



Investigation of cross-reactive
antibodies against *Plasmodium*
falciparum-infected erythrocytes

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Abstract

Cross-reactive antibodies have recently come into focus as promising tools for targeted vaccine design in the fields of HIV and influenza, but the identification of such antibodies that bind to *Plasmodium falciparum*-infected erythrocytes has proven difficult due to extensive clonal antigenic variation on the surface of these cells. In this study, I report the identification of cross-reactive human monoclonal antibodies that bind to *P. falciparum*-infected erythrocytes from most parasite strains tested. These antibodies gained broad reactivity through the insertion of a large piece of DNA between the V and DJ segments of the antibody heavy chain, which is encoded on chromosome 14. The core of the insert, which is the primary element required for binding to the infected erythrocytes, codes for the collagen-binding domain of LAIR1, an immunoglobulin superfamily inhibitory receptor that is encoded on chromosome 19. In each of the donors studied, the LAIR1-containing antibodies were derived from a single B cell clone and acquired mutations that increased binding to infected erythrocytes and reduced binding to collagen. The targets of these antibodies were identified as members of the RIFIN variant surface antigen family. The LAIR1-containing antibodies were able to opsonize and agglutinate *P. falciparum*-infected erythrocytes, suggesting that they may be useful in reducing parasite burden *in vivo*. Using newly developed assays, I show that the presence of LAIR1-containing antibodies is not limited to a particular place or population, but can be found quite commonly in individuals from different malaria-endemic areas. These findings demonstrate a novel pathway of antibody diversification by interchromosomal DNA transposition that results in the production of functional antibodies and suggest that RIFIN

epitopes that are recognized by the LAIR1-containing antibodies may be potential candidates for the development of a blood-stage malaria vaccine.

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Abbreviations

7-AAD	7-amino-actinomycin D
AID	Activation-induced cytidine deaminase
AMA1	Apical membrane antigen-1
aNHEJ	Alternative NHEJ
AP	Alkaline phosphatase
APRIL	A proliferation-inducing ligand
ARPP	Antigen Receptor Probabilistic Parser
ATS	Acidic terminal sequence
BAFF	B-cell activating factor
BCL	B-cell lymphoma
BCR	B cell receptor
BSA	Bovine serum albumin
CD	Cluster of differentiation
CDR	Complementarity-determining region
C _H	Heavy chain constant domain
ChAd	Chimpanzee adenovirus
CHO	Chinese hamster ovary
CIDR	Cysteine-rich inter-domain regions
C _L	Light chain constant domain
cNHEJ	Classical NHEJ
CSA	Chondroitin sulfate A
CSP	Circumsporozoite protein
CTCF	CCCTC-binding factor
DAPI	4',6-diamidino-2-phenylindole
DBL	Duffy-binding like
DC	Domain cassette
DMSO	Dimethyl sulfoxide
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DTT	Dithiothreitol
EBA	Erythrocyte binding antigens
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPCR	Endothelial protein C receptor
Fab	Fragment, antigen binding
FBS	Foetal bovine serum
Fc	Fragment, crystallisable
FcRL	Fc receptor-like
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate

FR	Framework
FSC	Forward scatter
GAP	Genetically attenuated sporozoite
gDNA	Genomic DNA
GLURP	Glutamate-rich protein
GPI	Glycophosphotidylinositol
HA	Haemagglutinin
HBEC	Human brain endothelial cell
HBS	HEPES-buffered saline
HCAb	Heavy chain-antibody
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
iBAQ	Intensity-based absolute quantification
ICAM-1	Intercellular adhesion molecule-1
IE	Infected erythrocyte
IgNAR	Ig new antigen receptor
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
LAIR1	Leukocyte-associated immunoglobulin-like 1
LC-MS	Liquid chromatography-mass spectrometry
LFA	Lymphocyte function-associated antigen
LFQ	Label-free quantification
LINE	Long interspersed nuclear element
mAb	Monoclonal antibody
ME-TRAP	Multiple epitope string with thrombospondin-related adhesion protein
MFI	Mean fluorescence intensity
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
MVA	Modified vaccinia Ankara
NCAM	Neural cell adhesion molecule
NHEJ	Non-homologous end joining
PARP	Poly(ADP-ribose) polymerase
Pax	Paired box
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PE	Phycoerythrin
PECAM-1	Platelet endothelial cell adhesion molecule-1
PEI	Polyethylenimine
PEXEL	<i>Plasmodium</i> export element
PfEMP1	<i>Plasmodium falciparum</i> erythrocyte membrane protein-1

PfMC-2TM	<i>Plasmodium falciparum</i> Maurer's cleft two transmembrane
PfRH	<i>Plasmodium falciparum</i> reticulocyte-binding protein homologue
PfRON2	<i>Plasmodium falciparum</i> rhoptry neck protein 2
PHA	Phytohaemagglutinin
<i>pir</i>	<i>Plasmodium</i> interspersed repeat
PVDF	Polyvinylidene difluoride
RAG	Recombination-activating gene
RIFIN	Repetitive interspersed family
RIPA	Radioimmunoprecipitation assay
RPMI-C	RPMI complete medium
RPMI-I	RPMI incomplete medium
RSS	Recombination signal sequence
RT	Reverse transcription
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SERA	Serine repeat antigen
SH2	Src homology 2
SHM	Somatic hypermutation
SP	Signal peptide
SSC	Side scatter
STEVOR	Subtelomeric variable open reading frame
SURFIN	Surface-associated interspersed gene family
S _μ	Switch region of IgM
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotidyl transferase
TEX	Trophozoite exported protein
TLR	Toll-like receptor
TM	Transmembrane region
TNF-α	Tumor necrosis factor alpha
TSI	Templated sequence insertion
TSIP	TSI polymorphism
UCA	Unmutated common ancestor
uE	Uninfected erythrocyte
UNG	Uracil-DNA glycosylase
VCAM-1	Vascular cell adhesion molecule-1
V _H	Heavy chain variable domain/region
V _L	Light chain variable domain/region
VSA	Variant surface antigen
XLF	XRCC4-like factor
XRCC	X-ray repair cross-complementing protein

Chapter 1. Introduction

This DPhil project began as an investigation into the serological response of malaria-exposed individuals to antigens on *Plasmodium falciparum*-infected erythrocytes, but has developed in an unexpected direction into a cross-disciplinary project that involves the fields of basic B cell immunology, malaria biology and DNA transposition. This calls for an introduction that is sufficiently broad to cover these different areas, and the purpose of this section is to focus on aspects of these fields as well as on special topics of interest that will be pertinent to the data that will be presented later. As this thesis may be of interest to readers from diverse research fields, I will try to cover these topics at a depth that will enable readers who specialise in particular aspects of this project but who may not have been formally trained in other aspects (for instance, non-immunologists or non-malariologists) to understand the findings of this study. I will also suggest suitable reviews for readers who desire more detailed information on selected topics.

This chapter will begin with an introduction to malaria, with particular emphasis on the characterization of variant surface antigens on the *P. falciparum*-infected erythrocyte surface and the development of malaria vaccine candidates. Next, I will describe the structure and function of antibodies and the generation of antibody diversity through V(D)J recombination and somatic hypermutation. I will also briefly cover the mechanisms of DNA double-strand break repair. I will then discuss the intersection between malaria and B cell immunology by describing antibody-mediated immune responses towards *P. falciparum* variant surface antigens and the effect of malaria infection on the B cell compartment.

This chapter will conclude with a discussion of the rationale and aims of this DPhil project.

1.1. Malaria

1.1.1. General introduction to malaria

Malaria is caused by protozoan parasites of the *Plasmodium* genus and is transmitted by female *Anopheles* mosquitoes. There are six *Plasmodium* species that are known to cause malaria in humans (Calderaro *et al.* 2013), but the most deadly form of the disease is caused by *P. falciparum*, which is especially prevalent in sub-Saharan Africa. *P. falciparum* has an ancient origin, with the earliest cases of malaria thought to occur at least thousands of years ago (Mu *et al.* 2002; Liu *et al.* 2010; Sundararaman *et al.* 2016). Correspondingly, research on malaria has a long and rich history, with the basic biology of malaria and the first vaccine trial on a *Plasmodium* species both described more than a hundred years ago (Sargent and Sargent 1910; Cox 2010). Research in this field has contributed substantially to a recent decrease in malaria cases (Bhatt *et al.* 2015). Nevertheless, malaria is still a major cause of morbidity and mortality today, accounting for approximately 400,000 deaths every year (World Health Organization 2016). While malaria has been effectively eliminated from North America and Europe, about half of the world's population live in tropical or subtropical areas and remain at risk of contracting the disease. Furthermore, there is currently no licensed vaccine for malaria. The most advanced vaccine candidate, RTS,S, which may be licensed soon, has been shown to have modest and short-term efficacy in a multi-centre Phase III clinical trial in Africa (RTSS Clinical Trials Partnership 2015). Therefore, there remains an urgent need to further our understanding of malaria and to develop more effective tools, primarily a vaccine with high efficacy, to combat the disease.

1.1.2. *Plasmodium* life cycle

A major factor that has contributed to the difficulty in combating malaria is the complex life cycle of the *Plasmodium* parasite in its mosquito vector and human host (Figure 1.1) (Crompton *et al.* 2014). The different stages of the life cycle are morphologically distinct, display different antigens and invade different parts of the human body that have unique immunological milieux. A malaria infection begins with the injection of motile *Plasmodium* sporozoites into the skin during a mosquito blood meal. These parasites rapidly enter blood vessels and travel to the liver, where they invade hepatocytes and undergo multiple rounds of cell division to form exoerythrocytic schizonts. These schizonts rupture to produce thousands of merozoites, which travel to the bloodstream and invade erythrocytes to initiate blood-stage infection. In erythrocytes, the merozoites sequentially mature into ring-stage and trophozoite-stage parasites. *P. falciparum* trophozoites express antigens on the surface of infected erythrocytes (IEs) that allow them to adhere to the endothelium of post-capillary venules and to avoid destruction in the spleen. The trophozoites undergo nuclear division to form schizonts, which rupture to release merozoites that reinvasion healthy erythrocytes. A complete life cycle of *P. falciparum* in IEs occurs in approximately 48 hours. A proportion of blood-stage parasites differentiate into sexual-stage gametocytes, which undergo further development in the bone marrow (Joice *et al.* 2014). Mature gametocytes are released into the bloodstream, where they are taken up by mosquitoes during a blood meal. In the mosquito, male and female gametocytes mature into gametes and fuse to form ookinetes, which subsequently divide and differentiate into sporozoites that invade the mosquito's salivary glands for subsequent rounds of infection.

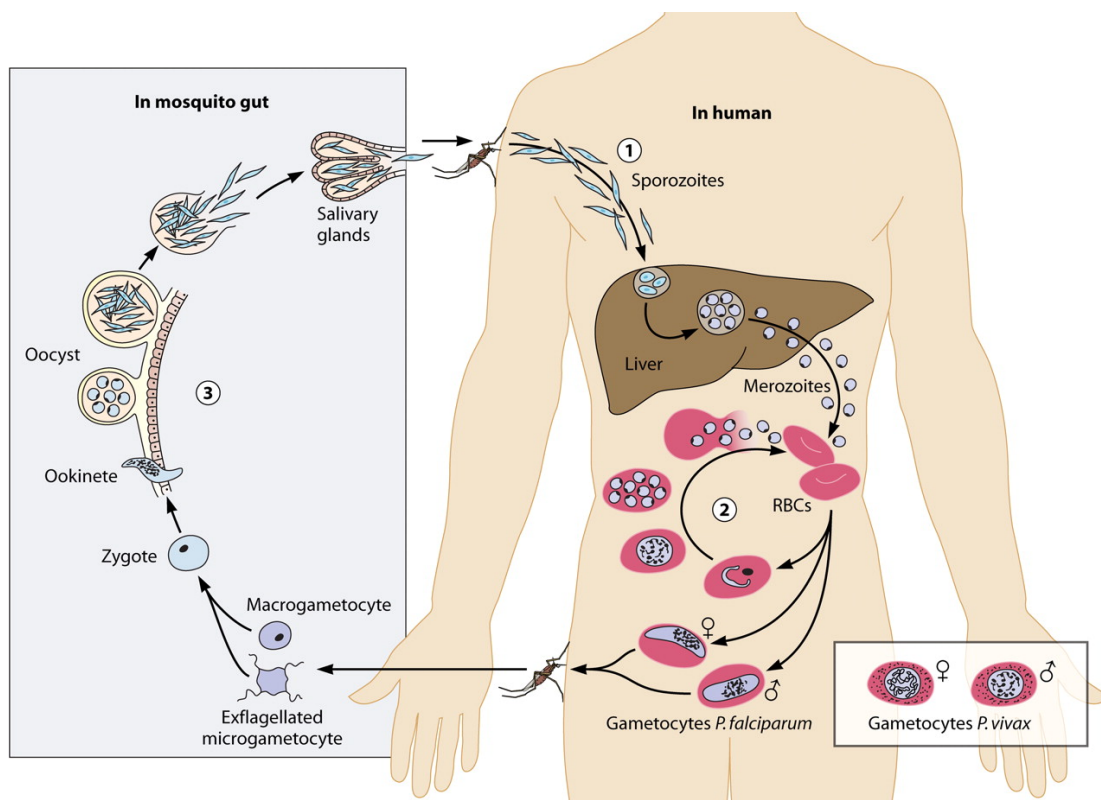


Figure 1.1. Life cycle of the *Plasmodium* parasite in its mosquito vector and human host. Malaria infection begins with the injection of motile sporozoites by female *Anopheles* mosquitoes into the skin. These sporozoites travel to the liver, where they invade hepatocytes and multiply to form exo-erythrocytic schizonts. Schizont rupture results in the release of thousands of merozoites, which invade healthy erythrocytes and begin a 48-hour asexual blood stage cycle. In this cycle, the merozoites mature and undergo nuclear division to form schizonts, which rupture to release many merozoites into the bloodstream. A percentage of blood stage parasites differentiate into sexual-stage gametocytes that can be taken up by mosquitoes. In the mosquitoes, male and female gametes fuse to form ookinetes, which divide and differentiate into sporozoites that can invade the mosquito's salivary glands for subsequent rounds of infection. Reprinted with permission from (Bousema and Drakeley 2011).

1.1.3. Malaria pathology

Most malaria cases involve non-specific febrile symptoms such as sweating, fever, and nausea, and are generally self-resolving or can be effectively treated with antimalarials. These symptoms are caused by a strong inflammatory response that is induced by *Plasmodium* products that are released during schizont rupture. For instance, the malarial waste pigment haemozoin and toxic products such as glycosphosphatidylinositol (GPI) activate Toll-like receptors (TLRs) and other innate immune receptors in macrophages, resulting in the release of pyrogenic cytokines such as TNF- α and IL-1 β (Naik *et al.* 2000; Coban *et al.* 2005; Parroche *et al.* 2007; Griffith *et al.* 2009). Accordingly, in *P. falciparum* infections, fevers occur in 48-hour cycles, mirroring the intraerythrocytic life cycle of the parasite.

