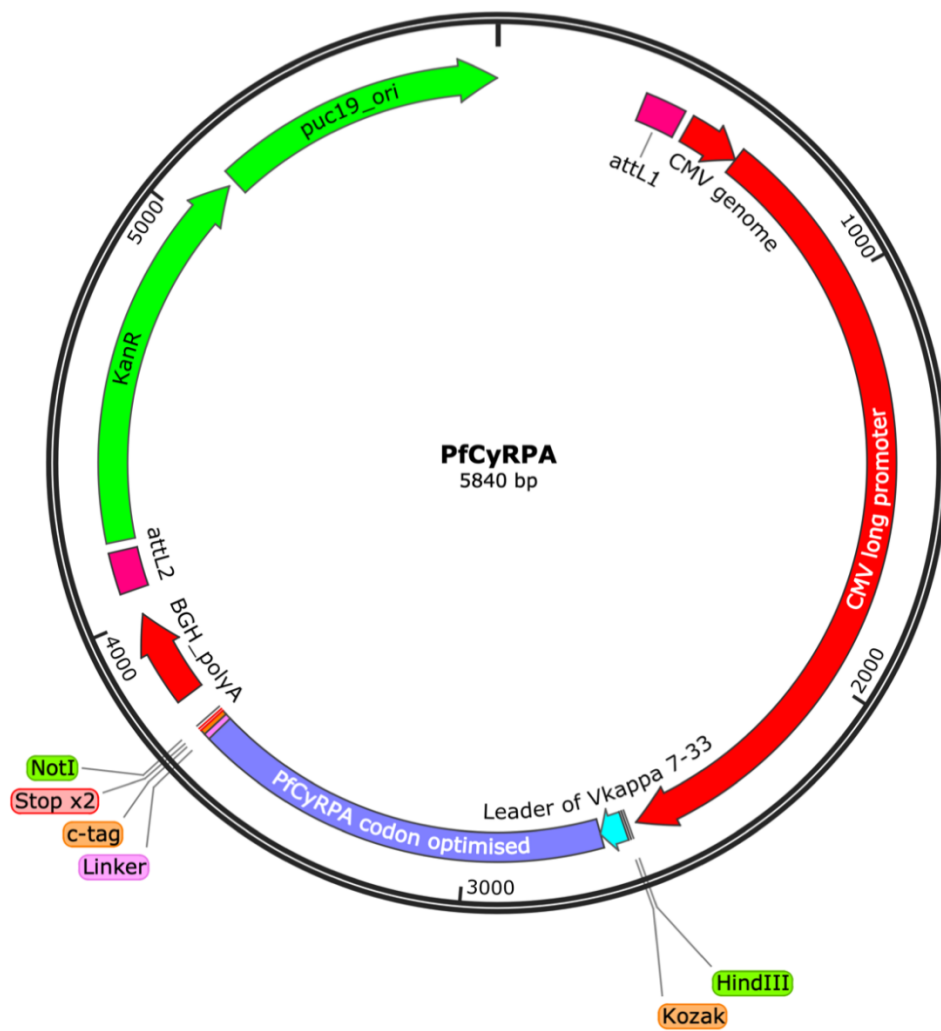


Figure 2.2 PfCyRPA expression vector map.

2.2.6 *PfRH5 Expression and purification*

Production of recombinant PfRH5 was previously optimized within the lab as part of the good manufacturing process development for the PfRH5 protein-in-adjuvant clinical trials (where the same protein was called 'RH5.1')¹⁴⁴. Briefly, PfRH5 was expressed in a *Drosophila* S2 line. S2 cells, stably expressing PfRH5, were grown at 25°C at 115 rpm in an Innova 44 shaking incubator (New Brunswick Scientific) in the presence of penicillin and streptomycin (100 IU/ml and 100µL/ml, respectively). The PfRH5 sequence here was based on the 3D7 clone of *P. falciparum*¹⁸⁷ with four mutations to remove putative glycosylation sites (T40A, T216A, T286A and T299A) and a C-terminal C-tag (EPEA). Cells were harvested in 2L batches and then subjected to tangential flow filtration (PALL) with a 10kDa cut-off. Subsequently, PfRH5 was purified using the same two-step purification as PfCyRPA: affinity purification using a 10ml CaptureSelect C-tag affinity resin (Thermo Fisher) and size exclusion chromatography using an S200 16/600 column and Äkta pure (GE Healthcare) in into TBS (150mM NaCl, 20mM Tris pH 7.4). For storage, protein samples were flash frozen in liquid nitrogen in 1ml aliquots.

2.2.7 *Fab digestion and purification*

Fab fragments (**Figure 2.3**) are typically made through one of two methods: recombinant expression of only the variable and first constant domains of the heavy chains or expression of the entire mAb and then proteolytic cleavage of the parental mAb into two Fab fragments and an Fc fragment. For genetic expression, these can then be purified through affinity purification with an affinity tag (if included in the construct); protein G has a weak affinity for the first constant domain, allowing for purification on protein G columns; or protein L, but protein L only binds to kappa light chains, making it useful for approximately

two thirds and half of the mouse and human antibody repertoire, respectively.¹⁸⁸ Here, for the experiments described in Chapter 3, Fabs were produced through papain digestion. Papain is a thiol-endopeptidase which cleaves between the threonine-histidine in the CDKTHT motif in the mAb heavy chain yielding two Fab arms and an Fc fragment¹⁸⁹.

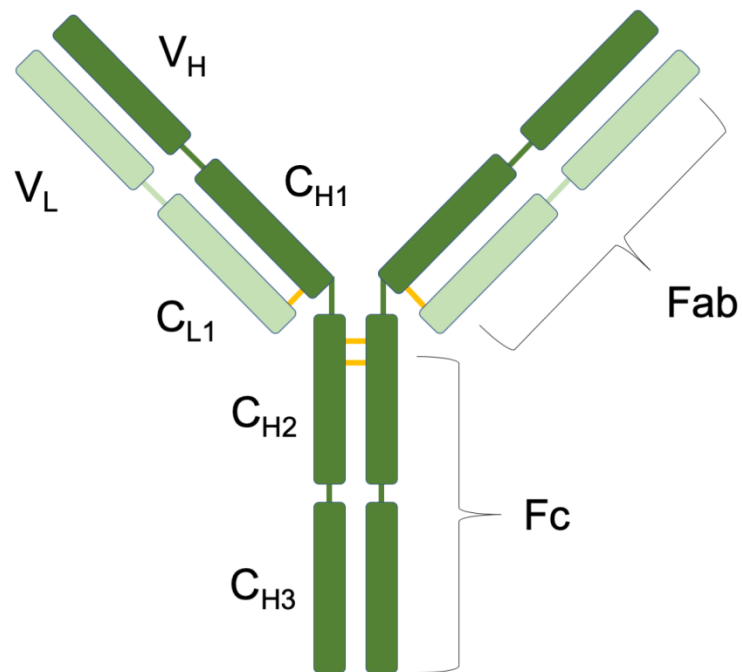


Figure 2.3 Illustration of the human igG1 structure indicating where the Fab and Fc arms are located.

Antibody Fabs were produced through papain digestion of parental mAb. 4mg of parental mAb was buffer exchanged into digestion buffer (20mM Na₃PO₄, 10mM EDTA, 25mM cysteine, pH 7.0) and then concentrated to 500μL, 8mg/ml. 250μL of agarose-resin-immobilized papain (Thermo Fisher Scientific) was washed twice in digestion buffer via centrifugation at 1000×g for 1 minute and then aspirated. Antibody was added to the immobilized papain and left shaking at 37°C for six hours. Next, the entire mixture was added to a 1ml spin column (Thermo Fisher Scientific) to remove the agarose resin. The agarose was washed twice with 1ml TBS and the flow through pooled. Next, a 1 ml protein A column (Thermo Fisher Scientific) was equilibrated in TBS (150mM NaCl, 20mM Tris pH 7.4) before the addition of the Fab flow through. Fab flow through was incubated with the protein A resin for 10 minutes at room temperature. Next, the flow through was collected through centrifugation and two subsequent washes with 1ml TBS. The bound Fcs and undigested mAbs were then eluted in 3ml glycine pH 2. Purified Fab was then subject to size exclusion chromatography using an S200 10/300 (GE Healthcare) into TBS.

2.2.8 SDS-PAGE

For reducing SDS-PAGE, samples were mixed with 25mM DTT (Thermo Fisher Scientific) and Laemelli buffer (BioRad) and then boiled for 10 minutes at 95°C. 15μL of sample was loaded into a 4-12% precast NuPAGE gradient polyacrylamide gel (Thermo Fisher Scientific) at 200V for 45 minutes in MES buffer pH 6.5. For non-reducing gels, the same procedure was followed with the omission of the DTT. After electrophoresis, protein gels were stained for 1-2 hours with Quick Coomassie stain (Generon), followed by washing in water for 2 hours, both carried out with continuous rocking.

2.2.9 Dot blot-based PfCyRPA binding

100ng of native state or denatured (boiled at 95°C for 5 minutes and reduced using 5mM dithiothreitol) PfCyRPA protein was blotted on to a nitrocellulose membrane (BioRad) and allowed to dry. The mAb of interest was prepared at a concentration of 1µg/ml in iBind Flex buffer (Thermo Fisher) and the secondary antibody, goat anti-human IgG alkaline phosphatase conjugate (Sigma-Aldrich), was diluted 1/1000 in iBind flex buffer (Thermo Fisher Scientific). The iBind blotting system was used for all incubation and wash steps, according to manufacturer's instructions (Thermo Fisher Scientific). After wash steps were completed, the membrane was rinsed in distilled water before the addition of SigmaFast BCIP/NBT (5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium, Sigma-Aldrich) to enable detection. The membrane was incubated with SigmaFast substrate for 120 seconds before washing with distilled water, air drying and scanning.

2.2.10 ELISA-based PfCyRPA binding

96-well NUNC immunoplates (Thermo Fisher Scientific) were coated with 50µL of either native state or denatured (boiled at 95°C for 5 minutes and reduced using 5mM dithiothreitol) PfCyRPA at 2µg/ml overnight at 4°C. The plates were then washed with PBS/Tween-20 (PBS-T) (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 0.05% Tween-20) 3 times. Next, plates were blocked with PBS (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄) with 1% casein for 60 minutes at room temperature. Plates were then washed with PBS-T before the addition of 50µL of mAb at 10µg/ml, diluted in PBS-casein. mAb solution was incubated for 60 minutes at room temperature before washing 3 times with PBS-T. Next, 50µL of goat anti-human whole IgG alkaline phosphate conjugate (Sigma-Aldrich), diluted 1/1000 in PBS with 1% casein, was added to each well and then incubated

for 60 minutes at room temperature. Then, plates were washed 6 times. Next, a 20mg tablet of 4-Nitrophenyl phosphate (pNPP, Sigma-Aldrich) was dissolved in 4ml of diethanolamine buffer (Thermo Fisher Scientific) with 16ml deionized water. 100µL of pNPP was then added to each well and plates were allowed to develop until the positive control (mAb c12) reached an OD₄₀₅ of approximately 2.0. The anti-PfRH5 mAb, R5.011⁶⁸, was used as a negative control as it should not bind to PfCyRPA. All samples were run in triplicate.

2.2.11 P. falciparum culture maintenance

Parasites were cultured in complete culture medium (RPMI, 10% heat inactivated human serum, 20 µg/ml gentamycin, 2mM L-glutamine, 0.05g/L hypoxanthine, 5.94g/L HEPES) at 37°C at 2% haematocrit in a 5% O₂, 5% CO₂, 90% N₂ atmosphere using unvented flasks. Parasites were split every Monday and Wednesday to 0.5% parasitaemia and Fridays to 0.3% parasitaemia.

2.2.12 P. falciparum assay of growth inhibition activity

On day one, parasite cultures with >2% parasitaemia were synchronised through incubation with 5% sorbitol at 37°C at 09:00 and again at 16:00. The synchronised ring stage parasites were then allowed to develop into mid-trophozoites over the course of 20 hours after the second synchronisation. On day two, the synchronized culture was used to make a solution of 2x complete culture medium with 0.4% parasitaemia and 2% haematocrit, which was then diluted 1:2 into the antibody containing culture media. All samples were run in triplicate with a positive control (anti-PfRH5 antibody 2AC7⁶⁷) at 200µg/ml, 25 g/ml and 5.25µg/ml and a negative control (anti-Ebola mAb EBL040¹⁹⁰), as well as 6x wells with no antibody, 6x wells with 5mM EDTA (100% GIA equivalent) and 3x wells with uninfected

erythrocytes. Parasites were then returned to the incubator where they stayed until day four. On the morning of the fourth day, erythrocytes were washed twice with cold PBS (4°C) before aspirating and freezing the cell pellets. Then, end parasitaemia was determined by measuring parasite lactate dehydrogenase activity. 3-acetylpyridine adenine dinucleotide (APAD, Sigma) stock solution was prepared at 10mg/ml alongside 2.5mg/ml nitro blue tetrazolium (NBT, Sigma) solution and diaphorase at 50units/ml (Sigma). 50µL of APAD stock solution and 200µL of Diaphorase stock solution were added to 10ml of NBT solution. 120µL of substrate was added to each well before being read on a Biorad ELISA Reader at 650nm until the OD was between 0.4-0.6 in the iRBC media-only control. % inhibition was calculated as:

$$\% \text{ inhibition} = 100 - \left(\frac{A650 \text{ sample of interest} - A650 \text{ RBC only}}{A650 \text{ negative antibody control} - A650 \text{ RBC only}} \times 100 \right)$$

Equation 2.13 Calculation of % GIA.

2.2.13 Antibody affinity and kinetics through SPR

Purified mAb was immobilized on a protein G chip through a 30s injection of 20nM mAb. PfCyRPA was diluted in running buffer (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 0.005% surfactant P20 (GE Healthcare)) to yield a top concentration of 500nM (or 1µM, where required), used to produce an 8-step, 2-fold dilution curve. After five start-up cycles, samples were injected for 30s at 30µL/min before allowing for dissociation for 600s. The chip was then regenerated with a 45s injection of 10mM glycine pH 1.5. In each curve, one concentration was run twice to ensure within-run consistency. Data was analysed using the Biacore X100 Evaluation sample. Where possible, a global Langmuir 1:1 interaction model was used to determine antibody kinetics. For mAbs with rapid dissociation (Cy.004 and

Cy.009), affinity was determined through steady-state affinity analysis using the same evaluation software.

2.2.14 SPR Competition Assays

In the absence of a dilution curve, SPR can also be used for detecting binding as a binary response (binds vs. does not bind). This approach was used to generate competition matrices where binding of one antibody was measured when the analyte was PfCyRPA bound to a different Fab or to an RCR complex member. Fab: PfCyRPA, PfRH5: PfCyRPA, PfCyRPA: PfRipr or RCR complexes were purified via incubation of PfCyRPA with complex members of interest for 30 minutes at room temperature, before size exclusion chromatography on a S200 10/300 into SPR running buffer (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄, 0.005% P20) run at 0.8ml/min. mAb was immobilized on a protein A or protein G (non-Fab complexes) chip via injection at 30μL/min for 30 seconds. 100nM PfCyRPA complex was then injected for 45s before allowing for 240s of dissociation. The protein A/G chip was regenerated through a 45s injection with 10mM glycine pH 1.5. For Fab competition evaluation, relative binding was quantified based on peak response of Fab: PfCyRPA alone divided by peak response of PfCyRPA on its own, which was then mapped on to a competition matrix.

2.2.15 Epitope binning through ELISA

A 96 well NUNC immune plates (Thermo Fisher) was coated with 50μL of mAb or PfCyRPA at 2μg/ml in PBS overnight at 4°C. The following day, each well was blocked with 100μL of PBS with 1% casein (Pierce) for one hour at room temperature. After blocking, well contents were discarded and the plate was washed with PBS-Tween 20 (PBS-T, 0.05% Tween-

20). Next, 50 μ L of PfCyRPA was added to each well that had been coated with a mAb at a concentration of 10 μ g/ml in PBS with 1% casein (wells already coated with PfCyRPA continued to be blocked with 1% casein). After 60 minutes, the well contents were discarded and then washed 3x with PBS-T. Next, 50 μ L of the 'competing' anti-PfCyRPA biotinylated mAb at 10 μ g/ml was added to each well and incubated for 60 minutes, before the well contents were discarded, followed by 3x wash steps with PBS-T. 50 μ L of streptavidin-alkaline phosphatase (Invitrogen) diluted 1/1000 in PBS-casein, was then added, incubated for 15 minutes, and washed 6x with PBS-T. Finally, a 20mg tablet of 4-Nitrophenyl phosphate (pNPP, Sigma) was dissolved in 4ml of diethanolamine buffer (Thermo Fisher) with 16ml deionized water. 100 μ L of pNPP was then added to each well and plates were allowed to develop until PfCyRPA coated directly on the plate detected with biotinylated c12⁷⁰ reached an OD₄₀₅ between 1.0 and 1.5 using a microplate reader (Tecan).

2.2.16 Assessment of haemolytic activity

In the presence of detergents or other haemolytic compounds, erythrocytes rupture and release haemoglobin. The quantity of released haemoglobin can then be measured spectrophotometrically and is proportional to the number of ruptured erythrocytes¹⁹¹. 100 μ L of red blood cells were diluted in 10ml PBS (137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 1.8mM KH₂PO₄), spun down at 500 $\times$ g for five minutes, then PBS was aspirated. 10ml PBS was then added and centrifugation repeated. Cells were then resuspended in 10ml PBS, and diluted down to 4 $\times$ 10⁶ cells per ml. 200 μ L of cells were aliquoted into each well in a 96-well plate. The plate was then spun down at 500 $\times$ g for five minutes and aspirated. Next, cells were resuspended in 200 μ L PBS with 1% Triton X-100 (positive control), PBS (negative control), 2 μ M PfCyRPA, 2 μ M PfRipr, 2 μ M PfRH5 or 2 μ M RCR complex. Cells were then incubated for

24 hours at 37°C. After incubation, cells were spun down at 500×g for five minutes and then 50µL of each solution moved to a new 96-well plate. Absorbance at 405nm was used to assess the degree of haemolysis using a microplate reader (Tecan). Background haemolysis (PBS control, OD₄₀₅ approximately equal to 0.3 compared to 1.6 for the positive control) was subtracted from each group and results reported as haemolysis relative to the positive control (1% Triton X-100).

2.2.17 Statistical analysis

All statistical analysis was carried out using Prism 8 (GraphPad Software Inc). The exact statistical tests used are specified in the relevant figure legends. In all cases, two-tailed tests were used with a level of significance of $\alpha < 0.05$ with corrections for multiple comparisons applied where indicated in figure legends.

2.3 Chapter IV methods

2.3.1 Preparation of protein samples for crystallization

Fab and PfCyRPA were mixed in a 1:1 ratio to yield a total of 4mg of protein and incubated at room temperature for 30 minutes. Next, the Fab:PfCyRPA mixture was injected into a S200 16/600 column using an Äkta pure (GE Healthcare) and underwent size exclusion chromatography (SEC) into low-salt TBS (50mM NaCl, 20mM Tris pH 8) in order to maximize contribution of crystallization screen components. Fractions containing assembled complex were pooled and concentrated to approximately 10mg/ml prior to plate set-up using an Amicon ultra centrifugal filers (Millipore) with a 50kDa molecular weight cut-off. The appropriateness of a 10mg/ml starting concentration was confirmed using a pre-crystallization test (Hampton Research).

2.3.1 Protein methylation for crystallography

For Cy.009:CyRPA complex, lysine methylation was explored as a method of routine crystallization rescue by decreasing protein solubility, as described by Walter *et al*¹⁹². Methylation changes crystallization properties of a protein by reducing the number of flexible, solvent exposed side chain that can prevent crystallization¹⁹². After allowing the complex to form at room temperature for 30 minutes, it was diluted to 1mg/ml in low salt TBS (50mM NaCl, 20mM Tris pH 8). 20µL/ml of 1M dimethylamine-borane complex (ABC, Sigma) was added alongside 40µL of 1M formaldehyde (Sigma) and incubated at room temperature for two hours. An additional 20µL/ml ABC and 40µL of formaldehyde was added and incubated for a further two hours. 10µL of ABC was added and the reaction was incubated

overnight. The following day, the protein solution was centrifuged at 3000×g for 10 minutes to remove any precipitated protein prior to SEC.

2.3.2 *Broad crystallization screens*

After concentration to approximately 10mg/ml, mAb:PfCyRPA crystallization screens were conducted using a sitting drop vapour diffusion approach. 200uL drops composed 50% of protein solution and 50% of crystallization condition were prepared using a Mosquito low volume nL pipetting instrument (TTP Labtech). All complexes were screened against the JCSG+, Proplex, PACT, Midas and Morpheus screens (Molecular Dimensions) at two temperatures, 4°C and 18°C for up to six months, representing 960 unique conditions. Cy.009:PfCyRPA was additionally screened against Morpheus II, JCSG II, JCSG III, and MemGold (Molecular Dimensions).

2.3.3 *Crystallization optimisation*

Cy.009:PfCyRPA crystals from wells that generated weakly diffracting ($<10\text{\AA}$) crystals were further optimized. Conditions of interest were screened again with the addition of 50μL of the Silver Bullets additive screen (Hampton Research) to the 100nL/100nL protein/crystallization mixture during plate set-up using the Mosquito low volume nL pipetting instrument (TPP Labtech). In the case of Cy.009:PfCyRPA, crystals were also grown in a matrix of increasing precipitant and salt concentration for the most promising conditions (**Appendix 1**). Further optimisation of additive screen grown crystals was attempted through dehydration. Crystals were dehydrated over three days using three-steps of increasing salt and precipitant in a hanging drop vapour diffusion set-up, EasyXtal 15-drop tool (Qiagen).

2.3.4 *Crystal freezing*

Crystals were 'fished' using a light microscope and a nylon loop between 50-300 μ M in diameter at either 4°C or 18°C, depending on the conditions that led to their growth. In order to prevent ice crystal formation, cryoprotection is necessary which enables ice vitrification instead of freezing, thereby preventing ice crystal formation. If they were not already cryoprotected, crystals were first transferred into a 2 μ L drop of mother liquid containing cryoprotectant (25% glycerol, 25% ethylene glycol, 25% polyethylene glycol (PEG) 400 or 25% 2-methyl-2,4-pentanediol (MPD)). After transferring the crystal into the cryoprotectant, crystals were again removed into a nylon loop, before plunging into liquid nitrogen and insertion into a 16-well UniPuck for transport to the synchrotron under cryogenic conditions.

2.3.5 X-ray diffraction and data collection

Data for Cy003:PfCyRPA, Cy.007:PfCyRPA and Cy.009:PfCyRPA were collected using a wavelength between 0.95-1Å from the Diamond Light Source on beamline i03 (Cy.003:PfCyRPA), i04 (Cy.007:PfCyRPA, Cy.009:PfCyRPA), SOLEIL PXI (Cy.009 Fab) or Swiss Light Source PXIII (Cy.002:PfCyRPA). All data were collected under cryogenic conditions at 100K to reduce the impact of beam damage. Three images were initially collected at 0°, 45° and 90° after centring the crystal on the beam. For well-diffracting crystals, based on the indexing of these images, a data collection strategy was suggested by mosflm or EDNA^{178,179} to determine starting angle and number of degrees of rotation to yield a complete dataset. For well-diffracting crystals, a full dataset was then acquired by rotating the crystal on a goniometer, allowing for the acquisition of all unique reflections. Data indexing, reduction and scaling were carried out automatically by FastDP, Xia2/3diii, DIALS, Xia2/Multiplex and autoproc, within the Diamond Light Source analytical pipeline¹⁹³. Datasets were selected on

the basis of superior resolution, overall completeness >95%, CC1/2 >0.5 (or in the case of Cy.009:PfCyRPA, >0.3) and $I/\sigma(I)$ cut-off of approximately 2.

2.3.6 *Phasing and refining crystal structures*

Due to the fact that the structures for both PfCyRPA and Fab fragments have been solved previously^{66,72}, experimental phasing was not required as there were appropriate search models to perform molecular replacement. For Cy.004:PfCyRPA, Cy.007:PfCyRPA, Cy.002:PfCyRPA and Cy.003:PfCyRPA, the structures were determined by molecular replacement using Phaser¹⁸⁴, part of the CCP4 suite¹⁹⁴. Two search models were used, one of PfCyRPA from PDB 5TIH and the other anti-PfRH5 mAb R5.016 (6RCS). In both cases, flexible regions such as CDR loops were removed from the search model prior to molecular replacement. The model of Fab:PfCyRPA complex was then refined using Coot¹⁸⁵, Phenix¹⁹⁵ and Buster¹⁸⁶ before validation with MolProbity¹⁹⁶. In the case of Cy.009:PfCyRPA, this structure was solved by first by obtaining the structure for the Cy.009 Fab on its own, in its methylated state, using the 6RCS search model and then using the refined Cy.009 structure alongside the PfCyRPA from the Cy.004:PfCyRPA structure as a search model to complete molecular replacement of the Cy.009:PfCyRPA complex. This was necessary because of the low-resolution dataset obtained, 5.1Å, despite the numerous optimization approaches described in 2.3.3 and outlined in **Appendix 1**.

2.4 Chapter V Methods

2.4.1 Synergy assay of growth inhibition activity

The GIA assay set-up followed the same broad protocol as described in 2.1.11. However, to assess synergy/antagonism/additivity, two antibodies were used in combination. In all cases, one mAb was held at a constant concentration that on its own would be predicted to yield approximately 30% inhibition. A second mAb was then titrated in at increasing concentrations to a maximum of 2mg/ml. In parallel, an inhibition curve of the titrated mAb alone was run in parallel to enable the calculation of Bliss additivity based on inhibition of each mAb on its own as compared to when delivered in combination. The full details of the synergy calculation can be found in **Chapter 5**.

2.4.2 Dual antibody binding kinetics via SPR

In order to determine how affinity of one mAb changed in the presence of a second mAb, mAb affinity/kinetics were compared between PfCyRPA alone and PfCyRPA bound to a Fab of interest. PfCyRPA in complex with Fab was prepared via incubation of the Fab of interest with PfCyRPA in equimolar concentrations for 30 minutes prior to purification on a S200 10/300 column (GE Healthcare) in PBS with 0.005% P20 surfactant. Approximately 300 response units of mAb were conjugated to a CM5 chip (GE Healthcare). 10 start-up cycles were run with PBS-P20 buffer prior to kinetic analysis. After completion of start-up cycles, Fab: PfCyRPA was injected for 60s followed by 240s of dissociation. Where possible, affinity was determined using a global Langmuir 1:1 interaction model. For mAbs with rapid dissociation, affinity was determined based on steady-state analysis using the Biacore X100 evaluation software.

2.4.3 PCR for recombinant expression of anti-PfCyRPA Fab fragments

Fabs for the triple Fab:PfCyRPA complex were purified after recombinant expression of only the Fab fragment. First, the variable regions and first constant domain (V-C1) of the antibody heavy chain were cloned out of the plasmid flanked with BsiWI and AgeI restriction sites. To do this, 0.5µL of template DNA was mixed with 1µL of 10µM forward primer (GCCGTACGGCCAACCATG), 1µL 10µM reverse primer (CCAAACCGGTTTCATCACTTGTCGAGCTCTTGGG), 10µL 2X Phusion Master Mix (New England BioLabs) and 7.5µL of DNase free water. Next, a thermocycler was used to execute a three-step PCR protocol, initial denaturation at 98°C for 30s, then 10s at 98°C, 30s at 67°C and 30s at 72°C, followed by a 10 minute final extension at 72°C. After amplification, samples were mixed 1:4 with DNA loading dye (New England BioLabs) and run on a 1% agarose gel at 100V for 40 minutes. Bands were excised and DNA was extracted using a gel extraction kit (Qiagen) according to the manufacturer's instructions.

2.4.4 Restriction digest and ligation for expression of Fab fragments

After successful amplification and purification of the PCR products, these were digested alongside the parental mAb using BsiWI and AgeI restrictions sites. These were the sites originally used to clone in the full length mAb, and were also added to the start and end of the Fab PCR product. For the restriction digest, 0.5µL of each restriction enzyme was mixed with 9µL of the PCR product or the parental plasmid at 100ng/µl alongside 2µL CutSmart buffer (New England BioLabs) and 8µL DNase free water. Samples were incubated at 37°C for 60 minutes before running on an agarose gel and excising bands, as described above.

After gel extraction of the bands of interest (plasmid backbone, digested PCR product), the plasmids were ligated using 1µL of plasmid backbone, 4µL PCR product, 1µL of

DNA ligase buffer (New England BioLabs) 0.5 μ L of DNA ligase (New England BioLabs) and 3.5 μ L of DNase free water. Ligation was carried out at room temperature for one hour. Then STELLAR electrocompetent *E. coli* (Takara Bio) were transformed by mixing 30 μ L of competent cells with 3 μ L of the ligation reaction, allowing them to sit for 15 minutes on ice, then heat shocking them at 42°C for 45s, adding 200 μ L of SOCS essential medium (New England BioLabs) and then incubating for an hour at 37°C prior to streaking on LB plates with ampicillin. These were incubated overnight. The resulting colonies were 'miniprep'd according to the manufacturer's instructions (Qiagen) and sequenced (GATC) in the forward (primer: GCTTCTGGTTACTGACTAC) and reverse (primer: CTTATCATGTCGCGGCC) direction.

2.4.5 Fab purification

Since protein G also has some, albeit weak, affinity for Fabs, the same columns could be used as for the mAb purification and followed the same protocol as described above. Ideally, protein L would be used which binds to the light chain. However, protein L only binds to kappa light chains, thus precluding its use here. After affinity purification, Fabs were purified through size exclusion chromatography using an S200 10/300 (GE Healthcare) at 0.8ml/min in TBS (150mM NaCl, 20mM Tris pH 7.4).

2.4.6 Triple Fab complex production

Purified Fabs and PfCyRPA were mixed together in equimolar ratios to obtain a total protein content of 4mg. After 30 minutes of incubation at room temperature, the triple Fab:PfCyRPA complexes were purified using an S200 16/600 in low salt TBS (50mM NaCl, 20mM Tris pH 8). After SEC, the fractions containing the triple complex were pooled and

concentrated to 10mg/ml using an Amicon centrifugal filter (Millipore) with a 100kDa cut-off. Protein concentration was confirmed using a nanodrop (Thermo Fisher Scientific).

2.4.7 Broad crystallization screens

Crystallization screens were carried out using the same approach described in **2.2.3**, except here the two triple Fab complexes (Cy.003:Cy.007:Cy.009:PfCyRPA and Cy.003:Cy.007:Cy.004:PfCyRPA) were screened against Proplex, Morpheus, PACT, Morpheus, JCSG+ and MemGold (Molecular Dimensions) at 4°C and 18°C. All of these broad screens were also done using methylated protein complex, as described in **Section 2.3.2**.

2.4.8 Crystallization optimisation

Crystallization for a single Morpheus condition was optimized in a Silver Bullets additive screen (Hampton Research). This condition contained 0.09M halogen mix, 0.1M buffer system 1 pH 6.5, 30% v/v precipitant mix 2.

2.4.9 Crystal freezing

The same method was used as in **2.3.4**.

2.4.10 X-ray diffraction and data collection

Data for Cy.003:Cy.007:Cy.004:PfCyRPA were collected at the Diamond Light Source on beamline i04 at a wavelength of 0.9795Å. The same data reduction and scaling approach was used as described in **2.3.5**.

2.4.11 Phasing and refining crystal structures

Molecular replacement used a search model consisting of superimposed Cy.003:Cy.007:Cy.004:PfCyRPA or Cy.003:Cy.007:Cy.009:PfCyRPA with the PfCyRPA from

Cy.004:PfCyRPA, as it was at the highest resolution. Molecular replacement initially failed to pack with all three Fabs bound. However, based on relatively promising log-likelihood gain and TFZ scores from Phaser, it seemed probable that the solution was partially correct. Molecular replacement was repeated with only two Fabs bound, only one Fab bound, and only PfCyRPA or only Fab. Through this approach, it was determined that the structures that had useable datasets were dual Fab structures (Cy.003: Cy.007: PfCyRPA) rather than triple Fab structures. Once this was determined, downstream refinement followed the same approach described in **2.3.6**.

2.4.12 Statistical analysis

All statistical analysis for non-crystallographic work was carried out using Prism (GraphPad Software Inc). The exact statistical tests used are specified in the relevant figure legends. In all instances, two-tailed tests were used and corrected for multiple comparison where required.

Chapter 3: Functional characterisation of a panel of anti-PfCyRPA mAbs

Authorship statement

The GIA assay is standardised. To minimise variability and enable direct comparison between different projects ongoing in the lab, all GIA assays were carried out by one of two dedicated research associates: Dr. Doris Quinket and Amelia Lias. This includes both regular parasite maintenance and the assay set-up and read-out. Further, Dr. Joe Illingworth made the hybridomas for Cy.002, Cy.005 and Cy.010. Dr. Lea Barfod made the hybridomas for Cy.001. Plasmids for Cy.003, Cy.004, Cy.007 and Cy.009 were generously provided by the National Institute for Biological Standards and Controls. Robert Donat cloned Cy.001, Cy.002, Cy.005 and Cy.010 into a human IgG backbone. The recombinant PfRH5 and PfRipr proteins were produced by either Dr. David Pulido-Gomez or Hannah Davies.

3.1 Introduction

Antibodies, whether induced through natural infection or through vaccination, play an important role in immunity against malaria^{14,43}. The discovery of antigens that are able to induce cross-strain inhibitory neutralizing antibodies against *P. falciparum* has been one of the great successes of recent years in malaria research. Antibodies that target PfCyRPA have been previously shown to be strain-transcending^{66,70,72}. However, compared to anti-PfRH5 mAbs, inhibitory mAbs against PfCyRPA are much less potent (as measured) than those that target PfRH5¹⁴.

The two most potent published neutralizing anti-PfCyRPA mAbs, c12 and 8A7, both target the same region of PfCyRPA, binding to blades 1-3^{66,72}(**Figure 1.4**). However, the panels from which these antibodies were discovered, 16 and 5 antibodies, respectively

, were limited by their relatively small size^{66,70-72}. Thus, it was of interest to continue to expand the search for more potent anti-PfCyRPA mAbs, as it was possible that with an expanded panel of anti-PfCyRPA antibodies, more potent clones would emerge. Furthermore, only the binding epitopes of c12 and 8A7 have been clearly mapped, and so it is unclear whether or not other parts of the six bladed β -propeller can elicit neutralizing antibodies, or whether mAbs against these other regions have simply not been isolated. By understanding which regions of PfCyRPA can elicit neutralizing antibodies, synthetic immunogens could be designed in the future that only incorporate the regions of PfCyRPA capable of eliciting neutralizing antibodies.

There is growing interest in using passively transferred mAbs as therapeutics against infectious disease¹⁹⁷. This is particularly relevant in malaria where it is difficult to obtain sufficiently high levels of antibody through conventional vaccination to confer lasting immunity. Recently, the first anti-sporozoite mAb targeting PfCSP (aimed at blocking infection) has entered clinical trial (ClinicalTrials.gov Identifier NCT04206332). Thus, isolating the most potent antibodies that block merozoite invasion during the blood-stage of infection

is of interest for prophylactic development, which could be an alternative to, or complement, a sporozoite-stage anti-infection mAb. To date the most potent clones have been anti-PfRH5 mAbs, which is consistent with the performance of polyclonal sera whereby anti-PfRH5 polyclonal IgG inhibits at lower concentrations than that of anti- anti-PfCyRPA^{102,106,107}.

It is also known that anti-PfCyRPA antibodies can act additively or synergistically with anti-PfRH5 antibodies, suggesting that an antibody cocktail targeting multiple members of the RCR complex may be more effective than an anti-PfRH5 mAb on its own.^{102,106,107}. However, these synergistic relationships appear to be clone specific¹⁴. Hence, a more thorough analysis of antibody combinations is required to identify the most potent combinations, but this first requires an expanded panel of well-characterized anti-PfCyRPA mAbs.

The final rationale for the work reported in this Chapter is that the role of the RCR complex in merozoite invasion remains unclear. Understanding how antibodies that target the RCR complex inhibit invasion, can also inform the underlying mechanisms of invasion and the role of the RCR complex. The research aim of this Chapter was therefore to determine the parameters that define a potent neutralizing anti-PfCyRPA mAb.

3.2 Results

3.2.2 Expression of a panel of anti-PfCyRPA mAbs

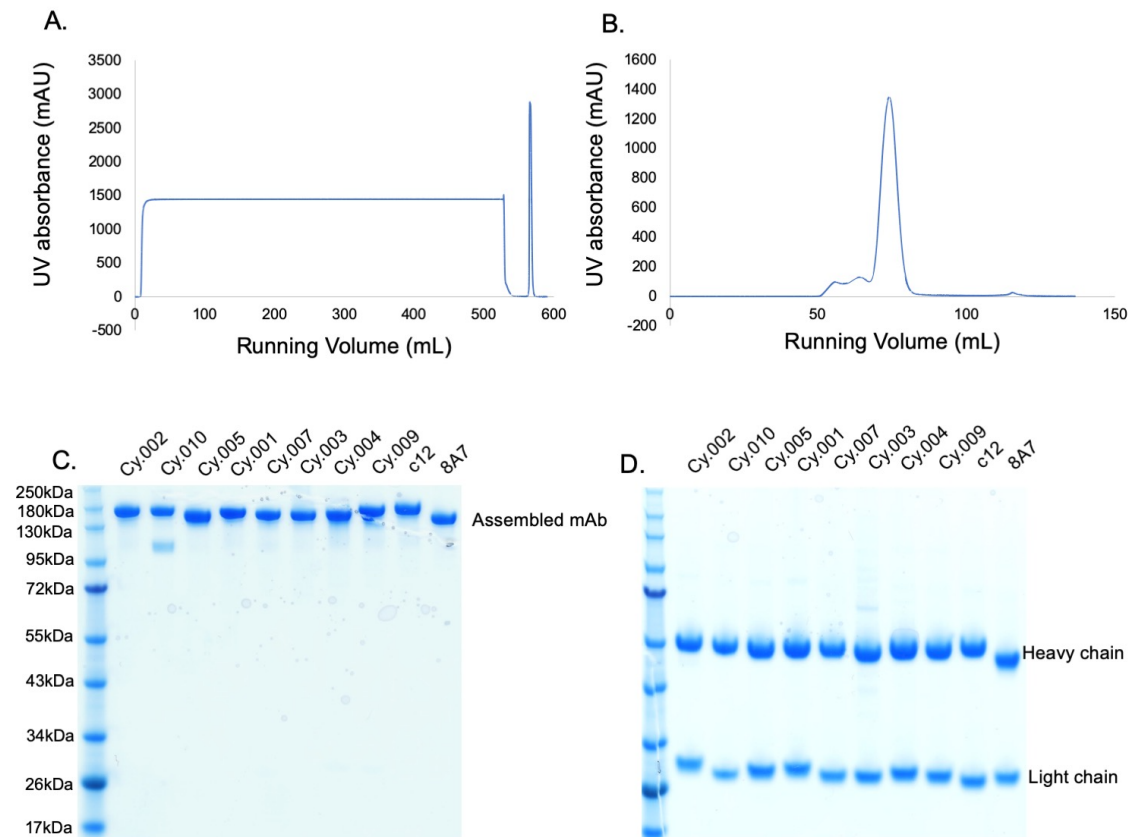


Figure 3.1 Recombinant antibody expression and purification. **A.** Protein G chromatogram showing binding to column, wash step and then elution with glycine. **B.** Size exclusion chromatography on an S200 16/600 column showing Cy.009 monoclonal antibody eluting at approximately 75ml. **C.** Non-reducing SDS page stained with Coomassie blue. **D.** Reducing SDS-PAGE stained with Coomassie blue.

A panel of anti-PfCyRPA mAbs was assembled from three sources. First, four anti-PfCyRPA mAbs, Cy.003, Cy.004, Cy.007 and Cy.009, were generously provided by the National Institute for Biological Standards and Control in the United Kingdom. These mAbs were generated through chicken immunization followed by cloning into a chimeric human IgG1 backbone by Icosagen using their proprietary HybriFree technology¹⁹⁸.

Four other mAbs, Cy.001, Cy.002, Cy.010 and Cy.005 were made from mouse derived hybridomas that were subsequently sequenced and the variable domains synthesized to be cloned into a chimeric human IgG1 backbone, retaining the plasmid's secretion peptide and constant domains.

The final two antibodies included in our panel were the published c12 and 8A7 antibodies. Though originally derived from mouse hybridomas, the variable domain sequences were retrieved from the relevant structures (PDBs: 5EZO, 5TIH respectively), codon optimized for human cell line expression and then cloned into the same human IgG1 backbone to make chimeric human antibodies equivalent to those of Cy.001, Cy.002, Cy.005 and Cy.010. Together, this yielded a panel of ten antibodies, of which eight were previously uncharacterized. All ten antibodies were expressed in Expi293 cells, affinity purified and then gel filtered to yield purified recombinant antibody (**Figure 3.1**).

Recombinant PfCyRPA was expressed with a murine IgG1 signal peptide on the protein's N-terminus and an EPEA affinity tag (C-tag) on its C-terminus. This expression construct contained three mutations to remove potential N-linked glycosylation sites (S147A, T324A and T340A). This enabled expression in Expi293 cells, where supernatant was collected and separated from the suspended cells through centrifugation, purified on a C-tag affinity column and then gel filtered to yield purified PfCyRPA (**Figure 3.2**). When run on an SDS-PAGE

the non-reduced sample ran at a lower molecular weight than expected, and at a lower molecular weight than the reduced sample. This is likely related to the smaller effective size when the disulphide bonds are oxidized and PfCyRPA can retain some tertiary structure.

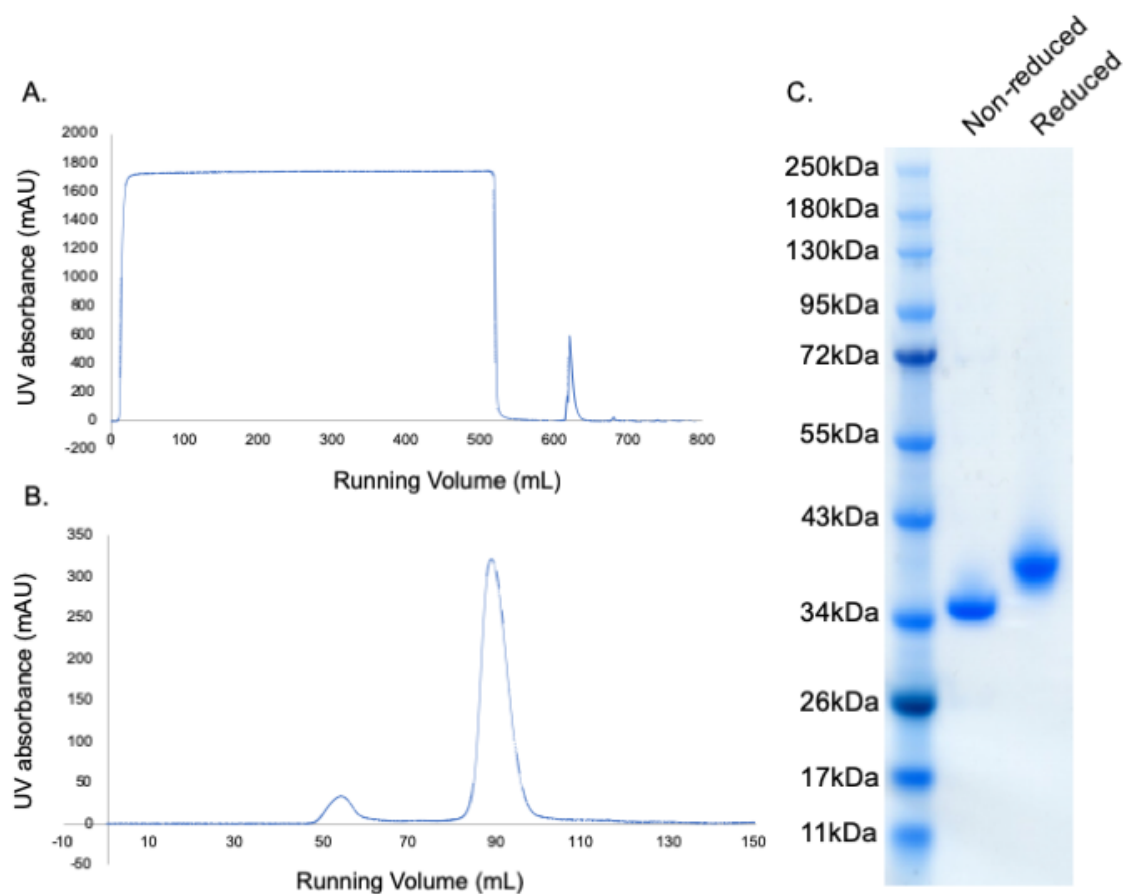


Figure 3.2 Production of recombinant PfCyRPA. A. C-tag chromatogram showing binding to column, wash and then elution. **B.** Size exclusion chromatography on an S200 16/600 column showing PfCyRPA eluting at approximately 90ml. **C.** SDS-PAGE run under reducing and non-reducing conditions stained with Coomassie blue

3.2.2 Binding properties of anti-PfCyRPA mAbs

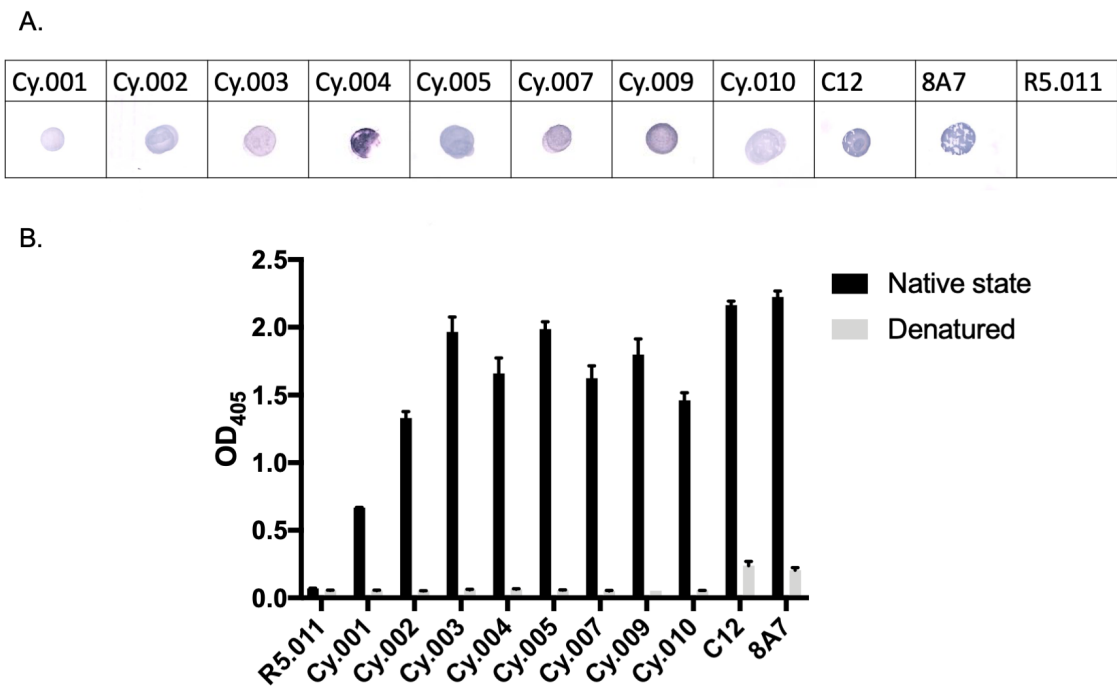


Figure 3.3 Anti-PfCyRPA antibodies binding to recombinant PfCyRPA. A. Anti-PfCyRPA dot blot of all the mAbs characterized in this Chapter. B. ELISA-based assessment of antibody binding to native state and denatured PfCyRPA showing that all antibodies bind to conformational epitopes. R5.011 is a fully human IgG1 anti-PfRH5 mAb which here is used as the negative control. Graph bars indicate the mean, error bars indicate standard deviation, n = 3.

All mAbs included in this analysis had their binding confirmed first by dot blot, where recombinantly expressed PfCyRPA was dotted onto a membrane, followed by incubation with test mAb and then visualization with an alkaline phosphatase linked goat anti-human antibody. In all cases, as expected, the antibodies bound to recombinant PfCyRPA (**Figure 3.3A**).

Next, binding to native or denatured PfCyRPA was measured by ELISA, in order to determine whether these mAbs bound to linear or conformational epitopes, as this is a fundamental property of mAbs (**Figure 3.3B**). Native state or heat-denatured and reduced PfCyRPA protein were coated on ELISA plates overnight followed by blocking with casein, then the antibody of interest was allowed to bind followed by detection with goat anti-human alkaline phosphatase antibody.

The results showed that all antibodies bound to a conformational epitope, including c12 and 8A7. This is consistent with the previously published data on c12 and 8A7, which showed that they bound to conformational epitopes^{66,72}. Importantly, this also showed that the process of making c12 and 8A7 into chimeric human antibodies did not result in the loss of PfCyRPA binding. Meanwhile, the anti-PfRH5 mAb R5.011 negative control did not bind to either native or denatured PfCyRPA (**Figure 3.3B**). Alongside this, all mAb VH and VL domain were sequenced to confirm that all ten mAbs were distinct.

3.2.3 Growth inhibition activity of anti-PfCyRPA mAbs

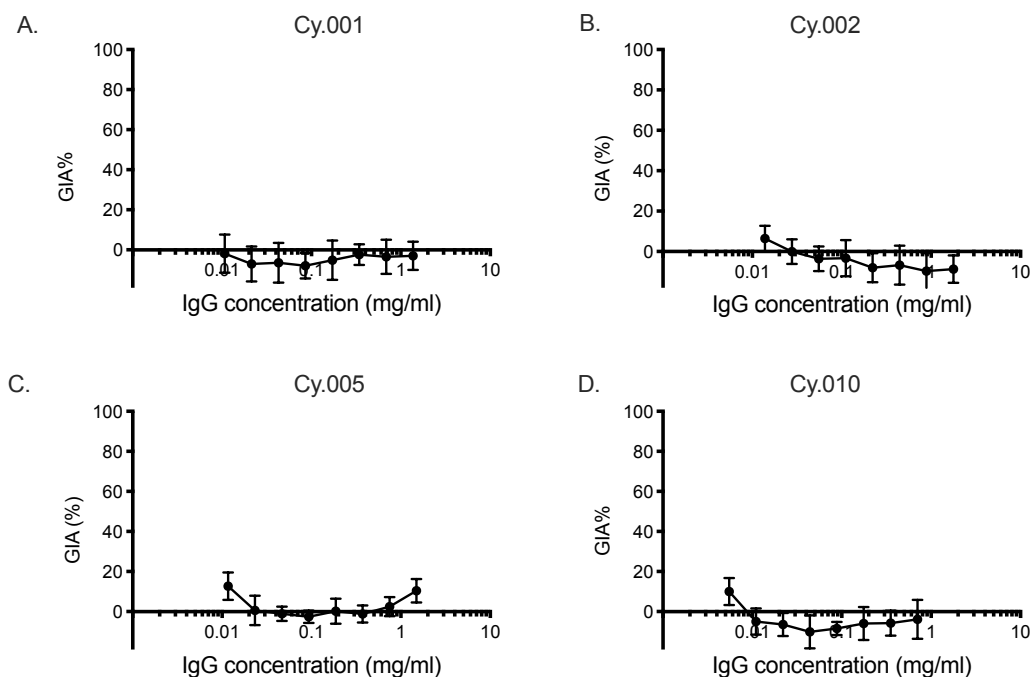


Figure 3.4 GIA of non-inhibitory anti-PfCyRPA mAbs using the 3D7 clone of *P. falciparum*. Point indicates mean inhibition and error bars indicate standard deviation across triplicates. A. Cy.001 B. Cy.002 C. Cy.005 D. Cy.010.

All ten mAbs were assessed for their GIA. A one-cycle GIA assay, as per the NIH Reference Laboratory standardized assay which measures parasite growth over 48 hours (equivalent to one invasion cycle), was used to determine how well each mAb blocked parasite growth using the 3D7 clone of *P. falciparum*. Tightly synchronized cultures of schizonts were allowed to burst and reinvade in the presence of each mAb individually. Initially, two-fold dilutions beginning at 2mg/ml were used, where material quantities permitted. However, in subsequent experiments this was changed to four-fold dilution so that there could be better resolution at low antibody concentrations.

Four of the mAbs, Cy.001, Cy.002, Cy.005 and Cy.010 were found to be GIA negative, based on a cut-off of <25% inhibition at 2mg/ml⁶⁸ (**Figure 3.4**). Five of the six remaining mAbs, Cy.003, Cy.004, Cy.007, Cy.009 and 8A7 were found to be GIA positive. c12 fell short of the 25% GIA at 2mg/ml cut-off, having only 12% GIA at 2mg/ml, but since this mAb has previously been published as an inhibitory

mAb of interest, it was included along with the 'GIA positive' group for more downstream analyses. The discrepancies between these results and the published data could simply be due to the fact that the assay here is a one-cycle GIA, while the GIA assay used to assess invasion inhibitory capacity of c12 and 8A7 originally was a two-cycle GIA^{66,72}. Two-cycle GIAs have previously been shown to yield higher apparent growth inhibition⁵⁹. Alternatively, it is possible that the cloning of these mouse mAbs into a human IgG1 backbone could have had a contributing role, but this is likely minimal since the binding kinetics remained comparable to published values (**Section 3.2.5**) and Fc effector functions should not impact measured GIA due to the absence of immune cells, alongside the heat inactivation of complement in the experimental system.

Compared to the previously published data, both c12 and 8A7 showed lower IC₅₀ values, the value at which parasite growth is 50% of the negative control (maximum growth), with the reported IC₅₀ for c12 and 8A7 being approximately 1mg/ml⁷⁰ and 1.5mg/ml⁷², respectively (**Figure 3.5**). In these experiments, IC₅₀ for c12 and 8A7 could not be interpolated, but based on a curve fit with a variable hill slope and a constrained upper bound of 100% GIA, IC₅₀ was estimated to be 20.05 mg/ml for c12 and 2.9mg/ml for 8A7 (**Table 3.1**). However, this value of c12 is subject to significant uncertainty due to the fact that the curve was only measured once.

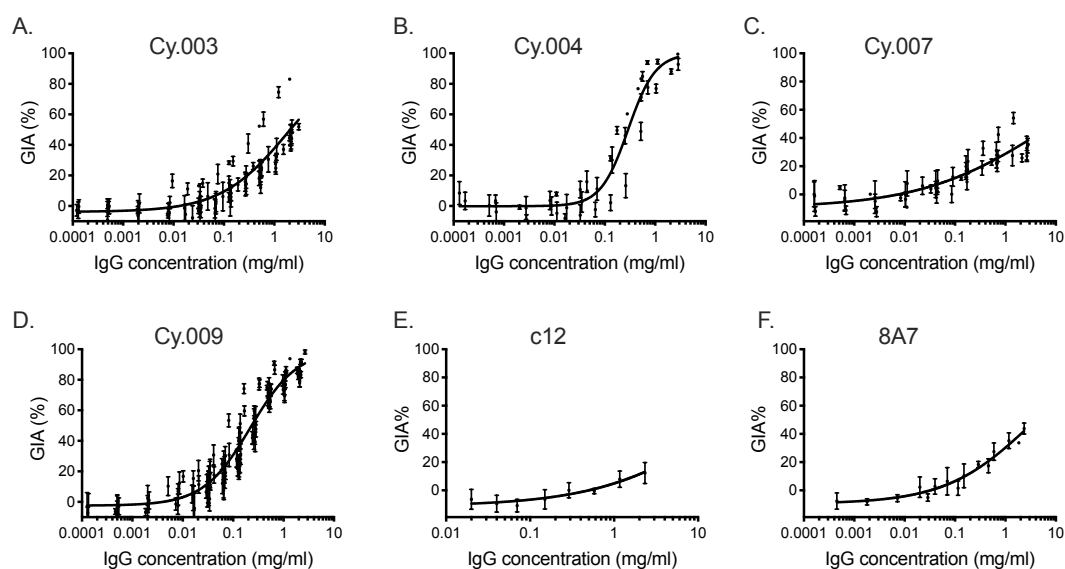


Figure 3.5 GIA of inhibitory anti-PfCyRPA mAbs using the 3D7 clone of *P. falciparum*. Point indicates the mean inhibition at a given concentration for a single repeat in triplicate, while error bars indicate the standard deviation of the triplicates. A. Cy.003, n=11 B. Cy.004, n=6 C. Cy.007, n=6 D. Cy.009, n=19 E. c12, n=1 F. 8A7, n=2 where n is an independent repeat. Curves were fit using a four-parameter logistic regression with a constrained upper bound of 100% GIA.

Table 3.1 50% inhibitory concentrations for anti-PfCyRPA mAbs. ^a indicates an extrapolated value.

mAb	IC ₅₀	95% Confidence
Cy.003	1.845	1.545 – 2.244
Cy.004	0.289	0.258 – 0.322
Cy.007	5.783 ^a	3.914 – 9.334
Cy.009	0.221	0.201 – 0.243
c12	20.05 ^a	6.326 – 467.1
8A7	2.917 ^a	2.197 – 4.107

Based on these results, Cy.004 and Cy.009 are significantly more potent than any previously published anti-PfCyRPA antibody (**Table 3.1**). In this head-to-head comparison with 8A7, the most potent known anti-PfCyRPA antibody, it is clear that Cy.004 and Cy.009 outperform 8A7 by approximately an order of magnitude based on IC_{50} . Cy.003 performs similarly to 8A7, whilst Cy.007 shows weaker inhibition, with a GIA titration curve resembling that of c12.

3.2.4 Epitope binning of anti-PfCyRPA mAbs

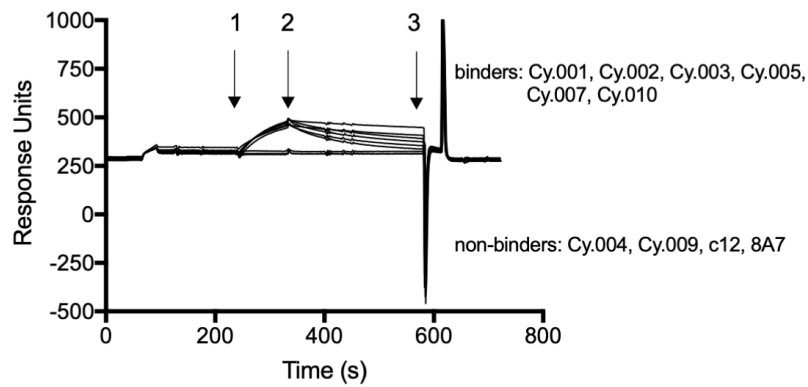
In order to inform relative binding positions of these mAbs on the PfCyRPA propeller, and whether there are distinct regions of PfCyRPA that can elicit neutralizing antibodies, two different methods of epitope binning were used to further characterize these antibodies. Helpfully, the crystal structures of c12 and 8A7 bound to PfCyRPA have already been solved^{66,72}, providing a useful orientating information (**Figure 1.4**).

First, epitope binning using SPR was attempted. Traditionally, this can be carried out by immobilizing the antigen of interest and then, step-wise, injecting mAb-1 until all the binding sites are saturated and then injecting mAb-2, following the same principle that is commonly used in bio-layer interferometry antibody competition assays^{68,199}. If mAb-2 is still able to bind, as determined by rising response units, it is said that these antibodies do not compete. If mAb-2 is unable to bind, then the antibodies are said to compete. However, this is dependent on relatively high affinity binding of mAb-1 to the target molecule. Otherwise, mAb-1 dissociates while mAb-2 is binding, confounding the results by providing unoccupied binding sites. Following various experimental tests, it was established that this method could not be used. This was because it was evident that Cy.001, Cy.004 and Cy.009 all underwent

rapid dissociation, which meant that there would be unoccupied PfCyRPA sites while mAb-2 was injected.

An alternative SPR approach was therefore used which involved immobilizing one mAb (hereafter referred to as immobilized mAb) with protein A and then injecting the competing Fab-of-interest in complex with PfCyRPA. It was necessary to use Fab so that the protein A chip did not bind to the Fab: PfCyRPA complex, given mAb-2: PfCyRPA complex would bind to protein A via the Fc on the second mAb. This avoided the issue of a multi-step SPR method that provided ample time for mAbs to dissociate. Consequently, binding of the immobilized antibody to PfCyRPA protein alone could be compared to Fab: PfCyRPA. If the binding of Fab: PfCyRPA was substantially reduced as compared to the binding of PfCyRPA protein alone, this would be indicative of two mAbs that bound to overlapping epitopes (**Figure 3.6A**).

A.



B.

		Immobilized antibody									
		Cy.001	Cy.002	Cy.003	Cy.004	Cy.005	Cy.007	Cy.009	Cy.010	c12	8A7
Fab:PfCyRPA complex	Cy.001	0.793	2.313	2.438	3.833	2.377	2.469	1.982	2.827	2.069	2.229
	Cy.002	3.615	0.080	1.982	3.699	1.977	2.008	2.053	2.198	1.751	1.848
	Cy.003	5.582	2.310	-0.196	3.654	-0.075	0.339	2.088	2.722	1.202	0.316
	Cy.004	3.841	2.114	1.808	0.851	1.807	1.768	0.006	2.294	0.757	0.733
	Cy.005	5.697	2.601	0.045	3.927	0.215	1.081	2.303	2.605	1.637	1.816
	Cy.007	4.889	2.231	0.759	3.018	1.203	0.483	1.607	1.773	0.409	0.319
	Cy.009	4.115	2.126	1.787	0.904	1.868	1.713	0.153	2.293	0.734	0.733
	Cy.010	3.726	2.438	2.279	4.065	2.304	1.992	2.387	0.536	0.814	2.086
	c12	2.995	2.040	1.156	0.546	1.183	0.624	-0.057	2.513	0.293	0.291
	8A7	6.760	2.442	-0.272	-0.006	1.755	-0.107	0.010	2.872	-0.063	-0.097

Competing antibodies

Non-competing

Non-reciprocal competition

Figure 3.6 SPR-based epitope binning of anti-PfCyRPA mAbs. A. Example experiment where Cy.009 was immobilized on protein A. 1. Indicates start of Fab:PfCyRPA injection 2. Indicates end of injection, start of dissociation 3. Indicates end of dissociation and start of regeneration. B. Summary matrix of all combinations of immobilized mAb against Fab:PfCyRPA complex. The number in each box indicates peak response units of the immobilized mAb interacting with the Fab:PfCyRPA complex relative to peak response units when interacting with PfCyRPA alone. mAb combination are said to compete when relative binding ($\frac{RU_{Fab:PfCyRPA}}{RU_{PfCyRPA\ alone}}$) is less than 1.0.

Unfortunately, this approach showed high background binding among some (Cy.001, Cy.004 and to a lesser extent Cy.010 and Cy.007) of the negative controls, where both the immobilized mAb and the Fab in complex with PfCyRPA were the same and thus by definition compete. For Cy.004, the negative control of Cy.004 Fab: PfCyRPA still showed nearly 80% binding compared to PfCyRPA on its own (**Figure 3.6B**). While this still meets the cut off of less than 1.0 relative binding compared to PfCyRPA alone, a more binary relationship between binding vs not binding was expected.

Despite these shortcomings, a complete competition matrix was still produced using these SPR data. Only one combination showed non-reciprocal competition (c12 and Cy.010) (**Figure 3.6B**). Non-reciprocal competition refers to mAbs that compete in one direction (mAb1 immobilized, Fab2: CyRPA binding) but not in the other (mAb2 immobilized, Fab1: CyRPA binding). This suggests that the data were largely in agreement. However, due to the issue of high background among some the negative controls, a second method was required to validate these results.

In order to address the problem of high background binding, an ELISA-based method was used whereby one antibody was captured in the well, followed by incubation with PfCyRPA, and then a second biotinylated antibody. The biotinylated antibody was then detected using streptavidin alkaline phosphatase.

In this experimental set-up, all detected antibody must be bound to both the coating antibody as well as PfCyRPA in order to be detected. If PfCyRPA dissociates from the fast-dissociating antibodies coated on the well, then no signal will be detected as PfCyRPA will be lost during the wash steps, precluding binding by the detection antibody. Thus, unlike the SPR

approach, this method should not be prone to false positives showing two antibodies binding when in fact, they are in competition.

This approach showed a more binary relationship between competing versus non-competing antibody pairs (**Figure 3.7**). The boundary between competing and non-competing antibodies was empirically set at an OD of 0.25, providing a clear distinction between the two groups. Within each group in **Figure 3.7**, the detection mAb is the same, while the different capture antibodies coated on the plate change, with each data point being a single unique capture/detection antibody combination. A competition matrix was assembled based on relative binding of a mAb when PfCyRPA was coated directly onto the plate compared to when it was captured with a specific mAb (**Figure 3.8**).

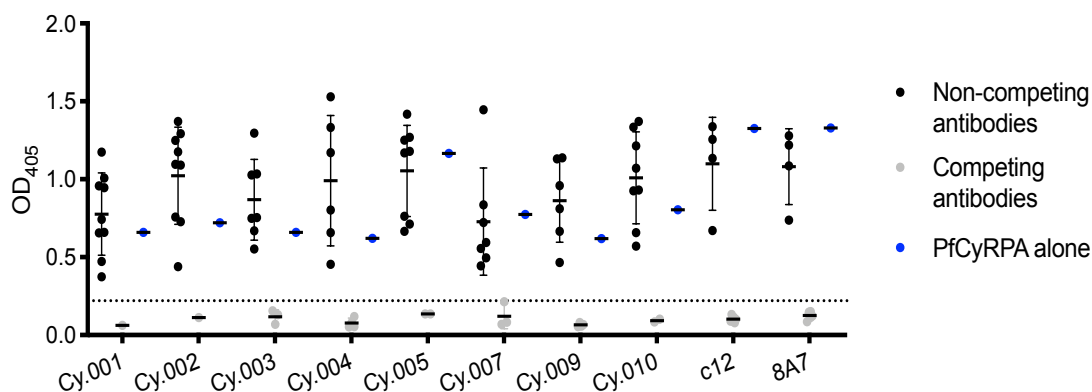


Figure 3.7 ELISA based assessment of dual antibody binding. Binding is reported as absorbance at 405nm. PfCyRPA alone (blue) shows absorbance when PfCyRPA was coated directly onto the plate. X-axis labels denote the detection antibody. Within each group, each data point is a different capture antibody from the panel. Error bars indicate the standard deviation within the competing (grey) vs. non-competing (black) groups.

		Detection antibody									
		Cy.001	Cy.002	Cy.003	Cy.004	Cy.005	Cy.007	Cy.009	Cy.010	c12	8A7
Capture antibody	Cy.001	0.09426	0.61001	0.83902	0.73055	0.61083	0.71861	0.75258	0.71048	0.50564	0.55485
	Cy.002	1.52961	0.15647	1.96661	2.46432	1.21628	1.86923	1.82775	1.70629	1.00862	0.96337
	Cy.003	1.78379	1.90263	0.23585	2.14649	0.11602	0.6416	1.83664	1.66169	0.94655	0.06453
	Cy.004	0.71808	1.01083	1.14448	0.12995	0.57171	0.93298	0.13079	0.81914	0.07645	0.08476
	Cy.005	0.99681	1.63333	0.19846	1.29378	0.11659	1.0796	1.31025	1.51062	0.85469	0.91765
	Cy.007	1.45454	1.79494	1.01487	1.88622	1.00297	0.10578	1.54905	1.15837	0.1004	0.11411
	Cy.009	0.56713	1.05114	1.1372	0.19326	0.65372	0.76883	0.11017	1.15144	0.08648	0.10131
	Cy.010	1.00147	1.51435	1.56832	1.05907	1.07315	0.57462	1.07597	0.10385	0.06773	0.81747
	c12	1.43687	1.73417	1.55835	0.08361	1.08931	0.277	0.09342	0.12684	0.07022	0.1114
	8A7	1.12635	1.52046	0.10376	0.0872	1.01161	0.08838	0.08863	1.33329	0.05936	0.09191

Figure 3.8 ELISA-based antibody competition assay of anti-PfCyRPA mAbs. The matrix indicates the relative signal from each detection antibody when PfCyRPA is captured by an antibody (left column) compared to when PfCyRPA is coated directly onto the plate. Antibodies that had less than 30% relative binding were taken to be competing.

Despite the concerns with the SPR-based method, the two methods largely agreed. The only differences were that the non-reciprocal competition observed between c12 and Cy.010 through SPR was found to be competition in both directions through the ELISA-based method. Meanwhile, Cy.003 and Cy.007, which were found to compete in the SPR-based assay, were found to not compete in the ELISA-based method. This result was subsequently confirmed in Chapter 5 of this thesis through crystallography (**Figure 5.19**), suggesting that the ELISA-based result is more reflective of binding in solution as compared to SPR.

Based on the ELISA competition data, a summary of competition relationships between the antibodies is shown in **Figure 3.9**. Among the previously uncharacterized inhibitory mAbs, (Cy.003, Cy.004, Cy.007 and Cy.009) there were three distinct epitope bins, as only Cy.004 and Cy.009 competed with each other, but there was no competition between Cy.003, Cy.007 and Cy.004/Cy.009. However, all four of these, along with c12, competed with 8A7. Meanwhile, none of the non-inhibitory antibodies competed with 8A7. This suggests that even though Cy.003, Cy.007 and Cy.004/009 do not compete, they are likely all binding the same region of PfCyRPA. The epitopes of these four mAbs bind likely overlap with the 8A7 epitope, while not competing directly with each other except in the case of Cy.004/Cy.009, thus explaining the observed binding competition data.

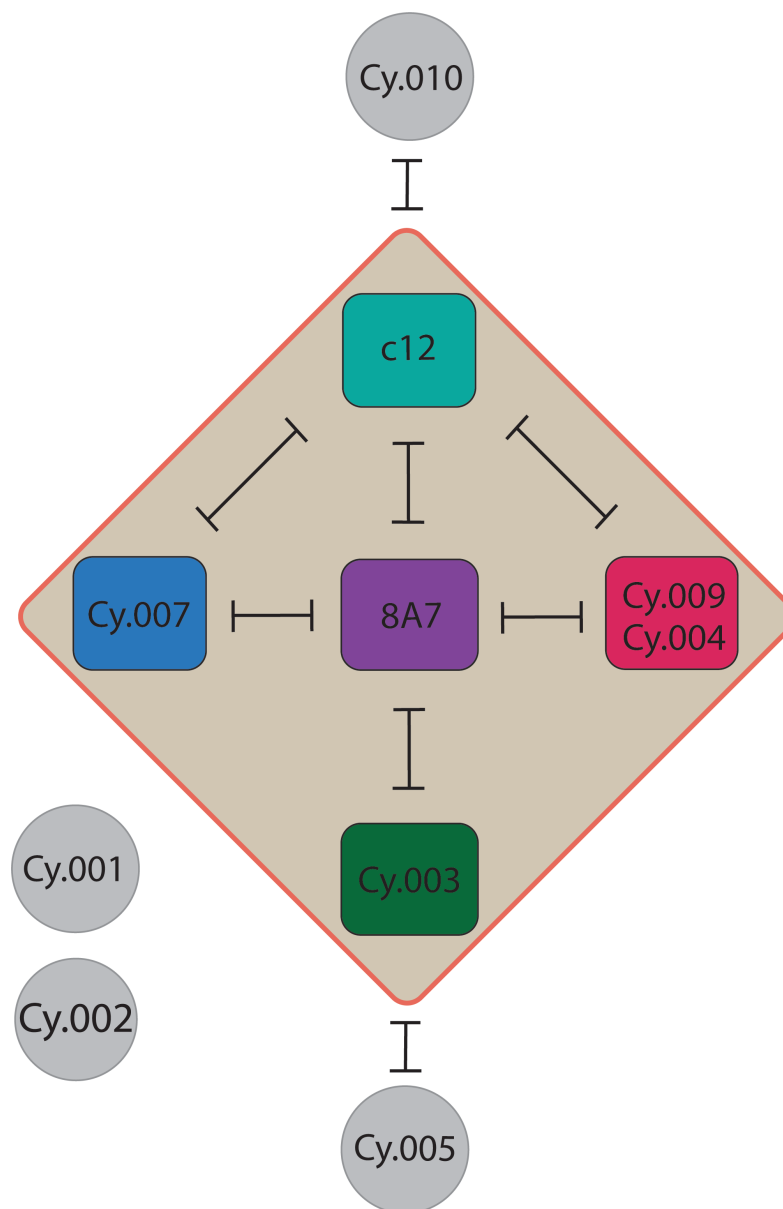


Figure 3.9 Summary of competition between antibodies as established through the ELISA-based method. All mAbs within the centre diamond are growth inhibitory *in vitro*, while mAbs in grey circles are non-inhibitory.

3.2.5 *Kinetic measurements of anti-PfCyRPA mAbs*

To better understand antibody functionality and perhaps explain some of the differences in biological activity, kinetic and/or affinity analyses of the panel of anti-PfCyRPA mAbs binding to PfCyRPA were undertaken using SPR. Kinetics/affinities were determined through multi-cycle SPR with mAb captured by protein G and regenerated using 0.2M glycine pH 1.5 (**Figure 3.10**). The two most potently inhibitory antibodies, Cy.004 and Cy.009, had remarkably poor kinetics, defined by rapid dissociation as seen by the short time to reach steady-state and the steep slope after the end of analyte injection. For this reason, steady-state affinity was used to determine the K_D of Cy.004 and Cy.009, as the k_d could not be reliably calculated (**Figure 3.11**).

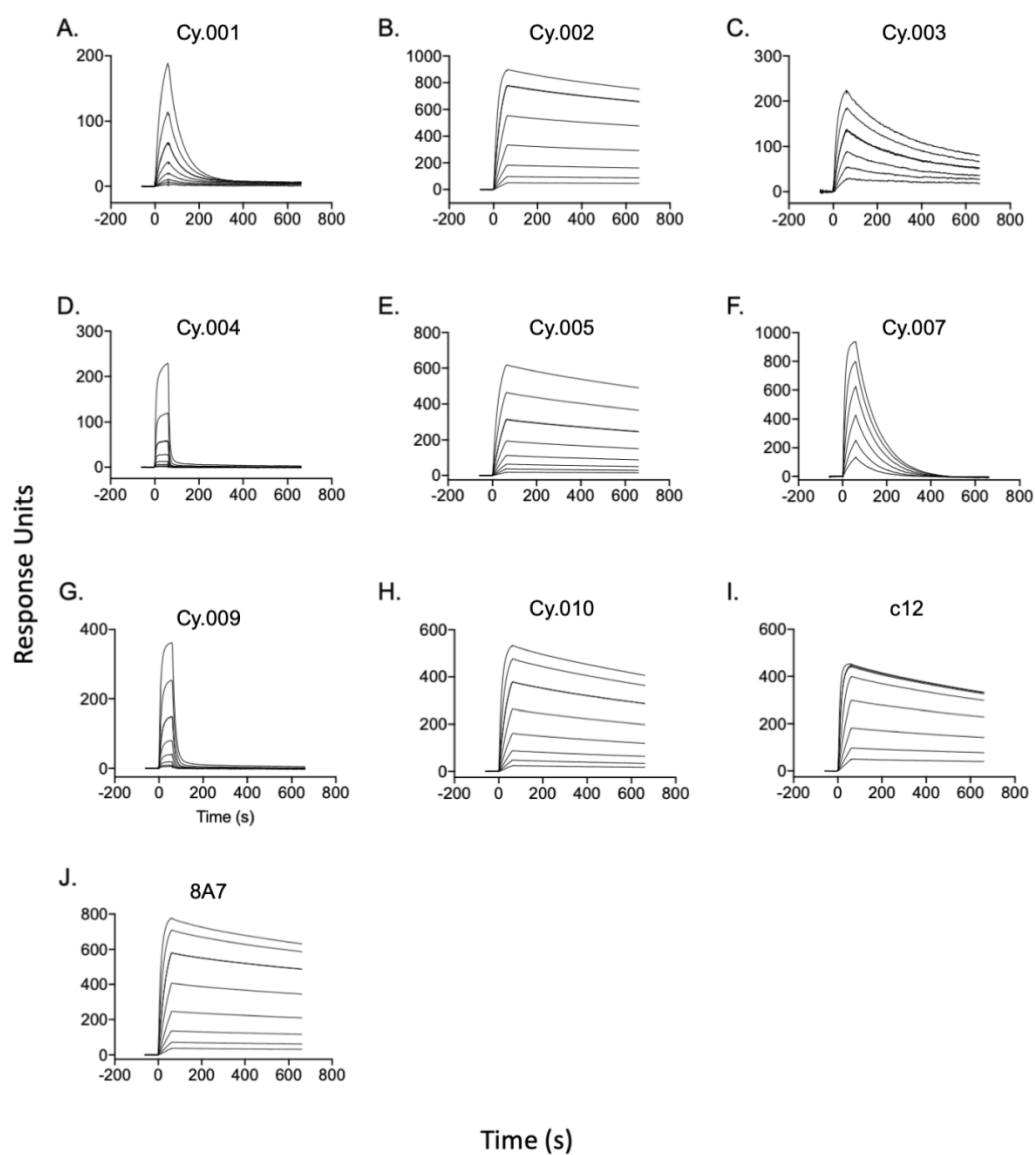


Figure 3.10 Antibody kinetics as determined by SPR using protein G chip. 2-step dilution curve with the top concentration for each antibody indicated in brackets. A. Cy.001 (500nM) B. Cy.002 (250nM) C. Cy.003 (250nM) D. Cy.004 (500nM) E. Cy.005 (500nM) F. Cy.007 (1000nM) G. Cy.009 (500nM) H. Cy.010 (500nM) I. c12 (500nM) J. 8A7 (500nM)

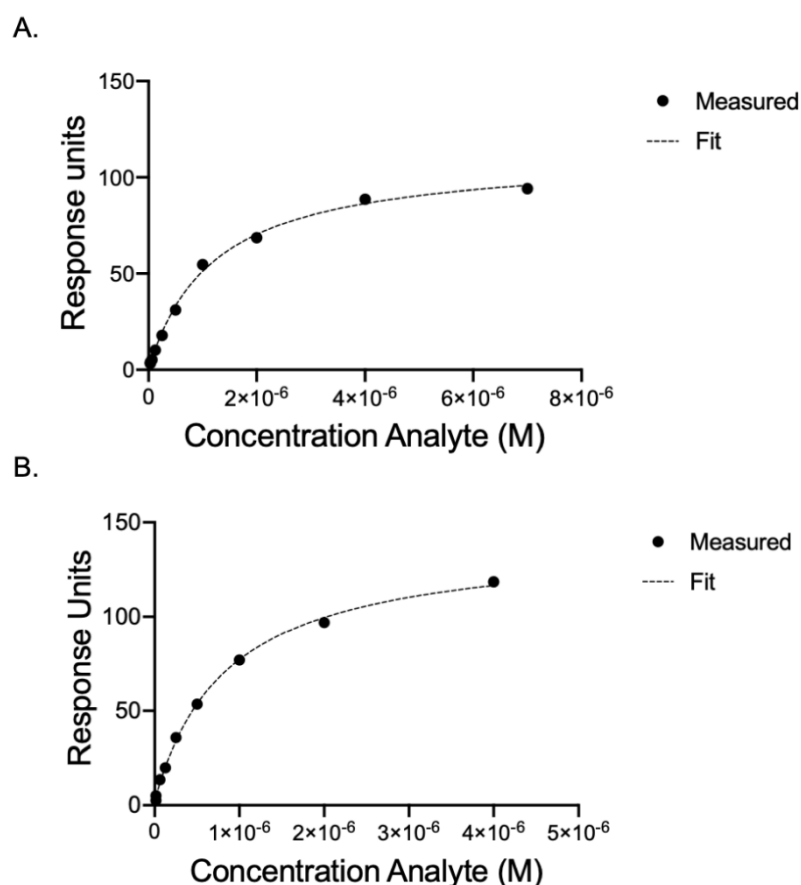


Figure 3.11 Steady state affinity measurements using CM5 immobilization. A. Cy.004. B. Cy.009.

It is well established that higher antibody affinity for its target is a desired property, as it typically improves antibody function²⁰⁰. In this case, there was no clear relationship between antibody affinity and GIA potency with no relationship found between k_a , k_d or K_D and GIA potency based on Pearson's correlation coefficient. The two most potent antibodies, Cy.004 and Cy.009, had the lowest affinity, up to four orders of magnitude worse than c12 (**Table 3.2**). From this, it was concluded that the reason for the superior GIA is not because of better binding kinetics. Intriguingly, Cy.004 and Cy.009 both competed with c12 and 8A7 and had much worse kinetics, yet inhibited invasion more effectively. This makes clear the crudeness of epitope binning for assessing antibody relationships. The relative contribution

of improved binding kinetics may be masked by significant differences in epitope-specific potency, despite the epitopes of each of these antibodies overlapping. It seems unlikely that worse binding kinetics would improve an antibody's potency, so in this case, it is likely that the specific epitope and/or angle of binding are more important factors in determining inhibitory capacity than kinetics.

Table 3.2 mAb kinetics and affinity SPR measurements. The U-value is the uniqueness of fit where a lower value indicates higher confidence in the results.

mAb	ka (10 ⁵ M ⁻¹ s ⁻¹)	kd (10 ⁻⁴ s ⁻¹)	KD (M)	U- value
Cy.001	0.572 ± 0.009	124.5 ± 0.660	2.18 × 10 ⁻⁷	4
Cy.002	3.07 ± 0.012	2.89 ± 0.007	9.42 × 10 ⁻¹⁰	1
Cy.003	2.73 ± 0.027	22.05 ± 0.160	8.08 × 10 ⁻⁹	1
Cy.004	N.D.	N.D.	1.18 × 10 ⁻⁶	N.A.
Cy.005	1.43 ± 0.004	4.06 ± 0.016	2.83 × 10 ⁻⁹	1
Cy.007	1.04 ± 0.038	109.6 ± 1.7	1.06 × 10 ⁻⁷	1
Cy.009	N.D.	N.D.	8.50 × 10 ⁻⁷	N.A.
Cy.010	2.24 ± 0.001	4.65 ± 0.005	2.08 × 10 ⁻⁹	1
c12	7.41 ± 0.025	5.33 ± 0.014	7.19 × 10 ⁻¹⁰	1
8A7	2.49 ± 0.009	2.88 ± 0.006	1.16 × 10 ⁻⁹	1

3.2.6 Assessment of anti-PfCyRPA mAb binding to the RCR complex

The only established role of PfCyRPA in *P. falciparum* invasion is architectural, bridging the interactions between PfRH5 and PfRipr¹⁴. Thus, it was of interest to determine whether or not there was a relationship

between the ability to block complex formation and the ability of an antibody to inhibit merozoite invasion.

In order to assess this, an SPR-based approach was used. mAb was immobilized on a protein G chip and then binding to PfCyRPA alone was measured compared to binding to the RCR complex. Due to the higher molecular weight of the RCR complex, given the same binding kinetics, one would expect a higher response unit peak for the RCR complex compared to

PfCyRPA alone, since this is dependent on mass concentration. Thus, a reduction in response units relative to PfCyRPA binding would be indicative of reduced binding to the PfCyRPA protein when in complex with PfRH5 and PfRipr.

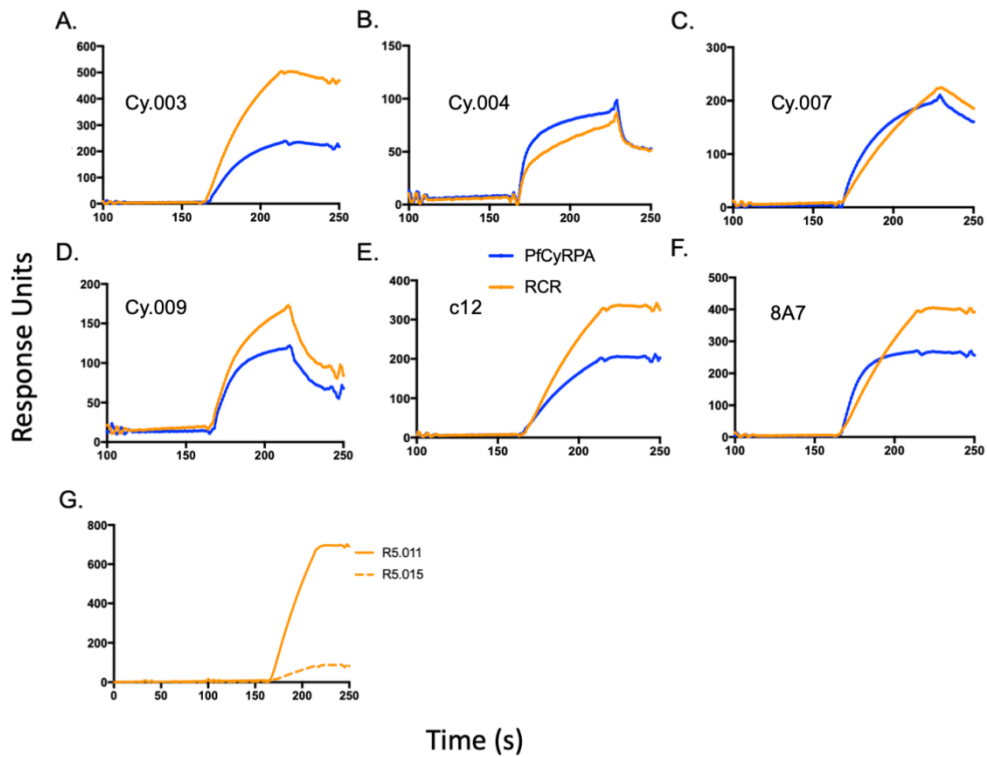


Figure 3.12 RCR binding profiling of neutralizing antibodies by SPR where PfCyRPA is shown in blue and RCR complex binding is shown in orange. A. Cy.003 B. Cy.004 C. Cy.007 D. Cy.009 E. c12 F. 8A7. G. RCR binding to anti-PfRH5 mAbs R5.011 and R5.015.

Among the neutralizing anti-PfCyRPA mAbs, all six appeared to bind to the assembled RCR complex (**Figure 3.12**). In this experiment R5.011 and R5.015 were used as the positive and negative controls, respectively, as the Fab:PfRH5 structures of both of these antibodies have been solved, with R5.011 binding away from the PfRH5:PfCyRPA interface, while R5.015 blocks PfCyRPA binding⁶⁸. As expected, R5.015 binding to RCR appears to show substantially reduced binding compared to R5.011. One antibody, Cy.004, showed marginally reduced

binding to the RCR complex as compared to PfCyRPA alone. This result is slightly ambiguous, but when combined with the data showing that binding to both dual complexes also follows a very similar curve to PfCyRPA alone as well, it does appear that Cy.004 binds to the RCR complex (**Figure 3.14**). Perhaps there are some restrictions on antibody orientation or angle of approach that reduces the binding efficiency slightly.

These data are in contrast to previously published results where 8A7 was reported to block the PfRH5 interaction with PfCyRPA¹⁰⁶. It is worth noting that based on the available structural data, it is not clear how this would occur as the PfRH5 and 8A7 sites are non-overlapping¹⁴. Further, similar RCR binding assays were carried out by our laboratory on the panel of anti-PfCyRPA mAbs reported here using SEC and co-immunoprecipitation; these analyses showed the same binding profiles shown here in this Thesis via SPR²⁰¹. These data therefore provide further support for this dataset, and deviate from the only published data evaluating antibody disruption of the RCR complex¹⁰⁶.

Among the non-neutralizing antibodies, only one, Cy.005, was able to bind to the assembled RCR complex as well as PfCyRPA alone (**Figure 3.13C**). For Cy.001, Cy.002 and Cy.010, binding to the RCR complex was substantially reduced compared to binding to PfCyRPA alone (**Figure 3.13**) This suggests that their binding sites overlap with either that of PfRH5 or PfRipr. Some low-level binding was still observed for all mAbs. This could be due to non-specific interactions of the RCR complex with the chip or disassembly of the tertiary complex into its components allowing free PfCyRPA to bind to the immobilized mAb.

To further parse the nature of the RCR complex binding profile, SPR-based binding was then compared between PfCyRPA alone and the two bipartite complexes, PfRH5: PfCyRPA and PfCyRPA: PfRipr. Again, all neutralizing mAbs bound both dual complexes, alongside

Cy.005. Among the non-neutralizing mAbs that could not bind the complex, all three showed reduced binding of one or both bipartite complexes compared to PfCyRPA alone. Notably, Cy.002 showed high levels of PfCyRPA:PfRipr binding, and reduced levels of PfRH5:PfCyRPA, suggesting its epitope overlaps with the PfRH5 binding site, but not that of PfRipr (**Figure 3.14A**). Here, higher level of background binding was observed likely due to the fact that the bipartite complexes seem to more rapidly dissociate than the tripartite complex (**Figure 3.14**).

Conversely, Cy.001 and Cy.010 both show reduced binding to PfRH5:PfCyRPA and PfCyRPA:PfRipr (**Figure 3.14A**); although, it is worth noting that the decrease in binding to the bipartite complexes by Cy.001 is only marginal. These antibodies do not compete with each other, yet presumably their epitopes are situated somewhere between the PfRH5 and PfRipr binding sites, allowing them to simultaneously compete with both complex PfRH5 and PfRipr. This would suggest that Cy.001 and Cy.010 bind between PfRipr and PfRH5 but from different directions, hence no competition is observed (**Figure 3.9**). Meanwhile, of the seven RCR complex binders, only c12 showed a slight decrease in binding to one of the bipartite complexes (PfCyRPA:PfRipr) compared to PfCyRPA alone, and this decrease was relatively small (<10%). Taken together, these data indicate that binding to the assembled RCR complex is necessary for *in vitro* growth inhibition, but complex binding alone is not sufficient for inhibition, as indicated by the lack of GIA for Cy.005. Thus, the mechanism of antibody inhibition does not appear to be disruption of the tertiary complex.