Severe malaria occurs only in about 1-2% of patients (Greenwood, Marsh and Snow 1991) and can be classified based on three overlapping syndromes: cerebral malaria, severe malarial anaemia, and respiratory distress (Marsh *et al.* 1995). It has been recognised that these syndromes do not merely involve specific organs but reflect more systemic dysfunction and may share common underlying causes. For instance, a key feature of severe cerebral and non-cerebral malaria is metabolic acidosis (Taylor, Borgstein and Molyneux 1993; Krishna *et al.* 1994; Allen *et al.* 1996; English *et al.* 1996; English 1997). An important factor that is likely to cause an increase in acidity during malaria infection is the release of pro-inflammatory cytokines such as TNF- α (reviewed in (Clark and Cowden 2003)). Several factors that may also contribute to the decline in pH include poor perfusion of tissues due to malaria-induced anaemia

and adhesion of *P. falciparum* IEs to blood vessel endothelial cells (Mackintosh, Beeson and Marsh 2004). Indeed, the sequestration of IEs in brain microvasculature through attachment of *Plasmodium falciparum* erythrocyte membrane protein-1 (PfEMP1) antigens to endothelial receptors such as intercellular adhesion molecule-1 (ICAM-1), E-selectin and vascular cell adhesion molecule-1 (VCAM-1) has long been associated with cerebral malaria (described more later)(Janota and Doshi 1979; Aikawa 1988; Riganti *et al.* 1990; Turner *et al.* 1994; Silamut *et al.* 1999; Ponsford *et al.* 2012). The sequestered IEs are thought to obstruct circulation and impair perfusion to the surrounding tissues, causing brain damage. Other groups have argued that other factors such as inflammation play a substantial role in the pathogenesis of cerebral malaria (Clark and Cowden 2003; Hunt *et al.* 2010; Riley *et al.* 2010). These two factors may be linked and indeed mutually reinforcing, as the co-culture of *P. falciparum* with human brain endothelial cells results in the upregulation of pro-inflammatory cytokines (Tripathi *et al.* 2009), which in turn upregulate endothelial receptors such as ICAM-1 that are critical for IE cytoadhesion (Turner *et al.* 1998; Bauer *et al.* 2002; Armah *et al.* 2005), creating a pathogenic positive feedback loop.

A subset of *P. falciparum*-infected individuals suffers from severe malarial anaemia. As can be expected, this is caused in part by the destruction of IEs during the parasite life cycle, but other parasite-induced mechanisms also act to reduce the overall level of uninfected erythrocytes. Severe anaemia is associated with a loss of complement regulatory proteins (Stoute *et al.* 2003) and *P. falciparum* infection is linked to increased deposition of members of the

complement pathway (Facer, Bray and Brown 1979) on the surface of uninfected erythrocytes, potentially leading to increased erythrocyte destruction. Moreover, uninfected erythrocytes in malaria patients are also more damaged by reactive oxygen species (Griffiths *et al.* 2001) and have reduced deformability (Dondorp *et al.* 2002), resulting in increased retention and destruction in the spleen. The erythrocyte loss is exacerbated by dyserythropoiesis in infected individuals, who have reduced reticulocyte counts (Phillips *et al.* 1986) that are probably due to decreased proliferation of erythroid precursors (Thawani *et al.* 2013).

A separate syndrome that uniquely affects primigravid women in malaria-endemic areas is pregnancy-associated malaria, which can occur even in individuals who were clinically immune to the disease prior to pregnancy. This syndrome affects both the mother and the foetus, with symptoms ranging from reduced birth weight, pre-term delivery and growth retardation to death of the foetus (Moya-Alvarez, Abellana and Cot 2014). The renewed pathogenicity of *P. falciparum* in these individuals is associated with the upregulation of the PfEMP1 variant VAR2CSA, which allows IEs to adhere to chondroitin sulfate A (CSA) in placental tissue (Salanti *et al.* 2004; Magistrado *et al.* 2008). Additionally, the infiltration of haemozoin-laden monocytes that secrete inflammatory cytokines such as TNF- α , IL-1 β and IL-8 in placental tissue is associated with poor outcome (Fried *et al.* 1998a; Moormann *et al.* 1999; Rogerson *et al.* 2003).

1.1.4. Age-specific immunity to malaria

Studies of the human immune response to malaria infection have revealed that the extent and nature of the immune response to the various stages of the *P. falciparum* life cycle can differ quite substantially. Adaptive immune responses can be generated against the pre-erythrocytic stages, but these responses are thought to play at best a minor role in protection from malaria, as they are not able to prevent blood-stage infection (Offeddu *et al.* 2012; Tran *et al.* 2013). Rather, naturally acquired immunity to malaria is primarily associated with an immune response against blood-stage parasites. This response is largely driven by antibodies and CD4⁺ T helper cells, although innate immune mechanisms may also play a role in parasite destruction (Langhorne *et al.* 2008). The role of the antibody response, which is an area of focus in this thesis, will be discussed further in a subsequent section.

Epidemiological studies of individuals living in malaria-endemic areas have shown that young infants up to 3-6 months of age are protected from severe malaria (McGuinness *et al.* 1998; Snow *et al.* 1998). Several reasons have been proposed for this protection, including the presence of maternal anti-*Plasmodium* antibodies, the use of foetal haemoglobin, the lack of riboflavin in the diet of these infants and the low levels of p-amino-benzoic acid (PABA) in breast milk (Snow *et al.* 1998; Riley *et al.* 2001). The risk of severe and non-severe malaria increases to its highest level after this period of protection but subsequently declines with age until adulthood (Figure 1.2). In malaria-endemic regions, immunity to severe malaria is obtained prior to protection from non-severe malaria, such that children older than five rarely get severe malaria

(Snow *et al.* 1997) while non-severe clinical malaria may persist until adulthood (Langhorne *et al.* 2008). Mathematical models have suggested that immunity to non-cerebral severe malaria may develop after one or two infections (Gupta *et al.* 1999), but recent data suggests that severe malaria can still occur after several non-severe infections but is unlikely to recur (Gonçalves *et al.* 2014). Nevertheless, the age-specific profile of malaria immunity changes with the intensity of malaria transmission. As the transmission intensity declines, the age at which immunity to clinical malaria is obtained increases (Snow *et al.* 1997; O'Meara *et al.* 2008; Griffin, Ferguson and Ghani 2014). An overall decline in the rate of malaria infection in sub-Saharan Africa over the past 15 years has already prompted this change (Griffin, Ferguson and Ghani 2014; Bhatt *et al.* 2015), suggesting that adjustments to age-specific strategies to treat malaria may be required.

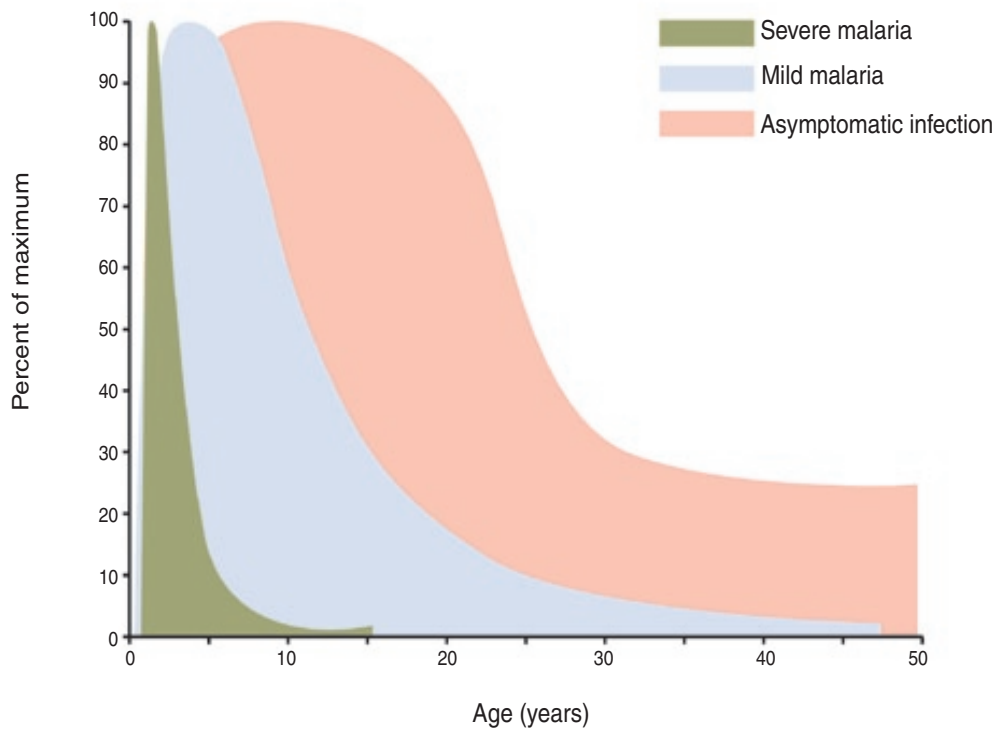


Figure 1.2. Age-specific acquisition of immunity to malaria. Immunity to severe malaria is typically acquired before the age of five, while immunity to non-severe malaria develops more gradually. Adults living in malaria-endemic areas are generally immune to clinical malaria but can have asymptomatic infections (reprinted with permission from(Langhorne *et al.* 2008)).

1.1.5. Variant surface antigens

The IE stage of the *Plasmodium* life cycle is characterised by immense antigenic diversity due to the ability of the parasites to employ clonal antigenic variation as a strategy to evade the immune response. This is possible as a sizeable proportion of the *Plasmodium* genome (~5% of genes in *P. falciparum*) is used to carry multiple genes encoding different variants of large antigen families (Gardner *et al.* 2002). Given the energy cost required by the parasite to maintain these large families, it is likely that clonal antigenic variation is essential to ensure parasite survival in its human host. The main variant surface antigen (VSA) families known to be expressed on the *P. falciparum* IE surface are PfEMP1 (Baruch *et al.* 1995; Smith *et al.* 1995; Su *et al.* 1995), repetitive interspersed family (RIFIN) proteins (Kyes *et al.* 1999; Fernandez *et al.* 1999), subtelomeric variable open reading frame (STEVAR) proteins (Kaviratne *et al.* 2002), surface-associated interspersed gene family (SURFIN) proteins (Winter *et al.* 2005) and PfMC-2TM proteins (Lavazec, Sanyal and Templeton 2006).

PfEMP1

PfEMP1 is the best-characterised *P. falciparum* VSA and is expressed during the trophozoite stage of the parasite life cycle. The members of this family are large (200-350 kDa) transmembrane proteins that contain multiple extracellular Duffy binding-like (DBL) domains or cysteine-rich inter-domain regions (CIDR) that are arranged in tandem (Figure 1.3). Each *P. falciparum* genome contains approximately 60 genes encoding PfEMP1, with the repertoire of genes differing substantially between parasite strains. Despite the large number of variants that can potentially be expressed, the expression of PfEMP1 is tightly regulated such that a single parasite only expresses one variant of the protein at any given time (Scherf, Lopez-Rubio and Riviere 2008). Several systems have been used to classify the different PfEMP1 variants, including a widely used system that divides the variants into several groups (groups A, B and C) based on conserved upstream regulatory sequences, chromosomal gene position (centromeric or telomeric) and the direction of gene transcription (Lavstsen *et al.* 2003). Other methods of classification are based on the sequence of an N-terminal DBL1 α domain that is conserved in most PfEMP1 variants (Bull *et al.* 2007) and the presence of so-called domain cassettes (DCs), or pairs of domains that are commonly found in different variants (Rask *et al.* 2010).

PfEMP1 has been closely linked to malaria pathology due to the ability of its different domains to bind to various endothelial receptors including ICAM-1, VCAM-1, platelet endothelial cell adhesion molecule-1 (PECAM-1), CD36, CSA and endothelial protein C receptor (EPCR) (Salanti *et al.* 2004; Rowe *et al.* 2009; Turner *et al.* 2013). For instance, VAR2CSA, a relatively conserved PfEMP1

variant that mediates binding of IEs to placental CSA, has been linked to pregnancy-associated malaria (Salanti *et al.* 2004; Magistrado *et al.* 2008). Moreover, the CIDR domains of DC8 and DC13 PfEMP1 subtypes have been linked to cerebral malaria, presumably through binding to the endothelial receptor EPCR on human brain endothelial cells (Claessens *et al.* 2012; Avril *et al.* 2012; Turner *et al.* 2013). Several PfEMP1 variants belonging to group A are able to mediate binding of IEs to uninfected erythrocytes to form rosettes, which have been linked to severe malaria in African children (Carlson *et al.* 1990; Rowe *et al.* 1995). More generally, parasites that express group A PfEMP1 have been associated with severe malaria (Warimwe *et al.* 2009). PfEMP1 molecules are key targets of the immune response during natural infection, and antibodies against PfEMP1 have been associated with protection from malaria (Chan *et al.* 2012). Furthermore, as will be discussed later, antibodies against VAR2CSA that are generated after the first pregnancy are thought to protect multigravid women from subsequent pregnancy-linked malaria episodes (Duffy and Fried 2003; Salanti *et al.* 2004). Nevertheless, the high degree of PfEMP1 variability has proven to be a serious obstacle to its use as a general malaria vaccine candidate, with the exception of VAR2CSA, which is a promising pregnancy-associated malaria vaccine candidate (Fried and Duffy 2015).

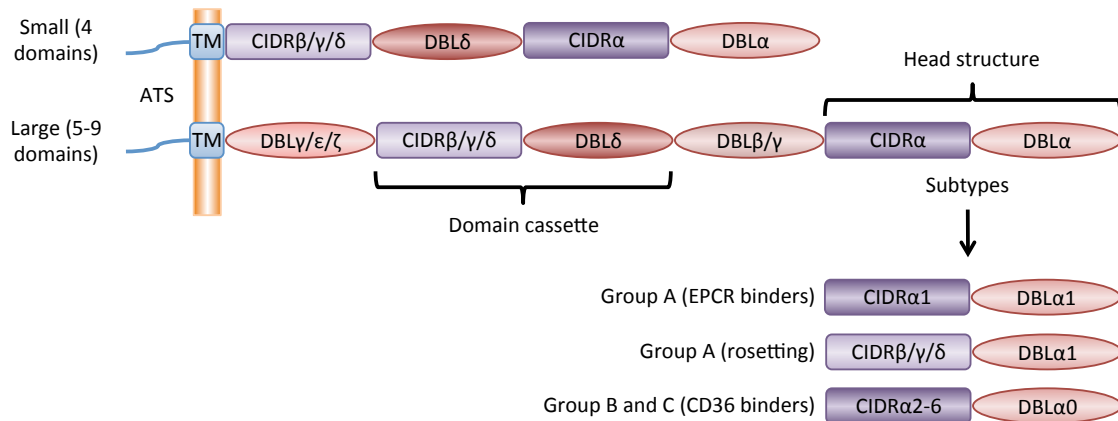


Figure 1.3. Domain architecture of PfEMP1 variants. PfEMP1 is made of Duffy binding-like (DBL) and cysteine-rich inter-domain regions (CIDR) domains that are arranged in tandem. Some domain types preferentially form pairs that are known as domain cassettes (DCs). Small and large PfEMP1 variants have similar architectures, but large variants have unique DBL domains that are absent in small variants. All PfEMP1 variants contain a transmembrane (TM) region and a conserved acidic terminal sequence (ATS). The PfEMP1 head structure is semi-conserved and is commonly used for binding to diverse receptors, depending on the exact domain subtypes present. Modified with permission from (Smith 2014).

RIFINs

RIFINs are small (30-45 kDa) transmembrane proteins that make up the largest *P. falciparum* VSA family (~150 variants). RIFINs have been studied most in association with their role as variant antigens on the IE surface, but these antigens also appear to be widely expressed at different stages of the life cycle including in sporozoites, gametocytes and merozoites (Florens *et al.* 2002; Le Roch *et al.* 2003). Nevertheless, their function and level of surface expression during these stages are not known. RIFINs are encoded by *rif* genes that contain two exons, with the first encoding a canonical signal peptide and the second encoding the protein proper. The second exon also contains a *Plasmodium* export element (PEXEL) motif that regulates the export of RIFINs from the parasitophorous vacuole to the IE membrane. RIFIN expression appears to be regulated by upstream repressor elements at the transcriptional level (Tham *et al.* 2007; Wang *et al.* 2009a; Goel *et al.* 2015), but it is unclear whether individual parasites are restricted to express a single RIFIN variant, as is the case for PfEMP1. *rifs* are considered to be part of the *pir* (*Plasmodium* interspersed repeat) family which includes the *stevor* genes in *P. falciparum* and gene families that are expressed in different *Plasmodium* species, including in *P. berghei* (*bir*), *P. vivax* (*vir*) and *P. knowlesi* (*kir*) (Janssen 2004).