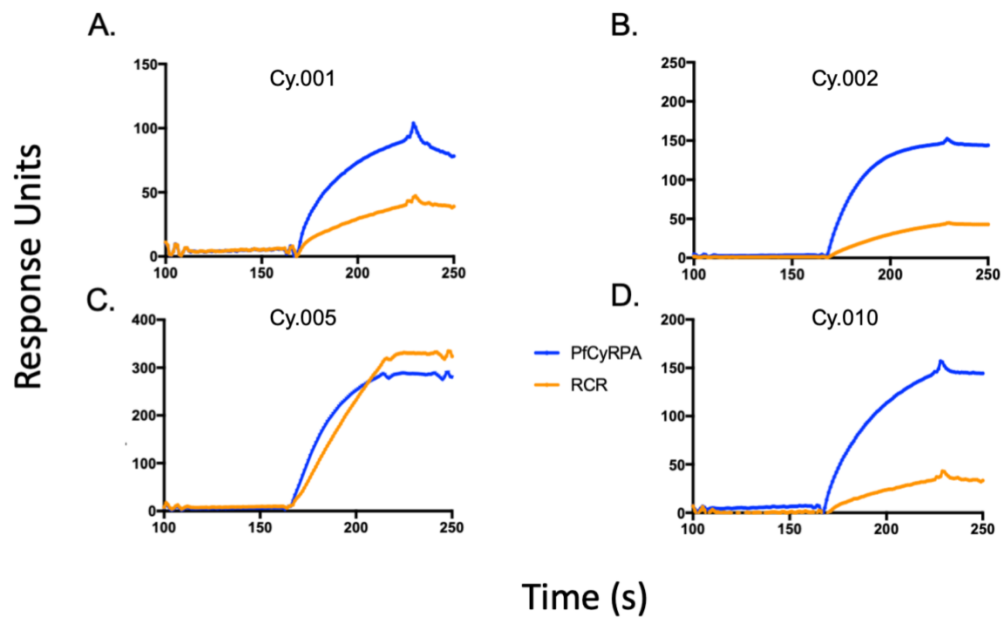
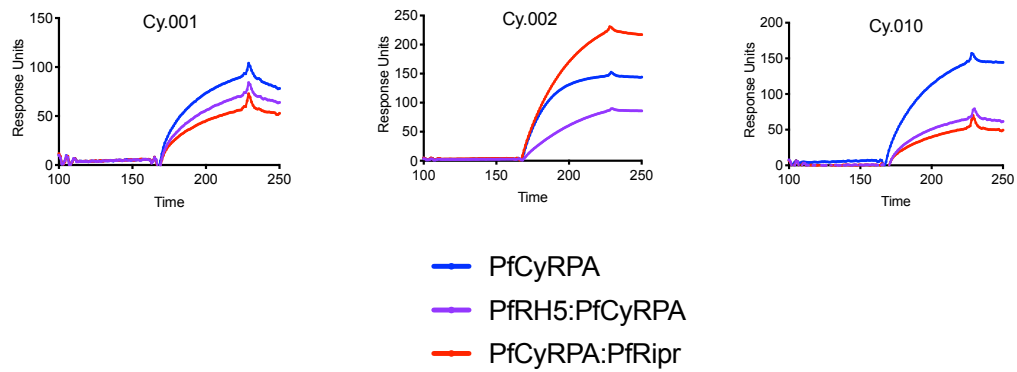


Figure 3.13 RCR binding profile of non-neutralizing antibodies by SPR where PfCyRPA binding is indicated in blue and RCR complex binding is in orange. A. Cy.001 B. Cy.002 C. Cy.005 D. Cy.010.

A. RCR non-binders



B. RCR binders

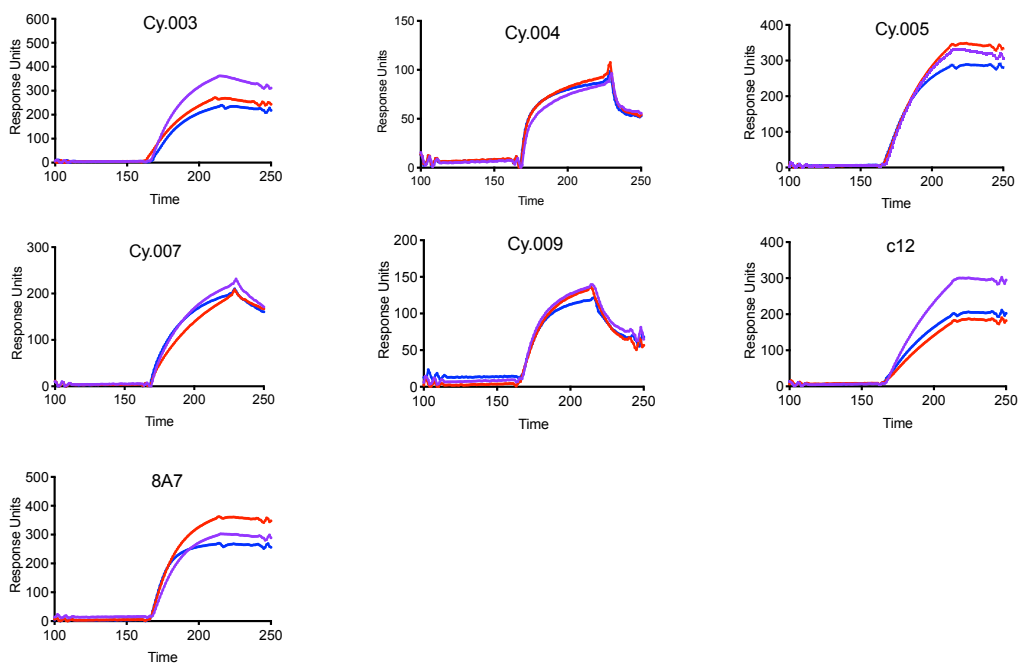


Figure 3.14 Anti-PfCyRPA mAb binding to bipartite PfRH5:PfCyRPA and PfCyRPA:PfRipr complexes by SPR. A. Binding of mAbs that did not bind to tripartite complex (RCR non-binders). B. Binding of mAbs that did bind to the tripartite complex (RCR binders).

3.2.7 Evaluation of RCR-mediated haemolysis

Given that it appears unlikely that inhibitory antibodies directly disrupt the tertiary complex, the next step was to evaluate whether inhibitory anti-PfCyRPA mAbs blocked the function of the RCR complex, even if the complex itself remained intact when the mAbs were bound. One proposed theory of the function of the RCR complex is that PfRH5 and PfRipr insert into the membrane after PfRH5 binds to basigin²³. One piece of evidence for this theory is that the RCR complex was shown to be haemolytic²³. If insertion into the erythrocyte membrane is important for the function of the RCR complex, then perhaps inhibitory antibodies targeting this complex could block this process. This was a proposed mechanism for how c12, which points toward the erythrocyte membrane when bound to PfCyRPA, could function. One way to evaluate this was to determine if incubation of the RCR complex with anti-PfCyRPA antibodies could abolish the RCR complex's haemolytic activity.

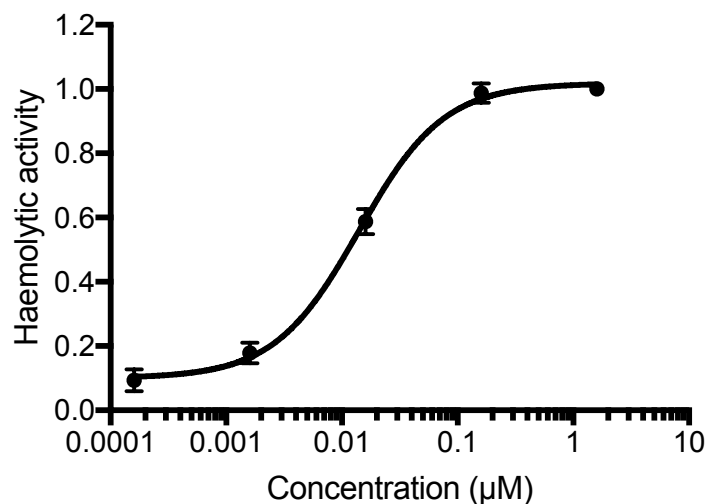


Figure 3.15 *S. aureus* α -haemolysin induced haemolysis relative to 1% Triton X-100. Triton X-100 treatment was taken to be 100% haemolysis (1.0 on Y-axis). Each data point is the mean of three independent replicates and the error bar indicates the standard deviation.

In order to assess this, the assay was first set-up comparing haemolysis induced by α -haemolysin using 1% Triton X-100 as a positive control and PBS as the negative control (**Figure 3.15**). The OD₄₀₅ for the negative control (PBS treatment) was 0.33 AU compared to 1.6 AU for the Triton X-100 treated group providing adequate dynamic range to assess haemolysis. The EC₅₀ for α -haemolysin in this experiment was 0.14 μ M (95% CI 0.10 μ M-0.18 μ M, n = 3). This aligns well with previously reported values for α -haemolysin haemolysis of human RBCs²⁰².

To determine the haemolytic activity of the RCR complex, erythrocytes were incubated with 2 μ M of RCR complex, or each of PfRH5, PfCyRPA or PfRipr on their own. This concentration was chosen to replicate the results of Wong *et al.* who first reported this finding (they used a concentration of 1.6 μ M)²³. As a positive control, 1 μ M α -haemolysin was used while PBS alone was used as the negative control. The original published experiments did not include a positive control, which makes it difficult to interpret the increase in lysis observed by incubation with the RCR complex.

There was no significant haemolysis induced by the RCR complex or any of its constitutive components (**Figure 3.16**). This is in contrast to the results reported by Wong *et al.*²³. While the data here show an increase above baseline (PBS-treated) haemolysis for the RCR-complex treated group, it is not significantly different from the PBS-treated group, nor from any of the groups treated with only one of the components of the complex. Thus, the ability of these inhibitory antibodies could not be assessed as the finding that the RCR complex induced haemolysis could not be replicated.

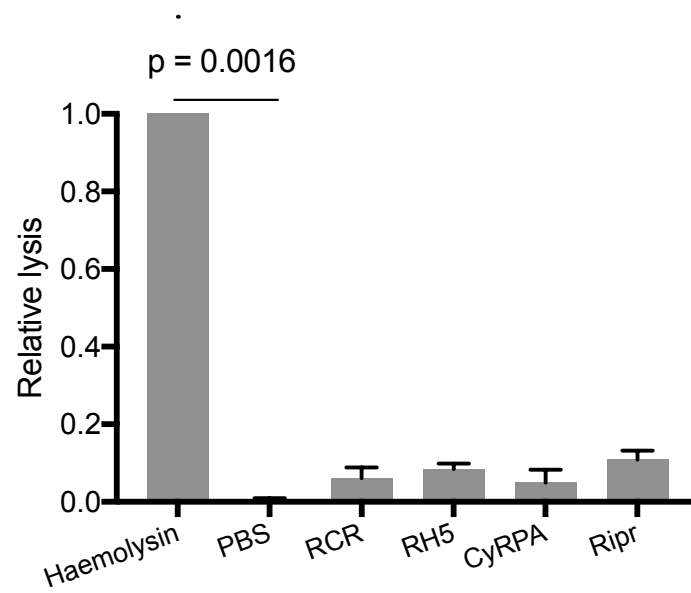


Figure 3.16 RCR-induced haemolysis. Kruskal-Wallis test was used to compare groups with a Dunnett's multiple comparison's post-test. Each bar indicates the mean with the error bars indicating the standard deviation across three independent replicates all run in triplicate.

3.2.8 GIA of anti-PfCyRPA Fabs

Another possible mechanism through which anti-PfCyRPA mAbs may inhibit invasion is through steric hindrance with the membrane, as was reported for c12. I sought to investigate this as another possible mechanism of anti-PfCyRPA invasion inhibition. It was previously noted that based on the orientation of the RCR complex when it binds to basigin, anti-CyRPA mAb c12 likely points towards the erythrocyte membrane^{23,106}. Based on this, the authors speculated that c12 could prevent the complex from getting close enough to the membrane to either bind basigin or for PfRH5/PfRipr to insert into the membrane²³.

In order to test this, it was reasoned that if the mechanism of c12 was indeed related to steric hindrance with the membrane, then the same binding module, but smaller, should decrease parasite growth inhibition. Therefore, to investigate this, the panel of inhibitory mAbs were cleaved into Fabs through papain digestion and then Fabs and Fcs were separated by protein A affinity followed by size exclusion chromatography. The GIA of these Fabs were then measured in a GIA assay to determine how this size reduction impacted inhibition potency. Cy.002 was included as a non-inhibitory mAb control, under the assumption that it should remain non-inhibitory since it cannot bind the RCR complex.

GIA between the mAb and Fab of the same antibody were compared at equimolar concentrations (**Figure 3.17**). The results show that for Cy.003, Cy.004, and Cy.009, the Fab and mAb perform similarly, albeit not identically, in the assay. However, Cy.007 and c12 (the two weakest inhibitory antibodies) showed dramatically increased potency. Based on extrapolated IC_{50} values, the IC_{50} reduced to 1.614 μ M from 111.8 μ M for c12 and 1.931 μ M from 86.43 μ M to Cy.007. Although the values for the mAb IC_{50} values are subject to a high degree of uncertainty due to extrapolation, it appears that the smaller Fabs are much more

potent than their mAb counterparts. In previous studies that examined how anti-PfRH5 Fabs compared to their mAb counterparts, no such difference was observed^{67,68}.

The observation that both Cy.007 and c12 demonstrate increased Fab potency, have similar mAb IC₅₀ values and compete with each other in the ELISA competition assays suggests they are likely very similar mAbs in terms of epitope and angle of approach. However, they are not identical, given c12 also competes with inhibitory mAbs Cy.003, Cy.004 and Cy.009, as well as non-inhibitory mAb Cy.010, suggesting there are some slight differences in the binding epitope.

Returning to the original hypothesis, and contrary to what has been previously published, if there is a role for steric hindrance in the invasion mechanisms of these antibodies, it is more likely to be the opposite of the theory originally proposed. Since the smaller Fab fragment is more potent than the much larger mAb, steric effects likely impede the function of Cy.007 and c12, rather than mediate invasion inhibition.

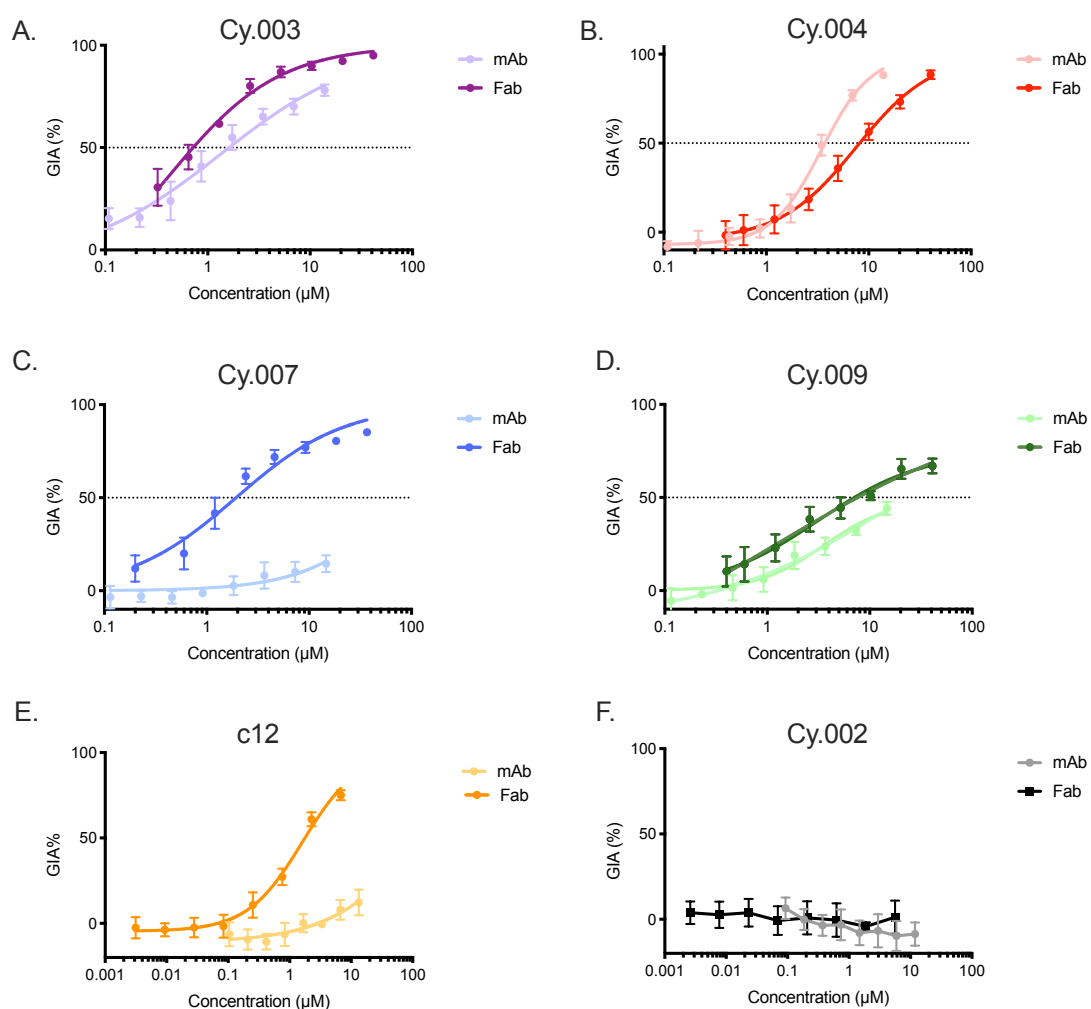


Figure 3.17 Anti-PfCyRPA Fab vs mAb GIA. mAb and Fab were run in the same assay except for c12 and Cy.002 where mAb and Fab data have been pooled from separate experiments. Points indicate mean and error bars denote the standard deviation across triplicate. Curves were fit using a four-parameter logistic regression with the upper limit constrained to 100% GIA. A. Cy.003 B. Cy.004 C. Cy.007 D. Cy.009 E. c12 F. Cy.002.

3.3 Discussion

3.3.1 Development of potent anti-PfCyRPA mAbs

In this Chapter, a panel of eight new anti-PfCyRPA mAbs were characterized based on their binding properties, ability to block merozoite invasion and ability to bind to the assembled RCR complex.

Cy.004 and Cy.009 outperform the most potent published anti-PfCyRPA mAbs, c12 and 8A7. This is in spite of having remarkably poor binding kinetics. This suggests that significant improvements could be made to these antibodies should kinetic parameters be improved. There are two broad approaches that could be taken to do this. The first is to simply expand the panel of anti-PfCyRPA antibodies. In particular, antibodies isolated from humans after a multi-dose vaccine regimen routinely have affinities below 10 nM^{42,199}. Given that PfCyRPA remains a vaccine target of interest, vaccinees would be an ideal source of fully human potentially neutralizing antibodies. Fully human anti-PfCyRPA mAbs would be difficult to isolate from individuals who were naturally infected due to the low immunogenicity of PfCyRPA over the course of natural infection^{70,97}, meaning that until the first humans are vaccinated with PfCyRPA, studies of anti-PfCyRPA mAbs will be confined to mAbs from other animal sources, as was the case in this Chapter.

This approach would require a high throughput method to screen antibodies for inhibitory activity. Ideally, this would be done without using a GIA assay, which is labour intensive and fairly low throughput for large antibody screens. One approach could be to screen based on an antibody's ability to compete for binding with 8A7. At present, all of the inhibitory antibodies compete with 8A7 for binding, perhaps indicating that 8A7 binds at the centre of a critical inhibitory region. This would then eliminate all antibodies that bind

elsewhere on PfCyRPA from the screen, thereby (hopefully) removing all of the non-inhibitory antibodies. However, this alone is not enough to determine antibody potency as both the strongest (Cy.004, Cy.009) and weakest (c12, Cy.007) inhibitory antibodies compete with 8A7 for binding. Thus, more extensive antibody characterization would be required to find the most potent inhibitory clones, likely through assays of GIA.

An alternative to this would be to directly improve Cy.004 and Cy.009 binding kinetics. There are many examples of successfully improving an antibody's binding kinetics *in vitro* through peptide display²⁰³, using random mutagenesis or more targeted mutagenesis to specific regions of the CDR loops, targeted based on alanine scanning to identify critical residues^{204–206}. Alternatively, if the interaction between PfCyRPA and Cy.004/Cy.009 can be determined at near-atomic resolution, the interface between the CDR loops and PfCyRPA should be clearly resolved. With this information, computational approaches to antibody maturation can be used²⁰⁶. These computational methods can screen a greater number of possible CDR variations (10^{40} compared to 10^{10} for *in vitro* approaches); the best candidates can then be selected for expression and assessment of binding affinity based on predicted favourable energetic properties²⁰⁷.

Improved binding kinetics do not necessarily improve function. Previous reports of antibody engineering to improve the binding kinetics of palivizumab, a respiratory syncytial virus prophylactic therapy, did not substantially improve the clinical performance of the drug^{208,209}. It is thought that germinal centres cannot differentiate between antibodies that have an affinity lower than 100 pM^{210,211}. This suggests, although is certainly not proof of, the idea that at some point no functional improvement is seen with better kinetics.

3.3.2 *Mechanisms of invasion inhibition*

The epitope binning carried out in this Chapter is a fairly crude way of determining where on PfCyRPA these antibodies bind. Although the data suggest that the four new inhibitory mAbs all bind the same region as 8A7, which binds to blades 2/3 of the PfCyRPA propeller, it does not explain why Cy.004 and Cy.009 are more potent antibodies. Relatively minor differences in the position of the epitope, or alternatively, a different angle of binding, may dramatically change antibody potency. This is one possible mechanism to explain the variation in potencies, which does not appear to be related to an antibody's kinetics or its ability to block PfRipr or PfRH5 binding.

With this in mind, determining the crystal structures of the Fab fragments of these mAbs bound to PfCyRPA would help to understand antibody function and to inform why Cy.004 and Cy.009 are more potent mAbs. This would also enable the rational design of more potent antibodies in the future and provide a detailed understanding of the structural determinants of inhibition. It could also potentially provide insight into the mechanism of action of these mAbs. This is explored further in **Chapter 4**.

It is not clear how anti-PfCyRPA antibodies function. Blocking the PfRH5 and PfRipr interfaces does not appear to be the mechanism of action for these antibodies. Of the antibodies that bound to regions of PfCyRPA that compete for binding with PfRH5 and PfRipr, none showed inhibitory activity. This is consistent with the complex emerging from the merozoite preformed, or forming quickly upon secretion from the merozoite. If the complex is already formed, then these epitopes would not be accessible. This is also consistent with the antibody analysis carried out for PfRH5 and PfRipr²⁰¹ whereby antibodies that bind epitopes that are inaccessible on the assembled RCR complex fail to block parasite invasion. However, this is not consistent with previously published reports by others of how anti-RCR

complex antibodies block invasion⁶⁹. Healer *et al.* showed that antibodies that block critical interactions between PfRH5 and PfRipr block invasion of the erythrocyte⁶⁹. There are some reasons to be sceptical of these results. The first is that the authors show 8A7 blocks the PfRH5 interaction, a finding that could not be replicated here. Then, the authors show that 2AC7, a well characterized anti-PfRH5 mAb, blocks the PfRH5-PfCyRPA interaction. However, 2AC7 binds the tip of PfRH5, adjacent to basigin, which is on the opposite side of the PfRH5 kite to where PfCyRPA interacts^{19,67,68}. Finally, many other PfRH5 antibodies that block the PfRH5-PfCyRPA interaction have been described, all of which fail to neutralize parasites⁶⁸.

The existing theory, that anti-PfCyRPA mAbs block membrane insertion or that they sterically hinder via the Fc interaction with the erythrocyte membrane^{23,69}, do not appear to be well supported based on the data presented here. Indeed, Fabs demonstrated similar or superior inhibition to their mAb counterparts, which suggests that Fc-mediated steric hindrance is not the mechanism of action. The observation that the RCR complex is haemolytic could also not be replicated, which meant that it could not be determined whether anti-PfCyRPA antibodies block this function of the RCR complex. An alternative approach to assessing membrane insertion of the RCR complex would be to explore differential solubilization of the RCR complex components after incubation with erythrocytes in the presence and absence of specific inhibitory anti-PfCyRPA clones. Previously, it was shown that PfRipr is associated with the membrane fraction, which was in part why the theory of membrane insertion was proposed²³. If the anti-PfCyRPA antibodies block complex function by preventing membrane insertion of PfRH5 and PfRipr in the presence of anti-PfCyRPA mAb, then PfRipr should not be associated with the membrane fraction.

Given the results of the haemolysis experiments, to date, the only function of the RCR complex that is seemingly certain is its interaction with erythrocyte basigin. Fundamentally,

we do not understand what this complex does biologically, if anything, beyond binding to its erythrocyte receptor. However, basigin binding is not dependent on PfCyRPA, as only PfRH5 interacts directly with basigin, which suggests the biological reality is more complex than a simple receptor-ligand interaction. It would be worthwhile to assess whether or not anti-PfCyRPA antibodies can indirectly block the RCR complex-basigin interaction, given that this is the only known function.

This could be tested by making RCR complex-based fluorescent tetramers and staining erythrocytes with or without the presence of anti-PfCyRPA antibodies. Conveniently, since PfRH5 antibodies that block this interaction (e.g. R5.004) or do not block basigin or RCR complex formation (e.g. R5.011) have been isolated, these could serve as useful negative and positive controls⁶⁸. One would expect that if a given mAb does not interfere with basigin binding, then the cells should be fluorescently stained, and if it does, then they should not be, as determined through flow cytometry. Tetramers are likely required due to the low affinity of the PfRH5-basigin interaction. This experiment would determine whether PfCyRPA blocks the PfRH5-basigin interaction in the context of the human erythrocyte so long as there are no other receptor-ligand interactions between the RCR complex and the erythrocyte, or non-specific interactions such as through heparin sulfate proteoglycans²¹². The recent observation that PfRipr binds to SEMA7A may complicate this experiment, as additional receptor interactions between the RCR complex and the erythrocyte would mean that blocking basigin would not block RCR complex binding to the erythrocyte.²¹³ This type of experiment has been done previously with some success, although the results do show high background binding among the negative controls²³. Other approaches, such as assessing quaternary RCR-mAb complex binding to basigin using techniques such as SPR or ELISA could also be used; however,

these approaches would remove the interaction from the context of a membrane, making it less physiologically relevant.

Another useful approach to determine how anti-PfCyRPA antibodies inhibit invasion would be to use video microscopy. This has been used previously to assess changes in *P. falciparum* egress and invasion.^{16,22,68,214} Through this approach, the phenotype of invading merozoites in the presence of an anti-PfCyRPA mAb could be compared to that of a basigin blocking anti-PfRH5 mAb and to that of an unrelated non-inhibitory mAb. This could provide useful insight as to whether or not anti-PfCyRPA mAbs function at the same steps of invasion as anti-PfRH5 mAbs, even if they do not directly block the PfRH5-basigin interaction. Given the presence of CyRPA in *P. knowlesi* but not RH5, it is possible that PfCyRPA could retain additional ancestral functions beyond bridging the PfRH5-PfRipr interaction.⁸³ It is possible then, that the mechanism of action could be unrelated to the RCR complex. Perhaps there is an additional interacting partner of PfCyRPA that binds on the free-face where c12/8A7 bind. This is entirely speculative simply to illustrate there are possibilities for mAb mechanisms that extend beyond the current scope of the RCR complex function.

Previously, it was shown that R5.011, a non-neutralizing mAb, can synergize with other anti-PfRH5 mAbs, as well as mAbs against PfCyRPA, PfRipr, and PfAMA1⁶⁸. Future experiments, notably those carried out in **Chapter 5**, should investigate the relationships between different anti-PfCyRPA mAbs, as it is of interest whether these antibodies can cooperate in a similar way as to what was seen with anti-PfRH5 mAbs.

In conclusion, the work in **Chapter 3** has shown that all of the characterized inhibitory anti-PfCyRPA mAbs appear to bind to the same region of PfCyRPA, which is accessible even when in complex with PfRH5 and PfRipr. The most potent antibody clones, Cy.004 and Cy.009,

do not appear to derive their improved potency based on kinetic parameters, but rather it likely lies in the precise position of the epitope relative to the other inhibitory anti-PfCyRPA mAbs. The mechanism of inhibition for anti-PfCyRPA antibodies remains unclear. In the following chapter I will therefore go on to use X-ray crystallography to carry out a detailed structural investigation of the Fab:PfCyRPA complexes for the inhibitory anti-PfCyRPA mAbs in hopes of understanding some of the structural features that are shared between potentially inhibitory mAbs.

Chapter 4: Structural basis of anti-PfCyRPA invasion inhibition

Authorship Statement

In this Chapter, some synchrotron data was collected by Dr. Edward Lowe, of the Oxford University's Department of Biochemistry. Dr. Abhishek Jamwal (Department of Biochemistry) built and refined the structure of Cy.007 bound to PfCyRPA, and assisted with the final refinement steps of Cy.004 bound to PfCyRPA.

4.1 Introduction

4.1.1 Relevance of structural biology to antibody-antigen interactions

The ease and rapidity with which three dimensional structures of proteins can be determined with atomic level accuracy is one of the great advances of modern biochemistry. These structures, when coupled with relevant functional data, can reveal the mechanism(s) underlying protein function. In the context of antibody-antigen interactions, there has been expanding interest in using these techniques to understand structural features of antibodies that protect against disease and antibodies which do not. These findings can be used to engineer antibodies with specific functions, usually enhanced potency, or design synthetic antigens that can elicit antibodies with a known epitope specificity, the latter of which is one type of structure-guided vaccine design, as described in the introduction to this Thesis.

These structural insights have advanced the field of vaccinology by informing the mechanism underlying antibody-based inhibition of certain pathogens. This includes revealing the utility of exceptionally long CDRH3 loops found in HIV broadly neutralizing antibodies, which enable the antibody to penetrate through the heavy glycosylation of the glycoprotein and interact directly with the viral membrane²¹⁵. As well, the finding that some

neutralizing epitopes are only accessible in either the pre- or post-fusion conformation for the RSV F-glycoprotein trimer led to the idea that structure-guided stabilization of this trimer could ensure these epitopes remained accessible to the immune system^{216–218}. Using these methods, key cross-species inhibitory epitopes of the Ebola spike protein have also been identified²¹⁹. And more recently, during the [ongoing at the time of writing] SARS-CoV-2 pandemic, structural insights have revealed that most neutralizing antibodies bind predominantly to the receptor binding domain of the SARS-CoV-2 spike protein, although they do not necessarily block ACE2 receptor binding directly^{220–222}. Taken together, understanding the structural underpinnings of antibody-antigen interactions can inform our understanding of protective immune responses and guide vaccine design.

4.1.2 Methods in protein structural biology

Broadly, there are three methods in structural biology used to solve protein structures: nuclear magnetic resonance (NMR), X-ray crystallography and cryo-electron microscopy (cryo-EM). NMR is predominantly used for highly disordered proteins. X-ray crystallography is a versatile technique, but relies on the ability of a protein to pack in a highly ordered manner, which enables crystal formation. This precludes structural determination of proteins with high intrinsic flexibility or large disordered regions. Cryo-EM has become widely used in the past decade as technical advances, in both hardware and software, have enabled structural determination at the atomic level, with the resolution record at the time of writing standing at 1.54Å²²³. However, structures of this resolution are not routinely described using cryo-EM, with new cryo-EM structures often having resolutions closer to 4Å²²⁴. Like X-ray crystallography, high intrinsic flexibility presents challenges with cryo-EM, limiting the resolution of the reconstructions. There is the added challenge that different views of the protein must be distinguished in order to enable image classification, which can be difficult

for pseudo symmetric proteins²²⁵. A benefit of cryo-EM is that it uses single dispersed particles for analysis, i.e. the requirement that a protein is capable of ordered packing for crystallization is no longer relevant. As such, cryo-EM is most useful for large proteins or protein complexes which are often difficult to crystallize. To date, there have been only a handful of successful structures determined through cryo-EM for proteins <100kDa²²⁶.

In this Chapter, X-ray crystallography is used for structural analysis. The reason for this is that the Fab:PfCyRPA complexes examined are only 90kDa and cryo-EM may have proven difficult on these relatively small protein complexes. Further, there have been two previous successes crystallizing PfCyRPA, reported by two different groups, revealing a 6-bladed propeller, which is a rigid structure that lends itself to crystallization^{66,72}. As such, I reasoned that X-ray crystallography had a reasonable probability of yielding better structural information than cryo-EM.

4.1.3 Chapter aims

The aims of this Chapter were to understand the structural basis for anti-PfCyRPA mAb inhibition through the use of X-ray crystallography. Using the data from Chapter 3 of this thesis, I sought to establish a detailed structural understanding of anti-PfCyRPA inhibitory mAbs, while considering these structural findings in the context of mAb GIA, kinetic and epitope binning data from the previous Chapter. The antibodies that were chosen for crystallization were done so on the basis of being from distinct epitope bins, with the exception of Cy.004/Cy.009. Both Cy.004 and Cy.009 were crystallized despite belonging to the same epitope bin because the more potent of the two, Cy.009, did not readily diffract. As a result Cy.004:PfCyRPA which yielded strongly diffracting crystals was crystallized as well to complement the lower resolution data from Cy.009:PfCyRPA. With this information, I aimed

to understand structural features of potent compared to weakly inhibitory antibodies and begin to understand what regions of the protein were capable of eliciting these potentially neutralizing antibodies. Ultimately, this work aims to guide future iterations of synthetic or modified PfCyRPA immunogens.

4.2 Results

4.2.1 Structure of Cy.003:PfCyRPA

Cy.003:PfCyRPA complex was purified by incubation of Cy.003 Fab, produced via papain digestion of the parental mAb, with PfCyRPA in a 1:1 molar ratio for thirty minutes prior to purification via size exclusion chromatography. The elution fractions containing the assembled complex were concentrated to 10 mg/ml prior to sitting-drop set-up. This same procedure was used for all Fab:PfCyRPA complex set-ups, unless otherwise specified. 480 conditions (**Appendix 1**) at two temperatures were screened, with crystal growth detected in numerous conditions at both temperatures after five days (**Figure 4.1**). Approximately 30 crystal conditions led to crystal growth representing a diverse set of crystallization conditions in which the complex could crystallize, usually forming rod-like or rhomboid crystals. Crystals from conditions that were not already cryoprotected were harvested and transferred into the crystallization condition with the addition of 25% glycerol (replacing the same volume of water).

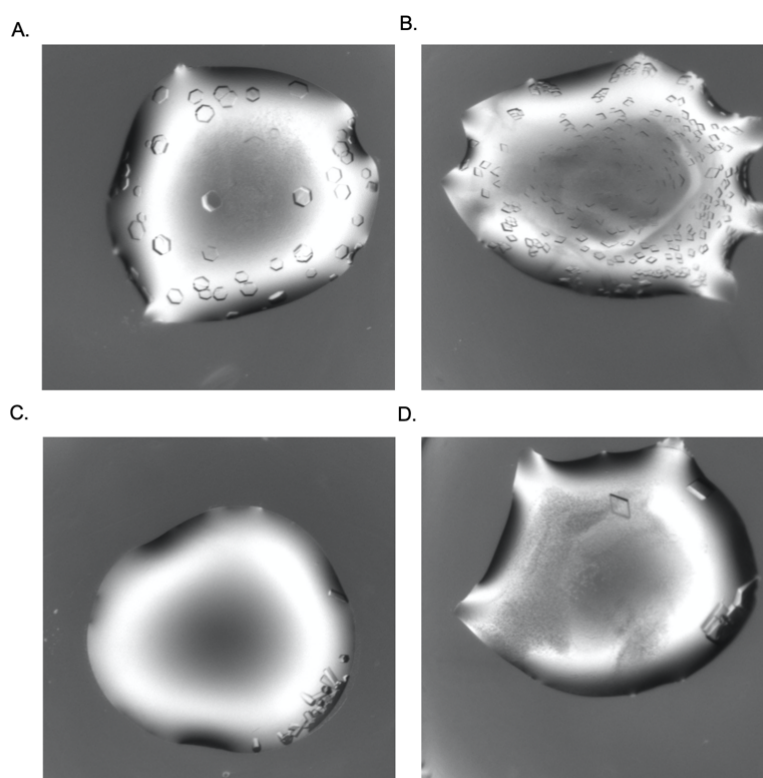


Figure 4.1 Crystal growth of Cy.003:PfCyRPA in four conditions. A. Pact A4, 18°C. B. Proplex A9, 18°C. C. Proplex A10, 18°C. D. Proplex B11, 18°C.

The best dataset collected for Cy.003:PfCyRPA was from Proplex A9 (0.2M NaCl, 0.1M MES pH 6, 20% PEG 2000 MME), harvested in 25% glycerol. One crystal from this condition diffracted to 3.1Å. Molecular replacement using a PfCyRPA search model from PDB 5TIH and a Fab search model from 6RCS, both with flexible loops removed, was successful in revealing a monoclinic lattice structure with space group C 1 2 1 and unit cell dimensions $a = 354.30\text{\AA}$, $b = 71.04\text{\AA}$, $c = 164.79\text{\AA}$, $\alpha = 90^\circ$, $\beta = 91.16^\circ$, $\gamma = 90^\circ$ (**Table 4.1**). The asymmetric unit contains four copies of Cy.003 bound to PfCyRPA. After refinement using Coot¹⁸⁵ and Buster¹⁸⁶, the $R_{\text{work}}/R_{\text{free}}$ were 21.9/23.9, indicative of strong agreement between the experimental data and the model.

Cy.003 contacts PfCyRPA using two stretches of amino acids between residues 135-146 and 203-206 of PfCyRPA with a buried surface area on PfCyRPA of approximately $650\text{\AA}^2$ (**Figure 4.2**). Residues 135-146 lie in the fourth strand of the second β -sheet (the second blade of the propeller), while the second contact point is in a loop between the second and third strand of the third β -sheet. All three light chain CDRs contact PfCyRPA, while on the heavy chain CDRH2 and CHRH3 are involved in PfCyRPA contacts (**Table 4.2**).

Table 4.1 Cy.003:PfCyRPA data collection and refinement statistics

Data collection	Cy.003:PfCyRPA
Space group	C 1 2 1
Cell dimensions	
a, b, c	354.30, 71.04, 164.79
α, β, γ (°)	90.00, 91.16, 90.00
Wavelength	0.97624Å
Resolution (Å)	164.76 – 3.14 (3.21 – 3.14)
Total Observations	239064 (15017)
Total Unique	72415 (4491)
R_{pim} (%)	13.6 (94.8)
R_{merge} (%)	13.9 (96.5)
R_{meas} (%)	19.5 (135.4)
$CC_{1/2}$	95.6 (42.5)
$I/\sigma(I)$	7.2 (1.3)
Completeness (%)	99.9 (99.9)
Multiplicity	3.3 (3.3)
Wilson B factor	59.8
Refinement	
Reflections	72368
$R_{\text{work}} / R_{\text{free}}$	21.9 (23.9)
Average B factor (Å ²)	89.0
Number of residues	
Protein	3002
R.m.s deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.06
Ramachandran plot	
Favored (%)	94.8
Allowed (%)	5.2
Outliers (%)	0

Table 4.2 Interacting residues at the Cy.003:PfCyRPA interface.

PfCyRPA			Cy.003			
Chain	Residue	Group	Chain	Residue	Group	Type of interaction
A/D/G/J	R135	Side chain	B/E/H/K	D124	Main chain	Hydrogen bond
A/D/G/J	Y137	Side chain	C/F/I/L	Y49	Side chain	Hydrogen bond
A/D/G	E143	Side chain	C/F/I	K84	Side chain	Hydrogen bond
A/D/G/J	N145	Side chain	B/E/H/K	W125	Side chain	Hydrogen bond
A/D/G/J	N145	Side chain	C/F/I/L	S48	Side chain	Hydrogen bond
A/D/J	N146	Main chain	C/F/L	R109	Side chain	Hydrogen bond
A/D/G/J	D203	Side chain	B/E/H/K	S72	Side chain	Hydrogen bond
A/D/G/J	K204	Side chain	B/E/H/K	N121	Main chain	Hydrogen bond
A/D/G/J	E206	Side chain	B/E/H/K	S126	Side chain	Hydrogen bond
G/J	E206	Side chain	H/K	S126	Main chain	Hydrogen bond

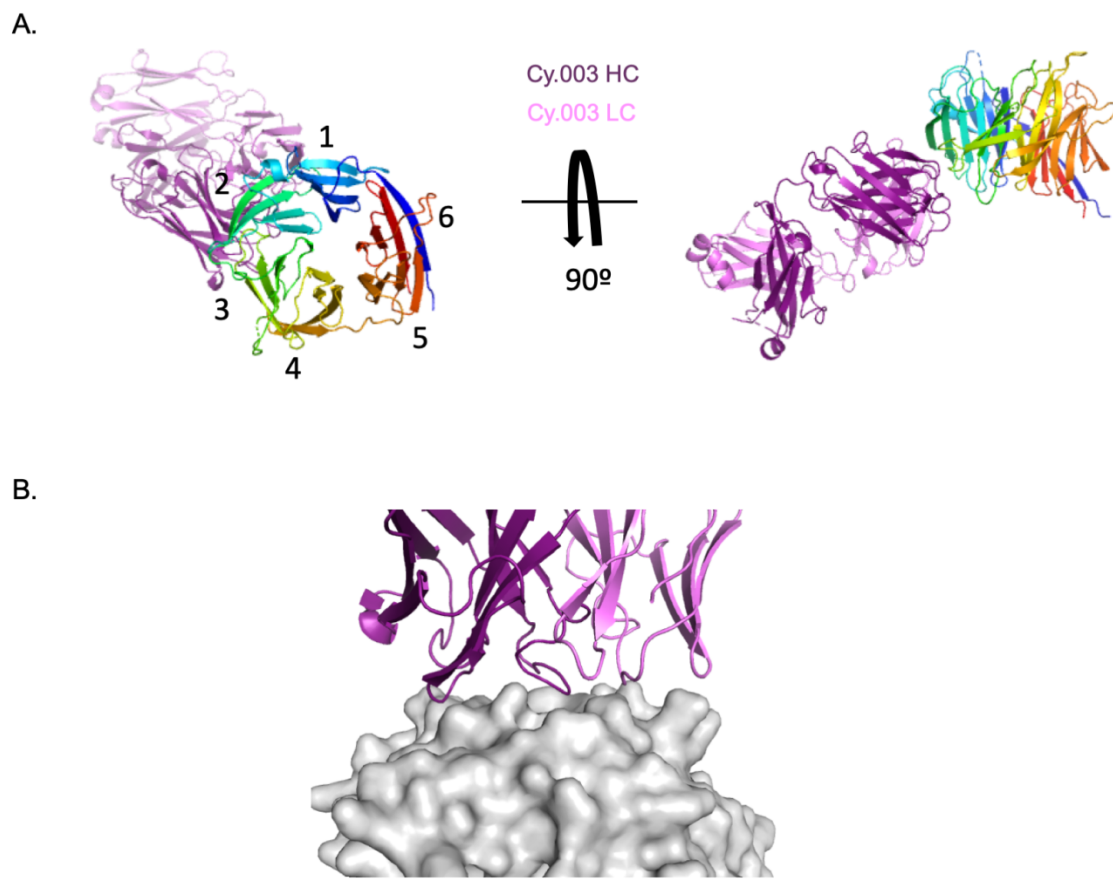


Figure 4.2 Crystal structure of Cy.003 bound to PfCyRPA. A. Position and angle of Cy.003 binding on PfCyRPA. Numbers indicate PfCyRPA blades. N- and C-termini come together to form blade 6. B. CDR loops contacting PfCyRPA. Both figures were produced using PyMol²²⁷.

4.2.2 Structure of Cy.004:PfCyRPA and Cy.009:PfCyRPA

Due to the moderately higher GIA of Cy.009 compared to Cy.004, initially, Cy.009 was selected from that epitope bin for crystallization. However, challenges with obtaining high resolution data resulted in attempting crystallization of Cy.004:PfCyRPA as well.

Cy.004:PfCyRPA complex was produced as described in **4.2.1**. 480 conditions at two temperatures (4°C and 18°C) were screened (**Appendix 1**). After three days, crystal growth was detected in fourteen drops (**Figure 4.3**). The crystals formed were predominantly rod-like, some with additional protrusions or clustering of multiple crystals together. The conditions that led to growth were, typically, a high molecular weight polyethylene glycol in combination with a neutral or slightly basic buffer (pH 7-8). The crystals were harvested and cryopreserved in the same condition that led to their growth, but with 25% of the water removed and replaced with glycerol.

The crystals grown in Proplex condition D12 (20% PEG 6000, 0.1M Tris pH 8.5) at 18°C successfully diffracted to 2.8Å in the $P 1 2_1 1$ space group with unit cell dimensions of $a = 103.06\text{\AA}$, $b = 80.32\text{\AA}$, $c = 114.91\text{\AA}$, $\alpha = 90^\circ$, $\beta = 115.23^\circ$, $\gamma = 90^\circ$, indicative of a monoclinic crystal lattice (**Table 4.3**). Molecular replacement using PfCyRPA (5TIH) and Fab (6RCS) search models with flexible loops removed was successful and revealed two copies of Cy.004:PfCyRPA in the asymmetric unit. After refinement, the R_{work} was 22.0 and the R_{free} 25.8, indicating strong agreement between the model and experimental data.