The sequence alignment of different RIFIN variants suggests that RIFINs consist of a conserved N-terminal region, a central hypervariable region, a transmembrane region and a basic cytoplasmic tail (Figure 1.4) (Joannin *et al.* 2008). It was initially thought that RIFINs have two transmembrane regions at each end of the protein, but recent evidence based on computational analysis, *in*

vitro translation models and enzymatic digest experiments suggests that RIFINs only contain a single C-terminal transmembrane region (Bultrini *et al.* 2009; Bachmann *et al.* 2015; Goel *et al.* 2015). RIFINs can be divided into type A and type B subgroups based on the number of conserved cysteines, overall sequence similarity and the presence of a 25-amino acid segment that is present in A-RIFINs but absent in B-RIFINs (Joannin *et al.* 2008). These different subgroups appear to have distinct functions and subcellular locations at the various stages of the parasite life cycle (Petter *et al.* 2007). During the IE stage, A-RIFINs are expressed in Maurer's clefts and on the IE surface, whereas B-RIFINs are expressed in the parasite and in the parasitophorous vacuole (Petter *et al.* 2007). In merozoites, A-RIFINs are expressed at the apex of the parasites while B-RIFINs are distributed evenly in the merozoite cytosol (Petter *et al.* 2007).

The function of the RIFIN family is still unclear and is under active investigation, but their diversity suggests that these antigens are involved in immune evasion. Furthermore, a recent study reported that specific RIFIN variants bind to the blood group A antigen to mediate rosetting with uninfected erythrocytes, suggesting that RIFINs play a role in malaria pathology and may have influenced blood group distribution in the human population (Goel *et al.* 2015). RIFINs are targets of the naturally acquired immune response, and individuals in malaria-endemic areas acquire anti-RIFIN antibodies that increase with age (Abdel-Latif *et al.* 2002). These antibodies were associated with a higher rate of parasite clearance after chemotherapy (Abdel-Latif *et al.* 2003) and appear to be a substantial component of the anti-VSA response (Abdel-Latif *et al.* 2004).

A-RIFINs



B-RIFINs

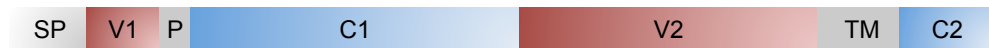


Figure 1.4. Representation of A-RIFINs and B-RIFINs. The C1A region is a 25-amino acid segment that is present in A-RIFINs but not B-RIFINs. SP, signal peptide; V, variable region; P, PEXEL motif; C, constant region; TM, transmembrane region. The images are not to scale. Based on information from (Joannin *et al.* 2008; Bultrini *et al.* 2009; Goel *et al.* 2015).

Other VSAs

Other VSAs expressed by *P. falciparum* include the STEVOR, SURFIN and PfMC-2TM families. STEVORs and PfMC-2TM are lower copy number gene families (~32 and 13 respectively) that have similar molecular weights to RIFINs (Bachmann *et al.* 2015). STEVORs have been reported to bind to glycophorin C on the erythrocyte surface to facilitate the formation of rosettes and merozoite invasion (Niang *et al.* 2014). SURFINs are low copy number (~10) high-molecular weight antigens that are expressed on the surfaces of merozoites and IEs. These antigens have some sequence similarity to PfEMP1 (Winter *et al.* 2005). Relatively little is known about the function of the SURFINs and PfMC-2TM antigens at present.

1.1.6. Merozoite surface antigens

This section will only briefly introduce merozoite surface antigens, which will be discussed later in the context of malaria vaccine development and the immune response against blood stage antigens. For a recent and comprehensive review, please refer to (Beeson *et al.* 2016). Merozoite surface antigens have primarily been studied with regard to their role in the invasion of host erythrocytes. These antigens are either constitutively expressed on the merozoite surface or are retained in apical organelles such as the rhoptries and micronemes and brought to the surface during merozoite release from IEs (Beeson *et al.* 2016). Merozoite antigens are associated with the membrane via GPI anchors (e.g. merozoite surface protein-1 (MSP1), MSP2), hydrophobic transmembrane domains (e.g. erythrocyte binding antigens (EBAs), PfrH1-4, apical membrane antigen-1 (AMA1)) or non-covalent associations (e.g. PfrH5, MSP3, MSP7) (Beeson *et al.* 2016). MSP1 is an abundant, polymorphic antigen on the merozoite surface that binds to erythrocyte spectrin to allow merozoite egress (Das *et al.* 2015) and is thought to play a role in erythrocyte invasion through binding to glycophorin A, band 3 and erythrocyte surface glycosaminoglycans (Beeson *et al.* 2016). MSP2 is a dimorphic antigen that is thought to be essential for invasion (Sanders *et al.* 2006), but its target receptor on the erythrocyte surface is still unknown. EBAs and the PfrH proteins (with the exception of PfrH5) are proteins that are derived from micronemes and rhoptries, respectively, and are thought to play overlapping roles in merozoite attachment to the erythrocyte surface (Tham, Healer and Cowman 2012). PfrH5 is a smaller member of the PfrH family that forms an interaction with erythrocyte basigin that is essential for invasion (Crosnier *et al.* 2011). AMA1 is a heavily disulphide-linked microneme-derived

protein that drives merozoite invasion through interactions with PfRON2 (Lamarque *et al.* 2011), which is parasite-expressed but is translocated to the erythrocyte membrane prior to invasion. MSP3 is a surface-associated protein that has an unknown function, but may be a promising malaria vaccine candidate (see below) (Sirima, Cousens and Druilhe 2011).

1.1.7. Malaria vaccines

Efforts to develop a malaria vaccine began more than 100 years ago, when Etienne and Edmond Sergent attempted to vaccinate birds with *P. relictum* sporozoites in 1910 (Sergent and Sergent 1910). Since then, much progress has been made in this area, but a vaccine with high efficacy has not yet been found. Factors that have hindered the development of a malaria vaccine include the inherent diversity of malaria antigens due to clonal variation, the absence of well-defined correlates of protection, and the difficulty in inducing robust and long-lasting immune responses in malaria-exposed individuals (Cunnington and Riley 2010; Riley and Stewart 2013). Nevertheless, several factors suggest that it is possible to produce a malaria vaccine that prevents disease. Adults living in malaria-endemic areas are immune to clinical symptoms, showing that it is possible to develop anti-disease immunity under natural conditions. The historical use of *Plasmodium* parasites to treat neurosyphilis demonstrated that the inoculation of parasites resulted in at least partial immunity against subsequent inoculation of parasites of the same strain (Jeffery 1966). Furthermore, the inoculation of adults with irradiated *P. falciparum* sporozoites through >1000 mosquito bites was shown to produce sterilizing immunity against homologous and heterologous strains in some individuals (Clyde *et al.* 1973a; 1973b; Edelman *et al.* 1993; Hoffman *et al.* 2002).

Pre-erythrocytic malaria vaccines

Due to the morbidity and mortality associated with *P. falciparum*, most vaccines under development have focused specifically on this organism. Vaccines based on the pre-erythrocytic stage of the life cycle are currently the most advanced in terms of clinical trial development. The RTS,S/AS01 vaccine, which is based on the sporozoite surface circumsporozoite protein (CSP), has been tested in a multi-centre phase III clinical trial in Africa (RTSS Clinical Trials Partnership 2015). This vaccine consists of the central repeat region (containing B cell epitopes) and C-terminal domain (containing T cell epitopes) of CSP mixed with the hepatitis B surface antigen to form virus-like particles. The vaccine had an efficacy of about 28% in children (5-17 months) and about 18% in infants (6-12 weeks) over the entire period of the trial (RTSS Clinical Trials Partnership 2015). The protection provided by the vaccine waned over time, but was increased by a booster dose provided halfway through the study (Olotu *et al.* 2013; RTSS Clinical Trials Partnership 2014; 2015). A second vaccine candidate that is based on a sporozoite surface protein is ME-TRAP, which contains the adhesion domain of the thrombospondin-related adhesion protein (TRAP) coupled with 19 epitopes from eight antigens (Moorthy 2003). Recently, the results of a phase 2b clinical trial in Kenya using this antigen were reported (Ogwang *et al.* 2015). In this trial, the vaccine was given in a prime/boost regimen, with chimpanzee adenovirus 63 (ChAd63) and modified vaccinia Ankara (MVA) viral vectors used to prime and boost, respectively. The study reported a 67% efficacy in reducing *P. falciparum* infection as determined by a PCR endpoint.

While subunit vaccines based on sporozoite antigens are currently the most advanced in terms of clinical trials, whole attenuated sporozoite vaccines have also been developed in an attempt to improve on the early studies that used irradiated sporozoites. Recent breakthroughs in producing large numbers of aseptic and pure sporozoites that meet regulatory safety requirements have advanced this field (Hoffman *et al.* 2010). Recent studies have shown that intravenous injection of a large number of irradiated sporozoites (five doses of 135,000 sporozoites or four doses of 270,000 sporozoites) can induce sterilizing immunity in >50% of malaria-naïve individuals tested (Seder *et al.* 2013; Ishizuka *et al.* 2016). Besides irradiated sporozoites, genetically attenuated sporozoites (GAPs), which are arrested at well-defined stages of sporozoite or liver-stage development through mutations of particular genes, have also been considered for use as vaccine candidates (Vaughan, Wang and Kappe 2010). GAPs are thought to have the advantage of containing more carefully defined alterations than irradiated sporozoites, which are attenuated by random DNA damage. Furthermore, while irradiated sporozoites are replication-incompetent and terminate at the early liver stage, GAPs can be designed to terminate at a later stage, allowing them to express a wider spectrum of pre-erythrocytic antigens. GAPs have been shown to induce protective immunity against homologous and heterologous challenge in mice (Aly *et al.* 2008; Butler *et al.* 2011), but are still at an early stage in human trials. GAPs with *p52* and *p36* gene deletions (preventing the development of parasitophorous vacuoles in hepatocytes) were recently tested in humans but one out of six volunteers developed blood-stage parasitemia, suggesting that further attenuation of the parasites needs to be conducted (Spring *et al.* 2013). An alternative approach to

sporozoite attenuation is the inoculation of infectious sporozoites under chemoprophylactic (usually chloroquine) cover. The rationale behind this approach is that chloroquine only acts on blood-stage parasites and thus allows the complete development of liver stages prior to parasite elimination. This method has been shown to induce protection in malaria-naïve individuals with fewer mosquito bites (36-45) required (Roestenberg *et al.* 2009; 2011) than with irradiated sporozoites (>1000). This protection is thought to be largely mediated by cytotoxic T cell responses against pre-erythrocytic stages (Bijker *et al.* 2013; 2014).

Pre-erythrocytic-based approaches are attractive candidates for vaccine development as protection at this stage would provide sterile immunity, which would block both malaria infection and transmission. Furthermore, the small number of sporozoites (~10-100) injected by mosquitoes during a blood meal makes this an ideal place to prevent infection (Crompton *et al.* 2014). Nevertheless, pre-erythrocytic vaccines run the risk of reducing the acquisition of natural immunity against blood-stage parasites, potentially leading to more severe consequences if a breakthrough infection occurs. For instance, when the RTS,S vaccine was administered to children in malaria-endemic areas without a booster dose, initial protection from severe malaria was reported, but higher rates of severe malaria occurred at later time points, especially in areas with high malaria transmission (RTSS Clinical Trials Partnership 2015). Whether this was due to the loss of naturally acquired immunity or was simply a stochastic event needs to be further investigated. A further challenge with pre-erythrocytic vaccines is to demonstrate that protection extends to heterologous strains of *P.*

falciparum, as most of the studies described used homologous strains for challenge.

Blood-stage malaria vaccines

Blood-stage malaria vaccines are designed to provide protection akin to that obtained through naturally acquired immunity, and generally aim to reduce parasite load and prevent disease rather than to provide sterile immunity (Riley and Stewart 2013). All seven blood-stage antigens that have been tested in (primarily early-stage) human clinical trials are merozoite surface antigens: AMA1, EBA175, glutamate-rich protein (GLURP), MSP1, MSP2, MSP3 and serine repeat antigen-5 (SERA5) (Beeson *et al.* 2016). Combinations of these antigens have often been used in these trials. In general, vaccines based on these antigens can induce high antibody titres but do not induce substantial protection in individuals from malaria-endemic areas, possibly due to the presence of multiple alleles of these antigens (Lyon *et al.* 2008; Biswas *et al.* 2014; Beeson *et al.* 2016). A clinical trial using MSP3 on children in Burkina Faso reported partial protection from clinical malaria, but this was an early-phase trial with a small cohort and a short follow-up period (Sirima, Cousens and Druilhe 2011).

A promising vaccine candidate that has emerged relatively recently is the merozoite surface antigen PfRH5. Unlike other merozoite antigens, PfRH5 has been shown to be essential for merozoite invasion as it forms an essential interaction with the Ok blood group antigen basigin on the surface of target erythrocytes (Crosnier *et al.* 2011). Furthermore, PfRH5 cannot be deleted and is critical for the survival of blood stage parasites (Baum *et al.* 2009). Individuals from malaria-endemic areas do not have high titres of antibodies against PfRH5 (Douglas *et al.* 2011; Tran *et al.* 2014), but when present, these antibodies are associated with protection against malaria (Tran *et al.* 2014). PfRH5 is conserved

in diverse *P. falciparum* strains (Manske *et al.* 2012; Bustamante *et al.* 2013) and antibodies from animals immunised with PfRH5 were able to cross-neutralize many different parasite strains (Douglas *et al.* 2011; Bustamante *et al.* 2013; Reddy *et al.* 2013). A recent vaccination trial on *Aotus* monkeys reported significant levels of protection in animals that correlated with the levels of anti-PfRH5 antibodies (Douglas *et al.* 2015). Human clinical trials with PfRH5 are currently under development.

VSAs on the IE surface are challenging targets for vaccine development due to extensive clonal antigenic variation, which leads to a predominantly variant-specific antibody response (Newbold *et al.* 1992). Nevertheless, specific variants of VSA families are being considered as vaccine candidates. For instance, the PfEMP1 variant VAR2CSA has emerged as a promising candidate to prevent pregnancy-associated malaria (Fried and Duffy 2015). VAR2CSA appears to have conserved epitopes that are targets of cross-reactive antibodies (Fried *et al.* 1998b; Gamain *et al.* 2004; Beeson *et al.* 2006; Bir *et al.* 2006), despite some polymorphism in VAR2CSA sequences between different *P. falciparum* strains (Barfod 2007, Bockhorst 2007). The level of antibodies against VAR2CSA increases with parity and is thought to contribute to protection in multigravid women (O'Neil-Dunne *et al.* 2001; Salanti *et al.* 2004; Staalsoe *et al.* 2004). These antibodies have been linked to positive outcomes such as increased birth weight, increased gestational age, higher maternal haemoglobin levels and reduced risk of infection (Duffy and Fried 2003; Salanti *et al.* 2004; Staalsoe *et al.* 2004; Ndam *et al.* 2015). Specific domains from the N-terminus of VAR2CSA are currently under development as malaria vaccine candidates (Fried and Duffy 2015).