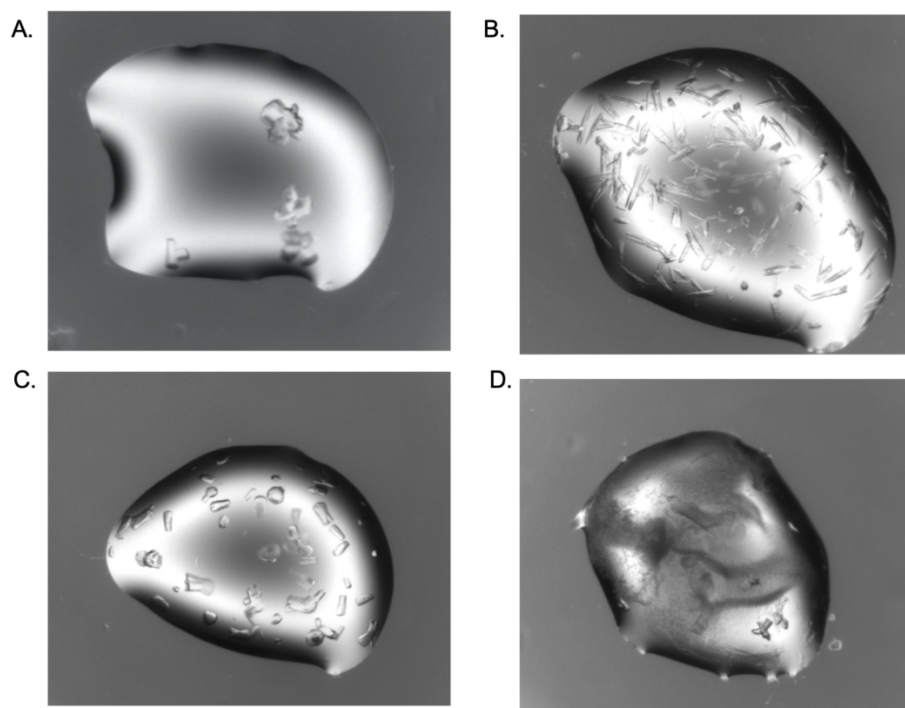


Figure 4.3 Cy.004:PfCyRPA crystals in multiple conditions. A. Proplex B5, 18°C. B. Proplex F5, 18°C. C. Midas B6, 18°C. D. Proplex D12, 18°C.

Table 4.3 Cy.004:PfCyRPA data collection and refinement statistics.

Data collection	Cy.004:PfCyRPA
Space group	P 1 21 1
Cell dimensions	
a, b, c	103.06, 80.32, 114.91
α , β , γ (°)	90.00, 115.23, 90.00
Wavelength	0.97857 Å
Resolution (Å)	47.11 – 2.79 (2.87 – 2.79)
Total Observations	284862 (13827)
Total Unique	42022 (2741)
R_{pim} (%)	10.4 (73.7)
R_{merge} (%)	16.7 (106.3)
R_{meas} (%)	19.7 (129.9)
$CC_{1/2}$	0.992 (0.757)
$I/\sigma(I)$	6.8 (0.9)
Completeness (%)	99.9 (87.3)
Multiplicity	6.8 (5.0)
Wilson B factor	61.1
Refinement	
Reflections	42008
$R_{\text{work}} / R_{\text{free}}$	22.0 (25.8)
Average B factor (Å ²)	85.0
Number of residues	
Protein	1494
Ca ²⁺	4
R.m.s deviations	
Bond lengths (Å)	0.009
Bond angles (°)	1.05
Ramachandran plot	
Favored (%)	94.9
Allowed (%)	5.1
Outliers (%)	0

Cy.004 binds to PfCyRPA predominantly on the second β -sheet of the 6-bladed propeller, though part of its epitope lies in a flexible loop between blades 1 and 2 (**Figure 4.4**). The buried surface area of PfCyRPA upon Cy.004 binding is approximately $900\text{\AA}^2$, with both the light and heavy chain of Cy.004 contacting PfCyRPA. A summary of the interacting residues can be seen in **Table 4.4**. The interaction is predominantly mediated by interactions between CDRH3 and PfCyRPA.

Two pockets of unexplained electron density within the Cy.004:PfCyRPA binding pocket were identified during refinement. In one instance, this pocket was flanked by E120 and E122 from PfCyRPA and D120/D121 from the heavy chain of Cy.004. In the absence of any other factor, these residues would not be expected to interact since both glutamate and aspartate are negatively charged at the pH of the crystallization condition (pH 8), given the pK of the carboxylic acid side chains are 4.25 and 3.65, respectively. Likewise, a second density pocket was observed flanked by D120, D127 and E131, all from the Cy.004 heavy chain. Both of these are consistent with the binding site for a divalent cation²²⁸. The source of a divalent cation is not the crystallization buffer, which lacks any salts. Speculatively, the source could be the PfCyRPA C-tag elution buffer (2M MgCl_2) or culture media which contains both calcium and magnesium.

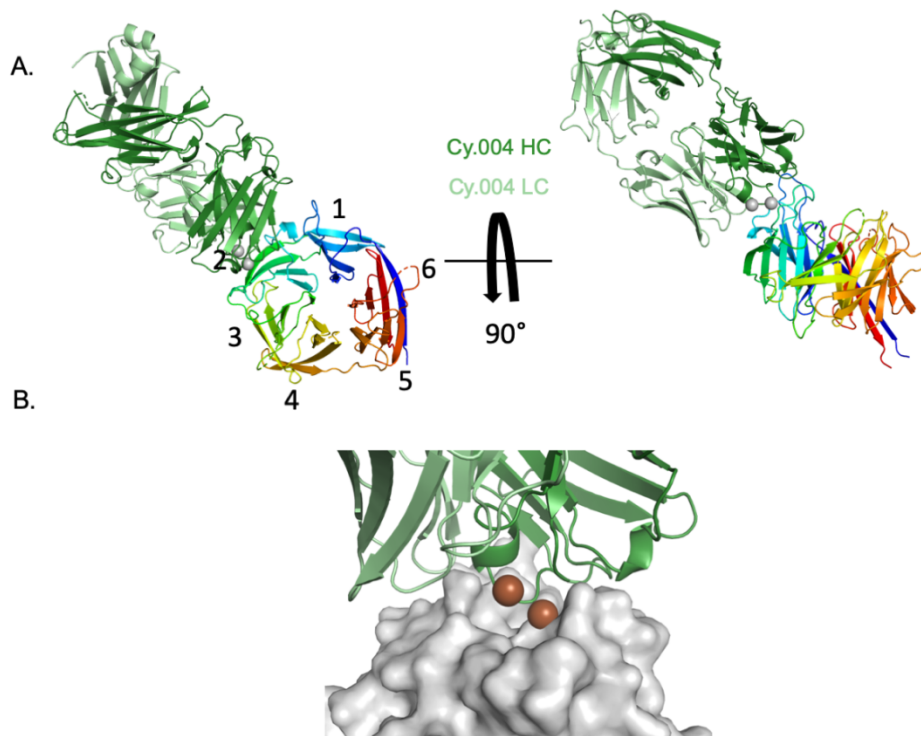


Figure 4.4 Crystal structure of Cy.004 bound to PfCyRPA. A. Position and angle of Cy.004 binding on PfCyRPA. Numbers indicate PfCyRPA blades. N- and C-termini come together to form blade 6. B. CDR loops contacting PfCyRPA. Brown spheres indicate putative divalent cation binding. Both figures produced using PyMol²²⁷.

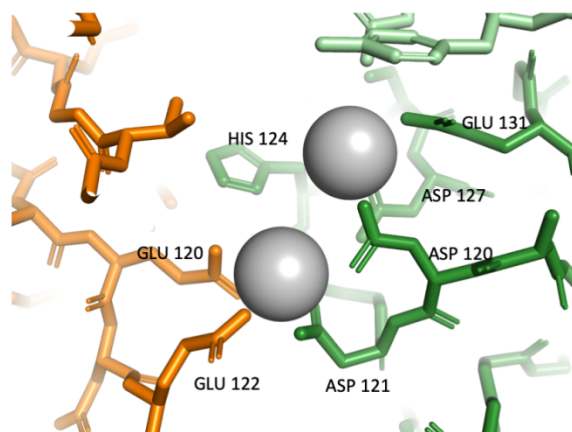


Figure 4.5 Putative divalent cation binding sites at the Cy.004: PfCyRPA interface. PfCyRPA (orange), Cy.004 HC (dark green), Cy.004 LC (light green).

The putative metal ion appears to be critical for antibody binding, occupying much of the antibody-antigen interface. The binding pocket consists of 'hard' side chains with low polarizability, with both glutamate and aspartate being carboxylates²²⁹. Both Mg^{2+} and Ca^{2+} prefer hard side chains in their coordination sphere, which is consistent with the presence of carboxylate side chains²²⁹. The two putative binding cations are both coordinated by D120, which appears to be engaged in monodentate binding to each cation (**Figure 4.5**). At the resolution achieved here (2.8Å), it is difficult to be certain of the exact orientation of the other coordinating amino acid side chain, and thus it is difficult to determine which are in monodentate interactions and which are in bidentate interactions, or to place waters to establish coordination numbers.

It was not possible to definitely identify this metal ion through the available crystallographic data alone. Importantly, given this structural information, this may explain why Cy.004 appears to have weak binding in the SPR experiments described in Chapter 3. In those experiments, the complex was purified without either magnesium or calcium in the SPR buffer. It is conceivable then that the addition of the appropriate divalent cation may reveal a higher antigen binding affinity. This would also provide further insight as to the identity of the putative metal ion, as if one of calcium or magnesium enhance binding, and the other does not, this would provide some clarity as to which is likely occupying the antibody-antigen interface.

Table 4.4 Interacting residues at the PfCyRPA: Cy.004 interface.

PfCyRPA			Cy.004			
Chain	Residue	Group	Chain	Residue	Group	Type of interaction
A	E96	Main chain	C	Y47	Side chain	Hydrogen bond
A/D	F94	Side chain	B/E	R111	Side chain	Cation- π
A/D	D98	Side chain	B/E	S126	Main chain	Hydrogen bond
A/D	D98	Main chain	B/E	Y125	Main chain	Hydrogen bond
A/D	D98	Side chain	C/F	S108	Side chain	Hydrogen bond
A/D	L99	Side chain	C/F	R111	Side chain	Hydrogen bond
A/D	T100	Side chain	B/E	G122	Main chain	Hydrogen bond
A/D	T100	Main chain	B/E	G123	Main chain	Hydrogen bond
A/D	E120	Side chain	B/E	D120	Side chain	Metal coordination
A/D	E120	Side chain	B/E	D121	Side chain	Metal coordination
A/D	E122	Side chain	B/E	D121	Side chain	Metal coordination
A/D	N126	Side chain	B/E	S50	Main chain	Hydrogen bond
D	F94	Main chain	F	R111	Side chain	Hydrogen bond
D	T97	Main chain	F	R111	Side chain	Hydrogen bond
D	D98	Side chain	E	S126	Side chain	Hydrogen bond

Cy.009:PfCyRPA underwent the same crystallization procedure as the other complexes, which included mixing 1:1 molar ratios of PfCyRPA and Cy.009 Fab before concentrating to 10 mg/ml and setting up sitting drop crystallization screens. In this case, the complex readily crystallized into hexagonal rods (**Figure 4.6**) but diffraction was consistently weak, with the best dataset only achieving resolutions of 7-8Å. These datasets were insufficiently resolved to enable molecular replacement, and thus further optimization was required. The complex most readily crystallized in conditions with a low concentration of salt and high molecular weight PEG, with a slightly acidic pH (5 - 6.5).

Multiple optimization strategies were used in parallel in the hope that one would result in well-diffracting crystals. The silver bullet additive screen was used in combination with the most promising conditions based on previous synchrotron data. Some of these crystals were then dehydrated in solutions of increasing precipitant concentration in a hanging drop set-up over 5 days. Methylation of the complex followed by broad crystallization screen was attempted¹⁹², but resulted in crystals that contained Fab alone, unbound to PfCyRPA. Optimization across a matrix of increasing salt and precipitant concentration was also used for some of the most promising crystallization conditions.

In the end, the optimization efforts were only marginally successful. The methylated broad screen yielded one dataset of Fab alone to approximately 2Å. This was refined and then used as a search model for the other low-resolution datasets to maximize the chance of a successful molecular replacement. Cy.009 Fab alone crystallized in C 1 2 1 space group with unit cells dimensions of $a = 184.59\text{\AA}$, $b = 38.38\text{\AA}$, $c = 143.01\text{\AA}$, $\alpha = 90^\circ$, $\beta = 119.07^\circ$, $\gamma = 90^\circ$, which is consistent with a monoclinic lattice structure (**Table 4.5**). Two copies of the Fab sit adjacently across their longest axis with CDR loops facing in opposite directions (**Figure 4.7**).

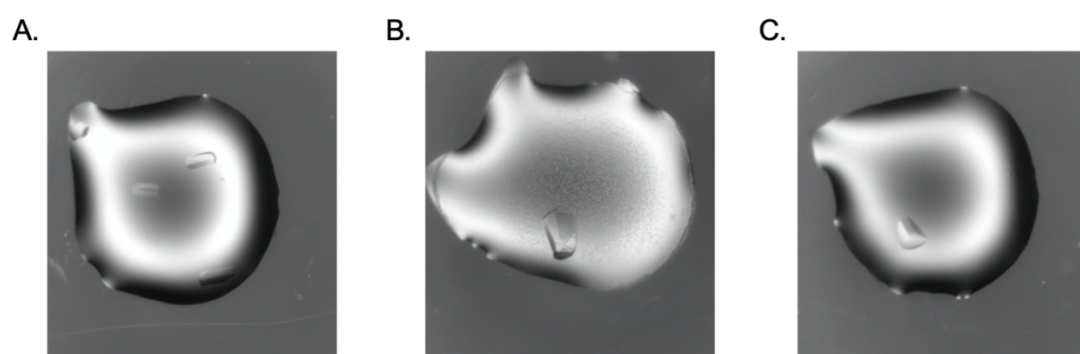


Figure 4.6 Cy.009:PfCyRPA crystals in multiple conditions. A. Proplex A5, 4°C. B. Proplex B4, 18°C. C. Proplex A8, 4°C.

Table 4.5 Data collection and refinement statistics for Cy.009 Fab

Data collection	Cy.009 Fab
Space group	C 1 2 1
Cell dimensions	
a, b, c	184.59, 38.38 143.01
α , β , γ (°)	90.00, 119.07, 90.00
Wavelength	0.9796Å
Resolution (Å)	40.74 – 2.06 (9.21 - 2.06)
Total Observations	182550 (13650)
Total Unique	55141 (4040)
R_{pim} (%)	10.4 (87.8)
R_{merge} (%)	10.6 (89.9)
R_{meas} (%)	14.9 (125.8)
$CC_{1/2}$	0.992 (0.722)
$I/\sigma(I)$	4.8 (1.2)
Completeness (%)	99.9 (99.9)
Multiplicity	3.3 (3.4)
Wilson B factor	31.7
Refinement	
Reflections	55008
$R_{\text{work}} / R_{\text{free}}$	22.6 (24.5)
Average B factor (Å ²)	57.0
Number of residues	
Protein	879
Water	267
R.m.s deviations	
Bond lengths (Å)	0.008
Bond angles (°)	1.06
Ramachandran plot	
Favored (%)	96.8
Allowed (%)	3.2
Outliers (%)	0

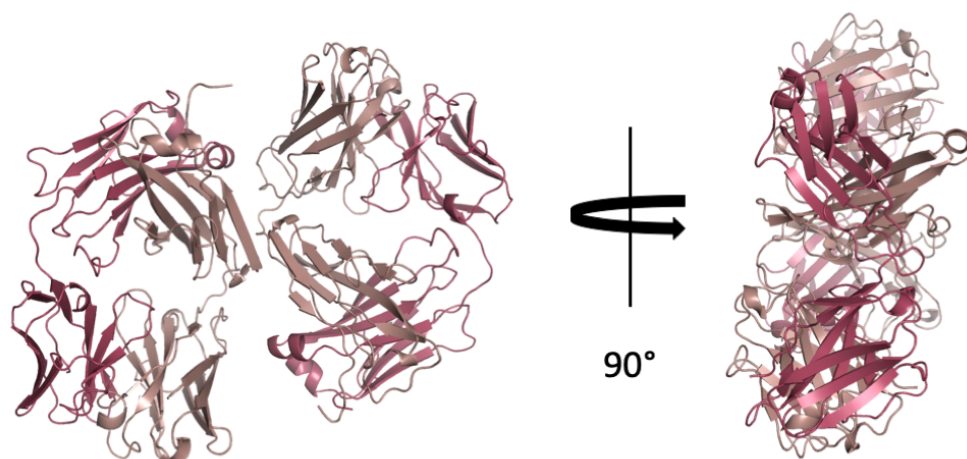


Figure 4.7 Asymmetric unit of Cy.009 Fab alone. Heavy chain (dark red) and light chain (light red).

For the Cy.009:PfCyRPA complex, one dataset from the silver bullets additive screen (1% tryptone, 0.02M Hepes sodium pH 6.8) using ProPlex condition A8 (0.2M ammonium sulphate, 10% PEG MME 2000 and 0.1M sodium acetate pH 5.5) yielded a dataset to 5.1Å. Molecular replacement using the PfCyRPA search model from the completed Cy.004 structure and the methylated Cy.009 Fab structure was successful and revealed four copies of Cy.009:PfCyRPA in the asymmetric unit. The complex crystalized in space group R 3 with unit cell dimensions $a = 288.42\text{\AA}$, $b = 288.42\text{\AA}$, $c = 184.80\text{\AA}$, $\alpha = 90^\circ$, $\beta = 90^\circ$, $\gamma = 120^\circ$, consistent with a trigonal lattice structure (**Table 4.6**). Some of the missing PfCyRPA and Cy.009 loops in the search model appeared during refinement, suggesting that the solution is likely to be correct, as these would not be expected to be visible without the presence of the correct molecular replacement solution.

Unfortunately, due to the low resolution of the dataset, specific interacting residues cannot be determined, although there is clear density for the main chain of the interacting CDR loops (**Figure 4.9**). What is clear based on this 5.1Å structure is that Cy.009 binds to PfCyRPA in the same location on blade two of the propeller as Cy.004 with a buried surface

area on PfCyRPA equal to approximately $620\text{\AA}^2$. Like Cy.004, it appears that CDRH3 contacts residues between strands two and three of the second β sheet (**Figure 4.8**). Superimposition of the two structures is consistent with antibodies with highly similar binding modalities (**Figure 4.10**).

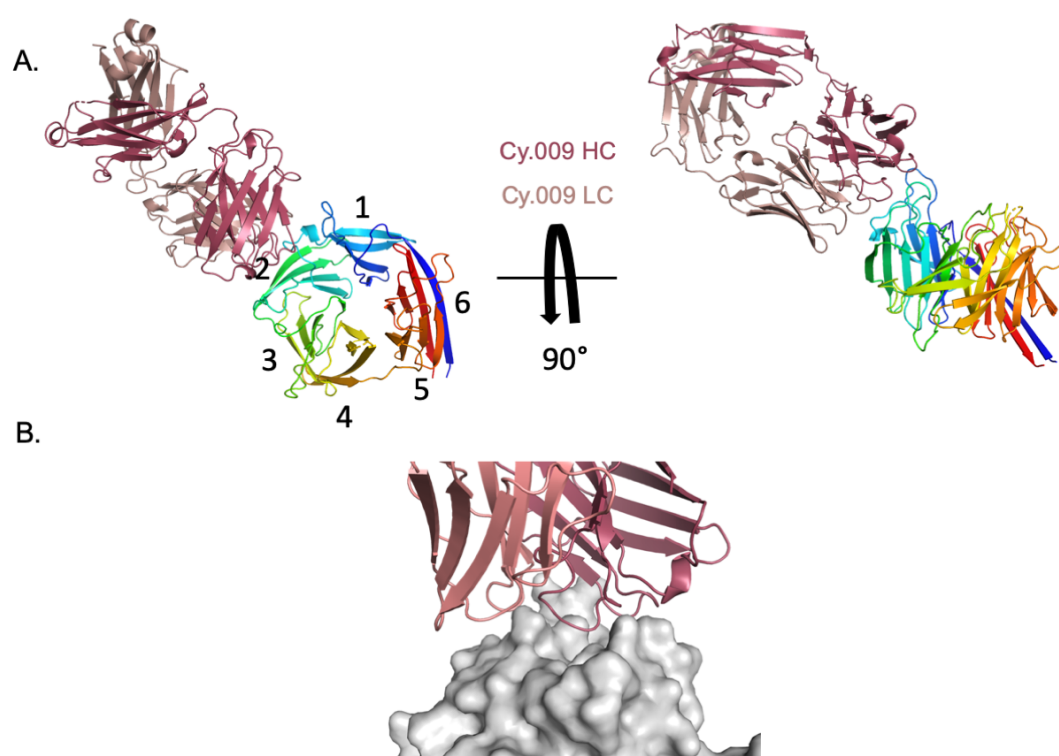


Figure 4.8 Crystal structure of Cy.009 bound to PfCyRPA. A. Position and angle of Cy.009 binding on PfCyRPA. Numbers indicate PfCyRPA blades. N- and C-termini come together to form blade 6. B. CDR loops contacting PfCyRPA. Both figures produced using PyMol²²⁷.

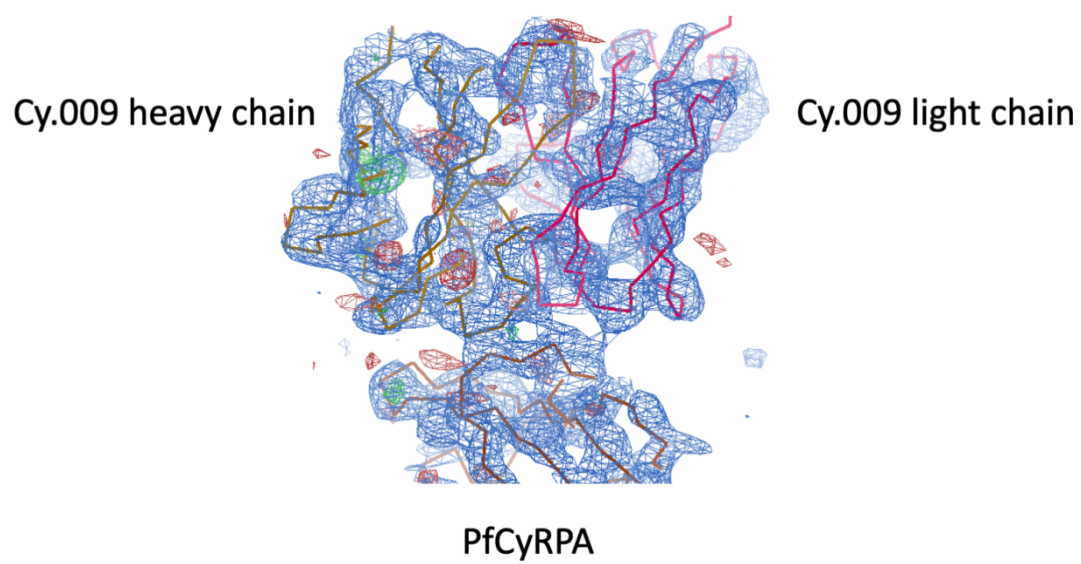


Figure 4.9 Electron density from unit A/B/C in the Cy.009:PfCyRPA structure. Figure produced using Coot¹⁸⁵.

Table 4.6 Data collection and refinement statistics for Cy.009:PfCyRPA.

Data collection	Cy.009:PfCyRPA
Space group	R 3
Cell dimensions	
a, b, c	288.42, 288.42, 184.80
α , β , γ (°)	90.00, 90.00, 120.00
Wavelength	0.97932 Å
Resolution (Å)	144.21 – 5.09 (5.18 – 5.09)
Total Observations	126250 (6394)
Total Unique	23466 (1183)
R_{pim} (%)	2.8 (94.4)
R_{merge} (%)	5.9 (198.7)
R_{meas} (%)	6.6 (220.1)
$CC_{1/2}$	1.000 (0.343)
$I/\sigma(I)$	10.4 (0.8)
Completeness (%)	99.9 (100.0)
Multiplicity	5.4 (5.4)
Wilson B factor	329.2
Refinement	
Reflections	23430
$R_{\text{work}} / R_{\text{free}}$	28.1 (30.2)
Average B factor (Å ²)	190.0
Number of residues	
Protein	3016
R m s deviations	
Bond lengths (Å)	0.007
Bond angles (°)	0.89
Ramachandran plot	
Favored (%)	94.5
Allowed (%)	5.5
Outliers (%)	0

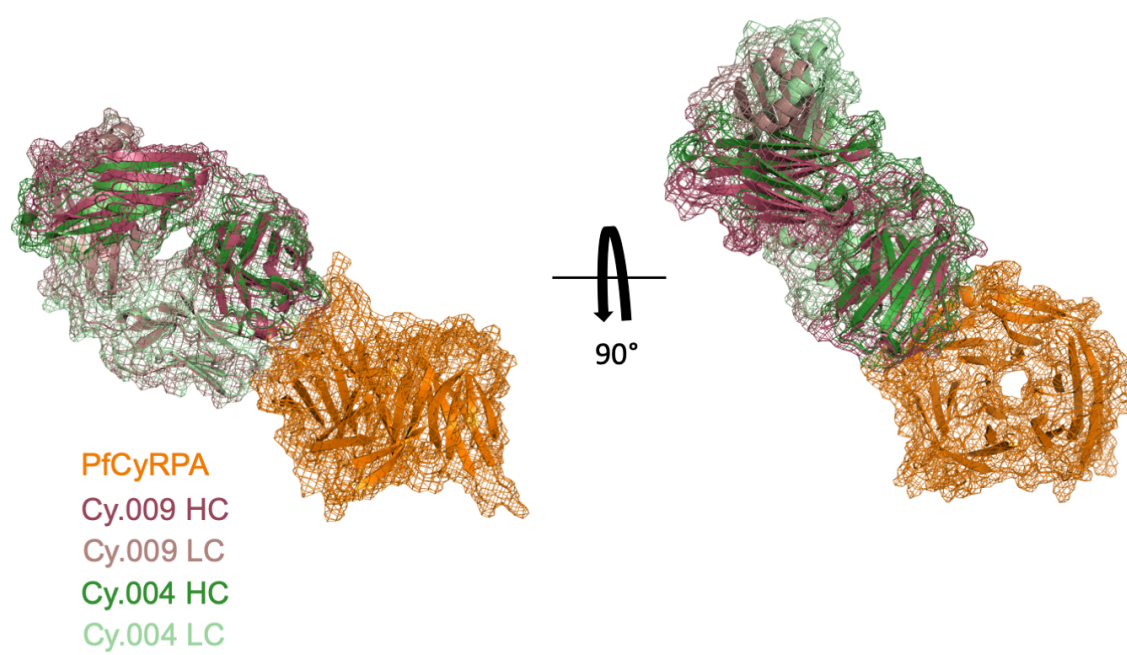


Figure 4.10 Cy.004:PfCyRPA and Cy.009:PfCyRPA superimposed. Figure produced using PyMol²²⁷.

Sequence comparison of the two antibodies revealed that they are highly similar, with equal length CDR loops and 1-2 point mutations per loop (**Figure 4.11**). Two of these mutation are in contact residues in the Cy.004:PfCyRPA structure (p.S50D and p.D121N). D121 lies in the putative metal binding site in the Cy.004:PfCyRPA structure. It is not clear from the available low-resolution structural data whether the Cy.009 retains the same putative cation binding site. However, the possibility should not be ignored, given the highly similar binding pockets present in Cy.004 and Cy.009 and because the D>N mutation does not preclude a cation binding site. In order to determine whether or not this same binding modality occurs between Cy.009 and PfCyRPA, the SPR approach described above for Cy.004:PfCyRPA should be used, which will inform whether or not Cy.009:PfCyRPA is also a metal coordinated interaction.

CDRH1 (Cy.004)	45	GFDFSSYA	52
CDRH1 (Cy.009)	45	GFTFSDYA	52
CDRH2 (Cy.004)	70	IRNDGSFT	77
CDRH2 (Cy.009)	70	IKSDGSFT	77
CDRH3 (Cy.004)	116	TKSADGGHYSDFSGEIDA	134
CDRH3 (Cy.009)	116	AKSDDNGGHYSDFSGEIDA	134
CDRL1 (Cy.004)	43	SGSTYN	48
CDRL1 (Cy.009)	43	SGSSYS	48
CDRL2 (Cy.004)	65	IYYNDK	70
CDRL2 (Cy.009)	65	IYYNDK	70
CDRL3 (Cy.004)	106	GNSDSRNVA	114
CDRL3 (Cy.009)	106	GNSDSRNA	114

Figure 4.11 Sequence alignment of Cy.004 and Cy.009 CDR loops. Red letters indicate sites where the two antibodies have different residues.

4.2.3 Structure of Cy.007:PfCyRPA

Cy.007:PfCyRPA crystallized in only a single condition (0.2M zinc acetate, 10% PEG 3000, 0.1M sodium acetate pH 4.5, with the addition of 25% glycerol for harvesting), after seven days, yielding two crystals. However, one of these crystals successfully diffracted to 3.0Å. Unusually, even after multiple rounds of refinement, the electron density for the variable domain side chains of the Fab was still poorly resolved. At 3.0Å, one would expect that side chains, particularly those at the antibody:antigen interface, would be well resolved. Further there was almost no electron density for the constant domain, although this has previously been reported and is likely the result of interdomain flexibility⁶⁸. More work is needed in order to improve the refinement of this dataset, but still preliminary interpretations of the structure can be explored.

Initial inspection of this suboptimal dataset revealed that PfCyRPA contacts are exclusively mediated via the heavy chain, predominantly CDRH2 and CDRH3 (**Figure 4.12**). Together, these form a buried surface area on PfCyRPA of approximately 650Å². Cy.007 interacts with PfCyRPA, primarily through binding to the fourth strand of the first β-sheet (**Figure 4.12**). As well, Cy.007 appears to make a single contact with the final strand of the sixth β-sheet (near the protein's N-terminus, as the sixth sheet is composed of both the N and C-termini).

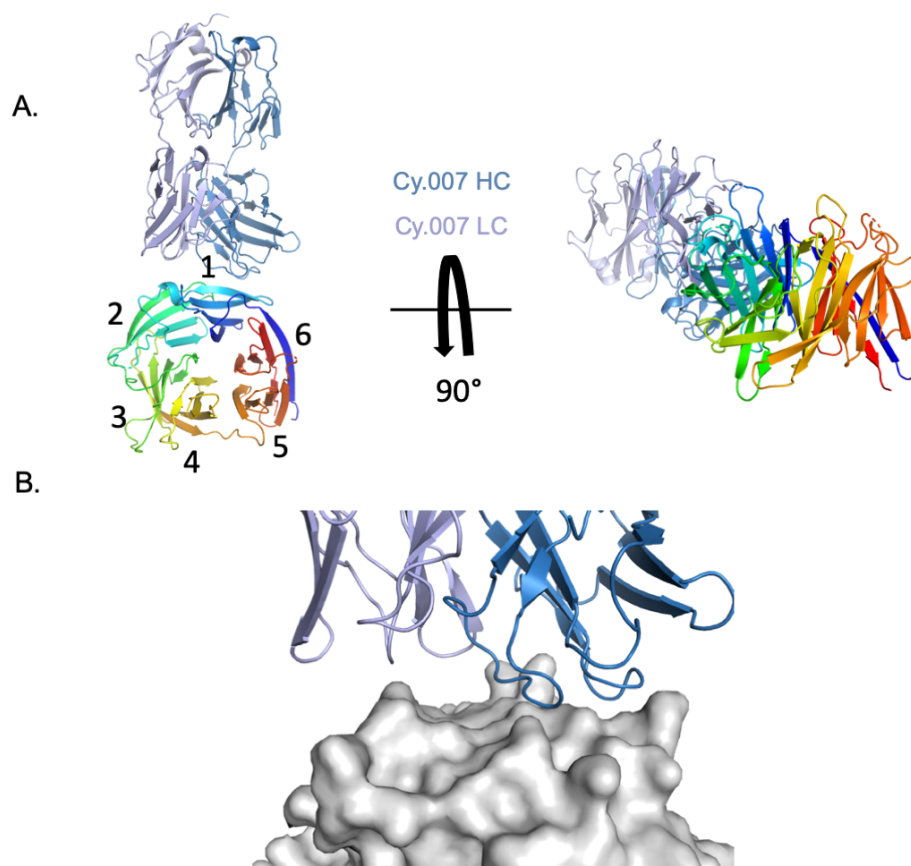


Figure 4.12 Crystal structure of Cy.007 bound to PfCyRPA. A. Position and angle of Cy.007 binding on PfCyRPA. Numbers indicate PfCyRPA blades. N- and C-termini come together to form blade 6. B. CDR loops contacting PfCyRPA. Both figures produced using PyMol²²⁷.

4.2.4 Structure of Cy.002:PfCyRPA

Crystal growth of Cy.002:PfCyRPA was noted after three days of incubation at 4°C and 18°C in a number of conditions characterized by sodium citrate pH 5 and 15-30% high molecular weight PEG. Some of the conditions that yielded crystals also included MgCl₂ or ammonium acetate (**Figure 4.13**). The crystals were harvested and cryopreserved with the addition of 25% glycerol to the crystallization buffer, replacing the same volume of water.

The best diffracting crystal yielded a dataset to 2.7Å in the C 1 2 1 space group with unit cell dimensions of $a = 205.05\text{\AA}$, $b = 80.69\text{\AA}$, $c = 117.36\text{\AA}$, $\alpha = 90^\circ$, $\beta = 108.73^\circ$, $\gamma = 90^\circ$, consistent with a monoclinic crystal lattice. Refinement of this dataset is still in progress and thus final refinement statistics are not available at the time of writing. However, even though it is not fully complete, the contact residues can be clearly resolved (**Table 4.7**). Cy.002 binds to PfCyRPA via a loop between the first and second strand of the third β -sheet, a loop between the third and fourth β -sheets and a loop between the second and third strand of the fourth β -sheet (**Figure 4.14**). Together, this forms a buried surface area on PfCyRPA of approximately 1150Å².

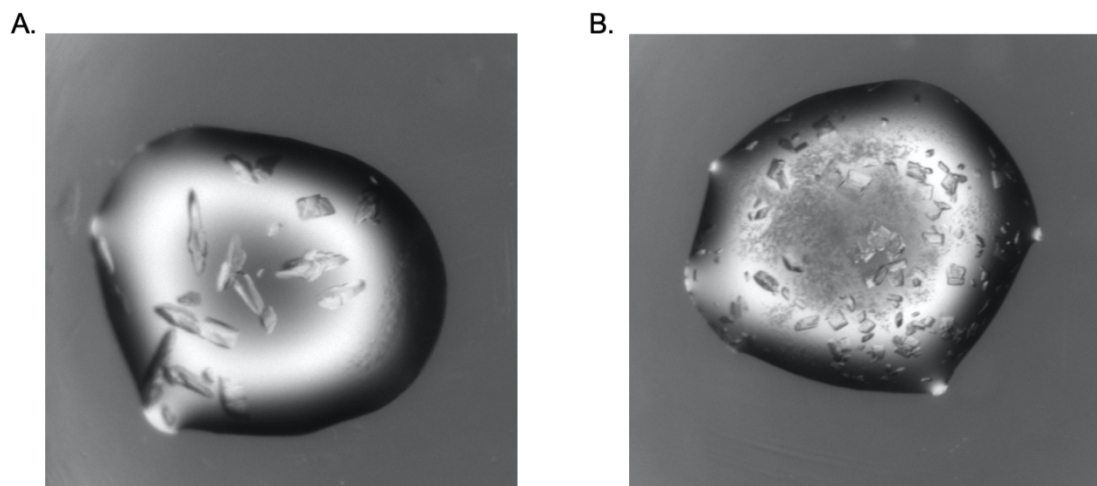


Figure 4.13 Cy.002:PfCyRPA crystals in multiple conditions. A. Proplex B8, 4°C. B. Proplex C3, 18°C.

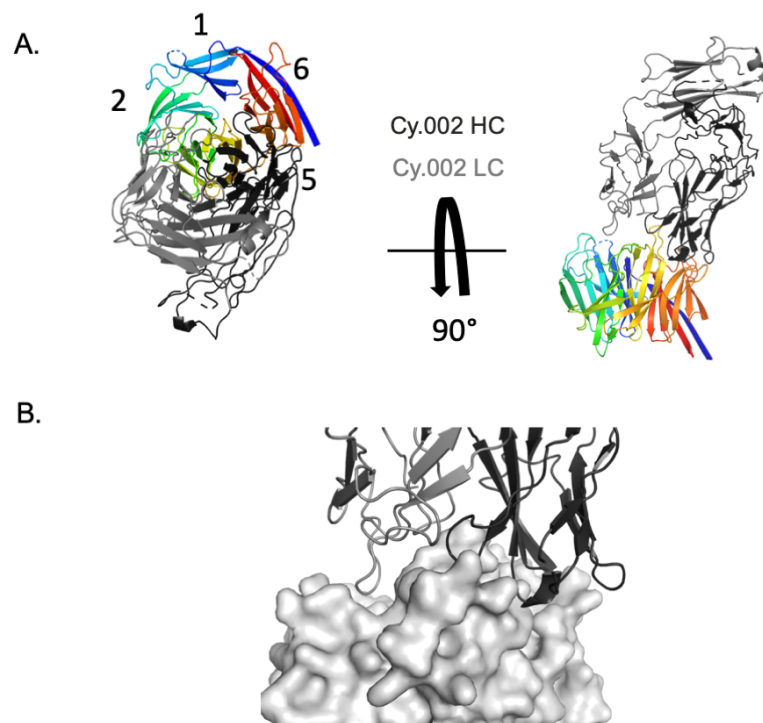


Figure 4.14 Crystal structure of Cy.002 bound to PfCyRPA. A. Position and angle of Cy.002 binding on PfCyRPA. Numbers indicate PfCyRPA blades. N- and C-termini come together to form blade 6. B. CDR loops contacting PfCyRPA. Both figures produced using PyMol²²⁷.

Table 4.7 Interacting residues at the PfCyRPA: Cy.002 interface.

PfCyRPA			Cy.002			
Chain	Residue	Group	Chain	Residue	Group	Type of interaction
A/D	Y185	Side chain	C/F	S51	Side chain	Hydrogen bond
A/D	K188	Side chain	B/E	D123	Side chain	Hydrogen bond
A/D	K188	Main chain	B/E	V119	Main chain	Hydrogen bond (via H ₂ O)
A/D	D189	Side chain	C/F	R74	Side chain	Hydrogen bond
A/D	K220	Side chain	B/E	T51	Main chain	Hydrogen bond
A/D	K220	Side chain	B/E	D71	Side chain	Hydrogen bond
A/D	L221	Main chain	B/E	D50	Main chain	Hydrogen bond
A/D	L221	Side chain	B/E	V118	Side chain	Hydrophobic
A/D	G222	Side chain	B/E	V119	Side chain	Hydrophobic
A/D	V223	Main chain	B/E	L120	Side chain	Hydrophobic
A/D	D245	Side chain	B/E	R69	Side chain	Hydrogen bond
A/D	D245	Main chain	C/F	R119	Side chain	Hydrogen bond
A/D	N246	Side chain	C/F	S115	Main chain	Hydrogen bond
A/D	N246	Main chain	C/F	D118	Main chain	Hydrogen bond
A/D	N248	Main chain	C/F	D118	Side chain	Hydrogen bond

4.2.5 *Understanding Fab binding in the context of known structures*

As discussed, two structures of inhibitory Fabs bound to PfCyRPA have already been solved^{66,72}. c12⁶⁶ is weakly inhibitory, with similar invasion inhibitory capacity to Cy.007. 8A7⁷² is moderately inhibitory, closest in potency to Cy.003. In both cases, with affinities in the high picomolar to low nanomolar range, c12 and 8A7 both have higher affinities than the four new inhibitory mAbs, which have affinities ranging from the mid nanomolar to low micromolar, as described in the previous Chapter.

Superimposition of c12 and 8A7 on the Fab:PfCyRPA structures described here revealed some important insights (**Figure 4.15**). The epitopes for c12 and Cy.007 are nearly entirely overlapping (**Figure 4.15A**). This similarity in epitope may help explain why both c12 and Cy.007 have very weak GIA. Despite c12 having much higher affinity, both show approximately 20% inhibition at 2mg/ml. This suggests that Cy.007's weak GIA does not lie in its weak affinity. c12 had a high-picomolar affinity as opposed to high nanomolar for Cy.007, but both show equivalently weak GIA. Instead, it suggests that the epitope targeted by these mAbs is not particularly potent in comparison with the other inhibitory anti-PfCyRPA mAbs.

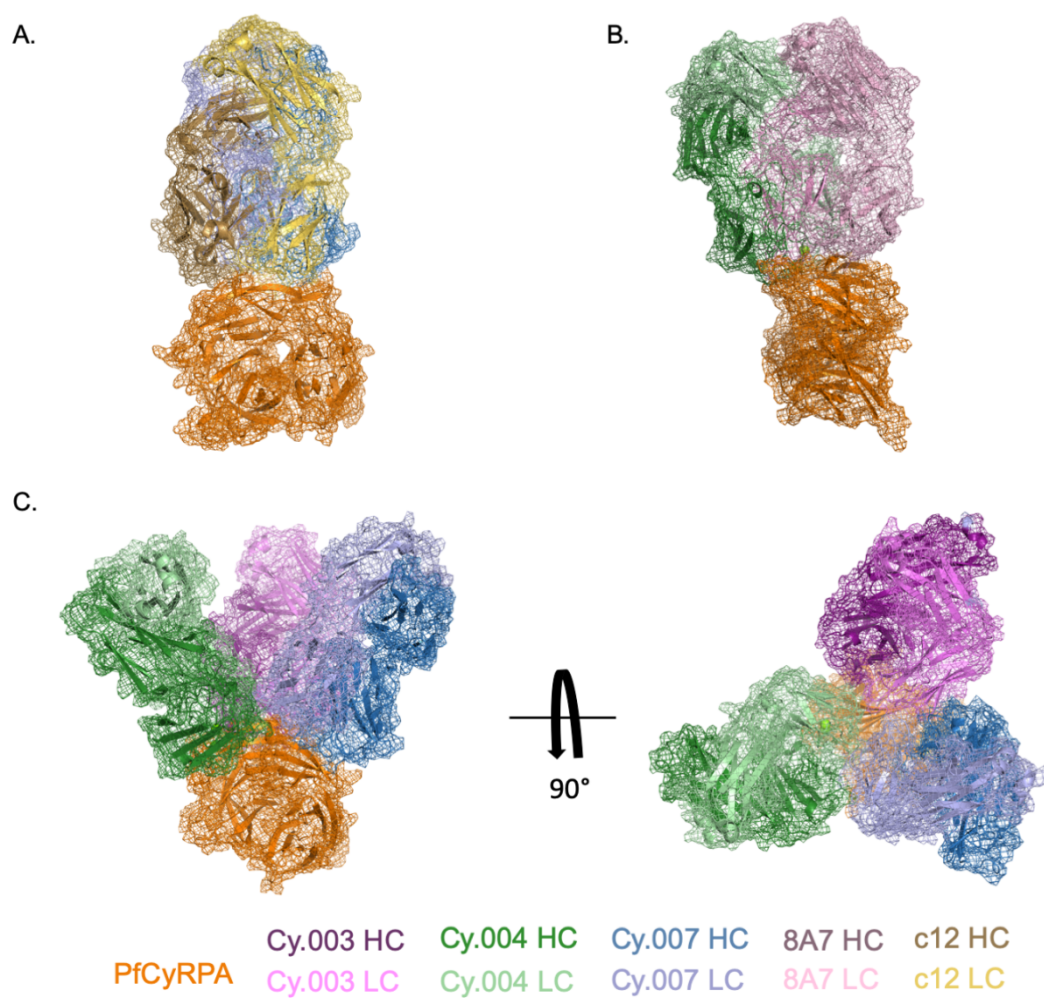


Figure 4.15 Multi-Fab superimposition on PfCyRPA. A. c12:FcCyRPA superimposed on Cy.007:FcCyRPA. B. 8A7:FcCyRPA superimposed on Cy.004:FcCyRPA. C. Cy.003:FcCyRPA, Cy.004:FcCyRPA and Cy.007:FcCyRPA superimposed on one another.

The similarity in binding epitopes also provides some insight into why only these two mAbs showed marked changes in GIA in the mAb as compared to their Fab form. In Chapter 3, I showed that none of Cy.002, Cy.003, Cy.004 or Cy.009 showed a significant increase or decrease in GIA when the Fab form was compared to the mAb form in equimolar concentrations. Conversely, c12 and Cy.007 showed dramatic increases in GIA when they were cleaved into Fabs. This suggests that the shared epitope between Cy.007 and c12 is not fully accessible to the 150kDa mAb. The reason for this lack of accessibility is not immediately clear. Previously, it was noted that when the RCR complex is bound to basigin, c12 likely points towards membrane²³. Cy.007 has the same angle of approach, an angle distinct from all of the other inhibitory mAbs. Thus, Cy.007 may have similar membrane constraints.