Transmission-blocking vaccines

As the focus of malaria vaccines has shifted to the prevention of disease transmission (Nunes *et al.* 2014), transmission-blocking vaccines against sexual-stage parasites and stages expressed in the mosquito have generated substantial interest. These vaccines do not protect immunized individuals, but aim to block transmission by eliciting the production of antibodies that target antigens that are unveiled in the mosquito stages. The sexual stage antigens that are under consideration as malaria vaccine candidates include Pfs25, Pfs230 and Pfs48/45 (van Dijk *et al.* 2001; Eksi *et al.* 2006; Wu *et al.* 2008; Kapulu *et al.* 2015). The latter three antigens are expressed on the gamete surface and can elicit antibodies in humans during natural infection (Healer *et al.* 1999; van Dijk *et al.* 2001; Eksi *et al.* 2006; Bousema *et al.* 2007; 2010), but the only antigen to have been tested so far in human clinical trials is Pfs25, which is only present in mosquito parasite stages (Ockenhouse *et al.* 1998; Wu *et al.* 2008). In one of these trials, Pfs25 was included as part of a multi-stage vaccine, NYVAC-Pf7 (Ockenhouse *et al.* 1998). High levels of anti-Pfs25 antibodies were observed, but these were unable to block transmission as determined by a membrane feeding assay, a standard test that can measure the inhibition of gametocyte infection of *Anopheles* mosquitoes.

Malaria vaccine outlook

The eradication of malaria is likely to require an efficacious malaria vaccine. While an extremely promising candidate has yet to emerge, the malaria research community has been working hard to produce an effective malaria vaccine based on virtually every stage of the *P. falciparum* life cycle. Given the unique advantages offered by the different stages as malaria vaccine candidates, it is likely that a successful vaccine will incorporate parasite antigens from multiple stages. Nevertheless, the rich diversity of *P. falciparum* antigens that is accessible due to the availability of the complete parasite genome sequence has not been fully utilized, as the vast majority of vaccine candidates in clinical trials are based on antigens that were identified prior to the publication of the genome (Conway 2015). Some of the yet-uncharacterized antigens could be promising vaccine candidates, but the sheer number of genes expressed by *P. falciparum* (~5400) presents a challenge for down-selection of candidate antigens. A potentially promising way to move forward is an antigen-agnostic approach that identifies antibodies that target *P. falciparum*, for instance in protected individuals, and that uses these antibodies to identify candidate antigens, as this technique allows the potential identification of antigens that elicit a protective immune response in malaria-exposed individuals.

1.2. B cells and antibodies

1.2.1. B cell receptor and signalling

B cells are a major class of lymphocytes that function to produce antibodies that target diverse antigens including lipids, proteins, peptides and carbohydrates. In humans, B cells develop in the bone marrow and subsequently enter the bloodstream for recirculation in secondary lymphoid organs such as lymph nodes and the spleen in search of target antigens. B cells detect antigens through their B cell receptors (BCRs), which are membrane-bound forms of antibodies that activate B cells upon antigen recognition (Figure 1.5) (Kurosaki, Shinohara and Baba 2010). BCRs initiate signal transduction through the accessory molecules Ig α and Ig β , which contain immunoreceptor tyrosine-based activation motifs (ITAMs) in their cytoplasmic regions. When BCRs are cross-linked by free antigens or antigens in immune complexes, Src-family kinases are activated and phosphorylate the tyrosine residues on the Ig α and Ig β ITAMs. The phosphorylated tyrosines act as binding sites for SH2 domains of other kinases, which then initiate a signalling cascade that leads to B cell activation. BCR signalling is enhanced by a coreceptor complex containing the membrane proteins CD21/CR2, CD19 and CD81. CD21 binds to C3d, a cleavage product of the complement protein C3 that attaches to surfaces targeted by the complement system. Hence, an antigen that is targeted by the complement system can strongly activate B cells due to the simultaneous binding of the antigen to the BCR and C3d to CD21 (Cherukuri, Cheng and Pierce 2001). CD81 is required for surface expression of CD19 and is thought to act as a chaperon to aid its secretion (Shoham *et al.* 2003). CD19 contains cytoplasmic tyrosine residues that

can recruit additional kinases to the BCR complex upon CD21 engagement (Wang *et al.* 2002). Therefore, simultaneous engagement of the BCR and coreceptor complex sends strong signals that favour B cell activation.

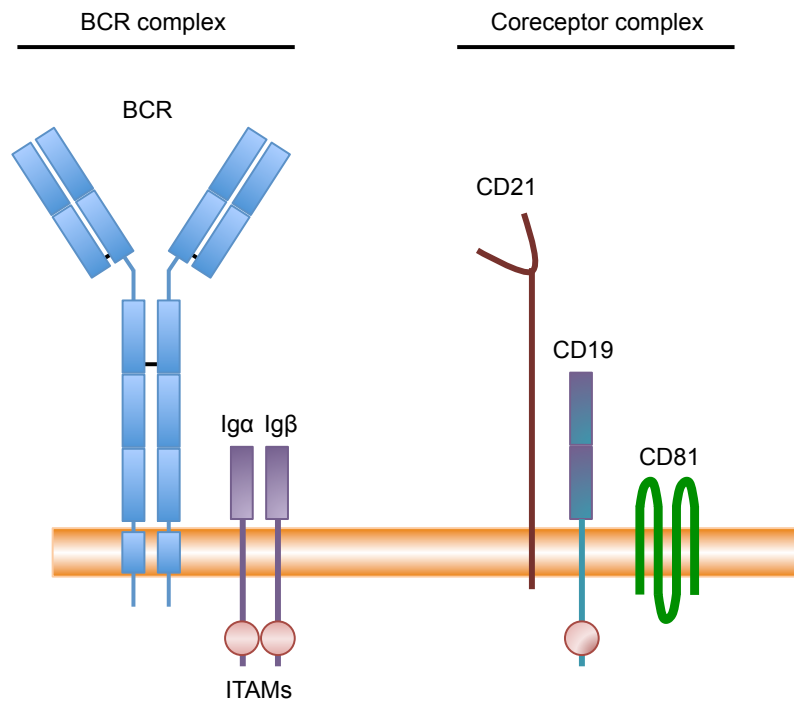


Figure 1.5. The B cell receptor (BCR) and B cell coreceptor complexes. The BCR complex is made of the BCR, which is a membrane-bound form of an antibody, and the signalling molecules Igα and Igβ, which contain immunoreceptor tyrosine-based activation motifs (ITAMs) that trigger downstream activation pathways. The coreceptor complex is made of the complement receptor CD21, the tetraspanin CD81, and CD19, which has cytoplasmic tyrosine residues that can augment B cell activation.

1.2.2. Antibody structure

Immunoglobulin domains of antibodies

Antibodies are symmetric molecules that consist of two identical functional units, each containing a heavy chain with four or five immunoglobulin domains and a light chain with two immunoglobulin domains (Figure 1.6). The heavy and light chains are encoded on different chromosomes – heavy chains are encoded on chromosome 14 while the kappa (κ) and lambda (λ) light chains are encoded on chromosomes 2 and 22, respectively. In both the heavy and light chains, the N-terminal domains are known as variable domains due to their relatively diverse sequences, while the remaining domains are more conserved and are known as constant domains. The variable and constant domains have a conserved fold that is known as the immunoglobulin-like fold as it was first discovered in antibodies (Williams and Barclay 1988). However, subsequent studies have revealed that this fold is extremely conserved not just in antibodies but also in many other proteins with unrelated functions including the T cell receptor and coreceptors (CD3, CD4, CD8), growth factor receptors (platelet-derived growth factor receptor or PDGFR), and adhesion molecules (neural adhesion molecule or NCAM; lymphocyte function-associated antigen 1 or LFA-1) (Williams and Barclay 1988). Each immunoglobulin-like domain contains about 100 amino acids and has a “sandwich” structure made of two opposing β -sheets that are held together by a conserved disulphide bond (Figure 1.7). The ends of the β -sheets are linked by flexible loops that are usually involved in binding to target molecules. Analysis of the structures revealed that the antibody constant and variable domains have slightly different structures, as the variable

domains have nine β -strands while the constant domains have seven. The vast majority of domains from other proteins with immunoglobulin-like folds are similar to either antibody variable domains (V-type) or constant domains (C-type), but some intermediate (I-type) domains that fall in between the two types have also been found (Harpaz and Chothia 1994). Examples of I-type domains includes the leukocyte-associated immunoglobulin-like 1 (LAIR1) collagen-binding domain (Brondijk *et al.* 2010), which will feature prominently in this study.

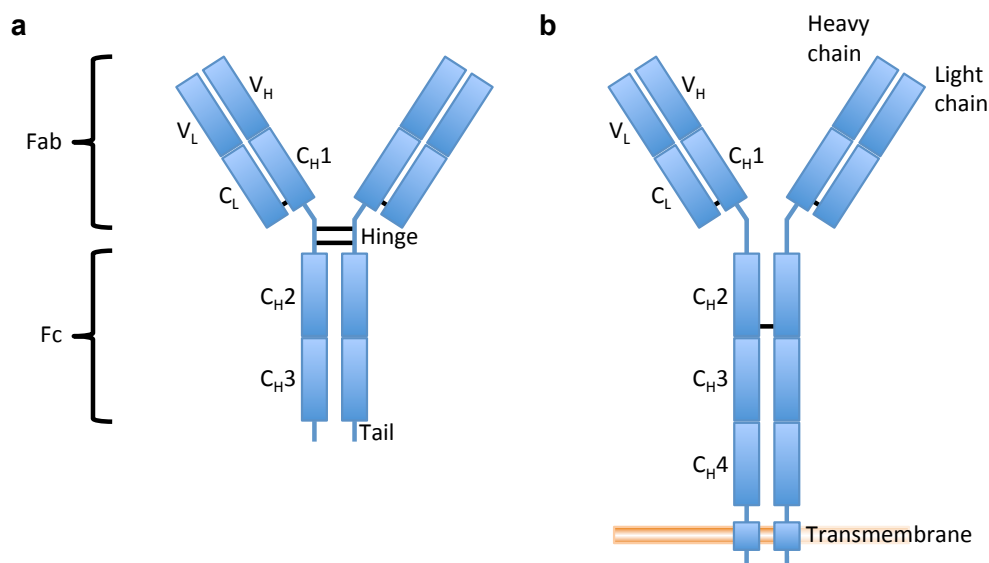


Figure 1.6. Representation of the secreted form of the IgG antibody isotype (a) and the membrane-bound form of IgM (b). IgG has three constant domains while IgM has four constant domains. The variable and first constant domains make up the Fab, while the remaining constant domains form the Fc region. The black lines show disulphide bonds between different antibody chains.

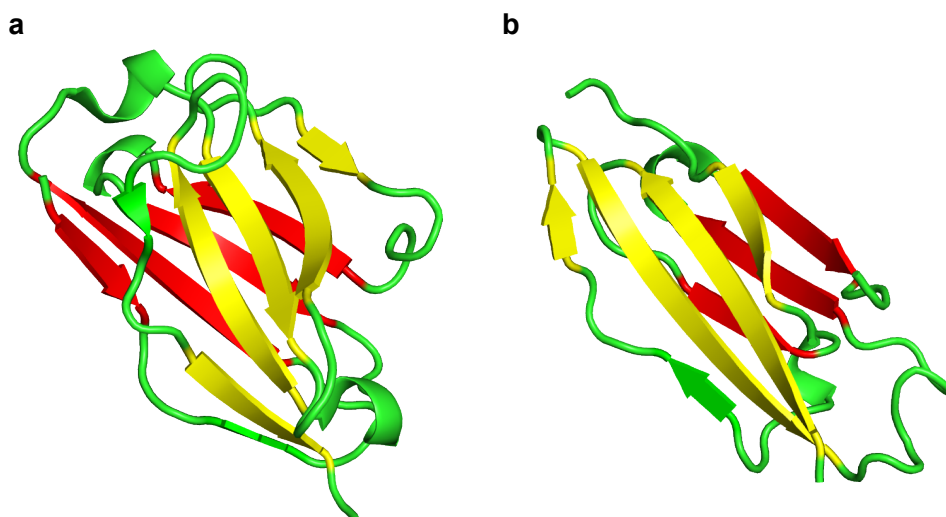


Figure 1.7. Examples of a V-type (a) and a C-type (b) immunoglobulin domain. The opposing β -sheets are highlighted with different colours. The images were self-made using PyMOL (Protein Data Bank accession 2zpk) (Nogi *et al.* 2008).

The antibody variable domain

The variable domains of the heavy and light chain (V_H and V_L , respectively) pair to form the antigen-binding site, which together with the N-terminal constant domains (C_H1 and C_L) is referred to as a Fab (fragment, antigen binding) (Figure 1.6) (Schroeder and Cavacini 2010). The Fab is followed by a hinge region, which confers flexibility on antibodies such that both antigen-binding sites can often bind to targets simultaneously, increasing the avidity of the antibody-antigen interaction. Each variable domain contains three loops protruding from the β -sheet sandwich that are known as complementarity-determining regions (CDRs) or hypervariable regions as they have the most diverse sequences in the variable domain. The hypervariable loops are supported by four adjacent, more conserved framework (FR) regions. Antibody heavy-light chain pairing brings together the six CDR loops from the two chains to form a large surface that is used for antigen binding. The flexibility of the loops enables different antibodies to form distinct antigen-binding surfaces, allowing the antibody repertoire as a whole to bind to antigens with different shapes and sizes. Moreover, in some cases, framework residues can also contribute to antigen binding (Sela-Culang, Kunik and Ofran 2013). The flexibility of the antigen-binding surface enables antibodies to recognize both conformational epitopes that are formed by folding of target proteins, and linear epitopes such as those that are made by consecutive amino acids in a polypeptide sequence.

The antibody constant region

The remaining C_H2 and C_H3 (and C_H4 if present) domains of the heavy chains are collectively known as the Fc (fragment, crystallisable) portion (Figure 1.6) (Schroeder and Cavacini 2010). The Fc region is used to distinguish between different antibody subclasses and confers unique effector functions on each subclass depending on the binding specificity of this region. Humans have five main antibody subclasses: IgD, IgM, IgA, IgE and IgG (Figure 1.8). IgD and IgM isotypes act as antigen receptors on naïve B cells, but the latter is also secreted as pentamers (or sometimes hexamers) that are generally formed through binding to a protein known as the J chain. Due to their multimeric structure, pentameric IgM antibodies are especially effective in activating the complement system. IgA antibodies are divided into IgA1 and IgA2 isotypes, with IgA1 antibodies containing a much longer hinge region than IgA2 antibodies (Woof and Kerr 2004). IgA molecules can pair with the J chain to form dimers that can be transported through epithelia as part of mucous secretions in the gut, tears and breast milk. IgA molecules in the blood lack the J chain and are monomeric. IgE molecules bind with high affinity to Fc receptors on basophils, mast cells and eosinophils and are involved in protection against helminths, but also play a role in allergic responses. IgG molecules are the main antibody subclass in the blood and can be divided further into IgG1, IgG2, IgG3 and IgG4 isotypes. IgG2 and IgG4 antibodies are thought to act mainly by neutralization of targets, while IgG1 and IgG3 antibodies are able to activate the complement pathway and bind to Fc receptors on macrophages, neutrophils and natural killer cells to mediate cellular responses such as opsonic phagocytosis and antibody-mediated cellular cytotoxicity. As is the case for the heavy chain Fc regions, the light chains are also

divided into isotypes (κ or λ), but these isotypes have differing variable regions as well as constant regions.