Superimposition of the Fab:PfCyRPA structures described here on the 8A7:PfCyRPA structures revealed that 8A7 binds between Cy.003, Cy.004/Cy.009 and Cy.007 (**Figure 4.15B,C**). This is consistent with the data described in Chapter 3 wherein 8A7 competes with all three groups of mAbs, which do not compete with each other. As predicted in Chapter 3, although they all bind to the same side of PfCyRPA, there is adequate spacing between Cy.003, Cy.004/Cy.009 and Cy.007 to enable simultaneous binding (**Figure 4.15C**). Due to minor shifts in binding position, the 8A7 binding position does not allow for simultaneous binding of any of these other mAbs.

8A7 binds closest to Cy.004/Cy.009. However, despite this similarity in epitope (**Figure 4.15B**), Cy.004/Cy.009 are considerably more potent in GIA (IC₅₀ of 2.9mg/ml versus approximately 0.25mg/ml). Since the difference in GIA cannot be attributed to binding affinity, it more likely lies in slight changes to the epitope and/or angle of mAb binding. Both bind to the loop between the first and second β -sheet (spanning residues 92-102 approximately). However, 8A7 also binds to the fourth strand of the second β -sheet (residues

145-148), whereas Cy.004 binds to a loop N-terminal of the 8A7 interacting sites, between strands two and three of the second sheet (this is where the putative cation binding site lies). It is possible then that it is the shift towards strands two and three from strand four that drives the difference in potency. Cy.003, which shows comparable potency to 8A7 binds to the same stretch of amino acids as 8A7 on the fourth strand of the second β -sheet. The reason why strands two and three of the second β -sheet may be more susceptible to neutralization remains unclear.

4.2.6 Understanding Fab binding in the context of the RCR complex

Superimposition of the structures described here are consistent with the hypothesis put forward in the previous Chapter: namely that neutralizing epitopes on PfCyRPA are accessible in the context of the larger tripartite RCR complex. Superimposition of the Cy.002:PfCyRPA structure (a non-inhibitory mAb) onto the RCR complex reveals that they would compete for binding. The PfRH5 binding is located on blades four and five of PfCyRPA. The extension of the Cy.002 epitope on to blade four of PfCyRPA would be expected to prevent binding in the presence of PfRH5. This is consistent with the PfRH5 competition data presented in Chapter 3.

All six inhibitory mAbs, Cy.003, Cy.004/Cy.009, Cy.007, c12 and 8A7 have epitopes that are accessible/exposed in the RCR complex (**Figure 4.16**). Cy.004/Cy.009 bind immediately adjacent to PfRH5, but there are no obvious clashes that would impede binding, consistent with both of these mAbs binding to the assembled RCR complex. Whether or not the close proximity of Cy.004/Cy.009 to PfRH5 has important functional implications remains unclear.

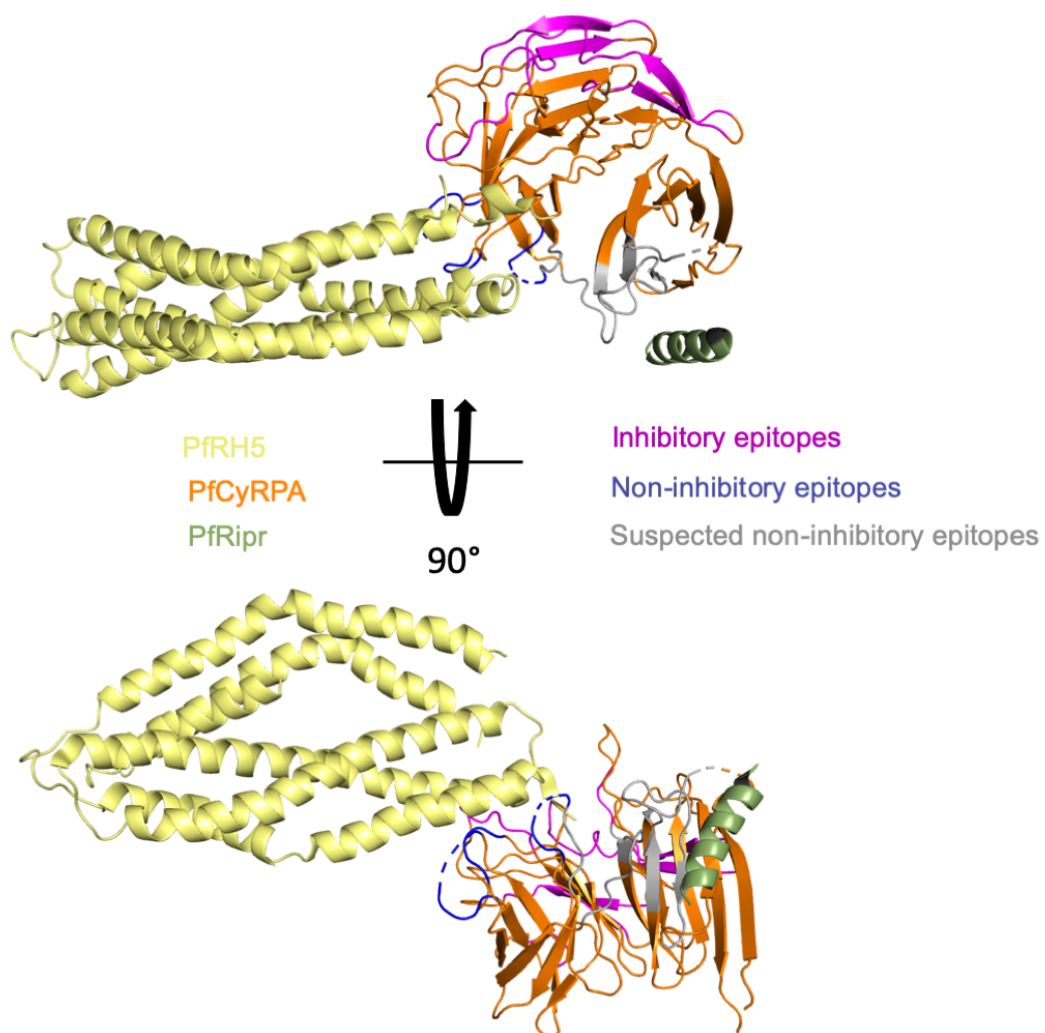


Figure 4.16 Mapped epitopes from structural studies onto the RCR complex. The blue region is where Cy.002 binds PfCyRPA. The pink region is where the panel of inhibitory mAbs bind. Grey region is where Cy.001 and Cy.010 could bind based on competing for PfCyRPA binding with PfRH5 and PfRipr.

4.3 Discussion

4.3.1 Shared structural properties of inhibitory antibodies

The addition of five new structures, three completed (Cy.003:PfCyRPA, Cy.004:PfCyRPA and Cy.009:PfCyRPA) and two in progress (Cy.007:PfCyRPA and Cy.002:PfCyRPA), to complement the two published structures (c12:PfCyRPA and 8A7:PfCyRPA), provides further insight regarding the key inhibitory epitopes on PfCyRPA^{66,72}. Notably, all of the inhibitory antibodies, whether weakly inhibitory (c12, Cy.007) or potentially inhibitory (Cy.004, Cy.009) bind to the same general region of PfCyRPA. This was anticipated based on the competition data from the previous Chapter that showed all inhibitory antibodies in this panel competed with 8A7 for binding to PfCyRPA, although they did not all compete with each other.

This shared binding region indicates that this face of PfCyRPA either plays a critical role in PfCyRPA function, or binding there provides a specific orientation to the antibodies that allows them to interfere with essential parasite invasion processes. Of the eight new mAbs tested, only one is able to bind to the assembled RCR complex but does not show GIA activity (Cy.005). Cy.005 does not compete with any inhibitory mAb other than Cy.003. Based on the structural data described in this Chapter, Cy.005 likely binds near the base of blades 2-3 of PfCyRPA, or alternatively, on the base of blade 4, opposite PfRH5, where an epitope could conceivably compete with Cy.003 binding but with neither any other mAb tested in the panel nor PfRH5/PfRipr. This would be the only identified region so far that is accessible in the assembled RCR complex, but does not elicit neutralizing antibodies.

One non-inhibitory antibody structure bound to PfCyRPA is shown here, Cy.002, which has its epitope masked by PfRH5 binding to PfCyRPA. It cannot be said definitively

whether the lack of Cy.002 functionality is the result of an inaccessible epitope, or because binding to blades 4/5 does not result in a useful antibody orientation to block downstream function. The preponderance of evidence suggests that this epitope must be inaccessible as the complex emerges either preformed, or forms rapidly upon secretion¹⁴. If the epitope is not accessible, the antibody cannot bind thus making the hypothetical orientation on a PfRH5-less PfCyRPA irrelevant.

Data from Chapter 3 showed non-inhibitory mAbs Cy.001 and Cy.010 block compete with both PfRipr and PfRH5, likely indicative of binding to blades 4 or 5. With these, an antibody targeting each blade of the PfCyRPA propeller other than blade 6 have been assessed for GIA capacity. Based on these data, it seems clear that the region of PfCyRPA that is capable of eliciting neutralizing antibodies is predominantly localized to blades 1-3 at the 'top' of the propeller (opposite where the N/C termini come together). **Figure 4.16** shows the established regions of PfCyRPA that induce neutralizing antibodies.

The observation that c12 and Cy.007 are more strongly inhibitory as Fabs compared to mAbs, described in the previous Chapter, also suggests that the epitope to which these two Fabs bind is susceptible to neutralization, but is not fully accessible during invasion. The reason(s) for the lack of accessibility remain unclear, but it may lie in the angle of approach of c12 and Cy.007, which is distinct from the other four inhibitory Fabs based on superimposition of the data shown here.

4.3.2 Heavy metal mediated antibody-antigen interactions

The observation that a divalent cation occupies much of the Cy.004 interface with PfCyRPA is unusual. Metal ions have a well-established role in mediating many protein-

protein interactions²³⁰, but it remains an unusual antibody binding modality that has been seldom reported previously²³¹.

With the data presented here, the presence of a metal ion in the Cy.004:PfCyRPA interface cannot be determined definitively. It will be necessary to repeat the SPR measurements which showed that Cy.004 has low affinity for PfCyRPA in the absence of divalent cations. It seems most likely that the cation would be Mg^{2+} or Ca^{2+} based on the fact that no additional ions were present during crystallization. Thus, the ion source would have to be expression media or purification buffers.

If the introduction of Mg^{2+} and/or Ca^{2+} improves the affinity of Cy.004 for PfCyRPA, this will be consistent with the structural data presented here. Other complementary approaches which will be attempted in the future include the use of micro particle induced X-ray emission (micro-PIXE)²³² to investigate the composition of the Cy.004:PfCyRPA complex. To do this, Cy.004:PfCyRPA complex would be prepared in the presence of the metal ion of interest, and then purified via SEC into a buffer without this ion, to remove any unbound material. Micro-PIXE could then provide the relative composition of the complex, including the presence of metal ions, based on characteristic wavelength emissions after exposure to a proton beam²³². Given that the composition of the complex is otherwise known (via the amino acid sequence), relative abundance of metal ions can be deduced.

These SPR experiments and micro-PIXE analysis are critical to answering the question of whether or not the putative metal ion is genuine, to determine its identity and to assess whether it has implications for antibody function. If mAb affinity for PfCyRPA is improved in the presence of Mg^{2+}/Ca^{2+} , then this may explain why the mAbs with the weakest affinity for PfCyRPA (Cy.004 and Cy.009) are the most inhibitory. This may simply be an artefact of the

experimental system, which lacks critical binding cofactors which are present in serum. It is important to note that Rosell Park Memorial Institute (RPMI) medium contains approximately 0.4mM of each MgSO_4 and $\text{Ca}(\text{NO}_3)_2$. This means that both Mg^{2+} and Ca^{2+} would be present in the media used to culture *P. falciparum* and conduct GIA assays. Further, in human plasma there is 0.55-0.75mM of Mg^{2+} and approximately 1mM of Ca^{2+} ^{233,234}. Thus, it is conceivable that antibodies with metals ions integral to their epitopes, though uncommon, may arise naturally during the course of infection or after vaccination.

In short, although it may not explain the mechanism of Cy.004/Cy.009, the dependence on cation coordination for PfCyRPA binding may explain why these antibodies that appear to bind weakly also inhibit potently. Their binding may actually occur with much higher affinity than originally measured in Chapter 3. Whether this hypothesized increase in affinity alone when $\text{Mg}^{2+}/\text{Ca}^{2+}$ are added could account for their enhanced potency as compared to 8A7 remains to be determined.

4.3.3 *Speculating on a mechanism of inhibition*

One of the lingering mysteries of this Chapter is why the six inhibitory antibodies differ drastically in their ability to block invasion, despite binding similar regions of PfCyRPA. Without knowing the underlying mechanism of invasion inhibition, it is difficult to speculate as to why one antibody may inhibit more effectively than a different antibody that binds a similar epitope. In order to understand this, a mechanism of action needs to be established.

As discussed at length in Chapter 3, the only thoroughly convincing function of the RCR complex is that it binds to basigin¹⁴. There is no evidence that suggests anti-PfCyRPA antibodies block complex assembly, let alone basigin binding. As such, we are left to speculate on other ways the antibodies could function.

One appealing possibility is an additional interacting partner that works downstream of the RCR complex and binds to PfCyRPA on the seemingly critical epitopes around the tops of blades two and three. Perhaps by binding to this region of PfCyRPA, the inhibitory antibodies described here block downstream processes in which PfCyRPA is involved. There is limited evidence to support this theory, but one line of evidence is that PfCyRPA is conserved between different *Plasmodium* species, and yet PfRH5 is not. Further, though PfRipr is conserved, the PfCyRPA/PfRipr interaction is not⁸³. The conservation of PfCyRPA but absence of PfRH5 suggests that, at least in other species, PfCyRPA has a non-PfRH5 related function. Perhaps then, it is this functionality that these mAbs interfere with, rather than with the RCR complex itself and/or PfRH5 binding to basigin. However, further studies into CyRPA, across *Plasmodium* species, would be needed to support this theory, which at present remains largely conjecture.

4.3.4 Designing an improved anti-PfCyRPA vaccine antigen

Currently, PfCyRPA is viewed as a less promising vaccine target in comparison to PfRH5. The reason for that is that both polyclonal sera targeting PfCyRPA, as well as monoclonal anti-PfCyRPA antibodies have been less potent than their PfRH5 counterparts in terms of GIA *in vitro*^{68,70,90,102,107}. Different strategies could be undertaken to improve the capacity of PfCyRPA, when delivered as a vaccine, to induce neutralizing antibodies, some of which would rely on the structural data produced in this Chapter. While on its own, it is still unlikely that even an optimized PfCyRPA immunogen could outperform PfRH5, the reported synergy between anti-PfRH5 and anti-PfCyRPA antibodies in GIA assays suggest that a multicomponent vaccine could be more effective than a PfRH5 vaccine on its own^{102,107}.

The possibility of simply expressing only blades 1-3 of PfCyRPA may instinctively be appealing, but is likely to be challenging. Although one study claims to have successfully expressed PfCyRPA fragments⁶⁶, there is little evidence shown that they were correctly folded or that they did not aggregate. As a secreted protein, the surface of PfCyRPA is relatively hydrophilic, but the regions between the blades remain largely hydrophobic. Thus, if only blades 1-3 were expressed, the hydrophobic regions of these blades that would normally associate with the hydrophobic regions of blades 6 and 4 would now be surface exposed. This would be problematic for protein stability and quality and would likely require more extensive protein engineering to produce a stable protein product.

Based on the available structural data, a few approaches could be taken. The first approach would insert glycosylation sites on the surface of PfCyRPA on the regions that did not induce neutralizing antibodies according to this study. During protein expression in mammalian cells, the non-neutralizing regions of PfCyRPA with the newly inserted glycosylation sites, would then be glycosylated with 'self' glycans, thereby avoiding detection by the vaccinee's immune system²³⁵. The non-glycosylated region of the protein would then be the focus of the host immune response. This approach would avoid the need to reengineer a PfCyRPA fragment that was stable in solution, while in principle obtaining the same result. There has been some limited success using this 'glycan masking' approach to guide immune responses away from the influenza HA head, towards the stalk²³⁶.

Lastly, one alternative approach would be epitope grafting, which was introduced previously. The challenge here is that PfCyRPA is already a relatively small protein at 40kDa, and thus whatever structure the key inhibitory epitopes were grafted onto would have to be considerably smaller in order to meaningfully reduce the proportion of neutralizing to non-neutralizing antibodies. A better approach may be to utilize the wide diversity of β -propellers

that exist in nature in order to develop a multi-dose vaccine regimen where each dose uses a different β -propeller backbone with the critical Cy.004/Cy.009 epitope grafted onto it, essentially a heterologous prime-boost vaccine regimen using different protein scaffolds²³⁷. That way, with each successive dose, the parts of the β -propeller that do not induce neutralizing antibodies would not be boosted by the next vaccine dose, while the key grafted regions, the only protein regions that are the same between the different backbones, would benefit from a pre-existing memory response to those regions that could be amplified. The benefits of this heterologous prime-boost approach has previously been shown for RSV²³⁸ and HIV²³⁹.

In practice, this approach is unlikely to be feasible, as the design, testing and large-scale manufacturing of one vaccine is challenging enough, but a vaccine where each dose is functionally a new product is unlikely to be seen as a viable option. But, as a concept, it remains scientifically intriguing.

In this Chapter, the key inhibitory region of PfCyRPA was found to be localized to the 'top' of the β -propeller on blades 1- 3. The evidence generated from these structural studies confirms what was hypothesized based on results from the previous Chapter: that all neutralizing antibodies bind to the same face of PfCyRPA, characterized by competition with 8A7, and neutralizing antibody epitopes do not compete for binding of PfCyRPA with PfRipr or PfRH5. In the next chapter, I will investigate how different combinations of neutralizing anti-PfCyRPA antibodies can combine to produce 'synergistic effects' on parasite neutralization.

4.4 Addendum

In the days immediately prior to the submission of this thesis, new data was acquired that helps to illuminate the role of metal ions in the Cy.004:PfCyRPA binding interface. These experiments could not be fully incorporated into this Thesis, but have been included here as they provide important supporting information to the hypotheses put forth in this Chapter.

In order to assess the role of metal ions in mediating the Cy.004:PfCyRPA interaction, approximately 500RU of Cy.004 was immobilized on a protein A chip. Then kinetics of PfCyRPA binding were measured in three different buffers: PBS with 25mM EDTA (**Figure 4.17A**), PBS with 1mM MgCl₂ (**Figure 4.17B**) and PBS with 1mM CaCl₂ (**Figure 4.17C**) beginning at 800nM of PfCyRPA and then following an 8-step two-fold dilution series.

The results show that the addition of calcium to the SPR buffer improved the K_D to 3.28×10^{-8} M as compared to 1.18×10^{-6} M for Cy.004 binding to PfCyRPA as measured in Chapter 3 (**Table 3.2**) (**Figure 4.17C**). Conversely, the addition of MgCl₂ only marginally improved the affinity to 2.03×10^{-7} M (**Figure 4.17B**). EDTA did not improve the binding affinity of Cy.004 for PfCyRPA.

These data suggest that there is indeed a role for Ca²⁺ in mediating the Cy.004:PfCyRPA interaction and this is likely the identity of the ion observed at the interface during these structural studies. Further follow-up investigations are planned with micro-PIXE and repeating this experiment with Cy.009.

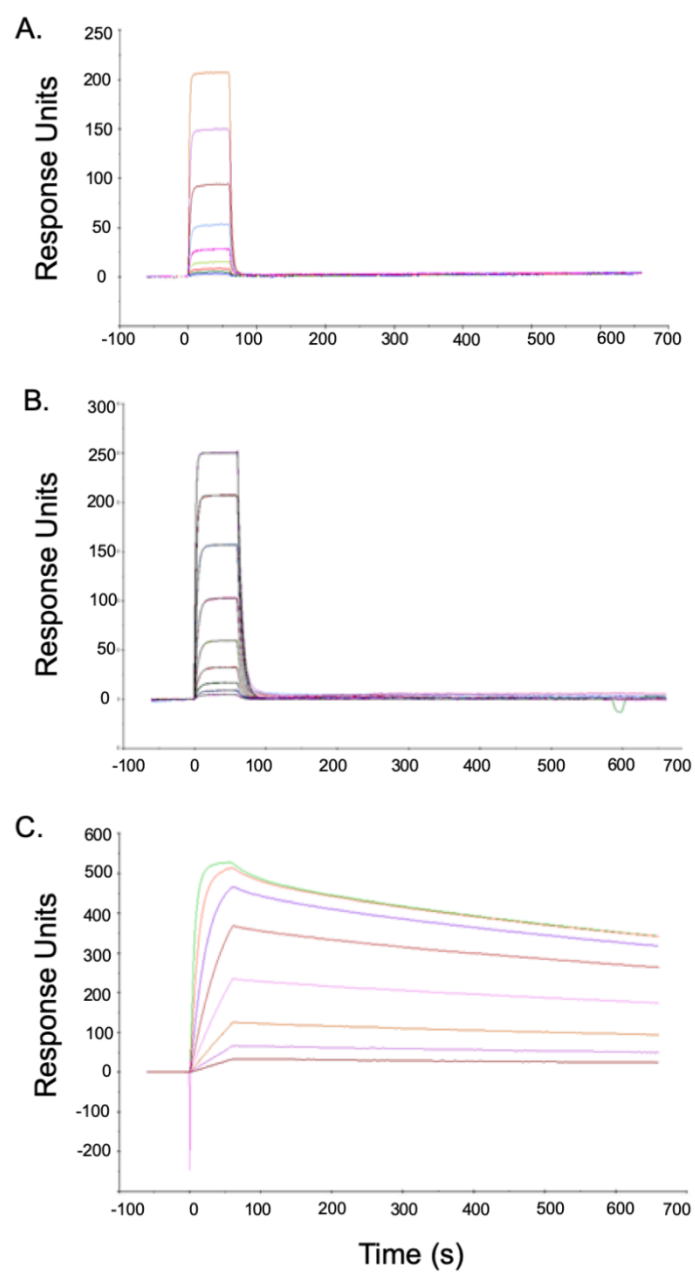


Figure 4.17 Kinetic assessment of Cy.004 binding to PfCyRPA in the presence of metal ions. A. PBS with 25mM EDTA B. PBS with 1mM $MgCl_2$ C. PBS with 1mM $CaCl_2$

Chapter 5: Synergy between pairs of anti-PfCyRPA neutralizing mAbs

Authorship Statement

As mentioned previously, the GIA assay is standardised. To minimise variability, all of the synergy GIA assays are carried out by Dr. Doris Quinkert or Amelia Lias, to enable direct comparison to other projects ongoing within the lab. This includes both regular parasite maintenance and the assay set-up and read-out.

5.1 Introduction

5.1.1 Defining synergy

As outlined in **Chapter 1** of this thesis, drug combinations that yield synergistic effects are of broad interest for therapeutic development in a number of diseases, perhaps most notably in treating cancer¹⁶⁵. For malaria, due to the challenge of achieving and maintaining sufficiently high antibody concentrations in the blood to protect against clinical disease, the idea of synergy, either between mAbs (in the case of an antibody cocktail) or between polyclonal antibodies targeting different antigens (in the case of vaccine), is an attractive approach to potentially reduce the amount of total antibody required for protection.

The concept of synergy, though intuitive in principle, is fraught with complications. Broadly, synergy is the idea of the whole being greater than the sum of its parts. That is to say, to determine if two antibodies are synergistic, one must first determine the expected effect of combining the antibodies *a priori*. These *a priori* additive effects can then be compared to the empirical outcomes. If two antibodies exceed the predicted activity, they

are said to behave synergistically, while if they have less activity than predicted, this is said to be antagonism. Though there are other terms used to describe the concepts of 'synergy' and 'antagonism', this work will use the terms 'synergy' and 'antagonism'.

Choosing a model to predict additive behaviour is non-trivial. One such model is Bliss independence²⁴⁰. Bliss independence, or Bliss additivity, is derived from the joint probabilities of independent events based on the responses to each drug individually (or in this case the level of antibody function). In other words, the probability of surviving a given treatment of two drugs is equal to the product of surviving each treatment on its own²⁴¹. Here, Bliss independence assumes that the two antibodies act independently and are non-mutually exclusive¹⁶³. In the case illustrated in **Equation 5.1**, if one anti-malarial antibody inhibits 50% of parasite invasion events and a second blocks 30% of invasion events, then the combined effect of the two antibodies behaving additively would be 65%.

$$\% inhibition_{comb} = \% inhibition_{mAb1} + \% inhibition_{mAb2} \times (1 - \% inhibition_{mAb1})$$

$$\% inhibition_{comb} = 0.5 + 0.3 \times (1 - 0.5)$$

$$\% inhibition_{comb} = 0.65$$

Equation 5.1: an example of the Bliss independence calculation using two monoclonal antibodies that block *P. falciparum* invasion

If the two mAbs have a combined effect that is less than 65%, then they are said to be acting antagonistically. Conversely, if a combination of the two antibodies has an effect that is greater than 65%, then they are said to be acting synergistically.

The shortcoming of this model is that it does not consider the shapes of dose response curves. This issue has been widely discussed in the literature, as Bliss additivity often

fails an intuitive test of synergy^{163,241–243}. In a sham synergy experiment, one would measure the synergy of a drug with itself. That is to say, if 1 unit of drug A is combined with a second unit of drug A, what is the expected outcome based on Bliss additivity, and what is actually seen.

This is illustrated in **Figure 5.1**, the dose response curve for Cy.004 alone shows that at dose 1 (D_1), 0.1mg/ml there is 15% inhibition. If D_1 is combined with a second dose of D_1 then one would expect the increase in inhibition to follow Bliss additivity. By definition, Cy.004 cannot antagonize or synergize with itself.

$$\% inhibition_{comb} = \% inhibition_{mAb1} + \% inhibition_{mAb1} \times (1 - \% inhibition_{mAb2})$$

$$\% inhibition_{comb} = 0.15 + 0.15 \times (1 - 0.15)$$

$$\% inhibition_{comb} = 0.27$$

Equation 5.2 Predicted Bliss additivity in sham synergy experiment.

Here, the Bliss additivity is expected to be 27% inhibition, (**Equation 5.2**) but instead 35% inhibition is measured, which gives the impression that Cy.004 synergizes with itself. This is due to the fact that Bliss additivity assume first order kinetics, or a slope of 1, whereas for Cy.004 it is 1.6, as determined by a four-parameter non-linear regression^{163,242}.

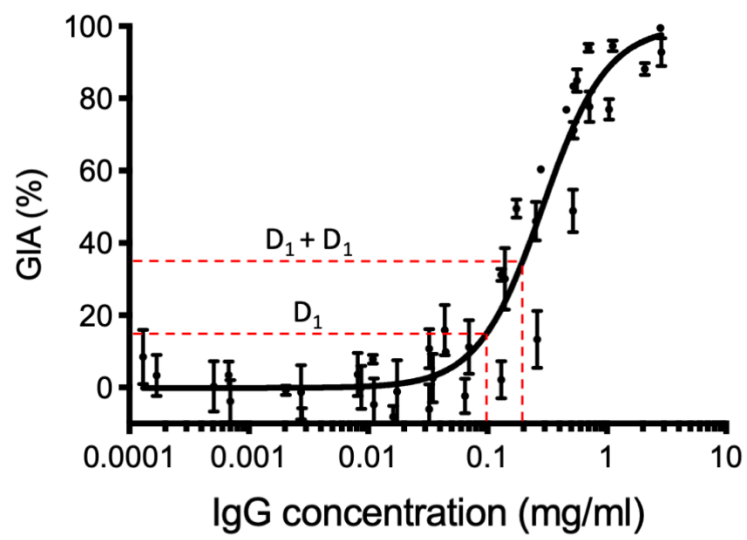


Figure 5.1 Bliss additivity fails to accurately predict sham synergy. The effect of $D_1 + D_1$ is greater than what would be expected if activity was predicted through Bliss independence.

A second model termed the Loewe additivity is rooted in the idea of dose equivalence and considers the shape of curves when predicting behaviour of combinations of antibodies^{244,245}. In this case, the Loewe model assumes that two different compounds that have the same response are equivalent. That is to say that if the IC₅₀ of drug A is 10μM and the IC₅₀ of drug B is 100μM then:

$$IC_{50} = 10\mu M \text{ drug A} = 100\mu M \text{ drug B} = 5\mu M \text{ drug A} + 50\mu M \text{ drug B}$$

Equation 5.3 Loewe additivity example.

The Loewe additivity uses this principle of dose equivalence to determine the concentration of each antibody that would be expected to yield a given response (**Equation 5.4**).

$$1 = \frac{A}{A_{50}} + \frac{B}{B_{50}}$$

Equation 5.4 Loewe additivity equation at the IC₅₀. A is the concentration of antibody A required to achieve 50% inhibition when in combination with a concentration (B) of antibody B relative to the IC₅₀ of both antibodies (A₅₀, B₅₀).

In the case of Loewe additivity, if the sum of the right side of the equation is less than one, this would indicate synergy, whereas a sum greater than one would indicate antagonism. Like Bliss additivity, Loewe additivity is not equally applicable in all circumstances. It assumes mutual exclusivity of binding, equal maximum responses between drugs and equal slopes,

which is not necessarily true for different inhibitory antibodies²⁴⁶. Loewe additivity is, therefore, often more appropriate for measuring drugs that target the same pathway¹⁶³.

The highest single agent model of synergy posits that if the effect of drug A and drug B together is higher than the same concentration of only the more potent drug, then the two drugs synergize²⁴⁷. This is summarized in **Equation 5.5**.

$$Inhibition_{A+B} > \max (Inhibition_A, Inhibition_B)$$

Equation 5.5 Highest single agent criteria are met if the above equation is satisfied where the inhibition of the combination is greater than the inhibition of both of its constitutive components.

The final widely-used model of synergy proposed by Chou and Talalay is another commonly used approach derived from the median effect principle (**Equation 5.6**)²⁴⁸. This method yields a combinatorial index similar to Loewe additivity. The Chou and Talalay additivity formula for mutually exclusive drugs is given in **Equation 5.7** and the formula for mutually non-exclusive drugs in **Equation 5.8** where there is an additional term to account for the interaction between the drugs. Like the Loewe definition of additivity, the sum of the right hand of these equations should be 1, with values lower indicating synergy and greater than 1 indicating antagonism. The value assigned to a given combination is known as its combinatorial index.

$$\frac{f_a}{f_u} = \left(\frac{D}{D_m} \right)^m$$

Equation 5.6 Median effect equation where f_a is the affected fraction, f_u is the unaffected fraction, D is a given dose, D_m is the dose required for the median effect (EC_{50}) and m is the slope of the curve.

$$\frac{f_a}{f_u} = \frac{D_1}{D_{m1}} + \frac{D_2}{D_{m2}}$$

Equation 5.7 Chou and Talalay formula for the combined effects of mutually exclusive drugs.

$$\frac{f_a}{f_u} = \frac{D_1}{D_{m1}} + \frac{D_2}{D_{m2}} + \frac{D_1 D_2}{D_{m1} D_{m2}}$$

Equation 5.8 Chou and Talalay formula for the combined effects of non-mutually exclusive drugs.

There are other synergy definitions that have been proposed and have been widely discussed^{163,241–243,245,247,249–251}. However, there is no consensus as to how synergy should be defined²⁵². As a result, the closest thing to consensus is an agreement that whatever definition of synergy is used in a given study should be clearly defined.

In this Chapter, ‘additivity’ refers to Bliss independence. The reason for this is three-fold. First, its simplicity was appealing as the model is intuitive, and results can be easily interpreted. Second, it is a widely used model and enables comparison to previous published literature¹⁰³. Third, when two anti-PfRH5 antibodies that both block basigin (but do not compete for binding with each other) are combined, the empirically measured growth inhibition curve closely matches the predicted Bliss additivity²⁰¹. This roots the Bliss independence definition in the biological reality of the system of interest. Fortunately, the cases explored in-depth here are not borderline cases, but rather very clear examples of outright synergy by any definition.

5.1.2 Synergy between anti-RCR mAbs

Synergy has been documented between antibodies targeting different members of the RCR complex^{59,69,107,201}. In one report of anti-PfRH5 mAbs, non-neutralising human anti-PfRH5 monoclonal antibodies were shown to potentiate the activity of invasion inhibitory

mAbs⁶⁸. The mechanism is thought to be that the non-neutralizing antibody slows invasion thereby providing more time for the neutralizing antibody to bind and enhancing its potency⁶⁸. How invasion is slowed (but not prevented) is not clear but it may relate to the fact that these 'non-neutralising clones' (e.g. mAb R5.011) bind adjacent to the recently identified PfRH5 N-terminal cleavage site, and cleavage is thought to be important for function²⁵³. Importantly, synergy is not seen between pairs of inhibitory PfRH5 mAbs, as synergy is limited to the R5.011-style mAb, which has no inhibitory activity on its own⁶⁸. In contrast, combinations of human mAbs that target the Duffy-binding protein of *P. vivax* appear to antagonize each other, although the mechanism underlying this observation remains unknown¹⁹⁹.

5.1.3 Chapter aims

In this Chapter, the first aim was to assess the functional relationships between different pairs of inhibitory and non-inhibitory anti-PfCyRPA mAbs. These were assessed through Bliss independence measures of synergy in the assay of GIA, with the intention of determining whether some combinations of mAbs showed enhanced potency. The second aim (in light of the observed data) was to then determine a plausible theory for how two inhibitory antibodies both targeting the same antigen could behave synergistically. Finally, I sought to validate this hypothesis experimentally.

5.2 Results

5.2.1 Assessing synergy between combinations of anti-PfCyRPA mAbs

In order to assess additivity/antagonism/synergy between different combinations of anti-PfCyRPA mAbs, a 'synergy GIA' assay was used. In this format, one concentration of antibody is held constant, while a second antibody is titrated in at increasing concentrations. The inhibition of the two mAbs can be compared with what would be predicted if the two mAbs functioned independently of each other, i.e. Bliss additivity (**Equation 5.1**). In the experimental set-up used here, one mAb was held constant at a concentration that would be predicted to give approximately 30% GIA. The second mAb was then added beginning at 2mg/ml, and then titrated down a 7-step 4-fold dilution curve. Each GIA assay was run alongside antibodies with a known GIA profile (2AC7, QA5, 9AD4) and a negative control which was an Ebola Zaire glycoprotein mAb¹⁹⁰, EBL040, that should not affect merozoite invasion. To minimize inter-assay variability, assays where the measured GIA for the internal controls was more than two standard deviations away from the mean were excluded, and repeated. A summary of mAbs investigated here, their relative potency and their binding location on PfCyRPA is shown in **Figure 5.2**.

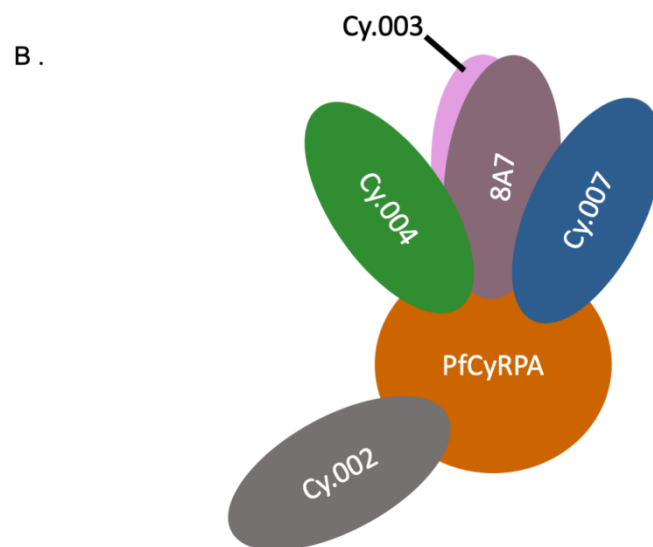
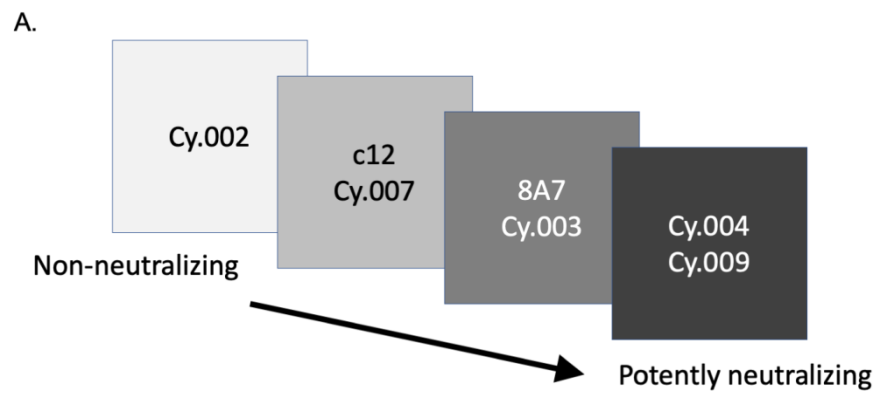


Figure 5.2 Summary of anti-PfCyRPA mAbs included in the synergy GIA assays. A. mAbs grouped based on their GIA potency alone with the least potent on left and most potent on right. B. An illustration depicting approximate binding positions on PfCyRPA where 8A7 competes with Cy.004, Cy.007 and Cy.003. Cy.009 binds in the same position as Cy.004.

Cy.002 has no functional activity in the GIA assay on its own, and was not anticipated to enhance the activity of other inhibitory mAbs. Accordingly, there appeared to be no increase in merozoite inhibition when Cy.002 was present alongside any of the tested inhibitory antibodies (**Figure 5.3**). The Bliss additivity prediction is lower than the measured response because on its own, Cy.002 appears to be below zero percent inhibition. This is frequently seen in GIA assays with non-inhibitory mAbs or at low concentrations of inhibitory mAb and is possibly an artefact of the experimental system. The functional significance, if any, is not known. It is clear that the measured inhibition in blue does not increase above the inhibition from the single inhibitory mAb held at a constant concentration (indicated in pink). It is worth noting that a calculation error meant that the concentration of Cy.004 was held above where it should optimally be to give 30% invasion inhibition. Despite this, it still is clear that there is no enhanced activity from Cy.002.

8A7 was not expected to show synergy with any of the other inhibitory mAbs due to the fact that it competes with them for binding. Indeed, no synergy was observed between 8A7 and any of the other neutralizing antibodies, with the possible exception of Cy.003 (**Figure 5.4**). It is difficult to determine if 8A7 is behaving additively or antagonistically. It appears that there could be weak antagonism at the higher concentrations where 8A7 has a measurable inhibitory effect on its own (1mg/ml and 2mg/ml). The reverse is true for Cy.003 where inhibition is higher than expected based on Bliss additivity at 1mg/ml and 2mg/ml of 8A7. However, the effect is very weak in both directions.

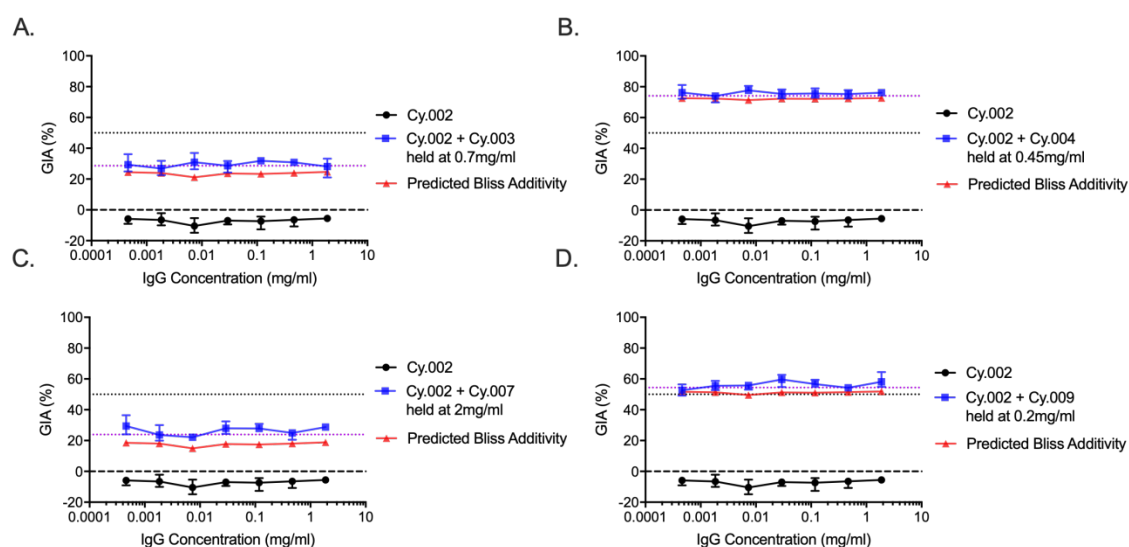


Figure 5.3 Cy.002 synergy GIA assays. Cy.002 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the mAb held at a constant concentration alone. The black line shows the inhibition activity of the mAb being titrated in alone (Cy.002). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation. A. Cy.002 synergy assessment with Cy.003 held constant. B. Cy.002 synergy assessment with Cy.004 held constant. C. Cy.002 synergy assessment with Cy.007 held constant. D. Cy.002 synergy assessment with Cy.009 held constant.

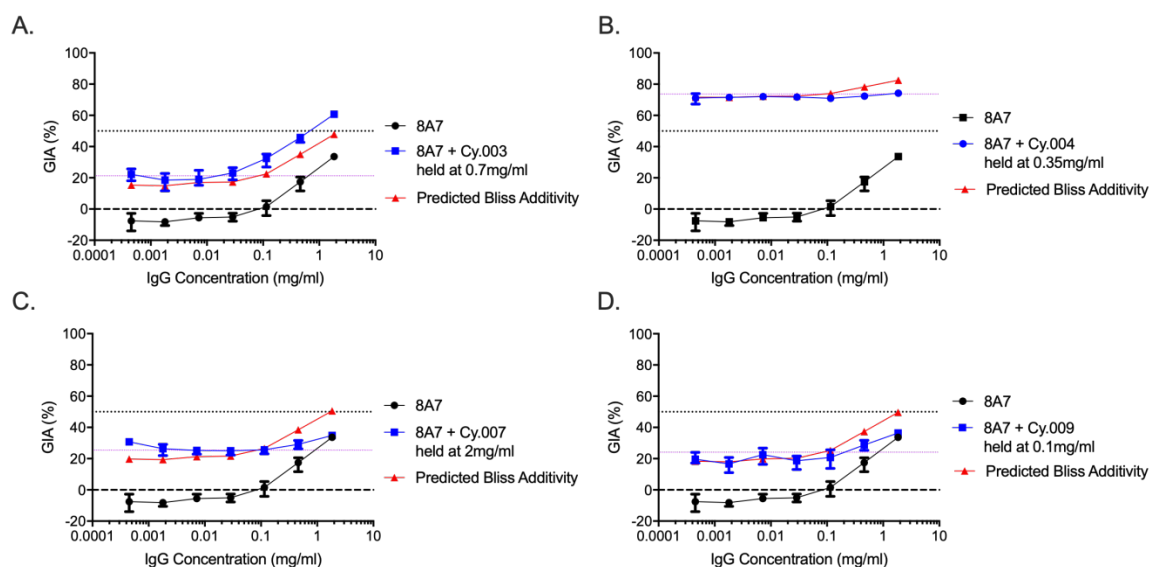


Figure 5.4 8A7 synergy GIA assays. 8A7 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the mAb held at a constant concentration alone. The black line shows the inhibition activity of the mAb being titrated in alone (8A7). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation.

A. 8A7 synergy with Cy.003 held constant B. 8A7 synergy with Cy.004 held constant C. 8A7 synergy with Cy.007 held constant. D. 8A7 synergy with Cy.009 held constant.

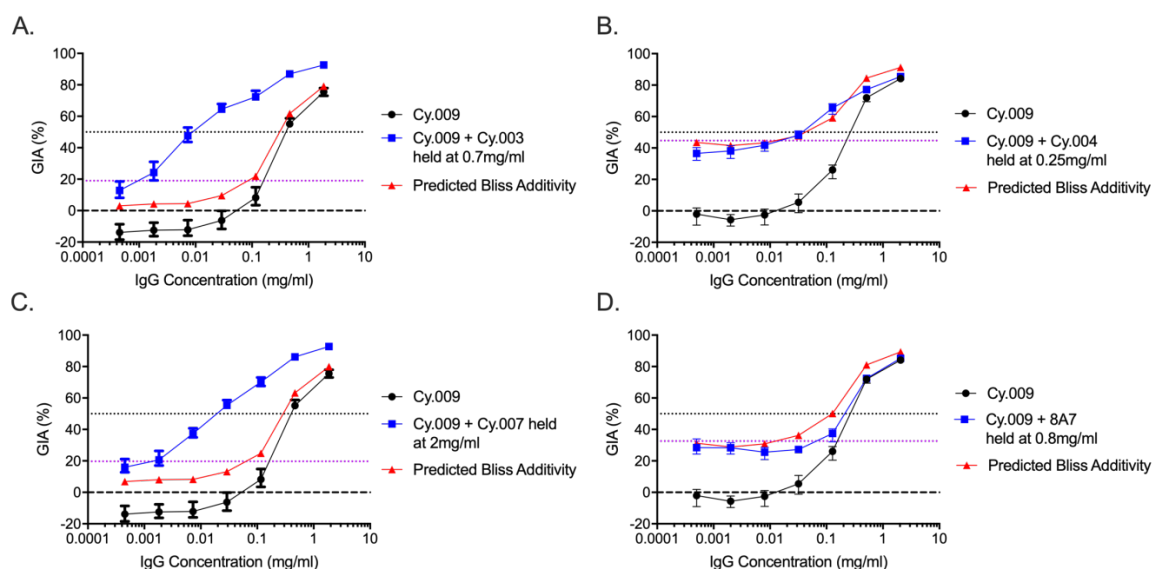


Figure 5.5 Cy.009 synergy GIA assays. Cy.009 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the mAb held at a constant concentration alone. The black line shows the inhibition activity of the mAb being titrated in alone (Cy.009). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation. A. Cy.009 synergy with Cy.003 held constant B. Cy.009 synergy with Cy.004 held constant C. Cy.009 synergy with Cy.007 held constant D. Cy.009 synergy with 8A7 held constant.

The synergy GIA assays for the most potently inhibitory mAb Cy.009, however, showed remarkable synergy outcomes when this clone was combined with both Cy.003 and Cy.007, two weakly to moderately inhibitory mAbs (**Figure 5.5A,C**). No such synergy was observed for 8A7 or Cy.004, which compete with Cy.009 for binding and thus do not bind simultaneously. Again, this combination seems to have a weakly antagonistic relationship, consistent with the same combination (8A7 and Cy.009) from **Figure 5.4D**, but in reverse (8A7 held constant).

In order to calculate the magnitude of the synergy a four-parameter non-linear regression curve was fit to the predicted Bliss additivity and the measured inhibition (**Figure 5.6**). From there, the IC_{50} , that is the concentration of antibody that achieves 50% inhibition was calculated (**Equation 5.9**) and the synergy quotient determined as described in **Equation 5.10**. If the held concentration of antibody alone exceeded 50% inhibition, this could not be calculated. The overall synergies of all combinations are given in **appendix 2**.

$$IC_{50} = EC_{50} \times \left(\frac{Minimum - Maximum}{50 - Maximum} - 1 \right)^{1/slope}$$

Equation 5.9 Formula to determine IC_{50} based on four parameter logistic regression.

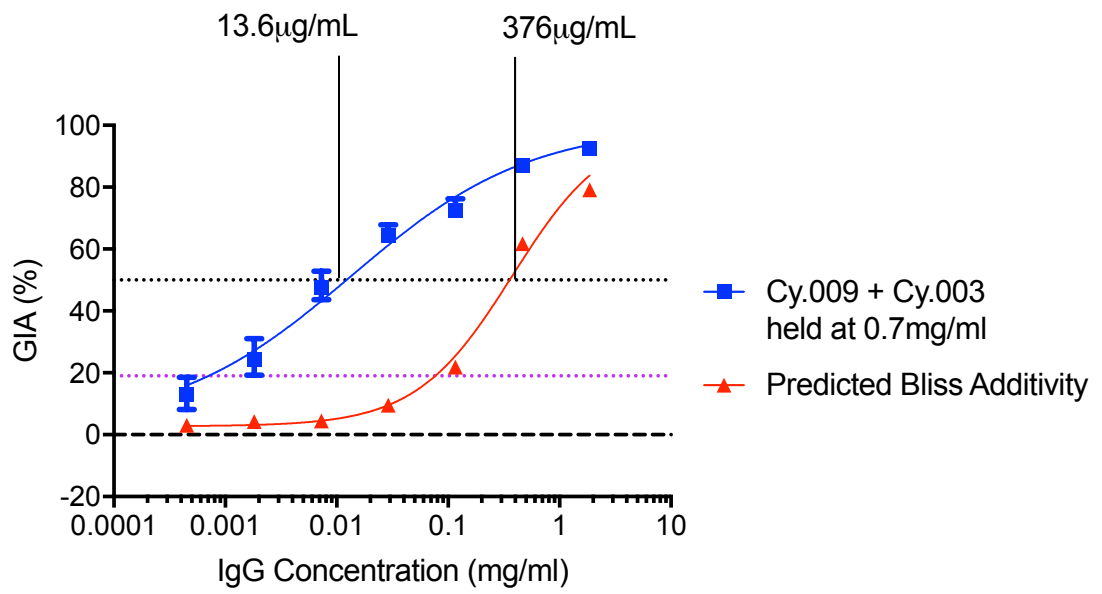


Figure 5.6 Sample curve fit for synergy assessment. These are the same data from Figure 5.5A, but Cy.009 alone has been removed for clarity.

$$\text{Fold increase} = \frac{IC_{50_{\text{predicted}}}}{IC_{50_{\text{actual}}}}$$

$$\text{Fold increase}_{\text{Cy.009} + \text{Cy.003}} = \frac{376 \mu\text{g/ml}}{13.6 \mu\text{g/mL}}$$

$$\text{Fold increase} = 27.6$$

Equation 5.10. Calculating fold increase in potency due to synergy for antibody pairs. Here, fold increase in synergy refers to the IC_{50} for Cy.009 when combined with 0.7mg/ml of Cy.003 relative to the Bliss additivity predicted IC_{50} .

In the case of Cy.009, the improvement in IC_{50} was 14-fold when in combination with a fixed concentration of Cy.007 and 28-fold when in combination with Cy.003. Given these intriguing results, the same experiment was run using Cy.004 instead of Cy.009. Since this clone binds to the same epitope as Cy.009, it was of interest to determine if this mAb shared the same synergy properties as well (**Figure 5.7**). Indeed, Cy.004 also synergized with Cy.003 and Cy.007 (**Figure 5.7A-B**), but not with Cy.009 or 8A7 (**Figure 5.7C-D**). When in combination with Cy.003 and Cy.007, the increase in potency was 109- and 24-fold, respectively.

To further confirm the observed synergy, the synergy GIA assay was next carried out in reverse with Cy.003 and Cy.007 titrated in (**Figure 5.8, Figure 5.9**). These results aligned well with what had already been observed, whereby Cy.003 and Cy.007 synergized potently with Cy.004 and Cy.009 (**Figure 5.8A,C, Figure 5.9B,C**) and not with 8A7 (**Figure 5.8D, Figure 5.9D**). Cy.003 and Cy.007 also synergized together (**Figure 5.8C, Figure 5.9A**). Quantifying the synergy in these assays could not be done reliably due to the fact that the predicted Bliss additivity never reached the IC_{50} . Further, Cy.004 was held at too high a concentration due to a calculation error, and thus the baseline inhibition was greater than 50% in some instances, thereby precluding the calculation of fold increase in potency. This is to be repeated; but has unfortunately not been possible before the completion of this Thesis due to COVID-19 related interruptions that have resulted in the closure of the parasitology lab.

However, based on these results, it appears that the most potent synergy occurs between Cy.004/Cy.009 and Cy.003/Cy.007, with the Cy.003/Cy.007 combination showing more modest synergy. This means that all inhibitory PfCyRPA antibodies tested (which do not compete for binding with each other) synergize. This is a surprising finding, as it suggests that the antibodies are not acting independently of one another. This is also in stark contrast to

anti-PfRH5 mAbs, whereby inhibitory antibodies only showed additivity when mixed together, even if they did not compete²⁰¹.

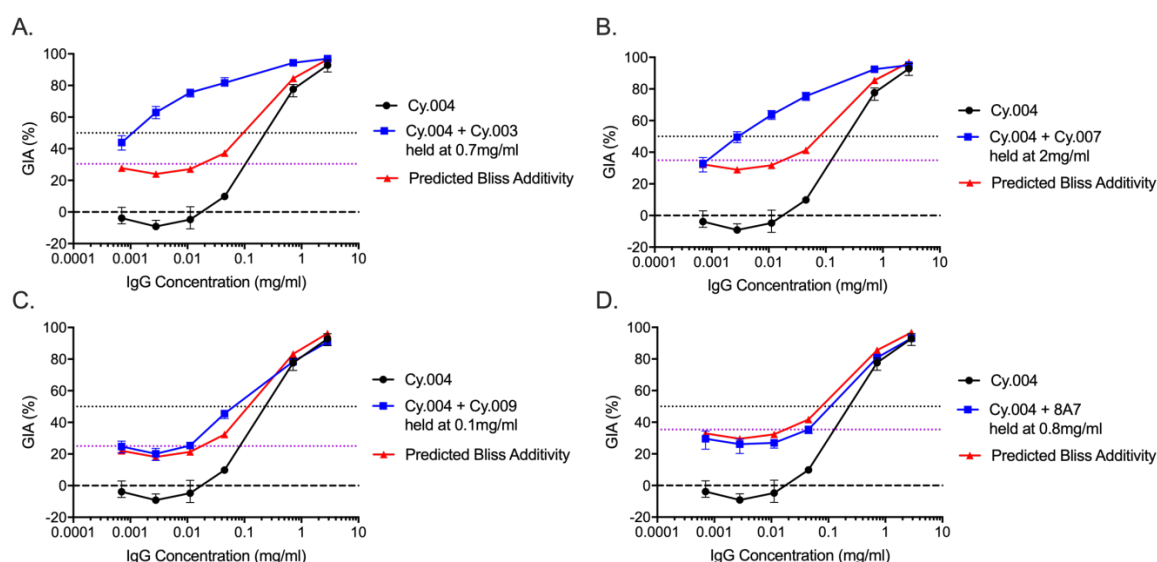


Figure 5.7 Cy.004 synergy GIA assays. Cy.004 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the mAb held at a constant concentration alone. The black line shows the inhibition activity of the mAb being titrated in alone (Cy.004). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation. The data point at 125 μ g/ml was excluded as no antibody was added to that well due to pipetting error. A. Cy.004 synergy with Cy.003 held constant B. Cy.004 synergy with Cy.007 held constant C. Cy.004 synergy with Cy.009 held constant D. Cy.004 synergy with 8A7 held constant.

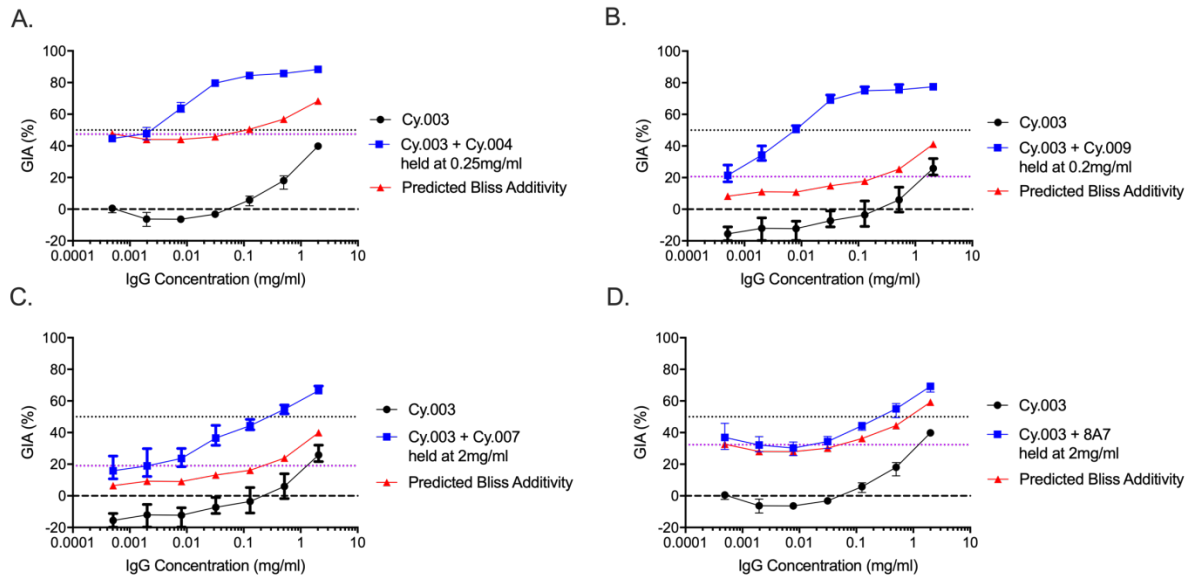


Figure 5.8 Cy.003 synergy GIA assays. Cy.003 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the mAb held at a constant concentration alone. The black line shows the inhibition activity of the mAb being titrated in alone (Cy.003). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation. A. Cy.003 synergy with Cy.004 held constant B. Cy.003 synergy with Cy.007 held constant C. Cy.003 synergy with Cy.009 held constant D. Cy.003 synergy with 8A7 held constant.

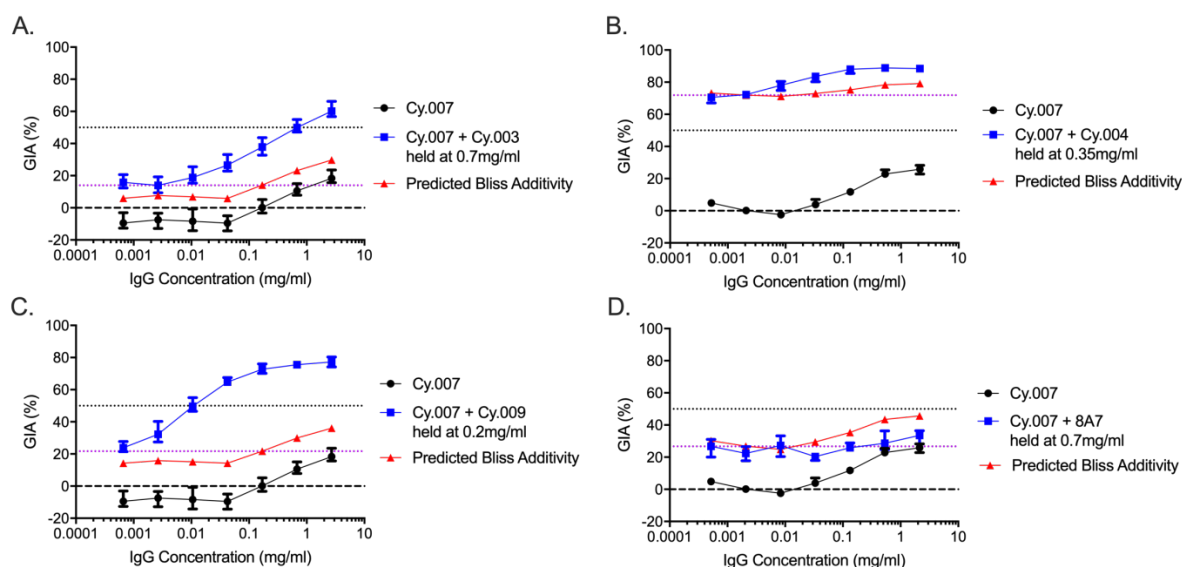


Figure 5.9 Cy.007 synergy GIA assays. Cy.007 was added in a four-fold dilution series with an upper concentration of 2mg/ml (black line). The pink dotted line shows the inhibitory effect of the second test mAb held at a constant concentration. The black line shows the inhibition activity of the mAb being titrated in alone (Cy.007). The red line is the predicted inhibition based on bliss additivity between the two mAbs. The blue line indicates the measured inhibition. Each data point is the mean across the triplicate with the error bars indicating the standard deviation A. Cy.007 synergy with Cy.003 held constant. B. Cy.007 synergy with Cy.004 held constant C. Cy.007 synergy with Cy.009 held constant D. Cy.007 synergy with 8A7 held constant.

The method of assessing synergy whereby one antibody is held constant and the other is added in increasing concentration, as used above, gives useful information regarding the relationships between different inhibitory antibodies. However, the usefulness of this synergy information is limited by the fact that it can mask the disadvantage of having one extremely potent antibody, and one less potent antibody. Even if the combination 'synergizes' it does not necessarily mean that it will be more potent than one antibody on its own, for example in a 50:50 antibody cocktail. This is because if the two mAbs that are being tested differ considerably in their potency, then the synergy between them may still be insufficient to make up for the lost potency that occurs when, for example, 50% of the more potent mAb is replaced in the cocktail by the less potent mAb. Sticking with this example, the 50:50 combination cocktail may still be synergistic, in that the measured inhibition exceeds the additive behaviour, but that does not necessarily make the combination useful therapeutically if overall potency is still less than using the same total concentration of the more potent mAb on its own²⁰¹. This is where the pharmacological ideas of synergy diverge from the principles of synergy being used here to try and reduce the amount of total antibody required for the prevention of infectious disease. This is perhaps best exemplified with experiments where Cy.004/Cy.009 were measured in GIA assays in combination with Cy.007. In order to achieve the 30% inhibition, Cy.007 was held at 2mg/ml. At 2mg/ml of Cy.007 and 0.125mg/ml of Cy.004, 60% inhibition was measured (**Figure 5.7B**), which was synergistic by the criteria used here, but 2.125mg/ml of only Cy.004 would be expected to have close to 100% inhibition.

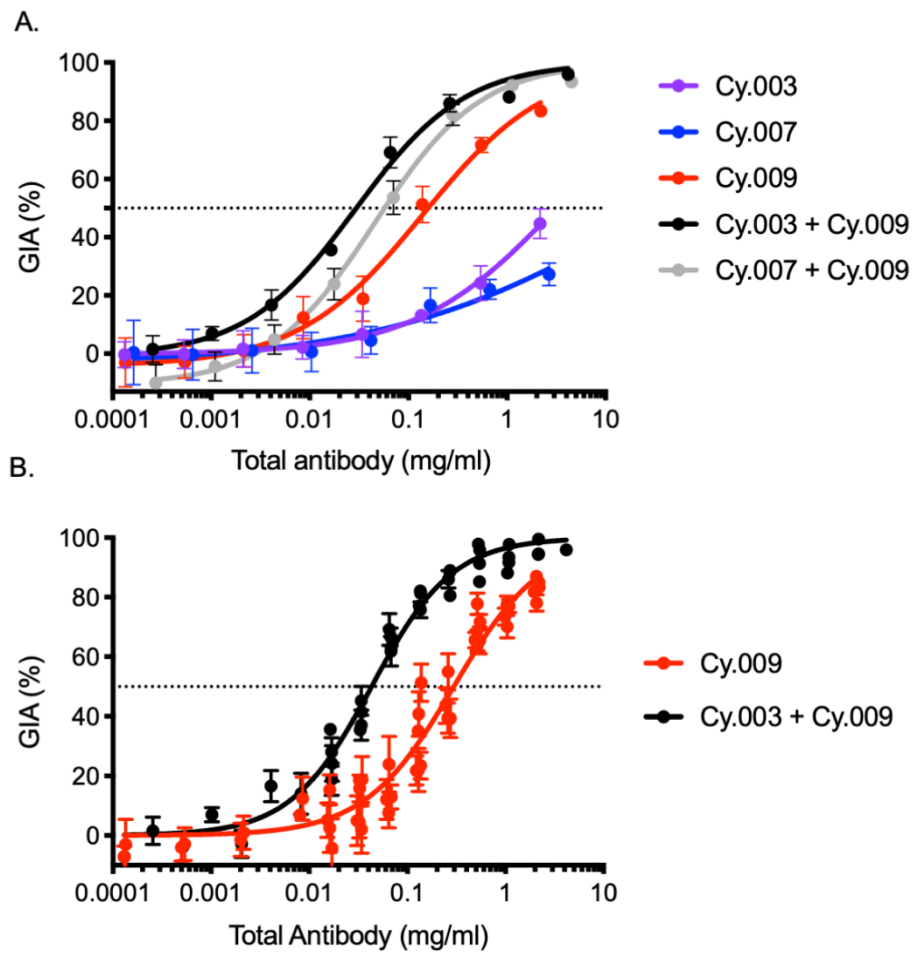


Figure 5.10 Evaluation of anti-PfCyRPA synergy in 1:1 antibody mix. Each data point is the mean of triplicate with errors bars indicating the standard deviation. A. Head-to-head comparison of mAb inhibition compared to 1:1 mix of two different mAbs. $n = 1$ for head-to-head comparison. B. Comparison of the most potent combination Cy.003 + Cy.009 compared to Cy.009 on its own, $n = 5$.

Therefore, to next determine whether the synergy I observed was functionally relevant in the sense that a combination of mAbs was more potent than a single mAb in equimolar concentrations, GIA assays were carried out with 1:1 mixtures of Cy.007 and Cy.003 with Cy.009 (**Figure 5.10**). Compared to Cy.009 alone, 1:1 mixtures decreased the IC₅₀ from 139.8µg/ml (95% CI: 98.8 – 192.7) to 44.9µg/ml (95% CI: 35.1 – 56.0) for Cy.009 and Cy.007 and to 28.8µg/ml (95% CI: 21.7 – 36.9) for Cy.009 and Cy.003 (**Figure 5.10A**). Due to the relatively high degree of variability of the GIA assay, the most potent combination, Cy.009 and Cy.003 was tested head-to-head against Cy.009 alone five times to confirm this finding, and to more accurately determine the IC₅₀ (**Figure 5.10B**). The IC₅₀ of the Cy.009 and Cy.003 combination across the five replicates was measured to be 43.1µg/ml, compared to 297.2µg/ml for Cy.009 alone. This improvement brings down the IC₅₀ of this 1:1 PfCyRPA mAb combination close to the most potent anti-PfRH5 mAbs, which have IC₅₀ values in the low tens of µg/ml^{67,68}. This shows that these mAb combinations clearly meet the highest single agent criteria for synergy. I therefore next sought to understand the potential mechanism(s) underlying this observed intra-antigen antibody synergy.

5.2.3 Mechanism of cooperative binding

The possibility that the binding of one antibody could change the affinity of the second antibody for the antigen was considered as a potential mechanism of synergy. To investigate the relationship between different mAbs, the binding kinetics of a given mAb were re-assessed when a second mAb was already bound to the antigen. In order to do this, Fab: PfCyRPA complex was purified through size exclusion chromatography. A mAb of interest was then amine conjugated to a CM5 chip whereby kinetics of binding could be compared for PfCyRPA on its own versus PfCyRPA bound to another Fab.

When Cy.004 was immobilized on a CM5 chip, both PfCyRPA alone and PfCyRPA in complex with Cy.002 Fab, derived from the non-synergistic mAb Cy.002, showed similar binding kinetics, characterized by rapid dissociation, faster than the limits of detection of the machine (**Figure 5.11A-C**). An approximate dissociation rate was calculated by determining the steady state affinity, and then using the k_a , which was within the limits of the machine, to calculate a k_d . This is only an approximation, as the measurement of k_a by the Biacore X100 is dependent on the k_d . However, based on this approximation, which gave a k_d of 0.45s^{-1} for PfCyRPA alone and 0.34s^{-1} for PfCyRPA: Cy.002 Fab, alongside visual inspection of the sensorgrams, no significant change in dissociation was detected.

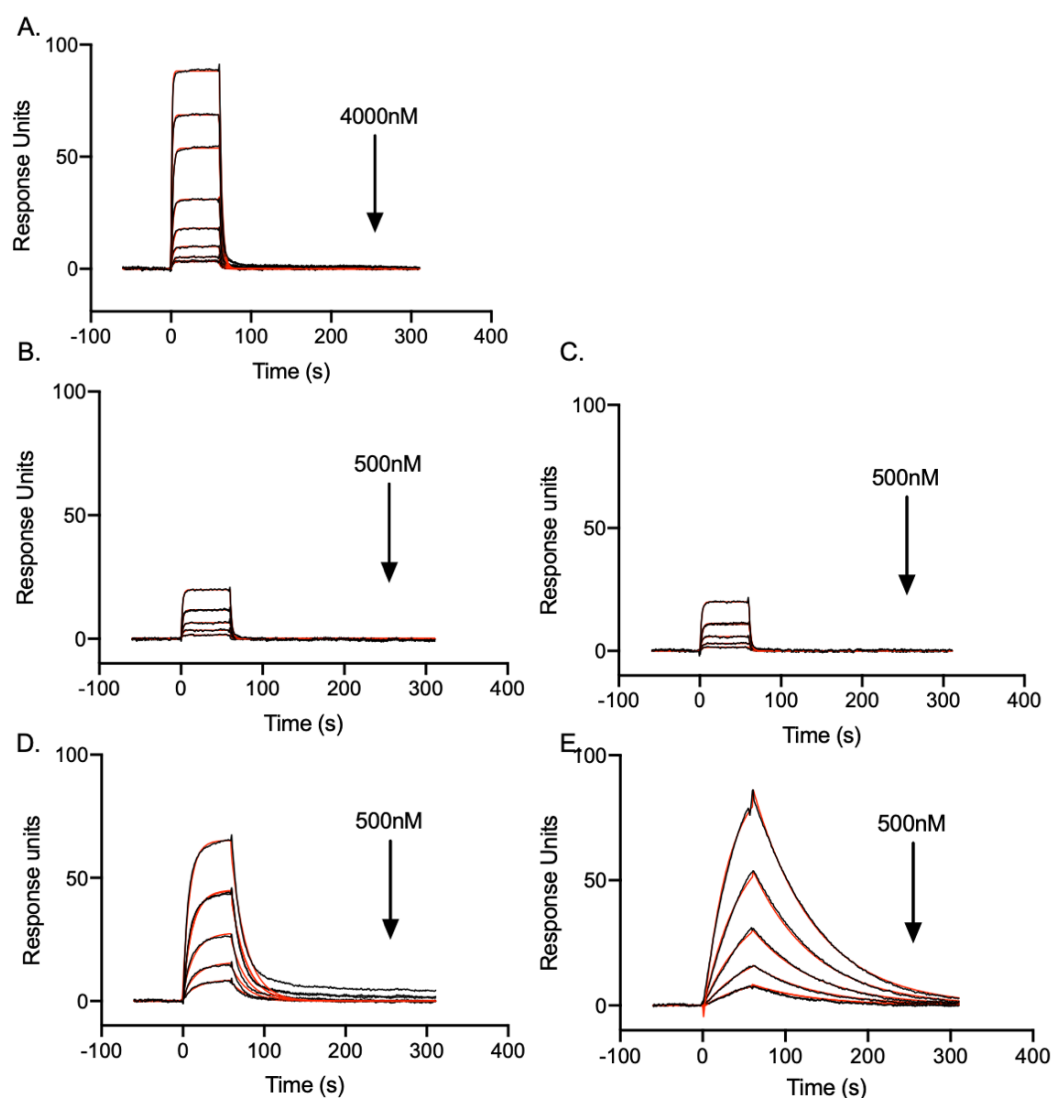


Figure 5.11 SPR analysis of Cy.004 binding to PfCyRPA alone or in complex with Fab. All curves start at concentration indicated and then are step-wise 2-fold dilutions. Black line indicates measured curve and red lines indicate the model fit. All assume 1:1 binding. A. B. Cy.004 binding to PfCyRPA alone, B is a truncated curve to enable direct comparison to the other panels. C. Cy.004 binding to PfCyRPA:Cy.002 Fab. D. Cy.004 binding to PfCyRPA:Cy.003 Fab. E. Cy.004 binding to PfCyRPA:Cy.007 Fab.

In contrast, when Cy.004 was immobilized and kinetics of Cy.003 Fab or Cy.007 Fab in complex with PfCyRPA were measured, there was a notable change in the shape of the sensorgrams, with a dramatic slowing of the dissociation. The dissociation was slowed enough to bring it within the measurement limits of the machine, which was approximately an order of magnitude improvement from 0.45s^{-1} of PfCyRPA alone to 0.059s^{-1} for PfCyRPA: Cy.003 Fab and 0.015s^{-1} for PfCyRPA: Cy.007 Fab (**Figures 5.11D,E and 5.13**). Similarly, there was an improvement of overall affinity from $1.3\mu\text{M}$ for PfCyRPA alone to approximately 400nM for PfCyRPA: Cy.003 Fab and PfCyRPA: Cy.007 Fab (**Figure 5.13**).

The same observation was made with Cy.009, where a dramatic slowing in the dissociation was observed when PfCyRPA was in complex with either synergistic Fab Cy.003 or Cy.007. Whereas no change was seen when PfCyRPA was in complex with non-synergistic Fab Cy.002 (**Figure 5.12**). From visual inspection of the graphs alone, it is clear there is a slowing in the dissociation for Cy.003 and Cy.007 PfCyRPA complexes, but not for Cy.002. Again, k_d had to be approximated for both PfCyRPA alone where it was found to be 0.1s^{-1} compared to 0.013s^{-1} for Cy.003 and 0.027s^{-1} for Cy.007 (**Figure 5.13**). Overall affinity decreased from 887nM for PfCyRPA to 257nM for PfCyRPA: Cy.003 Fab. Surprisingly, no increase in overall affinity for PfCyRPA: Cy.007 Fab was observed due to a simultaneous slowing of the rate of association, which may point to very slight competition for binding between these two Fabs.

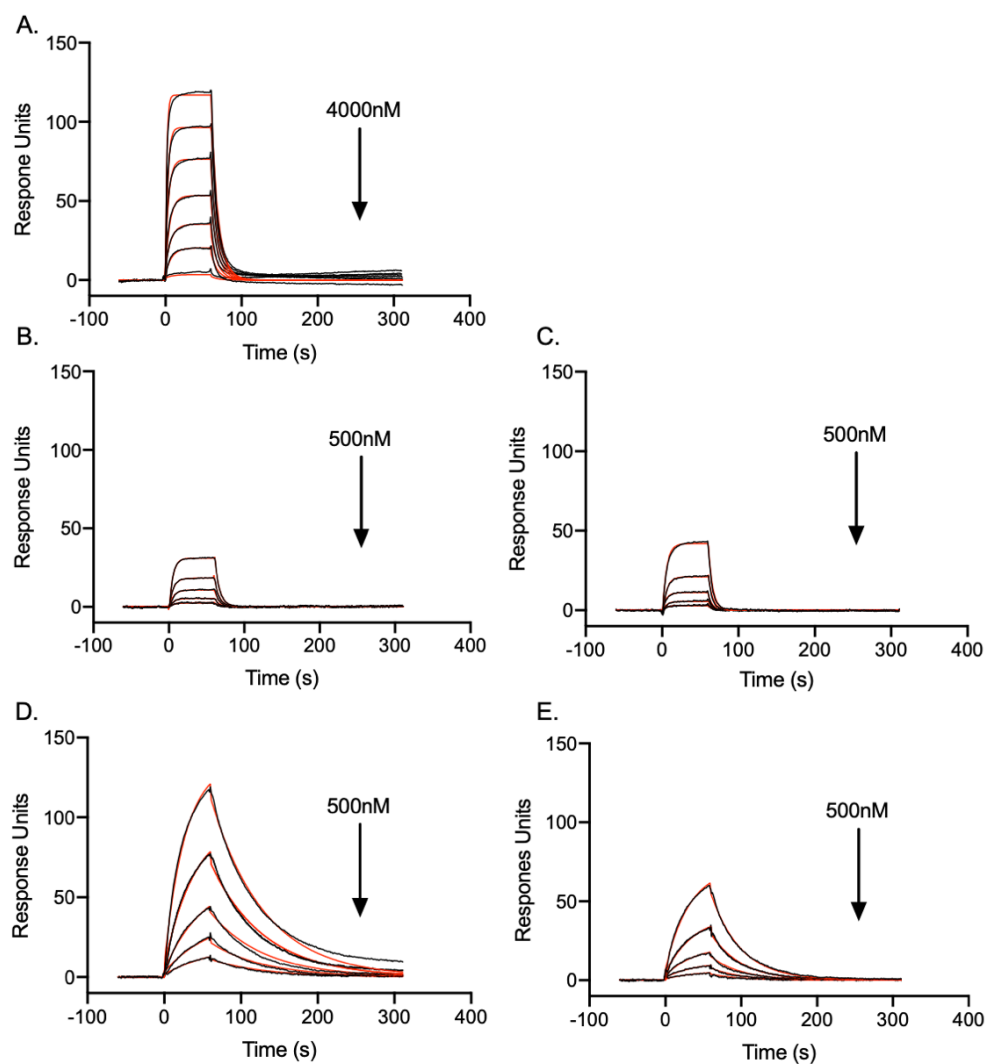


Figure 5.12 SPR analysis of Cy.009 binding to PfCyRPA alone or in complex with Fab. All curves start at concentration indicated and then are step-wise 2-fold dilutions. Black line indicates measured curve and red lines indicate the model fit. All assume 1:1 binding. A. B. Cy.009 binding to PfCyRPA alone, B is a truncated curve to enable direct comparison to the other panels. C. Cy.009 binding to PfCyRPA: Cy.002 Fab. D. Cy.009 binding to PfCyRPA: Cy.003 Fab. E. Cy.009 binding to PfCyRPA: Cy.007 Fab.

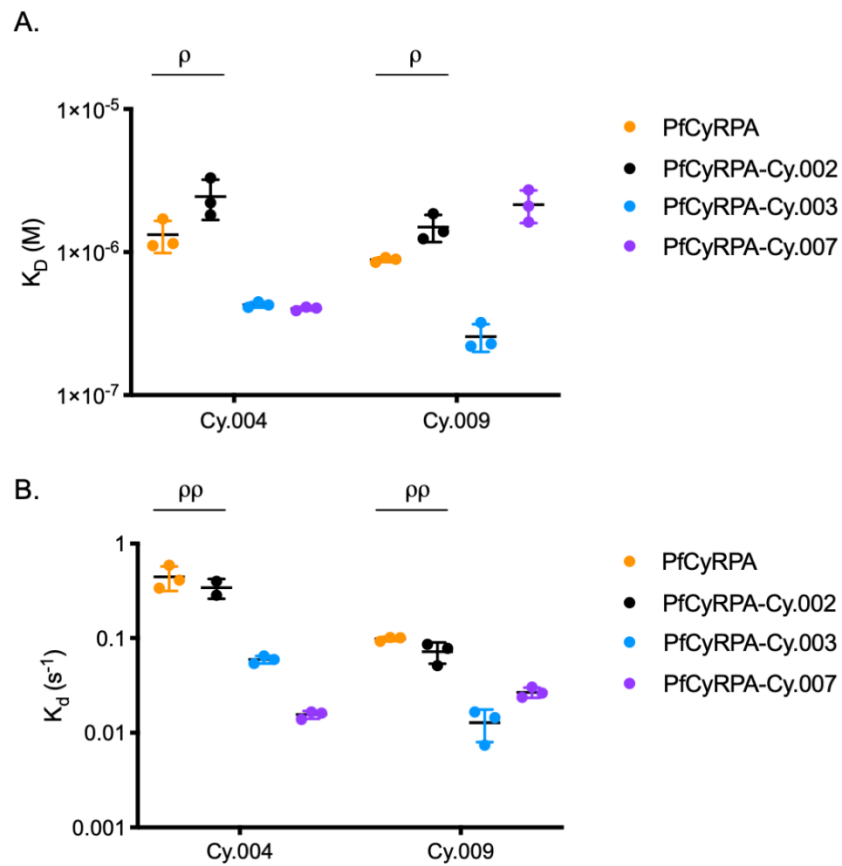


Figure 5.13 Summary of kinetic changes for PfCyRPA binding compared to PfCyRPA in complex with Fab. A. Overall affinity for Cy.004/Cy.009 binding to PfCyRPA, PfCyRPA: Cy.002, PfCyRPA: Cy.003 and PfCyRPA: Cy.007. B. Dissociation for Cy.004/Cy.009 binding to PfCyRPA, PfCyRPA: Cy.002, PfCyRPA: Cy.003 and PfCyRPA: Cy.007. Horizontal line indicates the mean, error bars show the standard deviation $n = 3$. ρ indicates values calculated from steady-state affinity, instead of k_a and k_d . $\rho\rho$ indicates values that are at or above the limits of the instrument.

When these combinations were run in reverse, with Cy.003 or Cy.007 immobilized on the CM5 chip, with Cy.004 and Cy.009 Fab in complex with PfCyRPA, no slowing of dissociation was observed (**Figure 5.14, Figure 5.15**). In the case of Cy.003 immobilized with PfCyRPA in complex with Cy.004 Fab, two distinct phases of dissociation were visible. During phase 1, the dissociation is driven by Cy.004 Fab rapidly dissociating from PfCyRPA, while during phase two the response is dominated by PfCyRPA dissociating from immobilized Cy.003 (**Figure 5.14D**). These two phases are very clear for Cy.003 binding to PfCyRPA: Cy.004 Fab because there is still a substantial discrepancy between the dissociation constants of Cy.003 for PfCyRPA (0.002s^{-1}) compared to 0.059s^{-1} for Cy.004 for PfCyRPA: Cy.003. These two phases are not clearly visible in the other sensorgrams as k_d for the immobilized antibody to the PfCyRPA: Fab complex is more similar to the affinity of the Fab to PfCyRPA (**Figure 5.14**). However, it remains important to note that in all cases, there are two dissociation events occurring simultaneously, which will affect the accuracy of the measurements – dissociation of mAb from PfCyRPA: Fab complex and dissociation of Fab from PfCyRPA. This is not visible when the lower affinity mAb is immobilized as the dissociation of mAb from PfCyRPA: Fab is much faster than the dissociation of Fab from PfCyRPA.

Unfortunately, the changes in kinetics for Cy.007 binding to Cy.003: PfCyRPA compared to PfCyRPA alone, or Cy.003 binding to Cy.007: PfCyRPA compared to PfCyRPA alone could not be assessed as the two mAbs do not bind simultaneously in SPR, as described in the SPR competition assays in Chapter 3. This appears to be artefactual, since they do both bind in ELISA, are not expected to compete based on their crystal structures, and synergize in GIA assays (which should not occur for two mAbs in competition with each other). As a result, Cy.003/ Cy.007 synergy cannot, at this point, be attributed to changes in binding kinetics as these have not been directly measured.

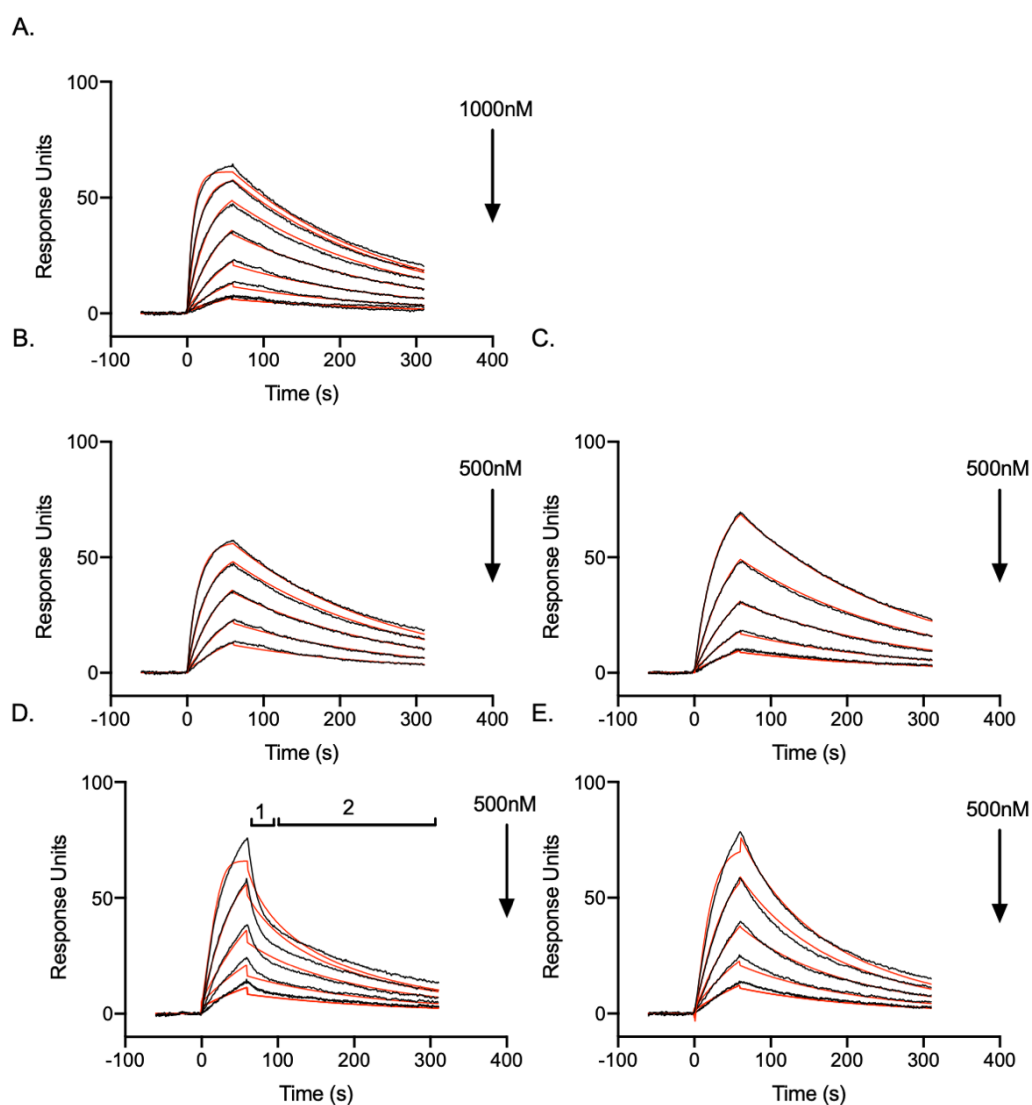


Figure 5.14 SPR analysis of Cy.003 binding to PfCyRPA alone or in complex with Fab. All curves start at concentration indicated and then are step-wise 2-fold dilutions. Black line indicates measured curve and red lines indicate the model fit. All assume 1:1 binding. A. B. Cy.003 binding to PfCyRPA alone, B is a truncated curve to enable direct comparison to the other panels. C. Cy.003 binding to PfCyRPA:Cy.002 Fab. D. Cy.003 binding to PfCyRPA:Cy.004 Fab Phase 1 and 2 are labelled. During phase 1, the curve is dominated by Cy.004 dissociation from PfCyRPA and during phase 2, the curve is dominated by PfCyRPA dissociation from Cy.003. E. Cy.003 binding to PfCyRPA:Cy.009 Fab.

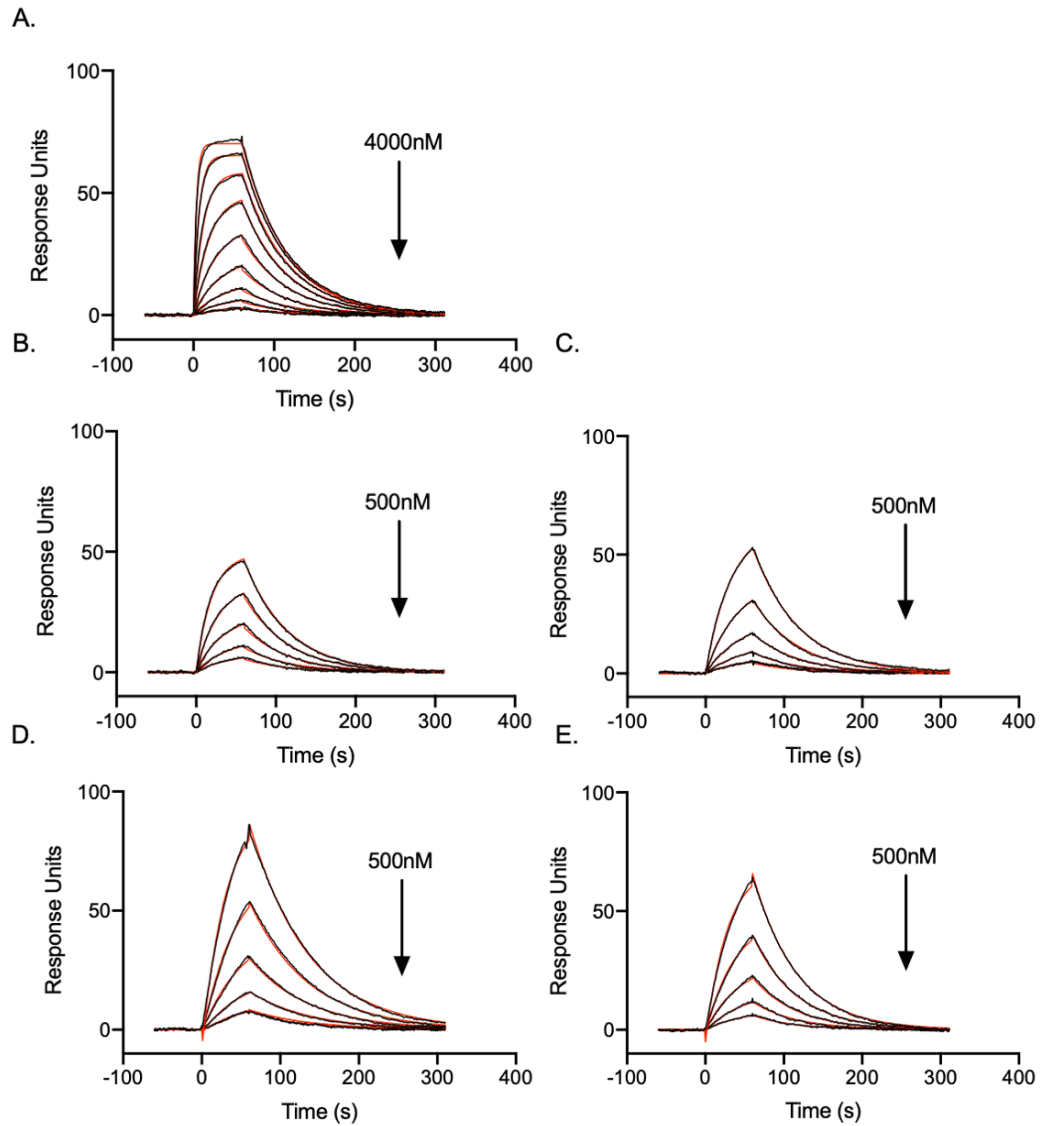


Figure 5.15 Cy.007 binding to PfCyRPA alone or in complex with Fab. All curves start at concentration indicated and then are step-wise 2-fold dilutions. Black line indicates measured curve and red lines indicate the model fit. All assume 1:1 binding. A. B. Cy.007 binding to PfCyRPA alone, B is a truncated curve to enable direct comparison to the other panels. C. Cy.007 binding to PfCyRPA: Cy.002 Fab. D. Cy.007 binding to PfCyRPA: Cy.004 Fab. E. Cy.007 binding to PfCyRPA: Cy.009 Fab.