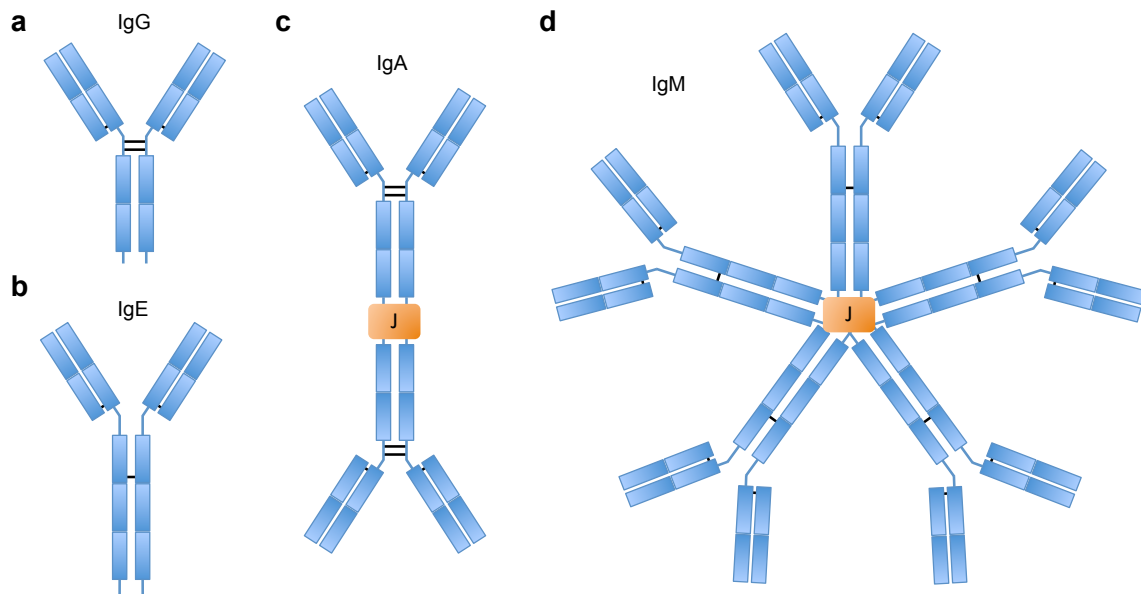


Figure 1.8. Representations of the different antibody isotypes. a, IgG. b, IgE. c, dimeric IgA held together by a J chain. d, pentameric IgM bound by a J chain.

1.2.3. V(D)J recombination and B cell development

Introduction to V(D)J recombination

Antibodies are potentially able to recognize millions of potential antigens from different macromolecule classes. The generation of a diverse antibody repertoire from a limited number of precursor genes relies mainly on the process of V(D)J recombination. V(D)J recombination occurs between variable (V), diversity (D) and joining (J) segments in the antibody heavy chain, and between V and J segments in the light chain. These segments are present in multiple copies: the human heavy chain locus contains about 38-46 V genes, 23 D genes and 6 J genes, the κ locus contains 34-38 V genes and 5 J genes, and the λ locus contains 29-33 V genes and 4-5 J genes (Lefranc *et al.* 2009). During V(D)J recombination, single V, D and J segments are chosen at random and combine to form a gene that encodes the antibody variable domain. Combinatorial diversity arises from the many possible combinations of V, D and J genes, as well as from the pairing of different antibody heavy and light chains (Figure 1.9). The number of possible antibodies that can arise from combinatorial diversity alone is about two million. When junctional diversity (described later) is also considered, the number of possible antibodies that can be made has been estimated to be an incredible 10^{16} (Schroeder and Cavacini 2010).

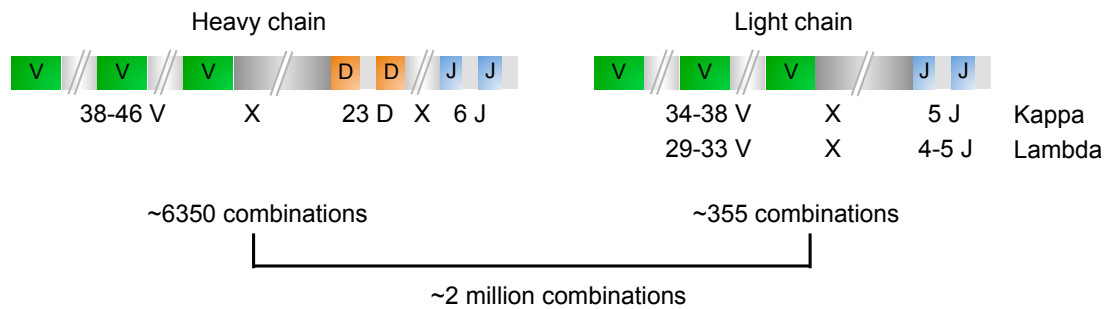


Figure 1.9. Combinatorial diversity. Heavy chains contain multiple copies of variable (V), diversity (D) and joining (J) segments while light chains contain multiple V and J segments. During V(D)J recombination, a single copy of each segment is chosen at random to combine to form a rearranged gene that codes for the antibody variable domain. Many different combinations can be made from these segments, giving rise to combinatorial diversity. It is estimated that two million combinations can be made when heavy-light chain pairing is also taken into account. Constant regions have been omitted for clarity. The number of genes belonging to each segment was obtained from the International Immunogenetics Information System (www.imgt.org) (Lefranc *et al.* 2009).

Mechanism of V(D)J recombination

V(D)J recombination is mediated by the recombination-activating gene 1 and recombination-activating gene 2 (RAG1 and RAG2) enzymes, which recognize conserved recombination signal sequences (RSSs) at the ends of the VDJ segments (Figure 1.10) (Schatz and Swanson 2011). These sequences are made of a conserved heptamer (usually 5'-CACAGTG-3', with the initial CAC reported to be essential (Lewis *et al.* 1997)) and an AT-rich nonamer separated by a variable sequence of 12 or 23 nucleotides. V(D)J recombination occurs between a segment containing a 12-nucleotide spacer and a segment containing a 23-nucleotide spacer, in what is known as the "12/23" rule. This avoids non-specific recombination between V and J segments in the heavy chain, as the V and J segments have 23-nucleotide spacers while D segments have 12-nucleotide spacers. Similarly, in the κ light chain, V segments contain 12-nucleotide spacers and J segments contain 23-nucleotide spacers, while in the λ light chain, V segments contain 23-nucleotide spacers and J segments contain 12-nucleotide spacers, ensuring specific V-to-J recombination. During V(D)J recombination, the RAG1/2 complex brings together the two relevant segments at the complementary heptamer sequences and creates double-strand DNA breaks, effectively cleaving the intervening sequence, which forms a closed loop and plays no further role in the process. The ends of the gene segments, which are known as coding ends, form hairpins after RAG activity. These are subsequently cleaved by a complex containing the endonuclease Artemis and DNA-dependent protein kinase (DNA-PK) (Ma *et al.* 2002). Single-strand overhangs are filled in by DNA polymerases, creating extra templated (P) nucleotides that modify the junction sequence. The coding ends can also be modified by exonucleases that

cleave terminal nucleotides and by terminal deoxynucleotidyl transferase (TdT), which randomly inserts a small number of non-templated (N) nucleotides into the ends of the fragments. These modifications introduce junctional diversity by greatly increasing the number of possible sequences that can be generated from a D-to-J, V-to-D, or V-to-J junction. The recombination process is terminated when enzymes in the non-homologous end joining (NHEJ) DNA repair pathway ligate the coding ends.

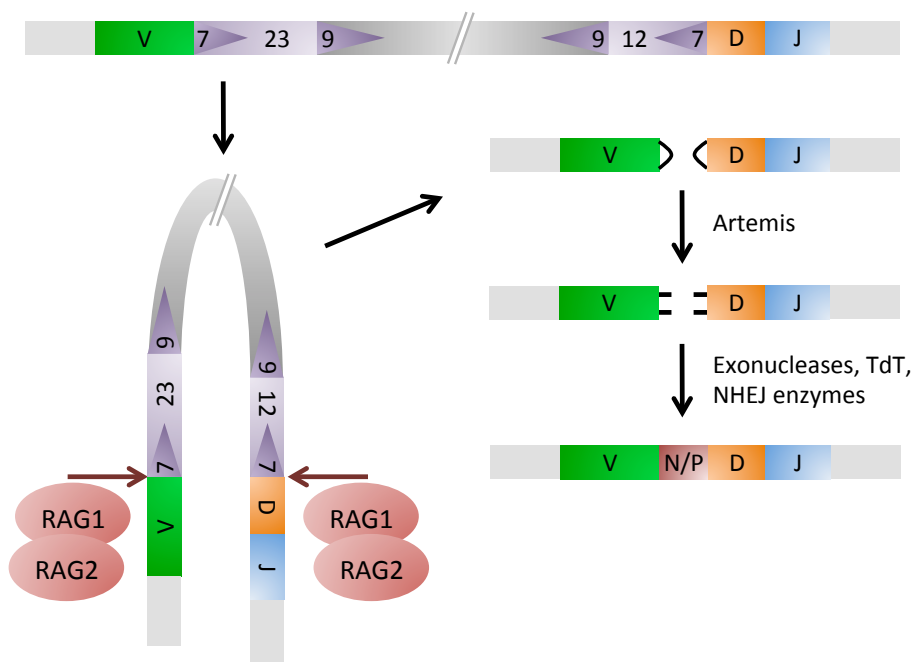


Figure 1.10. Scheme of V-to-D recombination in an antibody heavy chain. In this example, D-to-J recombination has already occurred, and a random V segment is chosen to pair with the rearranged DJ segment. The RAG1/2 complex aligns the recombination signal sequence (RSS) heptamers and cleaves the DNA just prior to the heptamers. The intervening sequence forms a closed loop and plays no further role in the process. The broken coding ends at the end of the V and D segments form hairpins that are cleaved by Artemis and subsequently modified by exonucleases and terminal deoxynucleotidyl transferase (TdT). Enzymes from the non-homologous end joining (NHEJ) pathway then ligate the broken ends, forming a junction bridged by new templated (P) and/or non-templated (N) nucleotides.

Recombination events during B cell development

V(D)J recombination occurs during B cell development in the bone marrow (Hardy and Hayakawa 2001). The process begins with heavy chain rearrangement in early-stage pro-B cells, with D-to-J recombination occurring prior to V-to-DJ recombination (Figure 1.11). The rearranged heavy chain then forms a pre-BCR complex with surrogate light chains and the accessory molecules Ig α and Ig β . In a process known as allelic exclusion, the pre-BCR complex inhibits recombination of the second heavy chain allele, allowing the production of only a single rearranged heavy chain. The successful expression of this complex on the B cell surface also allows the cell to pass a checkpoint from pro-B cells to pre-B cells, which then begin V-to-J recombination at the κ light chain locus. Successful κ rearrangement inhibits recombination at the λ locus, but if the κ rearrangement is non-productive, the cell can attempt rearrangement of the λ locus to produce a functional light chain. The rearranged light chain pairs with the heavy chain to form a complete BCR, which is expressed on the surface and is tested for binding to self antigens. It is thought that weak “tonic” BCR signalling allows the B cells to survive and migrate to the periphery, but strong reactivity for self antigens leads to an attempt to modify the BCR. In a pathway known as receptor editing, B cells reactivate the RAG machinery and undergo further rearrangement at the κ or λ locus to produce an alternative light chain that results in a non-self-reactive BCR (Gay *et al.* 1993; Tiegs, Russell and Nemazee 1993). If this process fails to mitigate self-reactivity, B cells may be rendered anergic or may die by apoptosis, depending on the strength of self-reactivity (Goodnow *et al.* 1988; Nemazee and Bürki 1989). It is thought that moderately self-reactive B cells become anergic to different degrees depending

on the level of constitutive BCR engagement, while strongly self-reactive B cells are deleted (Shlomchik 2008). These mechanisms promote B cell self-tolerance by limiting the number and activity of autoreactive B cells in the periphery.

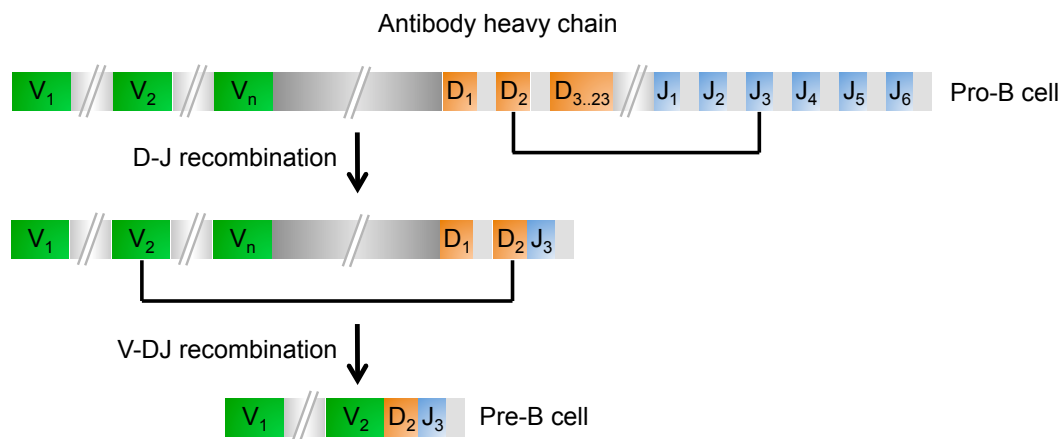


Figure 1.11. V(D)J recombination during B cell development. In early-stage pro-B cells, D-to-J recombination at the antibody heavy chain locus occurs first, followed by V-to-DJ recombination. The rearranged V(D)J locus codes for the variable domain of the pre-BCR in pre-B cells. Rearrangement at the light chain then occurs to allow the formation of a complete BCR (not shown).

Aberrant effects of RAG activity

While V(D)J recombination is limited to B and T lymphocytes due to the restricted expression of RAG1/2 in these cells, aberrant recombination events at sites other than the immunoglobulin and T cell receptor loci can still occur in these cells. This is due to the presence of numerous cryptic RSS sites (once every ~600 bp) that mimic canonical RSS sites in the human genome (Lewis *et al.* 1997). The presence of cryptic RSSs in a sequence of interest can be predicted with an online tool that also provides prediction scores that indicate the likelihood of a cryptic RSS being a legitimate target of the RAG machinery (<http://www.itb.cnr.it/rss/index.html>, (Merelli *et al.* 2010)). Furthermore, RAG1 and RAG2 can bind to thousands of transcriptionally active sites in the human genome, suggesting that there are numerous opportunities for aberrant recombination (Teng *et al.* 2015). Two recent studies has reported that widespread off-target recombination is limited due to the depletion of full cryptic RSS sites at the regions of RAG1 binding (Teng *et al.* 2015), as well as the requirement for the two cryptic RSS sites to be within loops flanked by convergent CTCF-binding elements (Hu *et al.* 2015). Nevertheless, aberrant RAG1/2-mediated recombination can still occur, and these enzymes has been implicated in the formation of chromosomal translocations found in human lymphomas (Kuppers *et al.* 1999; Kuppers and Dalla-Favera 2001; Papaemmanuil *et al.* 2014), as well as in inter-chromosomal genomic rearrangements involving cryptic RSS sites that have led to rare insertions of foreign sequences into the immunoglobulin and T cell receptor loci (Vaandrager *et al.* 2000; Messier 2003; Messier, O'Neill and Finette 2006).