The summary of these kinetic analyses is that it appears that the mAbs with superior binding kinetics (Cy.003 and Cy.007) are able to alter the kinetics of the inferior mAbs Cy.004 and Cy.009. However, the reverse is not true; whereby Cy.004 and Cy.009 do not improve the binding kinetics of Cy.003 and Cy.007. With respect to the **addendum in Chapter 5**, which shows that Ca^{2+} does have a mediating role in Cy.004 and Cy.009 binding to PfCyRPA, these experiments will need to be repeated with the addition of Ca^{2+} in the SPR buffer. It seems unlikely that the observed changes in kinetics described here relate to the role of Ca^{2+} , possibly being introduced during complex preparation. If that were the case, one would expect to see the same changes for all Fabs, including Cy.002, since all Fabs were prepared in the same way. Instead, the kinetics only change for Fabs that are non-competing and show synergy in GIA assays. Still, it will be necessary to repeat these experiments with Ca^{2+} present to show that these changes are not solely the result of a low affinity Cy.004/Cy.009: PfCyRPA interaction.

5.2.4 Heterotypic lateral antibody interactions

One possible mechanism is that Cy.003/Cy.007 could cause a conformational change in PfCyRPA. Having solved the crystal structures for these antibodies in complex with PfCyRPA, this question could be easily answered via superimposition of the CyRPA molecules from the different structures. No such conformational change was observed (**Figure 5.16**).

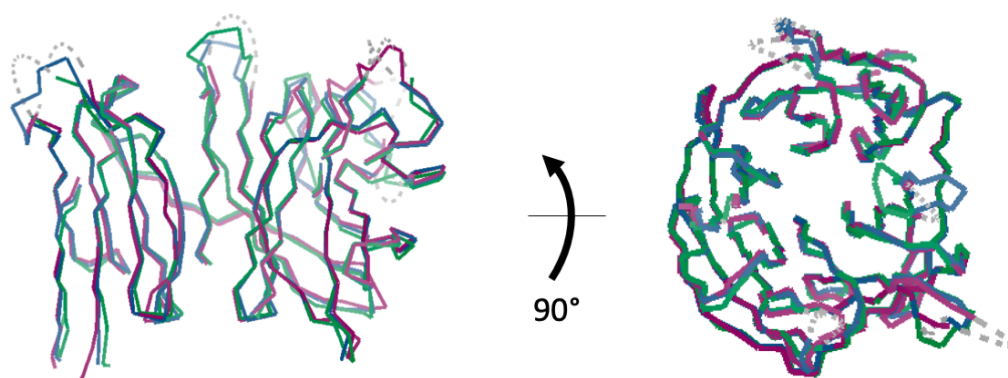


Figure 5.16 Superimposed PfCyRPA from Cy.003, Cy.004 and Cy.007 structures. PfCyRPA from Cy.003 is in blue, Cy.004 in purple and Cy.007 in green.

However, when the structures were superimposed, it became clear that Cy.003 and Cy.007 both bind close to Cy.004/Cy.009 and that there is the possibility of interactions occurring laterally between the Fabs (**Figure 5.17**). If this was the case, then it is conceivable that there could be lateral interactions between the different Fabs, allowing the higher affinity Fab to stabilize the binding of the lower affinity Fab. Due to the relative positions of the three Fabs on PfCyRPA, it is possible that there would be lateral contacts between all three Fabs. This would then provide a unifying model for synergy that explains why all three combinations show synergy.

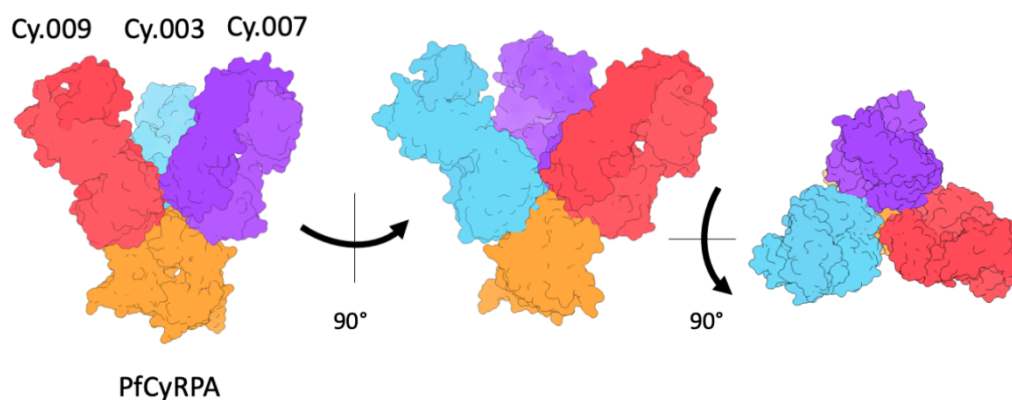


Figure 5.17 Superimposition of Cy.003, Cy.007 and Cy.009 bound to PfCyRPA.

Through superimposition it also became clear that Cy.007 has a more extensive interacting face with Cy.004 than Cy.003. This is also consistent with the more dramatic improvement in binding kinetics for Cy.004 when PfCyRPA is in complex with Cy.007 Fab. In order to determine if these lateral interactions are indeed occurring, the triple Fab complexes with PfCyRPA and Cy.003, Cy.007 and Cy.004/Cy.009²⁵⁴ were crystallized with the goal of determining their crystal structure (**Figure 5.18**).

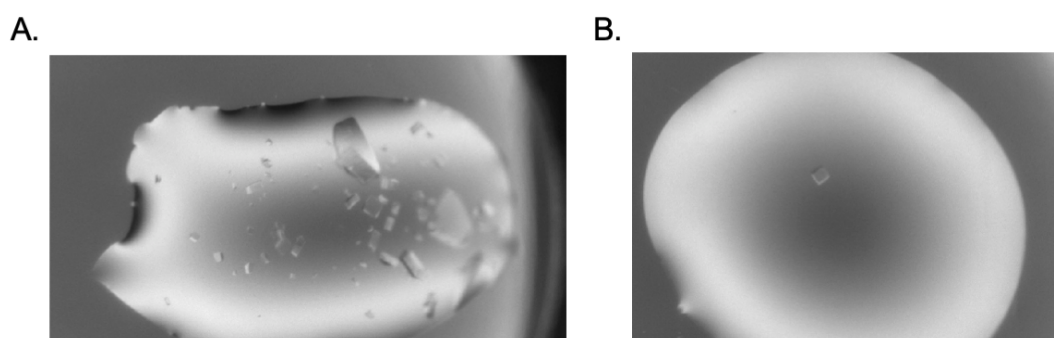


Figure 5.18 Crystals of triple Fab PfCyRPA complex. A. Cy.004: Cy.003: Cy.007: PfCyRPA which diffracted to 4.11Å
B. Cy.009: Cy.003: Cy.007: PfCyRPA, which diffracted to 4.5Å.

The crystallographic studies of these higher order complexes are still in the early stages, but have been included here to show that this line of inquiry that is still being pursued.

The best two collected datasets for the triple fab structures had resolutions of 4.11Å for PfCyRPA:Cy.003:Cy.007:Cy.004 and 4.5Å for PfCyRPA:Cy.003:Cy.007:Cy.009. Further optimization of these crystals using the silver bullets additive screen was successful and resulted in the collection of a dataset to 3.10Å. Indexing proved difficult due to suspected twinning, but a molecular replacement was successful and revealed four copies in the asymmetric unit in space group P 43 2 2.

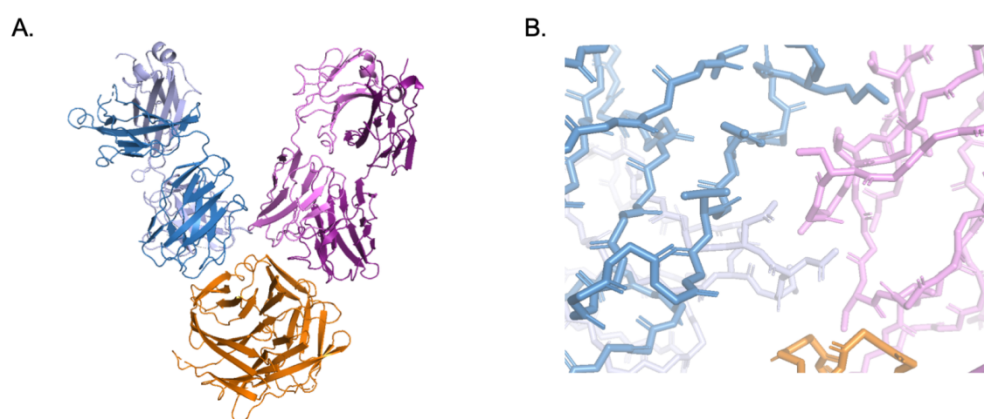


Figure 5.19 Unrefined molecular replacement solution (one copy only) of Cy.003 and Cy.007 bound to PfCyRPA.

A. Cy.007 (blue) and Cy.003 (purple) bound to PfCyRPA. B. Preliminary interface between Cy.003 and Cy.007 prior to refinement.

Unfortunately, molecular replacement revealed that it was not the triple Fab structure bound to PfCyRPA, but rather only Cy.003 and Cy.007. However, preliminarily, there does appear to be a contact interface between the two Fabs, consistent with what one would expect for stabilizing lateral interactions, as predicted by superimposition (**Figure 5.19**). The next steps will be to further refine this solution to identify the specific stabilizing interactions between the two Fabs.

One noteworthy gap in the data presented is that the kinetic changes for Cy.003 for PfCyRPA vs Cy.007:PfCyRPA (or, alternatively, Cy.007 for PfCyRPA and Cy.003:PfCyRPA) could not be assessed directly through SPR, as was done for Cy.004 and Cy.009 with Cy.003 and Cy.007 (see above). Nevertheless, here I have successfully co-crystallized the two Fabs bound to PfCyRPA, indicating that they do bind simultaneously. However, using SPR kinetic analysis to detect a change in binding affinity due to stabilizing lateral interactions cannot be done. An alternative approach to measure changes in kinetics, such as isothermal calorimetry will be necessary to complement the structure of Cy.003 and Cy.007 to conclusively show that these lateral interactions do indeed drive changes in affinity and thus drive synergy.

5.3 Discussion

5.3.1 Measuring synergy

In this Chapter, an analysis of additivity/synergy/antagonism between different anti-PfCyRPA mAbs was carried out. It was found that non-competing inhibitory anti-PfCyRPA mAbs were able to potentially synergize with each other to enhance growth inhibition activity.

As explored in the introduction to this Chapter, there is no consensus regarding the appropriate way to define synergy. In this Chapter, Bliss independence was used to define 'additivity' above and below which was synergy and antagonism, respectively. What is intriguing about the synergy observed here is that the epitopes of all of the inhibitory anti-PfCyRPA mAbs are localized to the same region of the PfCyRPA propeller, but they do not compete. Given the similarity in their binding positions, it would seem reasonable to assume that these mAbs would share a similar mechanism of action and thus should behave additively. Instead, potent synergy was observed between all inhibitory mAbs that did not compete with each other.

Though Bliss independence appears to be a reasonable model to measure enhanced activity when mAbs are delivered in combination, it does not necessarily provide therapeutically relevant information in the context of mAbs to prevent infectious disease. This is because there may be synergy between a potentially inhibitory mAb and a weakly inhibitory mAbs relative to the predicted Bliss additivity, but this may still be less inhibitory than the more potent mAb delivered alone at the same total concentration as the combination.

Here, 1:1 mixtures of synergistic combinations were compared to the inhibition of the single mAbs held at the same total concentration as the 1:1 mixture. That is to say, in the 1:1 mixture if there was 500µg/mL of each Cy.003 and Cy.009, this was compared to 1000µg/mL of either Cy.003 or Cy.009 alone. If the combination outperforms the individual agents then this is 'therapeutically relevant' synergy. This idea is similar to the highest single agent model of synergy; however, by that definition, 500µg/mL of each Cy.003 and Cy.009 would only have to outperform 500µg/mL of either Cy.003 or Cy.009²⁴².

Let us assume that the goal of a recombinant antibody therapy is to prevent an infectious disease for as long as possible, that all antibodies have an equal half-life, and that there is a ceiling to how much total recombinant antibody can be safely administered. The question becomes what combination of mAbs prevents infection at the lowest concentration, as this is equivalent to the mAbs that will continue to prevent infection for the longest length of time, given the concentration in the blood would decrease with time. Once the concentration drops below a given threshold, an individual would be susceptible again.

The highest single agent model of synergy posits that the two drugs synergize if their combined effect is greater than the effects of either drug on its own²⁴². This is perhaps a useful definition if the two drugs in question are fully distinct, with different mechanisms of action and importantly, different toxicity profiles. This is because the presence of one drug does not preclude the use of the other. However, for an antibody cocktail with multiple mAbs targeting the same pathway, there is no reason to think that using a cocktail of antibodies would change the ceiling on how much antibody can be safely administered. What matters is the total concentration of antibody. Thus, it does not matter whether 1000µg/mL of two antibodies outperforms each antibody in the combination individually at their respective concentrations

in the combination. The question is whether the combination outperforms the same total concentration of a single agent.

This approach to thinking about synergy is likely more helpful in the context of passively transferred antibody prophylaxis for infectious disease. This was the logic behind exploring 1:1 mixtures of antibodies and comparing them to an equivalent total concentration of each antibody in the combination. It is clear that select combinations of anti-PfCyRPA antibodies do indeed outperform the more potent clone alone, which would then justify their use in an antibody cocktail.

There are of course other non-potency reasons an antibody cocktail may be desirable over a single agent. Perhaps the most important of these would be the development of resistance to a single mAb via mutations in the epitope that would prevent mAb binding, particularly for mutation prone pathogens²⁵⁵. This may be one reason why a combination of anti-PfRH5 and anti-PfCyRPA mAbs may be more desirable than a single mAb or cocktail of mAbs targeting only one of these antigens.

5.3.2 Heterotypic lateral antibody interactions

The mechanism underlying the observed synergy was hypothesized to be rooted in the relative binding position of the inhibitory mAbs to one-another. The proposed mechanism is that the affinity for Cy.004 and Cy.009 for PfCyRPA is enhanced through lateral heterotypic antibody-antibody interactions with Cy.003 and Cy.007. The evidence presented here to support this mechanism is that Cy.004 and Cy.009 have slowed dissociation from PfCyRPA when Cy.007 or Cy.003 are also present. This effect cannot be explained by a conformational change in PfCyRPA. Moreover, preliminary dual-Fab crystal structures show that the relative positions of the bound Fabs are such that inter-Fab contacts are possible. If the specific lateral

stabilizing side chains be determined, the interacting residues could then be mutated to alanine, or another biochemically dissimilar amino acid from the original, to ablate the stabilizing interactions. Once ablated, it would be expected that synergy would be lost thereby demonstrating the biological significance of this mechanism.

One caveat to this model is that these experiments were completed prior to the realization that Cy.004/Cy.009 binding may be cation dependent, based on the unexplained density in the crystal structure described in **Chapter 4**. As a result, these kinetic experiments would need to be repeated in the presence of whatever cation is determined to lie at the Fab:PfCyRPA interface. This may have important kinetic implications for Cy.004/Cy.009 binding, and the changes in kinetics driven by suspected lateral interactions will need to be reassessed in this light.

Fab-to-Fab contacts have been described previously whereby head-to-head interactions create a solvent excluded interface between two copies of the same Fab^{256–258}. Most notably, this has been seen in the context of the malaria sporozoite-stage antigen CSP, whereby mAb 1210 binds on opposite sides of a 5-time NANP repeat (NANP₅)²⁵⁶. In that case, it was due to the highly repetitive NANP region of CSP, which allowed multiple molecules of the same mAb to bind to a single copy of CSP from opposite directions. This permitted the formation of six homotypic hydrogen bonds which increased the affinity to 286nM compared to 556nM when these critical interacting residues were mutated out²⁵⁶. In this case, the authors show that these mutations are selected for during affinity maturation, but they did not have a functional impact on parasite inhibition *in vivo*. Thus, these are selected for on the basis of more potent B-cell activation during affinity maturation rather than because they are functionally superior. Indeed, all antibodies are selected for *in vivo* on the basis of affinity, rather than functional parameters.

In the case of heterotypic interactions, it is more difficult to imagine how these would be selected for *in vivo*. It would require that two different B cells interact with the same antigen presented on follicular dendritic cells, resulting in enhanced affinity for the lower affinity of the two antibodies. Since the affinity of the pair of antibodies for PfCyRPA does not exceed the affinity of the higher affinity mAb alone for PfCyRPA, there is no real selection advantage. Thus, it is unlikely that these interactions have been selected for *in vivo*, as was the case with the homotypic interactions of the anti-CSP mAb 1210.

A second example of homotypic binding interactions was observed when the structure of CD20 was solved in complex with the drug Rituximab, which induces complement-dependent cytotoxicity²⁵⁹. In this case, the homotypic interactions are shown to enhance complement mediated cytotoxicity, which is critical for the function of the drug²⁵⁹. The stabilizing head-to-head interactions between different molecules of Rituximab had important implications for the drug's potency. In this case, and similar to mAb 1210, these homotypic interactions are made possible because of internal symmetry that provides multiple epitopes on the same antigen.

There is one example of head-to-head mAb binding to a non-symmetrical antigen²⁶⁰. In this case, the antigen is clasped between two of the same mAb, each binding to a different part of the antigen using a different combination of CDR loops²⁶⁰. In this case, the dual binding greatly enhances affinity for the antigen of each of the bound mAbs.

Lateral interactions between Fabs with functional implications have not, to my knowledge, been described previously. In the aforementioned study of antibody binding enhancement, it has been speculated that Fcs between two mAbs could interact to stabilize binding, but these interactions do not occur between the Fab fragments²⁶¹. In the case of the

work here, it is possible that this phenomenon was only observed due to the low affinity of Cy.004 and Cy.009. If these antibodies were high affinity, with slower dissociation, then perhaps the impact of heterotypic interactions slowing dissociation would be less dramatic, and thus be more difficult to detect.

5.3.3 Future investigations of anti-PfCyRPA mAb synergy

To summarize, here I have established that there is potent synergy between pairs of non-competing, inhibitory anti-PfCyRPA mAbs. This synergy may be driven by changes in binding kinetics that can be seen when two mAbs bind simultaneously compared to single mAb:PfCyRPA binding events. I hypothesize that this occurs due to lateral stabilizing interactions between simultaneously binding Fabs, although these need to be confirmed through crystallographic data do directly observe these interactions and/or mutagenesis studies to show that the loss of these stabilizing interactions reverses the kinetic changes and the GIA assay synergy.

The observation that slowing the dissociation of Cy.004 and Cy.009 may result in dramatically improved inhibition suggests that the upper limit of anti-PfCyRPA mAb inhibition has not been reached with Cy.004 and Cy.009. This justifies further attempts to isolate similar mAbs with improved binding kinetics as they may show similar potency as the Cy.004/Cy.009 Cy.003/Cy.007 combinations. In the context of passive transfer therapeutics, it would, of course, be preferable to have a single mAb as potent as Cy.004/Cy.009 and Cy.003/Cy.007 combinations, as manufacturing a single entity is more desirable compared to an antibody cocktail. However, at present, even in combination, anti-PfCyRPA mAbs are marginally less effective in GIA than the best anti-PfRH5 mAbs. Importantly, combinations of anti-PfCyRPA and anti-PfRH5 mAbs similarly demonstrate strong synergy, which outperforms the

combinations of anti-PfCyRPA or combinations of anti-PfRH5 antibodies²⁰¹. Thus, if an antibody cocktail was being manufactured, it would be more sensible to use a combination of anti-PfCyRPA and anti-PfRH5 antibodies due to the enhanced potency, in which case one would still want to use the most potent anti-PfCyRPA mAb possible.

Chapter 6: Conclusions and Future Directions

6.1 Key Findings

Through the work presented in this Thesis, several novel observations have been made that contribute to the body of literature on both antibody function and malaria vaccine design. The summary of the key findings of this Thesis are:

1. Monoclonal antibodies that target PfCyRPA are capable of potent neutralization of *P. falciparum* *in vitro* (IC₅₀ approximately 250µg/ml). Prior to this study, only weakly to moderately neutralizing antibodies had been described targeting PfCyRPA^{66,72}.
2. Vaccine-induced antibodies targeting PfCyRPA must bind epitopes that are accessible in the assembled RCR complex in order to neutralize *P. falciparum* parasite invasion in the GIA assay. This is in contrast to prior claims that suggested 8A7 functioned by blocking RCR complex assembly^{23,69}.
3. The hypothesized function of the RCR complex as a haemolytic protein complex²³ was not supported by the evidence generated here through haemolysis assays.
4. Structural studies indicate that neutralizing antibodies all bind to the same face of PfCyRPA, primarily centred on blade two of the propeller.
5. Preliminary structural evidence suggests that the potentially neutralizing antibody Cy.004 engages in Ca²⁺ mediated binding to PfCyRPA, a highly unusual antibody binding modality.
6. Non-competing neutralizing antibodies that target PfCyRPA are capable of potent synergy that enhances the blockade of *P. falciparum* invasion.

7. The simultaneous binding of two adjacent anti-PfCyRPA antibodies slows the dissociation of the lower affinity mAb, through what are believed to be lateral stabilizing interactions between antibodies. This mechanism may underlie the synergy observed between pairs of anti-PfCyRPA antibodies.

6.2 *Understanding the function of the RCR complex*

Understanding the function of the RCR complex is a significant gap in our knowledge of the mechanisms of *P. falciparum* invasion into erythrocytes. The purported role of the complex (i.e. insertion into the erythrocyte membrane to induce a calcium spike in the erythrocyte), remains, in my opinion, largely conjecture based on limited evidence¹⁴. Indeed, in an attempt to replicate some of the findings supporting this model, I was unable to do so. That is not to say the model is entirely wrong; however, I believe that the evidence supporting membrane insertion is weak and by extension that the role of the RCR complex in invasion remains largely unknown.

Without understanding the function of the complex, it is difficult to test possible mechanisms through which inhibitory anti-PfCyRPA mAbs function. Presumably, neutralizing mAbs prevent PfCyRPA from carrying out some essential invasion process, but in order to confirm this, the role of PfCyRPA in invasion must be better defined. One shortcoming of this Thesis is that it was unable to provide any further insight into the function of the RCR complex. As mentioned, the conservation of PfCyRPA across multiple species of *Plasmodium* that infect humans, but the lack of conservation of PfRH5, likely points to an important ancestral role/function of PfCyRPA⁸³. It is possible that this ancestral role could be retained in *P. falciparum* as well.

The binding of PfRH5 to basigin is the only established role for the RCR complex. However, this does not appear to be only for attachment of the merozoite to the erythrocyte, as the RCR complex is secreted. Thus, it may attach to basigin, but without an additional binding partner tethering it to the merozoite membrane, it is unclear what this binding event achieves. Recently, more evidence was published supporting the claim that P113 binds to the N-terminus of PfRH5. The report does not directly observe this interaction through structural studies, but it is detected through SPR and HDX and the authors show that antibodies targeting the PfRH5 N-terminus block P113 binding⁸¹. However, these antibodies are non-inhibitory which would point to either an inaccessible epitope or that this interaction is not critical for parasite invasion⁸¹. This evidence supports the finding that P113 does indeed tether PfRH5⁷⁹, consistent with a more classic receptor ligand interaction where both components are associated with their respective cell membranes. Still, all is not resolved, as if the only role of the PfRH5-basigin interaction is attachment to the erythrocyte, then there would be no need for the larger RCR complex since PfRH5 binds basigin and P113 without PfCyRPA or PfRipr.

Other insights into the function of PfCyRPA come from the study of plasmepsins, a class of aspartic proteases found in *P. falciparum*. Plasmepsin X (PMX) was recently shown to cleave both PfRH5 and PfRipr, the former towards its N-terminus and the latter near the middle of the protein²⁵³. Notably, PfCyRPA was not processed by PMX²⁵³. Separately, it was also found that the inhibition of plasmepsin IX (PMIX) results in the blockade of PfCyRPA secretion, but has no impact on PfRH5 or PfRipr²⁶². Given there is no known processing event undergone by PfCyRPA and none identified by the authors, it seems improbable that PMIX would cleave PfCyRPA directly. Perhaps instead, PfCyRPA secretion is dependent on a

different PMIX target, possibly PfRipr which may also be cleaved by PMIX. However, these new data still provide relatively little insight into the functional role of PfCyRPA.

One potential reason why blades 1-3 may be potent antibody targets is that PfCyRPA interacts with an additional binding partner. Although the present model of invasion suggests these blades are not actively involved in mediating known interactions, it is possible that they mediate an (as yet unknown) protein-protein interaction that occurs downstream of secretion of PfCyRPA and thus antibody binding to those blades prevents this process from advancing.

In order to investigate this possibility further, a few approaches were considered. First the use of BioID, a promiscuous biotinylating enzyme, linked to non-inhibitory anti-RCR mAbs was considered. Since the radius of BioID is relatively large (10nM)^{263,264}, a non-inhibitory mAb that binds a distal region of the RCR complex not involved in the invasion process (such as R5.011) could be used during invasion to detect new interacting proteins. Unfortunately, in attempting this experiment, it became clear that the method generated far too many biotinylated hits (>50) to be useful.

As a result, an approach that generates fewer false positives is required. One possible option is the use of cross-linking mass spectrometry, which enables unambiguous identification of protein-protein interactions. Using this method, whole cells (in this case merozoites) can be cross linked using a cleavable mass spectrometry compatible cross linker (such as disuccinimidyl sulfoxide (DSSO)²⁶⁵). After cross-linking, merozoite lysates can then be prepared, reduced and proteolytically digested before mass spectrometry. DSSO crosslinks primary amines (lysines of the protein N-terminus) that are in close proximity to each other (10-12.5Å). The challenge here is that in some cases, the interface between interacting proteins may not have any appropriate DSSO cross-linking sites. As well, high protein

coverage is a prerequisite. Unfortunately, none of PfRH5, PfCyRPA or PfRipr are highly abundant (based on preliminary experiments) and thus this may prevent identification of other interacting partners, should they exist. Based on the literature, some possibilities of other binding partners of the RCR complex include SEMA7A on the host RBC binding to PfRipr²¹³, or *P. falciparum* CSS and PTRAMP binding to PfRipr. In the case of CSS and PTRAMP, no direct evidence of this interaction has, to my knowledge, been published. This is speculation based on PfRipr binding to CSS and PTRAMP in *P. knowlesi*⁸³. These interactions may not be conserved across species, as is the case for the PfCyRPA:PfRipr interaction.

6.3 *Developing an effective malaria vaccine*

Today, the most significant challenge in malaria vaccine development remains inducing and maintaining functional antibodies at a sufficiently high concentration to block parasite invasion (in either the sporozoite- or blood-stage of infection) to protect against disease. Certainly, in the blood-stage of infection, the preponderance of evidence indicates that sufficiently high neutralizing antibody titres would protect against clinical disease.

There are many areas of vaccine design that can be improved to try and achieve this goal. This includes development of new and more potent adjuvants, optimizing the dosing schedule to increase the concentration of sustained antibody responses, and improving antigen selection or immunogen design. This Thesis focused on the latter. I have taken an established malaria vaccine target, PfCyRPA, and attempted to understand the features of neutralizing antibodies, so that rational vaccine immunogen design can now be undertaken.

However, even the most potent anti-PfCyRPA mAbs are less potent than the best PfRH5 mAbs. Consequently, if one were to design a single antigen vaccine, PfRH5 would still appear to be a more promising vaccine candidate; although, the inhibition mediated by

specific pairs of PfRH5 and PfCyRPA antibodies can surpass the best single anti-PfRH5 antibodies^{201,266}. Thus, a multi-antigen vaccine that focuses the immune response on these critical synergistic epitopes may reduce the levels of antibody required for protection. Such a vaccine could benefit from immune focusing strategies, as discussed in **Chapter 4**, whereby glycan masking or epitope grafting could be pursued to focus the immune response on only blades two and three of PfCyRPA.

Fortunately, the critical epitope region on PfCyRPA that generates antibody clones that display synergy when combined with anti-PfRH5 mAbs is the one that Cy.004/Cy.009 bind²⁰¹. This epitope region is not only capable of eliciting potent anti-PfCyRPA antibodies on its own, but these antibodies also synergize with those targeting PfRH5²⁰¹. Thus, the structure-function data generated here that will enable rational immunogen design for PfCyRPA should also inform the design of immunogens that can induce synergistic mAbs.

To overcome the parasite's highly evolved defences, a truly efficacious malaria vaccine will have to be highly optimized, potentially incorporating multiple antigens, demanding the use of a vaccine platform capable of inducing a potent immune response, and requiring multiple doses (in the case of RTS,S, four doses are required). Herein lies the paradox of malaria vaccine development: all of these requirements increase the cost, supply chain complexity and delivery challenges of vaccinating those most at risk of severe malaria, i.e. individuals who often live in rural areas with limited access to health care and often little ability to pay. As a result, the features that are necessary to make a hypothetical malaria vaccine highly effective may be the very same features that make it inaccessible to those who need it most. These are questions that regulators, health authorities, funding bodies and the pharmaceutical industry are undoubtedly grappling with. I mention this only to ground this discussion in the very real practical limitations of malaria vaccine development. I would be

remiss not to acknowledge that the strategies described here to create a more advanced, and indeed more effective, malaria vaccine could inadvertently reduce access, if these concerns are inadequately addressed.

6.4 *Antibody cocktails and synergy*

Unlike the synergy between different pairs of anti-PfCyRPA and anti-PfRH5 mAbs, whereby mAbs targeting different antigens show synergistic relationships, the focus of this Thesis work was to understand synergy between mAbs which target the same antigen. To that end, I proffer a new model of antibody synergy where one antibody is capable of physically stabilizing another via lateral interactions between adjacent Fab arms.

This finding helps to illustrate the diverse mechanisms through which synergy can occur. Interestingly, a preprint (non-peer reviewed literature) posted to BioRxiv (at time of writing) described a similar type of interaction where mAbs target a repetitive epitope²⁶⁷. In this case, two of the same mAb dimerized through contacts in the V_H domain and the loss of these contacts reduced biological activity²⁶⁷. This class of antibody was shown to be induced naturally through vaccination with a simian-human immunodeficiency virus (SHIV) glycopeptide²⁶⁷. This shows that this phenomenon of lateral Fab contacts may be more generalizable. In the HIV example, it is the same mAb dimerizing across a repetitive epitope whereas in the example presented here, it is two different mAbs binding immediately adjacent epitopes.

This suggests that the findings here could be applicable to antibody responses against other diseases. Whether it is possible to rationally design vaccine immunogens to specifically elicit these types of mAbs remains unclear. It would be challenging since the exact angle and orientation of the antibody becomes extremely important if one wishes to conserve these

lateral interactions, something that cannot currently be controlled by immunogen design or vaccination protocol. Rather, current immunogen design strategies elicit antibodies that bind to the immunogen at any angle or orientation.

However, these interactions may be more applicable for the development of antibody cocktails to provide passive immunity, whereby multiple mAbs are routinely combined for an enhanced biological effect. To my knowledge, the potential advantages of having two mAbs binding immediately adjacent epitopes are not currently recognized in antibody cocktail design, and this may be one future application of this finding. Before this is undertaken, more work is needed to confirm that these interactions do lead to enhanced biological effects, to understand what the critical stabilizing residues are and whether this is unique to certain antibody classes that have shared biochemical properties in these lateral interacting regions. It is also unclear whether the benefits of these putative stabilizing interactions would be relevant for higher affinity antibodies. It may be an artefact of the exceedingly low affinities of Cy.004 and Cy.009 (in the absence of Ca^{2+}). These low affinity antibodies may provide the appropriate circumstances for mAbs to benefit from stabilizing lateral interactions. In that case, an antibody cocktail may benefit more from developing higher affinity mAbs than by engineering these lateral interactions. I imagine that there are diminishing marginal returns on mAb affinity, whereby improving a mAb's affinity from 10^{-6} M to 10^{-7} M provides a greater enhancement of biological activity than one from 10^{-10} M to 10^{-11} M. It will be interesting to determine whether the hypothesized cation binding of Cy.004 and Cy.009 at the PfCyRPA interface enhances affinity for PfCyRPA and if it does, how this impacts these synergistic relationships.

With that in mind, immediate clinical utility was never the goal of these investigations. Rather, finalizing this model of antibody cooperativity through lateral Fab

interactions provides valuable scientific insight into the molecular mechanisms of antibody synergy.

6.5 *Final Remarks*

Reducing global mortality and morbidity due to malaria remains today, as it has for many thousands of years, a public health priority. Vaccination, for all of its challenges, is one of the most effective strategies to combat infectious disease. Indeed, a highly effective and durable malaria vaccine would radically change the malaria control landscape and would, for the first time, make malaria eradication in high burden settings an attainable goal. The biological and scientific barriers to achieving this are formidable, as outlined at length in this Thesis work. But, hopefully, some of the insights generated in this Thesis can contribute to the dismantling of these barriers and to make incremental progress towards a malaria-free world.

References

1. Carter, R. & Mendis, K. N. Evolutionary and historical aspects of the burden of malaria. *Clin. Microbiol. Rev.* **15**, 564–594 (2002).
2. WHO. World Malaria Report 2019. (2019).
3. WHO. *The potential impact of health service disruptions on the burden of malaria: a modelling analysis for countries in sub-Saharan Africa.* (2020).
4. Crompton, P. D. *et al.* Malaria immunity in man and mosquito: insights into unsolved mysteries of a deadly infectious disease. *Annu. Rev. Immunol.* **32**, 157–187 (2014).
5. Grau, G. E. *et al.* Tumor Necrosis Factor and Disease Severity in Children with Falciparum Malaria. *N. Engl. J. Med.* **320**, 1586–1591 (1989).
6. Rogerson, S. J., Hviid, L., Duffy, P. E., Leke, R. F. G. & Taylor, D. W. Malaria in pregnancy: pathogenesis and immunity. *Lancet Infect. Dis.* **7**, 105–117 (2007).
7. Maier, A. G., Matuschewski, K., Zhang, M. & Rug, M. Plasmodium falciparum. *Trends Parasitol.* **35**, 481–482 (2019).
8. Garcia, J. E., Puentes, A. & Patarroyo, M. E. Developmental biology of sporozoite-host interactions in Plasmodium falciparum malaria: implications for vaccine design. *Clin. Microbiol. Rev.* **19**, 686–707 (2006).
9. Kumar, H. & Tolia, N. H. Getting in: The structural biology of malaria invasion. *PLoS Pathog.* **15**, e1007943 (2019).

10. Silvie, O. *et al.* Hepatocyte CD81 is required for *Plasmodium falciparum* and *Plasmodium yoelii* sporozoite infectivity. *Nat. Med.* **9**, 93–96 (2003).
11. Manzoni, G. *et al.* *Plasmodium* P36 determines host cell receptor usage during sporozoite invasion. *Elife* **6**, e25903 (2017).
12. Josling, G. A., Williamson, K. C. & Llinás, M. Regulation of Sexual Commitment and Gametocytogenesis in Malaria Parasites. *Annu. Rev. Microbiol.* **72**, 501–519 (2018).
13. Aly, A. S. I., Vaughan, A. M. & Kappe, S. H. I. Malaria parasite development in the mosquito and infection of the mammalian host. *Annu. Rev. Microbiol.* **63**, 195–221 (2009).
14. Ragotte, R. J., Higgins, M. K. & Draper, S. J. The RH5-CyRPA-Ripr Complex as a Malaria Vaccine Target. *Trends Parasitol.* **36**, 545–559 (2020).
15. Gilson, P. R. & Crabb, B. S. Morphology and kinetics of the three distinct phases of red blood cell invasion by *Plasmodium falciparum* merozoites. *Int J Parasitol* **39**, 91–96 (2009).
16. Das, S. *et al.* Processing of *Plasmodium falciparum* Merozoite Surface Protein MSP1 Activates a Spectrin-Binding Function Enabling Parasite Egress from RBCs. *Cell Host Microbe* **18**, 433–444 (2015).
17. Cowman, A. F., Tonkin, C. J., Tham, W. H. & Duraisingh, M. T. The Molecular Basis of Erythrocyte Invasion by Malaria Parasites. *Cell Host Microbe* **22**, 232–245 (2017).
18. Lopaticki, S. *et al.* Reticulocyte and Erythrocyte Binding-Like Proteins Function Cooperatively in Invasion of Human Erythrocytes by Malaria Parasites. *Infect. Immun.*

- 79**, 1107–1117 (2011).
19. Wright, K. E. *et al.* Structure of malaria invasion protein RH5 with erythrocyte basigin and blocking antibodies. *Nature* **515**, 427–430 (2014).
 20. Crosnier, C. *et al.* Basigin is a receptor essential for erythrocyte invasion by *Plasmodium falciparum*. *Nature* **480**, 534–537 (2011).
 21. Volz, J. C. *et al.* Essential Role of the PfRh5/PfRipr/CyRPA Complex during *Plasmodium falciparum* Invasion of Erythrocytes. *Cell Host Microbe* **20**, 60–71 (2016).
 22. Weiss, G. E. *et al.* Revealing the sequence and resulting cellular morphology of receptor-ligand interactions during *Plasmodium falciparum* invasion of erythrocytes. *PLoS Pathog* **11**, e1004670 (2015).
 23. Wong, W. *et al.* Structure of *Plasmodium falciparum* Rh5–CyRPA–Ripr invasion complex. *Nature* **565**, 118–121 (2019).
 24. Aniweh, Y. *et al.* *P. falciparum* RH5-Basigin interaction induces changes in the cytoskeleton of the host RBC. *Cell Microbiol* **19**, e12747 (2017).
 25. Alexander, D. L., Mital, J., Ward, G. E., Bradley, P. & Boothroyd, J. C. Identification of the moving junction complex of *Toxoplasma gondii*: a collaboration between distinct secretory organelles. *PLoS Pathog* **1**, e17 (2005).
 26. Srinivasan, P. *et al.* Binding of *Plasmodium* merozoite proteins RON2 and AMA1 triggers commitment to invasion. *Proc Natl Acad Sci U S A* **108**, 13275–13280 (2011).
 27. Keeley, A. & Soldati, D. The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. *Trends Cell Biol* **14**, 528–532 (2004).

28. Junqueira, C. *et al.* Cytotoxic CD8+ T cells recognize and kill *Plasmodium vivax*-infected reticulocytes. *Nat. Med.* **24**, 1330–1336 (2018).
29. Wahlgren, M., Goel, S. & Akhouri, R. R. Variant surface antigens of *Plasmodium falciparum* and their roles in severe malaria. *Nat. Rev. Microbiol.* **15**, 479–491 (2017).
30. Saito, F. *et al.* Immune evasion of *Plasmodium falciparum* by RIFIN via inhibitory receptors. *Nature* **552**, 101–105 (2017).
31. Harrison, T. E. *et al.* Structural basis for RIFIN-mediated activation of LILRB1 in malaria. *Nature* (2020) doi:10.1038/s41586-020-2530-3.
32. Barnwell, J. W. *et al.* A human 88-kD membrane glycoprotein (CD36) functions in vitro as a receptor for a cytoadherence ligand on *Plasmodium falciparum*-infected erythrocytes. *J. Clin. Invest.* **84**, 765–772 (1989).
33. Turner, L. *et al.* Severe malaria is associated with parasite binding to endothelial protein C receptor. *Nature* **498**, 502–505 (2013).
34. Berendt, A. R., Simmons, D. L., Tansey, J., Newbold, C. I. & Marsh, K. Intercellular adhesion molecule-1 is an endothelial cell adhesion receptor for *Plasmodium falciparum*. *Nature* **341**, 57–59 (1989).
35. Dondorp, A. M. *et al.* Direct In Vivo Assessment of Microcirculatory Dysfunction in Severe *Falciparum* Malaria. *J. Infect. Dis.* **197**, 79–84 (2008).
36. Rowe, J. A., Moulds, J. M., Newbold, C. I. & Miller, L. H. P. *falciparum* rosetting mediated by a parasite-variant erythrocyte membrane protein and complement-receptor 1. *Nature* **388**, 292–295 (1997).

37. McQuaid, F. & Rowe, J. A. Rosetting revisited: a critical look at the evidence for host erythrocyte receptors in *Plasmodium falciparum* rosetting. *Parasitology* **147**, 1–11 (2020).
38. Kaul, D. K., Roth, E. F., Nagel, R. L., Howard, R. J. & Handunnetti, S. M. Rosetting of *Plasmodium falciparum*-infected red blood cells with uninfected red blood cells enhances microvascular obstruction under flow conditions. *Blood* **78**, 812–819 (1991).
39. Ghumra, A. *et al.* Identification of Residues in the C μ 4 Domain of Polymeric IgM Essential for Interaction with *Plasmodium falciparum* Erythrocyte Membrane Protein 1 (PfEMP1). *J. Immunol.* **181**, 1988 LP – 2000 (2008).
40. Rowe, J. A., Shafi, J., Kai, O. K., Marsh, K. & Raza, A. Nonimmune IgM, but not IgG binds to the surface of *Plasmodium falciparum*-infected erythrocytes and correlates with rosetting and severe malaria. *Am. J. Trop. Med. Hyg.* **66**, 692–699 (2002).
41. Barfod, L. *et al.* Evasion of immunity to *Plasmodium falciparum* malaria by IgM masking of protective IgG epitopes in infected erythrocyte surface-exposed PfEMP1. *Proc. Natl. Acad. Sci.* **108**, 12485–12490 (2011).
42. Alanine, D. G. W. *et al.* Human Antibodies that Slow Erythrocyte Invasion Potentiate Malaria-Neutralizing Antibodies. *Cell* doi:10.1016/j.cell.2019.05.025.
43. Draper, S. J. *et al.* Malaria Vaccines: Recent Advances and New Horizons. *Cell Host Microbe* **24**, 43–56 (2018).
44. Cohen, S., McGregor, I. A. & Carrington, S. Gamma-Globulin and Acquired Immunity

- to Human Malaria. *Nature* **192**, 733 (1961).
45. Pinkevych, M. *et al.* The Dynamics of Naturally Acquired Immunity to *Plasmodium falciparum* Infection. *PLoS Comput. Biol.* **8**, e1002729 (2012).
 46. Doolan, D. L., Dobano, C. & Baird, J. K. Acquired immunity to malaria. *Clin Microbiol Rev* **22**, 13–36, Table of Contents (2009).
 47. Good, M. F., Stanisic, D., Xu, H., Elliott, S. & Wykes, M. The immunological challenge to developing a vaccine to the blood stages of malaria parasites. *Immunol. Rev.* **201**, 254–267 (2004).
 48. Portugal, S., Pierce, S. K. & Crompton, P. D. Young lives lost as B cells falter: what we are learning about antibody responses in malaria. *J. Immunol.* **190**, 3039–3046 (2013).
 49. Walther, M. *et al.* Distinct roles for FOXP3 and FOXP3 CD4 T cells in regulating cellular immunity to uncomplicated and severe *Plasmodium falciparum* malaria. *PLoS Pathog.* **5**, e1000364–e1000364 (2009).
 50. Portugal, S. *et al.* Exposure-Dependent Control of Malaria-Induced Inflammation in Children. *PLoS Pathog.* **10**, e1004079 (2014).
 51. Rodriguez-Barraquer, I. *et al.* Quantification of anti-parasite and anti-disease immunity to malaria as a function of age and exposure. *Elife* **7**, e35832 (2018).
 52. Jensen, A. T. R. *et al.* *Plasmodium falciparum* associated with severe childhood malaria preferentially expresses PfEMP1 encoded by group A var genes. *J. Exp. Med.* **199**, 1179–1190 (2004).