1.2.4. The germinal centre reaction and AID activity

The germinal centre reaction

Following B cell development, mature B cells migrate to the follicles of secondary lymphoid organs such as the lymph nodes and the spleen where antigen encounter occurs. B cell recirculation to these regions is directed by the chemokine CXCL13, which is expressed by cells in the follicle such as follicular dendritic cells (De Silva and Klein 2015). This molecule binds to the chemokine receptor CXCR5 on B cells and acts as a chemoattractant to draw the cells into the follicle. Antigens are also brought into this region through lymphatic vessels or by innate immune cells such as macrophages and dendritic cells. In the follicle, B cells recognize antigens through their BCRs and internalize and process these antigens for presentation via MHC class II molecules to CD4⁺ follicular helper T cells (Lanzavecchia 1985). Signals from cytokines released by the T cells and CD40L-CD40 interactions with these cells integrate with stimulation of the BCR and Toll-like receptors on the B cells to initiate the germinal centre reaction at the B cell follicles (Ruprecht and Lanzavecchia 2006). In this reaction, the B cells undergo rapid proliferation and their antibodies gain increased affinity for their target antigens through the process of affinity maturation. Affinity maturation begins with B cell proliferation and somatic hypermutation of the BCR in the so-called dark zone of the germinal centre (Figure 1.12). The expanded and mutated B cells, which now possess modified affinities for their original targets, then migrate into the so-called light zone, where they compete for binding to a limited amount of antigen presented by follicular dendritic cells. B cells that out-compete their counterparts in binding to antigen receive survival signals,

resulting in the positive selection of B cells with the highest affinity. This process can occur for several cycles before positively selected cells differentiate into plasmablasts (that subsequently become either short-lived plasma cells or long-lived plasma cells that migrate to the bone marrow for long-term secretion of antibodies) or into memory B cells that can be reactivated upon subsequent antigen encounter. The high frequency of somatic hypermutation (about 10^{-3} per generation, (Rajewsky 1996)) can result in antibody sequences that are substantially different from their germline counterparts. For instance, in rare cases, extensive somatic hypermutation in human antibodies has generated extra-long CDR3 loops (>20 residues) that gain unusual binding abilities, such as the ability to bind to clefts in viral proteins (Saphire *et al.* 2001; Pejchal *et al.* 2010; McLellan *et al.* 2011; Ekiert *et al.* 2012). Nevertheless, a recent study has suggested that at least in some cases, only a few mutations are necessary to achieve optimal binding to antigen, and that subsequent mutations may be more useful to broaden the antibody response (Pappas *et al.* 2014).

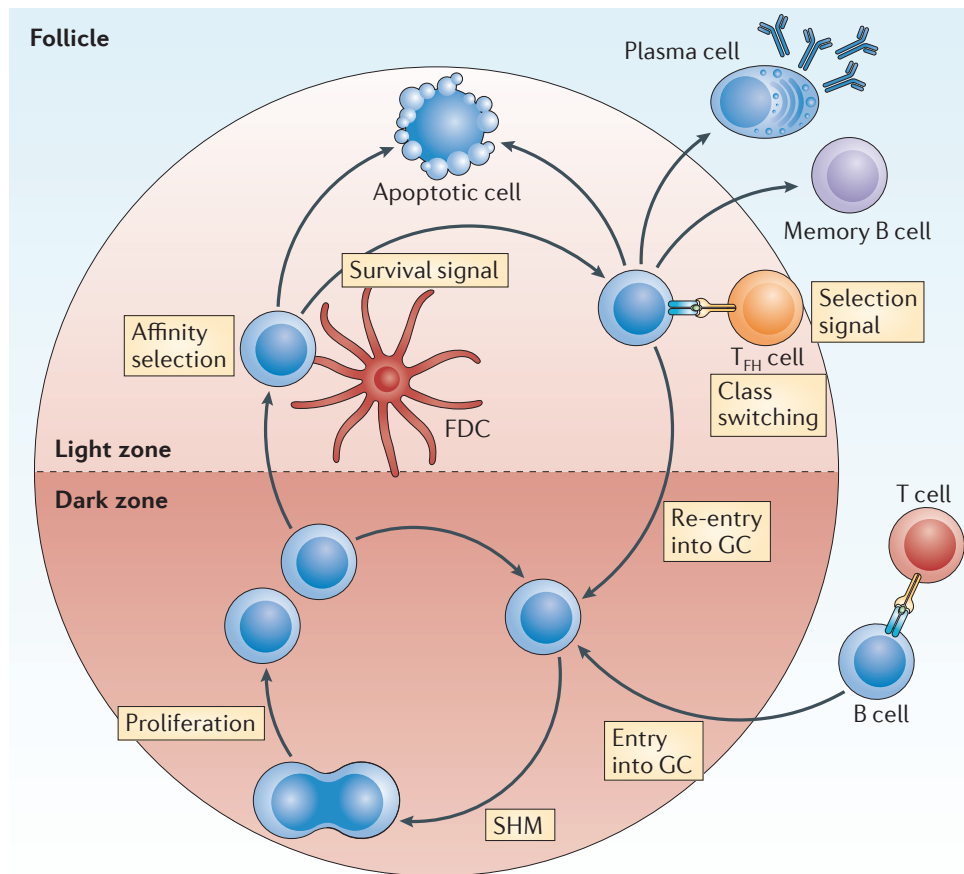


Figure 1.12. The germinal centre reaction. B cells that are activated by antigen binding and interactions with T cells enter the dark zone of the germinal centre, where they proliferate and undergo somatic hypermutation (SHM) to modify their BCR sequence. B cells with modified affinities for the target antigen enter the light zone, where they undergo affinity selection. In this process, the B cells compete for binding to antigen molecules that are presented by follicular dendritic cells (FDCs). B cells that do not bind with high affinity undergo apoptosis, while B cells that bind strongly receive survival signals. These cells can re-enter the dark zone for further rounds of proliferation and SHM, or differentiate into plasma cells that secrete antibodies or memory B cells that can be reactivated upon subsequent antigen encounter. Reprinted with permission from (Heesters, Myers and Carroll 2014).

Somatic hypermutation

The process of somatic hypermutation is mediated by the enzyme activation-induced cytidine deaminase (AID), which is expressed in activated B cells. AID preferentially acts on CDRs in the antibody V region to initiate somatic hypermutation and in the antibody heavy chain switch region to trigger class-switch recombination (see below). The selectivity of this enzyme is partly due to its propensity to bind to specific sequences, with the sequence WRCY (where W is A/T, R is A/G and Y is C/T) being a hotspot of AID activity (Di Noia and Neuberger 2007). AID is a mutagenic enzyme that converts cytosine bases (C) to uracil (U). During the process of somatic hypermutation, this mutation can lead to several outcomes, including normal DNA replication, which results in a C-to-T transition mutation (Figure 1.13). Alternatively, the uracil can be cleaved by the base excision repair enzyme uracil-DNA glycosylase (UNG) to form an abasic site. This site can be repaired by the DNA polymerase Rev1, which tends to add cytosine bases at the site opposite the abasic site, resulting in G-to-C transversions. Other polymerases such as DNA polymerase θ may also act on the site to give rise to transition or transversion mutations (Di Noia and Neuberger 2007). Besides that, the mismatch repair enzymes MSH2/MSH6, along with exonuclease I and DNA polymerase η , may also act on the G:U base pair. These enzymes can induce mutations in adjacent nucleotides, including at A:T pairs, through error-prone base incorporation in a mechanism that has yet to be clearly defined (Figure 1.13) (Di Noia and Neuberger 2007). Furthermore, AID-mediated mutation can also lead to insertions and deletions through a mechanism that was proposed to mimic replication slippage (Wilson *et al.* 1998).

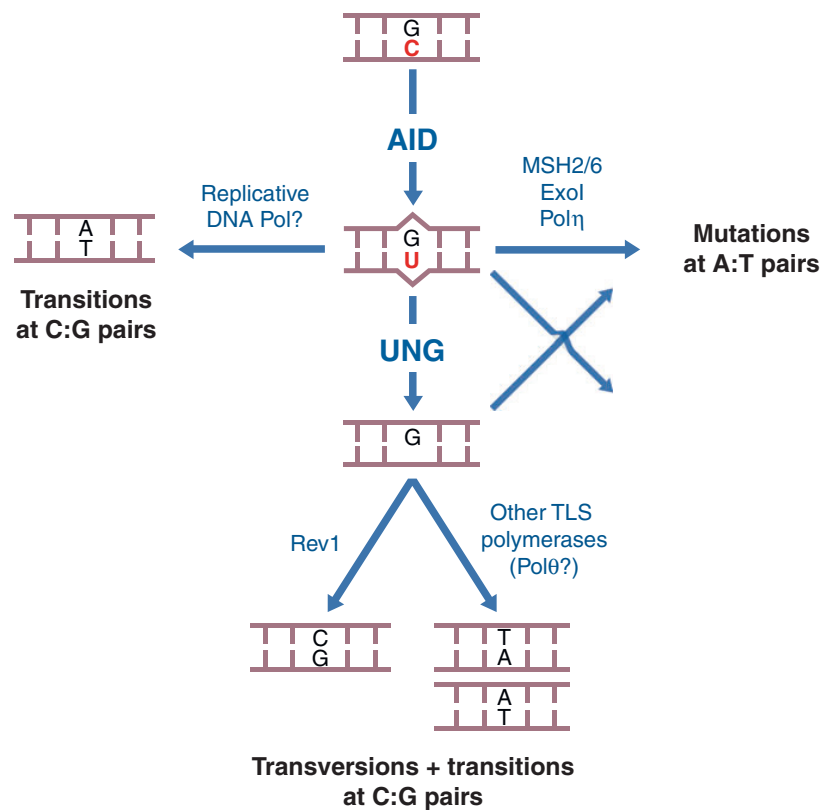


Figure 1.13. Mechanisms of AID-mediated somatic hypermutation. AID converts cytosines to uracils, resulting in a G:U mismatch. Regular DNA replication can be conducted on this site, resulting in a C-to-T transition (left). Alternatively, the uracil can be removed by the uracil-DNA glycosylase (UNG) enzyme, resulting in an abasic site. This site can be filled in by the DNA polymerase Rev1, resulting in a G-to-C transversion, or by other polymerases such as DNA Polθ that give rise to further mutations. The mismatch repair enzymes MSH2 and MSH6, together with accessory proteins, may also act to create mutations in nearby sites, including at A:T pairs. Modified with permission from (Di Noia and Neuberger 2007).

Class switch recombination

Besides being involved in somatic hypermutation, AID also initiates class-switch recombination (Figure 1.14), which converts the IgM isotype to the other isotypes to broaden antibody effector function. AID mediates this process by creating double-strand breaks at switch regions, which are located downstream of the variable region in every heavy chain isotype except for IgD. Specifically, AID creates breaks in the switch regions of IgM (S_μ) and a second isotype downstream of the S_μ region. The downstream switch region is then recombined with the S_μ region, resulting in a gene that encodes an antibody with the same specificity but with a different Fc region. The choice of the isotype that the antibody switches to is thought to be primarily driven by the specific cytokines that are present in the extracellular milieu.

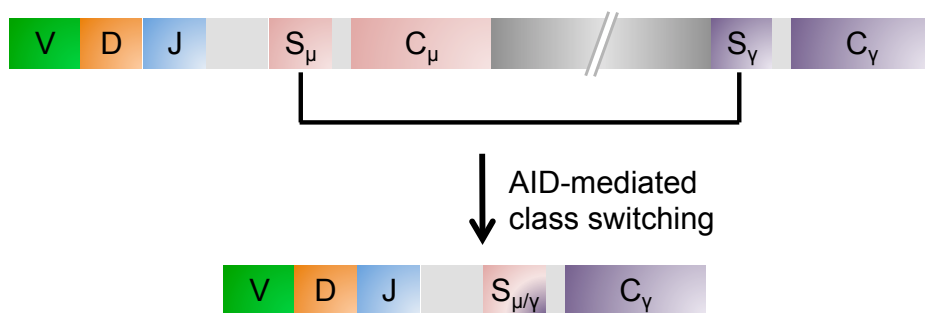


Figure 1.14. Representation of class-switch recombination from IgM to IgG. AID creates double-strand breaks at the switch regions upstream of the IgM and IgG constant regions. The switch regions then recombine, bringing the IgG constant domains adjacent to the variable region.

Aberrant effects of AID activity

AID activity is tightly regulated by several mechanisms to avoid the creation of off-target mutations and double-strand breaks. First, AID is only expressed in activated and germinal centre B cells that are stimulated by cytokines from T cells and/or B cell activators such as BAFF, APRIL and TLR agonists (Casellas *et al.* 2016). Even when expressed in B cells, AID is normally retained in the cytoplasm by members of the heat shock protein 90 pathway and by translation elongation factor eEF1A, with AID transport into the nucleus under tight regulation (Casellas *et al.* 2016). Furthermore, AID is preferentially directed to the V gene and heavy chain locus by several factors including the production and processing of sterile transcript at the heavy chain locus (Rajagopal *et al.* 2009; Wang *et al.* 2009b), the activity of the RNA exosome complex to render both DNA strands accessible to AID (Basu *et al.* 2011), the preference of AID for specific (WRCY) sequences, and the presence of superenhancers, which are assemblages of enhancers and promoters (Qian *et al.* 2014; Yeap *et al.* 2015; Casellas *et al.* 2016). Nevertheless, some of these factors also contribute to off-target AID activity, resulting in mutations that occur relatively commonly in specific non-immunoglobulin genes such as BCL6, CD95, CD79A and PAX5 (Casellas *et al.* 2016). Many of these genes are oncogenes that have been linked to B cell lymphomagenesis. Furthermore, legitimate AID activity in areas such as the switch region can lead to further mutations including the insertion of fragments of foreign DNA, potentially resulting in malignant B cell transformation (Nardini *et al.* 2000; Pratt *et al.* 2001; Lenz *et al.* 2007).

1.2.5. Unusual antibody formats

Camelid antibodies

While the antibodies of most mammals contain both heavy and light chains, organisms belonging to the Camelidae family, which includes camels, llamas and vicugna, also possess unusual ~90 kDa heavy chain-antibodies (HCAbs) that do not pair with light chains (Hamers-Casterman *et al.* 1993). The HCAbs are remarkably abundant in these organisms (~80% of total IgG in camels and ~10-25% in llamas) (De Simone *et al.* 2008; Blanc *et al.* 2009) and can be produced in response to viral vaccination (Daley *et al.* 2010), suggesting that they play important roles in the antibody response. HCAbs are homodimeric molecules, with each monomer containing a single variable VHH domain linked to two constant domains (Figure 1.15). These antibodies can be divided into IgG2 and IgG3 isotypes, with IgG2 antibodies containing a longer hinge region than IgG3 antibodies.

The camelid antibodies are characterized by a few unusual modifications that have enabled them to exist as heavy-chain only structures. First, these antibodies do not have C_H1 domains, which normally form a disulphide bond that facilitates heavy-light chain pairing. Correspondingly, the remaining constant domains are homologous to conventional C_H2 and C_H3 domains. An examination of the genomic DNA encoding these antibodies has shown that the C_H1 exon is actually intact in the genomic sequence, but that a single point mutation (5'-GT to 5'-AT) in the 5' splice donor site of the intron downstream of C_H1 results in the removal of the C_H1 region during pre-mRNA splicing (Nguyen *et al.* 1999; Woolven *et al.* 1999). A second hallmark of HCAbs is the presence of small, hydrophilic residues

instead of bulky, hydrophobic residues in the part of the FR2 region that usually associates with light chains (Desmyter *et al.* 1996). These mutations decrease the tendency of this region to associate with light chains and increase the solubility of the VHH domains without compromising overall backbone structure. Beyond these changes, the overall structure and gene organization of the VHH domain are similar to those of its conventional V_H counterpart (Desmyter *et al.* 1996).

It may seem that camelid antibodies have several disadvantages compared to conventional antibodies, as they cannot rely on heavy-light chain pairing to generate diversity and they only have three CDR loops that contribute to antigen binding, as opposed to the usual six loops. Nevertheless, these antibodies have several compensatory adaptations that increase diversity and allow high-affinity binding. Camelid antibodies are generated from diverse germline *VHH* genes and are extensively modified by somatic hypermutation (Nguyen *et al.* 2000). These antibodies have extra-long CDR loops that form a large surface area and that are constrained by conserved disulphide bonds (commonly found between the CDR1 and CDR3 loops) that stabilize the extended structure and minimize entropic loss upon binding to antigen (Govaert *et al.* 2012). These long loops and the overall smaller size of the HCAbs enable them to bind to structures that are usually inaccessible to conventional antibodies such as clefts in enzymes (Desmyter *et al.* 1996; De Genst *et al.* 2006). Indeed, the VHH domains of HCAbs, which are also known as nanobodies, are being widely investigated for use in various research, diagnostic and therapeutic applications (Muyldermans 2013).

Shark antibodies

Sharks and other cartilaginous fish, which are evolutionarily extremely divergent from camelids, appear to have generated strikingly similar antibodies through convergent evolution (Greenberg *et al.* 1995). These antibodies are known as Ig new antigen receptors (IgNARs). IgNARs contain five constant domains instead of two (Figure 1.15), but are otherwise quite similar to camelid antibodies. Like HCabs, IgNARs appear to lack C_H1-like domains, as their constant domains are homologous to the C_ω2- C_ω6 domains of more conventional IgW antibodies that are also present in sharks (Bernstein *et al.* 1996). IgNARs also have polar residues in the FR2 region that usually associates with light chains (Streltsov *et al.* 2004; Fennell *et al.* 2010) and have smaller FR2 regions overall (Greenberg *et al.* 1995; Flajnik, Deschacht and Muyldermans 2011). These antibodies have long CDR3 loops due to the presence of multiple D segments that are combined by several recombination events (Diaz, Greenberg and Flajnik 1998; Flajnik, Deschacht and Muyldermans 2011). IgNARs also have extra cysteine residues that either form disulphide bridges between the CDR1 and CDR3 loops, as in HCabs, or form more unusual CDR3-FW2 and CDR3-FW4 disulphide bonds that stabilize the extended CDR loops (Roux *et al.* 1998; Stanfield *et al.* 2004; Streltsov *et al.* 2004).

Cow antibodies

Cow antibodies represent another form of diversification from the conventional antibody structure. Cow antibodies contain heavy and light chains, but these antibodies have unusually long CDR3s that can contain more than 60 residues (Berens, Wylie and Lopez 1997; Wang *et al.* 2013). Recently solved structures of cow antibodies have revealed that cow V_H domains have an overall similar structure to conventional V_H domains except for the extended CDR3 region, which forms a “stalk” from two antiparallel β -strands that ends with a disulphide-linked knob domain that acts as the antigen-binding site (Figure 1.15) (Wang *et al.* 2013). The stalk and knob regions appear to be primarily derived from an extremely long D_{H2} segment. Unlike the CDRs of conventional antibodies, the remaining CDR loops of the heavy and light chains are relatively invariant and are thought to support this protruding structure. While the overall structures of the stalk and knob domains are conserved in different cow antibodies, these regions are extremely different in terms of sequence, stalk length and the number and pattern of disulphide bonds in the knob domain, potentially giving them the ability to bind to diverse antigens (Wang *et al.* 2013). The tendency of the knob domain to form multiple disulphide bonds is due to extreme codon bias in the D_{H2} region, which results in most residues being able to be mutated to cysteines with a single nucleotide substitution (Wang *et al.* 2013). Furthermore, this region contains many hotspots for somatic hypermutation, which occurs prior to antigen exposure in cows (Lopez, Perez and Wylie 1998), thus allowing the generation of a diverse antibody repertoire in naïve B cells. Like the shark and camelid antibodies, cow antibodies may be able to bind to targets that are not accessible by conventional antibodies, potentially

giving cows an advantage in dealing with their environment, which may present unique challenges such as the presence of rumen microorganisms (Wang *et al.* 2013).

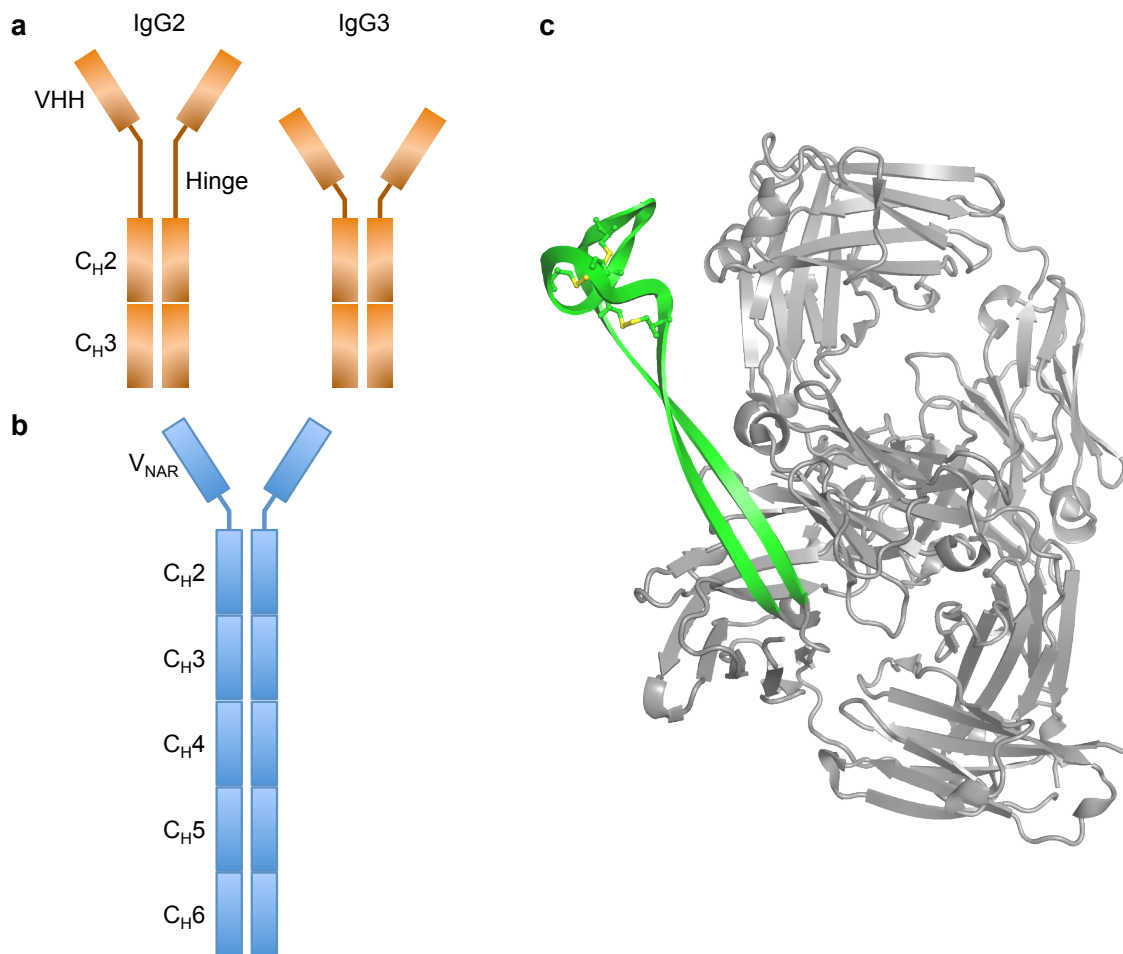


Figure 1.15. Unusual antibody formats. a, Schematic representation of camelid antibodies. These antibodies are made of two heavy chains, each containing a variable VHH domain linked to two constant domains through a hinge region. Camelid antibodies are divided into two isotypes: IgG2 antibodies (left) have a longer hinge than IgG3 antibodies (right). b, Schematic representation of a shark IgNAR. Like camelid antibodies, IgNARs consist of two heavy chains, but these antibodies have five constant domains instead of two. c, Cartoon representation of a cow antibody. These antibodies have heavy and light chains and are generally similar to conventional antibodies, except for an extra-long (potentially >60 aa) CDR3 region that forms a stalk and a disulphide-bonded knob domain (highlighted in green). Disulphide bonds in the knob domain are shown in yellow. The image was self-made using PyMOL (Protein Data Bank accession 4k3d) (Wang *et al.* 2013).

1.2.6. Generation of human monoclonal antibodies

Since the pioneering work of Köhler and Milstein (Kohler and Milstein 1975) describing the generation of mouse monoclonal antibodies using hybridomas, new techniques have continually been developed to produce large amounts of monoclonal antibodies. While the efficient production of mouse monoclonals was achieved relatively quickly, the stable generation of human monoclonal antibodies has proven more challenging. Initial experiments attempted to use human hybridomas and Epstein-Barr virus (EBV) immortalization of B cells for monoclonal antibody production, but these methods generally had low efficiencies (Traggiai *et al.* 2004). Other methods that have been developed include phage-display libraries containing human antibody heavy and light chain sequences and the generation of transgenic mice with human antibody genes, but these techniques have not been widely applied to antibodies that are generated in response to infection (McCafferty *et al.* 1990; Green 1999; Traggiai *et al.* 2004). Several methods are currently being used to generate human monoclonal antibodies against infectious agents. Antibody heavy and light cDNA can be obtained from individual B cells by RT-PCR and cloned into expression vectors for production of recombinant monoclonal antibodies (Tiller *et al.* 2008). This technique, combined with the collection of antibody-secreting B cells at the time of peak circulation (e.g. 7 days after vaccination) and single-cell sorting, is a powerful method to obtain large numbers of different monoclonal antibodies against the vaccinating antigen (Smith *et al.* 2009). Alternatively, the method of EBV immortalization of memory B cells to generate human monoclonal antibodies was greatly improved by the addition of a TLR agonist, which increases the efficiency of immortalization of memory B cells to >30% (Traggiai

et al. 2004). This method has successfully been used to obtain monoclonal antibodies against the SARS coronavirus (Traggiai *et al.* 2004), the influenza virus (Pappas *et al.* 2014) and paramyxoviruses (Corti *et al.* 2013). This technique has several advantages, especially with regard to generating monoclonal antibodies against infectious agents. First, the high efficiency of immortalization combined with high-throughput screening of B cell supernatants allows the investigation of a large proportion of the memory B cell repertoire at the clonal level. Second, this approach probes the entire history of the individual's response to prior antigens and does not require collection of blood at a precise time. Third, this method can detect antibodies that were generated due to natural infection rather than in response to a vaccinating antigen or an experimentally induced infection. Fourth, this approach enables the monoclonal antibodies to be screened for specificity to a target antigen prior to cloning, thus avoiding the need to clone large numbers of antibodies that may not be of interest. Fifth, this technique works especially well as an antigen-agnostic approach to first identify monoclonal antibodies that target a pathogen, and then to use these antibodies to identify target antigens through biochemical methods. This technique has the added advantage of allowing the B cells producing the antibodies to be maintained in culture, facilitating the extraction and characterization of the genomic DNA of the B cell clones of interest. As will be described later, this has proven useful in the investigation of the antibodies identified in this study.

1.3. Repair of double-strand DNA breaks

This section will briefly introduce different mechanisms that can be used to repair double-strand DNA breaks, as this will be relevant to the findings that will be reported later. For more details, the readers are referred to the following reviews: (Sung and Klein 2006; Deriano and Roth 2013). The formation of double-strand DNA breaks is essential for many cellular processes, including sister chromatid exchange during the first meiotic division, V(D)J recombination, and antibody isotype switching. These breaks can also be caused inadvertently by endogenous errors, such as the stalling of replication forks during DNA replication, or by exogenous factors such as chemical agents, ionizing radiation and reactive oxygen species (Shrivastav, De Haro and Nickoloff 2008). It is essential that these breaks be repaired accurately to avoid giving rise to large-scale chromosomal translocations or genomic instability, which can lead to the development of cancer. Accordingly, the cell has developed a range of pathways to repair double-strand DNA breaks that fall into two main categories: homologous recombination and non-homologous end joining (NHEJ).

1.3.1. Homologous recombination

Homologous recombination can only be used when a template is available, and hence is employed during repair of replication forks, during the S and G2 phase of mitosis when the sister chromatid is available, and during meiosis when the homologous chromosome is available (Sung and Klein 2006). In this process, the broken DNA ends are first resected by nucleases to produce single-stranded DNA overhangs. The recombinase RAD51 then binds to these ends, forming nucleoprotein filaments that can invade homologous double-stranded DNA sequences (Sung and Klein 2006). The RAD54 protein then mediates the exchange of the DNA strands, resulting in the pairing of a complete DNA strand with each broken strand (Solinger *et al.* 2001). DNA synthesis then occurs to complete the missing sequences and the strands are resolved, resulting in crossover or non-crossover events (Sung and Klein 2006).

1.3.2. Non-homologous end joining (NHEJ)

The NHEJ pathway is less accurate than homologous recombination as it does not use a template to facilitate the repair of double-strand DNA breaks. This mechanism can be divided into classical NHEJ (cNHEJ) and the more recently discovered alternative NHEJ (aNHEJ) pathways (Deriano and Roth 2013). Classical NHEJ, which is primarily used to repair double-strand DNA breaks during class-switch recombination and V(D)J recombination, begins with the attachment of the Ku70/Ku80 heterodimer to the broken DNA ends (Figure 1.16) (Deriano and Roth 2013). The DNA-Ku complex then recruits several other proteins that aid in DNA repair including the DNA-dependent protein kinase catalytic subunit (DNA-PKcs). DNA-PK then self-phosphorylates and phosphorylates other proteins including Ku, Artemis, and the ligation complex consisting of DNA ligase IV, XRCC4 and XLF. Artemis plays a role in cleaving hairpins formed during V(D)J recombination or damaged nucleotides in single-stranded DNA (Deriano and Roth 2013). The broken DNA ends are then ligated by DNA ligase IV, which is stabilized by XRCC4 and possibly aided by XLF. This process of ligation does not precisely preserve the original sequence, potentially leading to mutations in the repaired sequence.

While V(D)J recombination completely relies on cNHEJ to repair induced double-strand DNA breaks, class-switch recombination can occur at about 50% of wild type levels through aNHEJ in the absence of the critical cNHEJ elements Ku70 and DNA ligase 4 (Yan *et al.* 2007; Boboila *et al.* 2010a; 2010b). The ability of aNHEJ to function without these elements has led to its classification as a separate pathway from cNHEJ. The mechanism of aNHEJ repair is not well

understood, but this pathway appears to be even more error-prone than cNHEJ (Deriano and Roth 2013). aNHEJ preferentially ligates broken DNA ends using microhomologies, or short sequence homologies between the ends, but it can also mediate direct ligation (Boboila, Alt and Schwer 2012). The aNHEJ repair pathway is thought to begin with the binding of the single-strand DNA break repair enzyme PARP1 to the broken ends, followed by binding of the MRE11 nuclease and the DNA repair factor CtIP to process the ends (Figure 1.16) (Sartori *et al.* 2007; Boboila, Alt and Schwer 2012). Ligation of the broken ends is thought to be mediated by DNA ligase 3 and possibly DNA ligase 1 (Boboila, Alt and Schwer 2012).