53. Hviid, L. The immuno-epidemiology of pregnancy-associated *Plasmodium falciparum* malaria: a variant surface antigen-specific perspective. *Parasite Immunol.* **26**, 477–486 (2004).
54. Boyle, M. J. *et al.* Human antibodies fix complement to inhibit *Plasmodium falciparum* invasion of erythrocytes and are associated with protection against malaria. *Immunity* **42**, 580–590 (2015).
55. Bouharoun-Tayoun, H., Oeuvray, C., Lunel, F. & Druilhe, P. Mechanisms underlying the monocyte-mediated antibody-dependent killing of *Plasmodium falciparum* asexual blood stages. *J. Exp. Med.* **182**, 409–418 (1995).
56. Arora, G. *et al.* NK cells inhibit *Plasmodium falciparum* growth in red blood cells via antibody-dependent cellular cytotoxicity. *Elife* **7**, e36806 (2018).
57. Crompton, P. D. *et al.* In vitro growth-inhibitory activity and malaria risk in a cohort study in Mali. *Infect. Immun.* **78**, 737–745 (2010).
58. Dent, A. E. *et al.* Antibody-mediated growth inhibition of *Plasmodium falciparum*: relationship to age and protection from parasitemia in Kenyan children and adults. *PLoS One* **3**, e3557–e3557 (2008).
59. Reddy, K. S. *et al.* Multiprotein complex between the GPI-anchored CyRPA with PfRH5 and PfRipr is crucial for *Plasmodium falciparum* erythrocyte invasion. *Proc Natl Acad Sci U S A* **112**, 1179–1184 (2015).
60. Malkin, E. M. *et al.* Phase 1 clinical trial of apical membrane antigen 1: an asexual blood-stage vaccine for *Plasmodium falciparum* malaria. *Infect. Immun.* **73**, 3677–

- 3685 (2005).
61. Stephens, R. *et al.* Malaria-specific transgenic CD4⁺ T cells protect immunodeficient mice from lethal infection and demonstrate requirement for a protective threshold of antibody production for parasite clearance. *Blood* **106**, 1676–1684 (2005).
 62. Douglas, A. D. *et al.* A PfRH5-based vaccine is efficacious against heterologous strain blood-stage *Plasmodium falciparum* infection in aotus monkeys. *Cell Host Microbe* **17**, 130–139 (2015).
 63. Mahdi Abdel Hamid, M. *et al.* Vaccination with *Plasmodium knowlesi* AMA1 formulated in the novel adjuvant co-vaccine HTTM protects against blood-stage challenge in rhesus macaques. *PLoS One* **6**, e20547–e20547 (2011).
 64. Douglas AD Jin J, Miura K, Diouf A, Zenonos ZA, Ventocilla JA, Silk SE, Marshall JM, Alanine DGW, Wang C, Edwards NJ, Leiva KP, Gomez-Puerta LA, Lucas CM, Wright GJ, Long CA, Royal JM, Draper SJ., B. G. C. A defined mechanistic correlate of protection against *Plasmodium falciparum* malaria in non-human primates. *Nat Commun* **In press**, (2019).
 65. Pearson, R. D., Amato, R. & Kwiatkowski, D. P. An open dataset of *Plasmodium falciparum* genome variation in 7,000 worldwide samples. *bioRxiv* 824730 (2019) doi:10.1101/824730.
 66. Favuzza, P. *et al.* Structure of the malaria vaccine candidate antigen CyRPA and its complex with a parasite invasion inhibitory antibody. *Elife* **6**, (2017).
 67. Douglas, A. D. *et al.* Neutralization of *Plasmodium falciparum* merozoites by

- antibodies against PfRH5. *J Immunol* **192**, 245–258 (2014).
68. Alanine, D. G. W. *et al.* Human Antibodies that Slow Erythrocyte Invasion Potentiate Malaria-Neutralizing Antibodies. *Cell* **178**, 216–228.e21 (2019).
 69. Healer, J. *et al.* Neutralizing antibodies block the function of Rh5/Ripr/CyRPA complex during invasion of *Plasmodium falciparum* into human erythrocytes. *Cell. Microbiol.* **21**, e13030 (2019).
 70. Dreyer, A. M. *et al.* Passive immunoprotection of *Plasmodium falciparum*-infected mice designates the CyRPA as candidate malaria vaccine antigen. *J Immunol* **188**, 6225–6237 (2012).
 71. Favuzza, P. *et al.* Generation of *Plasmodium falciparum* parasite-inhibitory antibodies by immunization with recombinantly-expressed CyRPA. *Malar J* **15**, 161 (2016).
 72. Chen, L. *et al.* Structural basis for inhibition of erythrocyte invasion by antibodies to *Plasmodium falciparum* protein CyRPA. *Elife* **6**, (2017).
 73. Rodriguez, M., Lustigman, S., Montero, E., Oksov, Y. & Lobo, C. A. PfRH5: a novel reticulocyte-binding family homolog of *plasmodium falciparum* that binds to the erythrocyte, and an investigation of its receptor. *PLoS One* **3**, e3300–e3300 (2008).
 74. Hayton, K. *et al.* Erythrocyte binding protein PfRH5 polymorphisms determine species-specific pathways of *Plasmodium falciparum* invasion. *Cell Host Microbe* **4**, 40–51 (2008).
 75. Chen, L. *et al.* An EGF-like protein forms a complex with PfRh5 and is required for invasion of human erythrocytes by *Plasmodium falciparum*. *PLoS Pathog* **7**, e1002199

- (2011).
76. Baum, J. *et al.* Reticulocyte-binding protein homologue 5 - an essential adhesin involved in invasion of human erythrocytes by *Plasmodium falciparum*. *Int J Parasitol* **39**, 371–380 (2009).
 77. Chen, L. *et al.* Crystal structure of PfRh5, an essential *P. falciparum* ligand for invasion of human erythrocytes. *Elife* **3**, (2014).
 78. Gruszczyk, J. *et al.* Structurally conserved erythrocyte-binding domain in *Plasmodium* provides a versatile scaffold for alternate receptor engagement. *Proc Natl Acad Sci U S A* **113**, E191-200 (2016).
 79. Galaway, F. *et al.* P113 is a merozoite surface protein that binds the N terminus of *Plasmodium falciparum* RH5. *Nat Commun* **8**, 14333 (2017).
 80. Elsworth, B. *et al.* Proteomic analysis reveals novel proteins associated with the *Plasmodium* protein exporter PTEX and a loss of complex stability upon truncation of the core PTEX component, PTEX150. *Cell Microbiol* **18**, 1551–1569 (2016).
 81. Campeotto, I. *et al.* The Structure of the Cysteine-Rich Domain of *Plasmodium falciparum* P113 Identifies the Location of the RH5 Binding Site. *MBio* **11**, (2020).
 82. Galaway, F., Yu, R., Constantinou, A., Prugnolle, F. & Wright, G. J. Resurrection of the ancestral RH5 invasion ligand provides a molecular explanation for the origin of *P. falciparum* malaria in humans. *PLoS Biol.* **17**, e3000490–e3000490 (2019).
 83. Knuepfer, E. *et al.* Divergent roles for the RH5 complex components, CyRPA and RIPP in human-infective malaria parasites. *PLoS Pathog.* **15**, e1007809 (2019).

84. Sundararaman, S. A. *et al.* Genomes of cryptic chimpanzee *Plasmodium* species reveal key evolutionary events leading to human malaria. *Nat Commun* **7**, 11078 (2016).
85. Otto, T. D. *et al.* Genomes of all known members of a *Plasmodium* subgenus reveal paths to virulent human malaria. *Nat. Microbiol.* **3**, 687–697 (2018).
86. Wanaguru, M., Liu, W., Hahn, B. H., Rayner, J. C. & Wright, G. J. RH5-Basigin interaction plays a major role in the host tropism of *Plasmodium falciparum*. *Proc Natl Acad Sci U S A* **110**, 20735–20740 (2013).
87. Hayton, K. *et al.* Various PfRH5 polymorphisms can support *Plasmodium falciparum* invasion into the erythrocytes of owl monkeys and rats. *Mol Biochem Parasitol* **187**, 103–110 (2013).
88. Arevalo-Pinzon, G. *et al.* A single amino acid change in the *Plasmodium falciparum* RH5 (PfRH5) human RBC binding sequence modifies its structure and determines species-specific binding activity. *Vaccine* **30**, 637–646 (2012).
89. Plenderleith, L. J. *et al.* Adaptive Evolution of RH5 in Ape *Plasmodium* species of the *Laverania* Subgenus. *MBio* **9**, (2018).
90. Douglas, A. D. *et al.* The blood-stage malaria antigen PfRH5 is susceptible to vaccine-inducible cross-strain neutralizing antibody. *Nat. Commun.* **2**, 601 (2011).
91. Bustamante, L. Y. *et al.* A full-length recombinant *Plasmodium falciparum* PfRH5 protein induces inhibitory antibodies that are effective across common PfRH5 genetic variants. *Vaccine* **31**, 373–379 (2013).

92. Reddy, K. S. *et al.* Bacterially expressed full-length recombinant *Plasmodium falciparum* RH5 protein binds erythrocytes and elicits potent strain-transcending parasite-neutralizing antibodies. *Infect. Immun.* **82**, 152–164 (2014).
93. Payne, R. O. *et al.* Human vaccination against RH5 induces neutralizing antimalarial antibodies that inhibit RH5 invasion complex interactions. *JCI Insight* **2**, (2017).
94. Tran, T. M. *et al.* Naturally acquired antibodies specific for *Plasmodium falciparum* reticulocyte-binding protein homologue 5 inhibit parasite growth and predict protection from malaria. *J Infect Dis* **209**, 789–798 (2014).
95. Chiu, C. Y. *et al.* Association of antibodies to *Plasmodium falciparum* reticulocyte binding protein homolog 5 with protection from clinical malaria. *Front Microbiol* **5**, 314 (2014).
96. Richards, J. S. *et al.* Identification and prioritization of merozoite antigens as targets of protective human immunity to *Plasmodium falciparum* malaria for vaccine and biomarker development. *J Immunol* **191**, 795–809 (2013).
97. Partey, F. D. *et al.* Kinetics of antibody responses to PfRH5-complex antigens in Ghanaian children with *Plasmodium falciparum* malaria. *PLoS One* **13**, e0198371 (2018).
98. Osier, F. H. *et al.* New antigens for a multicomponent blood-stage malaria vaccine. *Sci Transl Med* **6**, 247ra102 (2014).
99. Patel, S. D. *et al.* *Plasmodium falciparum* merozoite surface antigen, PfRH5, elicits detectable levels of invasion-inhibiting antibodies in humans. *J Infect Dis* **208**, 1679–

- 1687 (2013).
100. Julien, J.-P. & Wardemann, H. Antibodies against *Plasmodium falciparum* malaria at the molecular level. *Nat. Rev. Immunol.* **19**, 761–775 (2019).
 101. Hu, G. *et al.* Transcriptional profiling of growth perturbations of the human malaria parasite *Plasmodium falciparum*. *Nat Biotechnol* **28**, 91–98 (2010).
 102. Illingworth, J. J. *et al.* Functional Comparison of Blood-Stage *Plasmodium falciparum* Malaria Vaccine Candidate Antigens. *Front. Immunol.* **10**, 1254 (2019).
 103. Azasi, Y. *et al.* Bliss’ and Loewe’s additive and synergistic effects in *Plasmodium falciparum* growth inhibition by AMA1-RON2L, RH5, RPR and CyRPA antibody combinations. *Sci. Rep.* **10**, 11802 (2020).
 104. Tamborrini, M. *et al.* Vaccination with virosomally formulated recombinant CyRPA elicits protective antibodies against *Plasmodium falciparum* parasites in preclinical in vitro and in vivo models. *NPJ vaccines* **5**, 9 (2020).
 105. Ntege, E. H. *et al.* Identification of *Plasmodium falciparum* reticulocyte binding protein homologue 5-interacting protein, PfRipr, as a highly conserved blood-stage malaria vaccine candidate. *Vaccine* **34**, 5612–5622 (2016).
 106. Healer, J. *et al.* Neutralising antibodies block the function of Rh5/Ripr/CyRPA complex during invasion of *Plasmodium falciparum* into human erythrocytes. *Cell. Microbiol.* **21**, e13030 (2019).
 107. Bustamante, L. Y. *et al.* Synergistic malaria vaccine combinations identified by systematic antigen screening. *Proc Natl Acad Sci U S A* **114**, 12045–12050 (2017).

108. Prakash, P. *et al.* Human Cyclophilin B forms part of a multi-protein complex during erythrocyte invasion by *Plasmodium falciparum*. *Nat Commun* **8**, 1548 (2017).
109. Sherling, E. S. *et al.* The *Plasmodium falciparum* rhoptry protein RhopH3 plays essential roles in host cell invasion and nutrient uptake. *Elife* **6**, (2017).
110. Satchwell, T. J. *et al.* Genetic manipulation of cell line derived reticulocytes enables dissection of host malaria invasion requirements. *Nat. Commun.* **10**, 3806 (2019).
111. Zhang, M.-Y. *et al.* Disrupting CD147-RAP2 interaction abrogates erythrocyte invasion by *Plasmodium falciparum*. *Blood* **131**, 1111–1121 (2018).
112. Griffin, J. T. *et al.* Reducing *Plasmodium falciparum* malaria transmission in Africa: a model-based evaluation of intervention strategies. *PLoS Med* **7**, (2010).
113. Ranson, H. *et al.* Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol* **27**, 91–98 (2011).
114. Ashley, E. A. *et al.* Spread of Artemisinin Resistance in *Plasmodium falciparum* Malaria. *N Engl J Med* **371**, 411–423 (2014).
115. Casares, S., Brumeanu, T.-D. & Richie, T. L. The RTS,S malaria vaccine. *Vaccine* **28**, 4880–4894 (2010).
116. Potocnjak, P., Yoshida, N., Nussenzweig, R. S. & Nussenzweig, V. Monovalent fragments (Fab) of monoclonal antibodies to a sporozoite surface antigen (Pb44) protect mice against malarial infection. *J. Exp. Med.* **151**, 1504–1513 (1980).
117. Nussenzweig, V. & Nussenzweig, R. S. Circumsporozoite proteins of malaria parasites. *Cell* **42**, 401–403 (1985).

118. Vanderberg, J., Nussenzweig, R. & Most, H. Protective Immunity Produced by the Injection of X-Irradiated Sporozoites of *Plasmodium berghei*: V. In vitro Effects of Immune Serum on Sporozoites. *Mil. Med.* **134**, 1183–1190 (1969).
119. Suscovich, T. J. *et al.* Mapping functional humoral correlates of protection against malaria challenge following RTS,S/AS01 vaccination. *Sci. Transl. Med.* **12**, eabb4757 (2020).
120. Scally, S. W. *et al.* Rare PfCSP C-terminal antibodies induced by live sporozoite vaccination are ineffective against malaria infection. *J. Exp. Med.* **215**, 63–75 (2018).
121. Tan, J. *et al.* A public antibody lineage that potently inhibits malaria infection through dual binding to the circumsporozoite protein. *Nat. Med.* **24**, 401–407 (2018).
122. Kisalu, N. K. *et al.* A human monoclonal antibody prevents malaria infection by targeting a new site of vulnerability on the parasite. *Nat. Med.* **24**, 408–416 (2018).
123. Murugan, R. *et al.* Evolution of protective human antibodies against *Plasmodium falciparum* circumsporozoite protein repeat motifs. *Nat. Med.* (2020) doi:10.1038/s41591-020-0881-9.
124. Collins, K. A., Snaith, R., Cottingham, M. G., Gilbert, S. C. & Hill, A. V. S. Enhancing protective immunity to malaria with a highly immunogenic virus-like particle vaccine. *Sci. Rep.* **7**, 46621 (2017).
125. Seder, R. A. *et al.* Protection Against Malaria by Intravenous Immunization with a Nonreplicating Sporozoite Vaccine. *Science* **341**, 1359 LP – 1365 (2013).
126. Lyke, K. E. *et al.* Attenuated PfSPZ Vaccine induces strain-transcending T cells and

- durable protection against heterologous controlled human malaria infection. *Proc. Natl. Acad. Sci.* **114**, 2711–2716 (2017).
127. Olotu, A. *et al.* Advancing Global Health through Development and Clinical Trials Partnerships: A Randomized, Placebo-Controlled, Double-Blind Assessment of Safety, Tolerability, and Immunogenicity of PfSPZ Vaccine for Malaria in Healthy Equatoguinean Men. *Am. J. Trop. Med. Hyg.* **98**, 308–318 (2018).
 128. Roestenberg, M. *et al.* Long-term protection against malaria after experimental sporozoite inoculation: an open-label follow-up study. *Lancet* **377**, 1770–1776 (2011).
 129. Walk, J. *et al.* Modest heterologous protection after *Plasmodium falciparum* sporozoite immunization: a double-blind randomized controlled clinical trial. *BMC Med.* **15**, 168 (2017).
 130. Ewer, K. J. *et al.* Protective CD8⁺ T-cell immunity to human malaria induced by chimpanzee adenovirus-MVA immunisation. *Nat. Commun.* **4**, 2836 (2013).
 131. Ogwang, C. *et al.* Prime-boost vaccination with chimpanzee adenovirus and modified vaccinia Ankara encoding TRAP provides partial protection against *Plasmodium falciparum* infection in Kenyan adults. *Sci. Transl. Med.* **7**, 286re5-286re5 (2015).
 132. Mensah, V. A. *et al.* Safety, Immunogenicity and Efficacy of Prime-Boost Vaccination with ChAd63 and MVA Encoding ME-TRAP against *Plasmodium falciparum* Infection in Adults in Senegal. *PLoS One* **11**, e0167951–e0167951 (2016).
 133. Halbroth, B. R. *et al.* Preclinical Development and Assessment of Viral Vectors

- Expressing a Fusion Antigen of Plasmodium falciparum LSA1 and LSAP2 for Efficacy against Liver-Stage Malaria. *Infect. Immun.* **88**, e00573-19 (2020).
134. Sheehy, S. H. *et al.* ChAd63-MVA-vectored blood-stage malaria vaccines targeting MSP1 and AMA1: assessment of efficacy against mosquito bite challenge in humans. *Mol Ther* **20**, 2355–2368 (2012).
 135. Ellis, R. D. *et al.* Phase 1 Trial of the Plasmodium falciparum Blood Stage Vaccine MSP142-C1/Alhydrogel with and without CPG 7909 in Malaria Naïve Adults. *PLoS One* **5**, e8787 (2010).
 136. Malkin, E. *et al.* A Phase 1 trial of PfCP2.9: An AMA1/MSP1 chimeric recombinant protein vaccine for Plasmodium falciparum malaria. *Vaccine* **26**, 6864–6873 (2008).
 137. Lyon, J. A. *et al.* Protection induced by Plasmodium falciparum MSP1(42) is strain-specific, antigen and adjuvant dependent, and correlates with antibody responses. *PLoS One* **3**, e2830 (2008).
 138. Thera, M. A. *et al.* A Field Trial to Assess a Blood-Stage Malaria Vaccine. *N. Engl. J. Med.* **365**, 1004–1013 (2011).
 139. Dutta, S. *et al.* Overcoming antigenic diversity by enhancing the immunogenicity of conserved epitopes on the malaria vaccine candidate apical membrane antigen-1. *PLoS Pathog.* **9**, e1003840–e1003840 (2013).
 140. Miura, K. *et al.* Overcoming allelic specificity by immunization with five allelic forms of Plasmodium falciparum apical membrane antigen 1. *Infect. Immun.* **81**, 1491–1501 (2013).

141. Srinivasan, P. *et al.* Immunization with a functional protein complex required for erythrocyte invasion protects against lethal malaria. *Proc Natl Acad Sci U S A* **111**, 10311–10316 (2014).
142. Srinivasan, P. *et al.* A malaria vaccine protects Aotus monkeys against virulent *Plasmodium falciparum* infection. *NPJ Vaccines* **2**, (2017).
143. Jin, J. *et al.* Accelerating the clinical development of protein-based vaccines for malaria by efficient purification using a four amino acid C-terminal ‘C-tag’. *Int J Parasitol* **47**, 435–446 (2017).
144. Jin, J. *et al.* Production, quality control, stability, and potency of cGMP-produced *Plasmodium falciparum* RH5.1 protein vaccine expressed in *Drosophila* S2 cells. *NPJ Vaccines* **3**, 32 (2018).
145. Campeotto, I. *et al.* One-step design of a stable variant of the malaria invasion protein RH5 for use as a vaccine immunogen. *Proc Natl Acad Sci U S A* **114**, 998–1002 (2017).
146. Bliss, C. M. *et al.* Viral Vector Malaria Vaccines Induce High-Level T Cell and Antibody Responses in West African Children and Infants. *Mol. Ther.* **25**, 547–559 (2017).
147. Regules, J. A. *et al.* Fractional Third and Fourth Dose of RTS,S/AS01 Malaria Candidate Vaccine: A Phase 2a Controlled Human Malaria Parasite Infection and Immunogenicity Study. *J. Infect. Dis.* **214**, 762–771 (2016).
148. Pallikkuth, S. *et al.* A delayed fractionated dose RTS,S AS01 vaccine regimen mediates protection via improved T follicular helper and B cell responses. *Elife* **9**, e51889

- (2020).
149. Hon, C. & Matuschewski, K. Malaria According to GARP: A New Trail towards Anti-disease Vaccination. *Trends Parasitol.* **36**, 653–655 (2020).
 150. Raj, D. K. *et al.* Anti-PfGARP activates programmed cell death of parasites and reduces severe malaria. *Nature* **582**, 104–108 (2020).
 151. Beeson, J. G., Chan, J.-A. & Fowkes, F. J. I. PfEMP1 as a target of human immunity and a vaccine candidate against malaria. *Expert Rev. Vaccines* **12**, 105–108 (2013).
 152. Lau, C. K. Y. *et al.* Structural conservation despite huge sequence diversity allows EPCR binding by the PfEMP1 family implicated in severe childhood malaria. *Cell Host Microbe* **17**, 118–129 (2015).
 153. Gardner, M. J. *et al.* Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**, 498–511 (2002).
 154. Baruch, D. I. *et al.* Immunization of Aotus monkeys with a functional domain of the *Plasmodium falciparum* variant antigen induces protection against a lethal parasite line. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 3860–3865 (2002).
 155. Salanti, A. *et al.* Evidence for the involvement of VAR2CSA in pregnancy-associated malaria. *J. Exp. Med.* **200**, 1197–1203 (2004).
 156. Sirima, S. B. *et al.* PRIMVAC vaccine adjuvanted with Alhydrogel or GLA-SE to prevent placental malaria: a first-in-human, randomised, double-blind, placebo-controlled study. *Lancet Infect. Dis.* **20**, 585–597 (2020).
 157. Hill, A. V. S. Vaccines against malaria. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **366**,

- 2806–2814 (2011).
158. Kulp, D. W. & Schief, W. R. Advances in structure-based vaccine design. *Curr. Opin. Virol.* **3**, 322–331 (2013).
 159. Steichen, J. M. *et al.* A generalized HIV vaccine design strategy for priming of broadly neutralizing antibody responses. *Science* **366**, 4380 (2019).
 160. Scarselli, M. *et al.* Rational design of a meningococcal antigen inducing broad protective immunity. *Sci. Transl. Med.* **3**, 91ra62-91ra62 (2011).
 161. Bajic, G. *et al.* Structure-Guided Molecular Grafting of a Complex Broadly Neutralizing Viral Epitope. *ACS Infect. Dis.* **6**, 1182–1191 (2020).
 162. Goldin, A. & Mantel, N. The Employment of Combinations of Drugs in the Chemotherapy of Neoplasia: A Review. *Cancer Res.* **17**, 635–654 (1957).
 163. Greco, W. R., Faessel, H. & Levasseur, L. The Search for Cytotoxic Synergy Between Anticancer Agents: a Case of Dorothy and the Ruby Slippers? *JNCI J. Natl. Cancer Inst.* **88**, 699–700 (1996).
 164. Diamant, E., Torgeman, A., Ozeri, E. & Zichel, R. Monoclonal Antibody Combinations that Present Synergistic Neutralizing Activity: A Platform for Next-Generation Anti-Toxin Drugs. *Toxins (Basel)*. **7**, 1854–1881 (2015).
 165. Jia, J. *et al.* Mechanisms of drug combinations: interaction and network perspectives. *Nat. Rev. Drug Discov.* **8**, 111–128 (2009).
 166. Gilchuk, P. *et al.* Analysis of a Therapeutic Antibody Cocktail Reveals Determinants for Cooperative and Broad Ebolavirus Neutralization. *Immunity* **52**, 388-403.e12

- (2020).
167. Williams, A. R. *et al.* Enhancing blockade of *Plasmodium falciparum* erythrocyte invasion: assessing combinations of antibodies against PfRH5 and other merozoite antigens. *PLoS Pathog* **8**, e1002991 (2012).
 168. Trager, W. & Jensen, J. B. Human malaria parasites in continuous culture. *Science* **193**, 673–675 (1976).
 169. Schuster, F. L. Cultivation of plasmodium spp. *Clin. Microbiol. Rev.* **15**, 355–364 (2002).
 170. Lambros C Fau - Vanderberg, J. P., Vanderberg, J. P. & Parasitol, J. Synchronization of *Plasmodium falciparum* erythrocytic stages in culture.
 171. Hill, D. A. *et al.* A blasticidin S-resistant *Plasmodium falciparum* mutant with a defective plasmodial surface anion channel. *Proc. Natl. Acad. Sci.* **104**, 1063–1068 (2007).
 172. Duncan, C. J. A., Hill, A. V. S. & Ellis, R. D. Can growth inhibition assays (GIA) predict blood-stage malaria vaccine efficacy? *Hum. Vaccin. Immunother.* **8**, 706–714 (2012).
 173. Sinha, S., Sarma, P., Sehgal, R. & Medhi, B. Development in Assay Methods for in Vitro Antimalarial Drug Efficacy Testing: A Systematic Review. *Front. Pharmacol.* **8**, 754 (2017).
 174. Gomez, M. S. *et al.* Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasite *P. falciparum*. *Mol. Biochem. Parasitol.* **90**, 235–246 (1997).

175. Schuck, P. USE OF SURFACE PLASMON RESONANCE TO PROBE THE EQUILIBRIUM AND DYNAMIC ASPECTS OF INTERACTIONS BETWEEN BIOLOGICAL MACROMOLECULES. *Annu. Rev. Biophys. Biomol. Struct.* **26**, 541–566 (1997).
176. Roost, H. P. *et al.* Early high-affinity neutralizing anti-viral IgG responses without further overall improvements of affinity. *Proc. Natl. Acad. Sci.* **92**, 1257–1261 (1995).
177. de la Mora, E. *et al.* Radiation damage and dose limits in serial synchrotron crystallography at cryo- and room temperatures. *Proc. Natl. Acad. Sci.* **117**, 4142–4151 (2020).
178. Powell, H. R. The Rossmann Fourier autoindexing algorithm in MOSFLM. *Acta Crystallogr. Sect. D* **55**, 1690–1695 (1999).
179. Incardona, M.-F. *et al.* EDNA: a framework for plugin-based applications applied to X-ray experiment online data analysis. *J. Synchrotron Radiat.* **16**, 872–879 (2009).
180. Evans, P. Scaling and assessment of data quality. *Acta Crystallogr. Sect. D* **62**, 72–82 (2006).
181. Evans, P. R. An introduction to data reduction: space-group determination, scaling and intensity statistics. *Acta Crystallogr. D. Biol. Crystallogr.* **67**, 282–292 (2011).
182. Assmann, G., Brehm, W. & Diederichs, K. Identification of rogue datasets in serial crystallography. *J. Appl. Crystallogr.* **49**, 1021–1028 (2016).
183. Rhodes, G. *Crystallography made crystal clear: a guide for users of macromolecular models*. (Elsevier, 2006).
184. McCoy, A. J. *et al.* {it Phaser} crystallographic software. *J. Appl. Crystallogr.* **40**, 658–

- 674 (2007).
185. Emsley, P., Lohkamp, B., Scott, W. G. & Cowtan, K. Features and development of Coot. *Acta Crystallogr. D. Biol. Crystallogr.* **66**, 486–501 (2010).
 186. Bricogne, G. *et al.* Buster version X.Y.Z. (2017).
 187. Aurrecochea, C. *et al.* PlasmoDB: a functional genomic database for malaria parasites.
 188. Sheng, S. & Kong, F. Separation of antigens and antibodies by immunoaffinity chromatography. *Pharm. Biol.* **50**, 1038–1044 (2012).
 189. Wang, A. C. & Wang, I. Y. Cleavage sites of human IgG1 immunoglobulin by papain. *Immunochemistry* **14**, 197–200 (1977).
 190. Rijal, P. *et al.* Therapeutic Monoclonal Antibodies for Ebola Virus Infection Derived from Vaccinated Humans. *Cell Rep.* **27**, 172-186.e7 (2019).
 191. Evans, B. C. *et al.* Ex vivo red blood cell hemolysis assay for the evaluation of pH-responsive endosomolytic agents for cytosolic delivery of biomacromolecular drugs. *J. Vis. Exp.* e50166–e50166 (2013) doi:10.3791/50166.
 192. Walter, T. S. *et al.* Lysine methylation as a routine rescue strategy for protein crystallization. *Structure* **14**, 1617–1622 (2006).
 193. Winter, G. & McAuley, K. E. Automated data collection for macromolecular crystallography. *Methods* **55**, 81–93 (2011).
 194. Winn, M. D. *et al.* Overview of the CCP4 suite and current developments. *Acta*

- Crystallogr. Sect. D* **67**, 235–242 (2011).
195. Liebschner, D. *et al.* Macromolecular structure determination using X-rays, neutrons and electrons: recent developments in {it Phenix}. *Acta Crystallogr. Sect. D* **75**, 861–877 (2019).
 196. Williams, C. J. *et al.* MolProbity: More and better reference data for improved all-atom structure validation. *Protein Sci.* **27**, 293–315 (2018).
 197. Hooft van Huijsduijnen, R. *et al.* Reassessing therapeutic antibodies for neglected and tropical diseases. *PLoS Negl. Trop. Dis.* **14**, e0007860 (2020).
 198. Kivi, G. *et al.* HybriFree: a robust and rapid method for the development of monoclonal antibodies from different host species. *BMC Biotechnol.* **16**, 2 (2016).
 199. Rawlinson, T. A. *et al.* Structural basis for inhibition of Plasmodium vivax invasion by a broadly neutralizing vaccine-induced human antibody. *Nat. Microbiol.* **4**, 1497–1507 (2019).
 200. Hoogenboom, H. R. Selecting and screening recombinant antibody libraries. *Nat. Biotechnol.* **23**, 1105–1116 (2005).
 201. Pulido-Gomez, D. *et al.* Characterisation of antimalarial monoclonal antibodies reveals synergistic epitopes in the Plasmodium falciparum RH5-CyRPA-Ripr invasion complex. *Prep.*
 202. Hildebrandt, A. B. S. Staphylococcus aureus alpha-Toxin. *J. Biol. Chem.* **265**, 17195–17200 (1991).
 203. Hanes, J., Schaffitzel, C., Knappik, A. & Plückthun, A. Picomolar affinity antibodies

- from a fully synthetic naive library selected and evolved by ribosome display. *Nat. Biotechnol.* **18**, 1287–1292 (2000).
204. Wark, K. L. & Hudson, P. J. Latest technologies for the enhancement of antibody affinity. *Adv. Drug Deliv. Rev.* **58**, 657–670 (2006).
 205. Betz, A. G., Rada, C., Pannell, R., Milstein, C. & Neuberger, M. S. Passenger transgenes reveal intrinsic specificity of the antibody hypermutation mechanism: clustering, polarity, and specific hot spots. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 2385–2388 (1993).
 206. Tabasinezhad, M. *et al.* Trends in therapeutic antibody affinity maturation: From in-vitro towards next-generation sequencing approaches. *Immunol. Lett.* **212**, 106–113 (2019).
 207. Clark, L. A. *et al.* Affinity enhancement of an in vivo matured therapeutic antibody using structure-based computational design. *Protein Sci.* **15**, 949–960 (2006).
 208. Wu, H. *et al.* Development of Motavizumab, an Ultra-potent Antibody for the Prevention of Respiratory Syncytial Virus Infection in the Upper and Lower Respiratory Tract. *J. Mol. Biol.* **368**, 652–665 (2007).
 209. Carbonell-Estrany, X. *et al.* Motavizumab for Prophylaxis of Respiratory Syncytial Virus in High-Risk Children: A Noninferiority Trial. *Pediatrics* **125**, e35 LP-e51 (2010).
 210. Foote, J. & Eisen, H. N. Kinetic and affinity limits on antibodies produced during immune responses. *Proc. Natl. Acad. Sci. U. S. A.* **92**, 1254–1256 (1995).
 211. Batista, F. D. & Neuberger, M. S. Affinity Dependence of the B Cell Response to

- Antigen: A Threshold, a Ceiling, and the Importance of Off-Rate. *Immunity* **8**, 751–759 (1998).
212. Sharma, S., Bartholdson, S. J., Couch, A. C. M., Yusa, K. & Wright, G. J. Genome-scale identification of cellular pathways required for cell surface recognition. *Genome Res.* **28**, 1372–1382 (2018).
 213. Nagaoka, H. *et al.* Antibodies against a short region of PfRipr inhibit Plasmodium falciparum merozoite invasion and PfRipr interaction with Rh5 and SEMA7A. *Sci. Rep.* **10**, 6573 (2020).
 214. Hale, V. L. *et al.* Parasitophorous vacuole poration precedes its rupture and rapid host erythrocyte cytoskeleton collapse in Plasmodium falciparum egress. *Proc. Natl. Acad. Sci.* **114**, 3439–3444 (2017).
 215. McLellan, J. S. *et al.* Structure of HIV-1 gp120 V1/V2 domain with broadly neutralizing antibody PG9. *Nature* **480**, 336–343 (2011).
 216. Ngwuta, J. O. *et al.* Prefusion F-specific antibodies determine the magnitude of RSV neutralizing activity in human sera. *Sci. Transl. Med.* **7**, 309ra162 LP-309ra162 (2015).
 217. McLellan, J. S. Neutralizing epitopes on the respiratory syncytial virus fusion glycoprotein. *Curr. Opin. Virol.* **11**, 70–75 (2015).
 218. McLellan, J. S. *et al.* Structure-Based Design of a Fusion Glycoprotein Vaccine for Respiratory Syncytial Virus. *Science* **342**, 592–598 (2013).
 219. Flyak, A. I. *et al.* Cross-Reactive and Potent Neutralizing Antibody Responses in Human Survivors of Natural Ebolavirus Infection. *Cell* **164**, 392–405 (2016).

220. Shang, J. *et al.* Structural basis of receptor recognition by SARS-CoV-2. *Nature* **581**, 221–224 (2020).
221. Yuan, M. *et al.* A highly conserved cryptic epitope in the receptor binding domains of SARS-CoV-2 and SARS-CoV. *Science* **368**, 630–633 (2020).
222. Yan, R. *et al.* Structural basis for the recognition of SARS-CoV-2 by full-length human ACE2. *Science* **367**, 1444–1448 (2020).
223. Kato, T. *et al.* CryoTEM with a Cold Field Emission Gun That Moves Structural Biology into a New Stage. *Microsc. Microanal.* **25**, 998–999 (2019).
224. Beckers, M., Mann, D. & Sachse, C. Structural interpretation of cryo-EM image reconstructions. *Prog. Biophys. Mol. Biol.* (2020)
doi:<https://doi.org/10.1016/j.pbiomolbio.2020.07.004>.
225. Higgins, M. K. & Lea, S. M. On the state of crystallography at the dawn of the electron microscopy revolution. *Curr. Opin. Struct. Biol.* **46**, 95–101 (2017).
226. Herzik, M. A., Wu, M. & Lander, G. C. High-resolution structure determination of sub-100 kDa complexes using conventional cryo-EM. *Nat. Commun.* **10**, 1032 (2019).
227. Schrödinger, LLC. *The {PyMOL} Molecular Graphics System, Version~1.8.* (2015).
228. Harding, M. M. The architecture of metal coordination groups in proteins. *Acta Crystallogr. Sect. D* **60**, 849–859 (2004).
229. Dudev, T. & Lim, C. Principles Governing Mg, Ca, and Zn Binding and Selectivity in Proteins. *Chem. Rev.* **103**, 773–788 (2003).

230. Lu, Y. Metal ions as matchmakers for proteins. *Proc. Natl. Acad. Sci.* **107**, 1811 LP – 1812 (2010).
231. Zhou, T., Hamer, D. H., Hendrickson, W. A., Sattentau, Q. J. & Kwong, P. D. Interfacial metal and antibody recognition. *Proc. Natl. Acad. Sci. U. S. A.* **102**, 14575–14580 (2005).
232. Garman, E. F. & Grime, G. W. Elemental analysis of proteins by microPIXE. *Prog. Biophys. Mol. Biol.* **89**, 173–205 (2005).
233. Bazydlo, L. A. L., Needham, M. & Harris, N. S. Calcium, Magnesium, and Phosphate. *Lab. Med.* **45**, e44–e50 (2014).
234. Jahnen-Dechent, W. & Ketteler, M. Magnesium basics. *Clin. Kidney J.* **5**, i3–i14 (2012).
235. Hariharan, V. & Kane, R. S. Glycosylation as a tool for rational vaccine design. *Biotechnol. Bioeng.* **117**, 2556–2570 (2020).
236. Eggink, D., Goff, P. H. & Palese, P. Guiding the Immune Response against Influenza Virus Hemagglutinin toward the Conserved Stalk Domain by Hyperglycosylation of the Globular Head Domain. *J. Virol.* **88**, 699–704 (2014).
237. Sesterhenn, F. *et al.* Boosting subdominant neutralizing antibody responses with a computationally designed epitope-focused immunogen. *PLoS Biol.* **17**, e3000164 (2019).
238. Boyington, J. C. *et al.* Structure-Based Design of Head-Only Fusion Glycoprotein Immunogens for Respiratory Syncytial Virus. *PLoS One* **11**, e0159709 (2016).
239. Briney, B. *et al.* Tailored Immunogens Direct Affinity Maturation toward HIV

- Neutralizing Antibodies. *Cell* **166**, 1459–1470.e11 (2016).
240. Bliss, C. I. THE TOXICITY OF POISONS APPLIED JOINTLY¹. *Ann. Appl. Biol.* **26**, 585–615 (1939).
 241. Wooten, D. J., Meyer, C. T., Quaranta, V. & Lopez, C. A Consensus Framework Unifies Multi-Drug Synergy Metrics. *bioRxiv* 683433 (2019) doi:10.1101/683433.
 242. Geary, N. Understanding synergy. *Am. J. Physiol. Metab.* **304**, E237–E253 (2012).
 243. Roell, K. R., Reif, D. M. & Motsinger-Reif, A. A. An Introduction to Terminology and Methodology of Chemical Synergy-Perspectives from Across Disciplines. *Front. Pharmacol.* **8**, 158 (2017).
 244. Loewe, S. & Muischnek, H. Über Kombinationswirkungen. *Naunyn. Schmiedeberg's. Arch. Exp. Pathol. Pharmacol.* **114**, 313–326 (1926).
 245. Foucquier, J. & Guedj, M. Analysis of drug combinations: current methodological landscape. *Pharmacol. Res. Perspect.* **3**, e00149–e00149 (2015).
 246. Sinzger, M., Vanhoefer, J., Loos, C. & Hasenauer, J. Comparison of null models for combination drug therapy reveals Hand model as biochemically most plausible. *Sci. Rep.* **9**, 3002 (2019).
 247. Berenbaum, M. C. What is synergy? *Pharmacol. Rev.* **41**, 93–141 (1989).
 248. Chou, T.-C. & Talalay, P. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**, 27–55 (1984).

249. Zimmer, A., Katzir, I., Dekel, E., Mayo, A. E. & Alon, U. Prediction of multidimensional drug dose responses based on measurements of drug pairs. *Proc. Natl. Acad. Sci.* **113**, 10442–10447 (2016).
250. Greco, W. R., Bravo, G. & Parsons, J. C. The search for synergy: a critical review from a response surface perspective. *Pharmacol. Rev.* **47**, 331–385 (1995).
251. Kashif, M. *et al.* Bliss and Loewe interaction analyses of clinically relevant drug combinations in human colon cancer cell lines reveal complex patterns of synergy and antagonism. *Oncotarget* **8**, 103952–103967 (2017).
252. Tang, J., Wennerberg, K. & Aittokallio, T. What is synergy? The Saariselkä agreement revisited. *Front. Pharmacol.* **6**, 181 (2015).
253. Favuzza, P. *et al.* Dual Plasmepsin-Targeting Antimalarial Agents Disrupt Multiple Stages of the Malaria Parasite Life Cycle. *Cell Host Microbe* **27**, 642–658 (2020).
254. WHO. Standardized protocol: Natural history protocol for zika virus infection. (2017).
255. Wagner, E. K. & Maynard, J. A. Engineering therapeutic antibodies to combat infectious diseases. *Curr. Opin. Chem. Eng.* **19**, 131–141 (2018).
256. Imkeller, K. *et al.* Antihomotypic affinity maturation improves human B cell responses against a repetitive epitope. *Science* **360**, 1358–1362 (2018).
257. Fuh, G. *et al.* Structure-function studies of two synthetic anti-VEGF Fabs and comparison with avastinTM-Fab. *J. Biol. Chem.* **281**, 951–961 (2005).
258. Hillig, R. C. *et al.* Fab MOR03268 Triggers Absorption Shift of a Diagnostic Dye via Packaging in a Solvent-shielded Fab Dimer Interface. *J. Mol. Biol.* **377**, 206–219

- (2008).
259. Rougé, L. *et al.* Structure of CD20 in complex with the therapeutic monoclonal antibody rituximab. *Science* **367**, 1224–1230 (2020).
 260. Hattori, T. *et al.* Antigen clasping by two antigen-binding sites of an exceptionally specific antibody for histone methylation. *Proc. Natl. Acad. Sci. U. S. A.* **113**, 2092–2097 (2016).
 261. Ehrlich, P. H., Moyle, W. R., Moustafa, Z. A. & Canfield, R. E. Mixing two monoclonal antibodies yields enhanced affinity for antigen. *J. Immunol.* **128**, 2709–2713 (1982).
 262. Pino, P. *et al.* A multistage antimalarial targets the plasmepsins IX and X essential for invasion and egress. *Science* **358**, 522–528 (2017).
 263. Roux, K. J., Kim, D. I., Raida, M. & Burke, B. A promiscuous biotin ligase fusion protein identifies proximal and interacting proteins in mammalian cells. *J Cell Biol* **196**, 801–810 (2012).
 264. Varnaitè, R. & MacNeill, S. A. Meet the neighbors: Mapping local protein interactomes by proximity-dependent labeling with BioID. *Proteomics* **16**, 2503–2518 (2016).
 265. Piersimoni, L. & Sinz, A. Cross-linking/mass spectrometry at the crossroads. *Anal. Bioanal. Chem.* **412**, 5981–5987 (2020).
 266. Singh, H. *et al.* Antibody combinations targeting the essential antigens CyRPA, RH5 and MSP-119 potentially neutralize Plasmodium falciparum clinical isolates from India and Africa. *J. Infect. Dis.* (2020) doi:10.1093/infdis/jiaa608.

267. Williams, W. B. *et al.* Fab-dimerized glycan-reactive antibodies neutralize HIV and are prevalent in humans and rhesus macaques. *bioRxiv* 2020.06.30.178921 (2020)
doi:10.1101/2020.06.30.178921.

Chapter 7: Appendices

Appendix 1: Crystallization screens and optimisation table. All complexes began with a 10-plate broad screen of 480 unique conditions at two temperatures (Proplex, JCSG+, Midas, Morpheus, Pact).

<i>mAbs in complex</i>	<i>Temperatures (°C)</i>	<i>Methylation screen</i>	<i>Silver bullets screen</i>	<i>Matrix optimization</i>	<i>Other plates</i>
<i>Cy.002</i>	4,18	No	No	No	None
<i>Cy.003</i>	4,18	No	No	No	None
<i>Cy.004</i>	4,18	No	No	No	None
<i>Cy.007</i>	4,18	No	No	No	None
<i>Cy.009</i>	4,18	Yes	Yes, 10 conditions	Yes, 3 conditions	JCSG II JCSG III Memgold Morpheus II
<i>Cy.009:Cy.002</i>	4,18	No	No	No	None
<i>Cy.009:Cy.003</i>	4,18	Yes	No	No	None
<i>Cy.009:Cy.007</i>	4,18	Yes	No	No	None
<i>Cy.009:Cy.001</i>	4,18	No	No	No	None
<i>Cy.009:Cy.003:Cy.007</i>	4,18	Yes	No	No	Memgold
<i>Cy.004:Cy.003:Cy.007</i>	4,18	Yes	Yes, 2 conditions	No	Memgold

Appendix 2: Synergy data summary of antibody pairs from Chapter 5. *indicates where an IC50 was extrapolated as the combination did not reach 50% inhibition.

		Held Constant			
		Cy.003	Cy.004	Cy.007	Cy.009
Titrated	Cy.003		59	19*	777*
	Cy.004	109		24	2
	Cy.007	22	ND		1250*
	Cy.009	28	2	14	