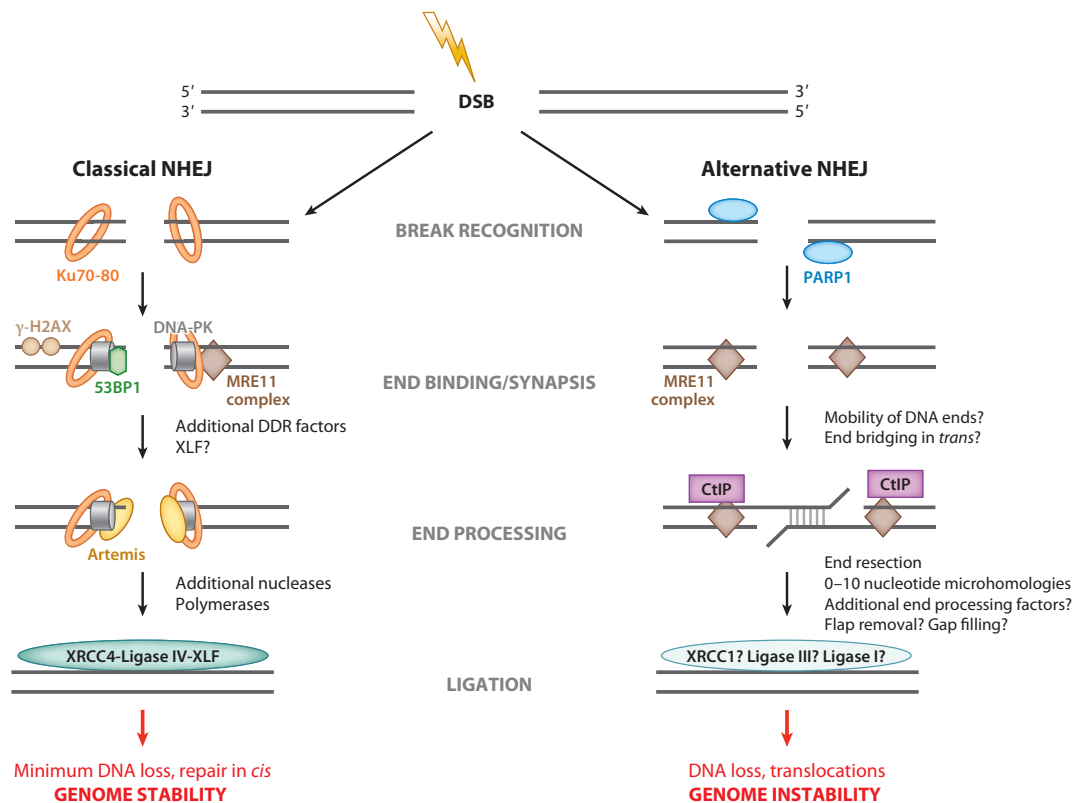


Figure 1.16. Repair of double-strand DNA breaks (DSB) by the classical NHEJ (cNHEJ) or alternative NHEJ (aNHEJ) pathways. In the cNHEJ pathway, the Ku70/Ku80 heterodimer binds to the broken DNA ends and recruits a number of proteins that aid processing of the broken ends. Ligation of the ends is mediated by DNA ligase IV, in complex with the DNA repair factors XRCC4 and XLF. The aNHEJ pathway is less clear, but is thought to be initiated by binding of the DNA repair enzyme PARP1 to the double strand breaks, followed by recruitment of the MRE11 nuclease complex. DNA ligation is thought to be performed by DNA ligase III or DNA ligase I. The aNHEJ pathway is less accurate than the cNHEJ pathway and potentially results in genomic rearrangements. Reprinted from (Deriano and Roth 2013).

1.3.3. Templated sequence insertion

Recently, a new mechanism of double-strand DNA break repair, dubbed templated sequence insertion (TSI), was proposed (Onozawa *et al.* 2014). In this mechanism, RNA (including pre-mRNA) that originates from distant parts of the genome can be reverse-transcribed by endogenous retrotransposons and used as “patches” to repair DNA double-strand breaks. The authors described two classes of TSI; class 1 TSIs were described as those likely to be mediated by the endogenous retrotransposon LINE-1 due to frequently observed insertions at LINE-1 target sites (5'-TTTT/A-3'), the presence of target-site duplications and the presence of a polyA tail in the insert. Class 2 TSIs appeared more similar to aNHEJ, with the presence of microhomology, small deletions at the insertion site and non-templated nucleotide additions. Subsequent work has shown that these insertions can occur in individuals from different parts of the world (Onozawa, Goldberg and Aplan 2015) and that some TSIs can be considered as polymorphisms (TSIPs) that are relatively common in the human population.

1.4. The B cell response to blood-stage malaria

Given the importance of the *P. falciparum* blood stage in causing disease, it is unsurprising that the development of an antibody response against blood stage parasites has been associated with protection against malaria. Seminal passive transfer experiments in the 1960s demonstrated that IgG antibodies from malaria-exposed adults in West Africa could substantially reduce parasite loads and decrease malaria symptoms in children from the same area (Cohen, Carrington and McGregor 1961). Follow-up experiments showed that the protective effect of these antibodies was maintained when they were given to children from East Africa (McGregor, Carrington and Cohen 1963) or Thailand (Sabchareon *et al.* 1991), suggesting that the antigenic targets of these antibodies were similar in these different regions. Subsequent studies have suggested that antibodies against merozoites (Osier *et al.* 2008) and antigens on the IE surface may both play a role in protection from malaria. In the following sections, I will focus on the antibody response to VSAs on the IE surface.

1.4.1. Serological studies of VSAs

Introduction to the agglutination assay

The agglutination assay is a technique that is used to test the ability of antibodies to bind to *P. falciparum* IEs (Marsh, Sherwood and Howard 1986; Newbold *et al.* 1992). In this assay, a parasite isolate is stained with a dye that labels parasite DNA, enabling the differentiation of IEs from uninfected erythrocytes. The stained parasite sample is rotated with a test serum or antibody of interest, which may cross-link multiple IEs to form agglutinates upon binding to surface antigens. The number and size of agglutinates are counted using fluorescence microscopy to obtain a semi-quantitative measure of antibody recognition of the IE surface. The mixed agglutination assay is an adaptation of the original assay that tests the ability of antibodies within a single serum sample to recognize several different parasite isolates simultaneously (Newbold *et al.* 1992). In this assay, two different isolates are stained with distinct DNA-binding dyes, rotated with serum and observed by fluorescence microscopy. The presence of dual-colour agglutinates indicates the presence of cross-reactive antibodies against the two parasites tested.

The advent of flow cytometry as a more quantitative approach to measure antibody binding to the IE surface has reduced the use of the agglutination assay, but this assay is still useful for specific applications and may offer different experimental outcomes from flow cytometry. For instance, the results obtained by flow cytometry do not always correlate with those obtained by agglutination, possibly as agglutination measures not only antibody-antigen interaction but also the ability of antibodies to bridge multiple IEs (Giha *et al.* 1999).

Furthermore, in contrast to flow cytometry, the mixed agglutination assay can be used to determine if the reactivity of a serum for multiple parasite isolates is due to true cross-reactivity i.e. the presence of broadly reactive antibodies, or if it is mediated by different groups of variant-specific antibodies.

Antibody responses to VSAs

The agglutination assay played an instrumental role in key experiments in the 1980s and 1990s that aimed to characterize the antibody response of individuals in malaria-endemic areas to VSAs on the IE surface. These experiments showed that sera from malaria-exposed children only recognized a limited number of parasite isolates (Marsh and Howard 1986; Forsyth *et al.* 1989; Iqbal, Perlmann and Berzins 1993; Bull *et al.* 1999). Furthermore, in children, antibodies produced after infection by a particular isolate could bind to IEs of the homologous isolate but not of heterologous isolates, suggesting that antibodies against IEs were obtained in a piecemeal fashion (Marsh and Howard 1986; Forsyth *et al.* 1989). In contrast, sera from malaria-exposed adults could recognize many different isolates (Marsh and Howard 1986; Forsyth *et al.* 1989), but were mostly unable to form mixed agglutinates when tested in the mixed agglutination assay (Newbold *et al.* 1992). These observations suggested that antibodies against VSAs are predominantly variant-specific, and that the observed broad reactivity of adult sera is due to gradual exposure-driven accumulation of variant-specific antibodies rather than to cross-reactive antibodies (Newbold *et al.* 1992). Several lines of evidence suggest that this accumulation of anti-VSA antibodies contributes to the protection of malaria-exposed adults from clinical symptoms. Out of a series of assays of the host immune response against blood-stage parasites, only the level of antibodies against IEs as measured by erythrocyte agglutination correlated with protection (Marsh *et al.* 1989). Subsequent work has shown that this association holds true for some isolates but not others (Bull *et al.* 1998; Giha *et al.* 2000; Dodoo *et al.* 2001). Perhaps more convincingly, several studies have shown that parasites

expressing a particular set of VSAs are more likely to infect individuals who do not have antibodies against that set of variants (Bull *et al.* 1998; Giha *et al.* 2000). Collectively, these findings suggest that antibodies against VSAs contribute to protection against malaria. The mechanism of antibody-mediated protection remains unclear, but antibodies may trigger destruction of IEs through opsonisation for phagocytosis by monocytes (Groux and Gysin 1990), activation of the complement pathway (Stanley *et al.* 1984), induction of antibody-dependent cellular cytotoxicity (Brown and Smalley 1980), and reversal of trophozoite adhesion to the endothelial wall (Fried *et al.* 1998b; Adams *et al.* 2014).

While most antibodies generated against VSAs appear to be variant-specific, several pieces of data suggest that some anti-VSA antibodies may be at least partially cross-reactive. First, parasites that cause severe malaria or mediate pathological phenotypes such as rosetting appear to express a restricted set of PfEMP1 variants that is more commonly recognized by sera from malaria-exposed individuals (Bull *et al.* 1999; Jensen *et al.* 2004; Warimwe *et al.* 2009; Cham *et al.* 2009; Claessens *et al.* 2012; Ghumra *et al.* 2012). In line with these findings, immunization of mice with multiple PfEMP1 CIDR1 domains generated a partially cross-reactive response (Gratepanche *et al.* 2003), and a trial involving the immunization of *Aotus* monkeys reported potentially cross-reactive epitopes in the DBL domain of PfEMP1 (Patarroyo *et al.* 2014). Restricted epitopes or PfEMP1 variants such as these may be targets of cross-reactive antibodies, as indicated by a study reporting the generation of cross-reactive antibodies against specific rosetting parasites (Ghumra *et al.* 2012). Second,

studies using the agglutination assay have suggested that some adults living in malaria-endemic areas may possess cross-reactive antibodies. Early work investigating the ability of Gambian adult sera to agglutinate parasites from Gambian children showed that antibodies that were eluted after binding to one parasite isolate were able to recognize several other parasite isolates (Marsh and Howard 1986). A subsequent study that found predominance of variant-specific antibodies also reported the presence of a single cross-reactive Gambian adult serum (Newbold *et al.* 1992). A more recent study investigating sera from malaria-exposed adults in India reported the existence of both variant-specific and cross-reactive antibodies (Chattopadhyay *et al.* 2003). In this study, sera from convalescent adults in an area with low malaria transmission and from clinically immune adults living in a hyperendemic area were able to form mixed agglutinates with specific parasite combinations. Third, studies of populations in Indonesia that have recently migrated to a malaria-endemic area have suggested that adults acquire protective immunity more rapidly than children (Baird *et al.* 1991; 1993). A component of this protection could be cross-reactive antibodies against VSAs. Collectively, these findings suggest that broadly reactive antibodies against VSAs on the IE surface may be present in some adults who live in malaria-endemic areas. However, to our knowledge, a large-scale screen of immune adult sera or plasma for broadly reactive antibodies against VSAs has not been performed to date. Hence, despite its potential significance for the development of a malaria vaccine, the possibility that such cross-reactive antibodies exist has yet to be fully investigated.

Antibody isotypes in responses to IEs

Studies aiming to characterize the specific antibody isotypes induced in responses to IEs have reported that the cytophilic IgG1 and IgG3 isotypes are generally dominant in malaria-infected individuals (Piper, Roberts and Day 1999; Kinyanjui *et al.* 2003; Cabrera *et al.* 2004). Agglutination of IEs has also been associated with IgG antibodies, particularly those of the IgG1 isotype (Piper, Roberts and Day 1999). IgM responses against IEs are observed following *P. falciparum* infection (Kinyanjui *et al.* 2003), but the role of antigen-specific IgM in protection against malaria remains unclear (Ghumra *et al.* 2008; Pleass *et al.* 2016). Non-immune IgM molecules that do not recognize *P. falciparum* antigens can also coat IEs of specific strains through binding of the IgM Fc portions to specific PfEMP1 variants (Rowe *et al.* 2002). This binding has been associated with a higher risk of severe malaria (Rowe *et al.* 2002) and is postulated to provide an advantage to *P. falciparum* by two mechanisms. First, non-specific IgM binding has been shown to be required for rosetting in a subset of parasites (Rowe *et al.* 2002; Ghumra *et al.* 2008), probably due to the ability of the antibody to cluster multiple PfEMP1 molecules (Stevenson *et al.* 2015; Akhouri *et al.* 2016). Second, non-specific IgM binding to the PfEMP1 variant VAR2CSA has been shown to inhibit binding by specific IgG molecules that may trigger parasite destruction (Barfod *et al.* 2011).

1.4.2. Effects of malaria infection on B cells

Longevity of antibodies and memory B cells

Anecdotal reports that immune individuals who leave malaria-endemic areas regain susceptibility to malaria have led to the suggestion that the B cell response to blood-stage malaria antigens is defective or short-lived (Struik and Riley 2004). However, studies investigating the antibody response to well-defined merozoite antigens have indicated that the picture is far from simple. In terms of antibody longevity, merozoite-specific antibodies decay relatively rapidly in children and in malaria-naïve adults who are transiently exposed to malaria infection or vaccination, but are more stable in adults living in malaria-endemic areas (Taylor *et al.* 1996; Cavanagh *et al.* 1998; Kinyanjui *et al.* 2007; Akpogheneta *et al.* 2008; Crompton *et al.* 2009; Ndungu *et al.* 2013). These observations may reflect a difference in the ability of these individuals to generate short-lived versus long-lived plasma cells. A study comparing a village with recently reduced malaria transmission to a nearby village with high transmission but otherwise similar conditions reported that memory B cells are longer lasting than antibodies in plasma (Ndungu *et al.* 2012). Accordingly, memory B cells specific for merozoite antigens have been consistently detected in a percentage of malaria-exposed individuals (Dorfman *et al.* 2005; Weiss *et al.* 2010; Nogaro *et al.* 2011). A study investigating the malaria-specific memory B cell compartment before and after a well-delineated malaria season in Mali reported that this compartment expands sharply during an acute malaria attack but contracts following the attack to a level slightly higher than before the malaria season, resulting in a small net increase in memory B cell levels with age

(Weiss *et al.* 2010). Nevertheless, it was observed in this study, as well as in others, that the percentage of adults in malaria-endemic areas with memory B cells that recognize specific malaria antigens is only about 30-50% (Crompton *et al.* 2014). Studies on individuals who are no longer substantially exposed to malaria due to a decrease in malaria transmission (Wipasa *et al.* 2010; Nogaro *et al.* 2011; Ndungu *et al.* 2012) or emigration to non-malaria-endemic countries (Ndungu *et al.* 2013) reported that a proportion maintained memory B cells against malaria antigens. Collectively, these findings suggest that the memory B cell response against blood-stage parasites is long lasting, but that only a proportion of individuals are capable of producing memory B cells against specific antigens. The reason for this is currently unclear, but this phenomenon may be linked to the ability of *Plasmodium* to polyclonally activate B cells and/or to the high proportion of atypical memory B cells in malaria-exposed individuals.

Polyclonal B cell activation by malaria antigens

Malaria infection results in a sharp increase of IgG and IgM levels (Abele *et al.* 1965; Banic *et al.* 1991), and individuals living in malaria-endemic areas have higher levels of antibodies (Rowe *et al.* 1968), many of which are not malaria-specific (Curtain *et al.* 1964). Early experiments also reported that *P. falciparum* culture supernatants and crude *P. falciparum* extract could stimulate non-specific B cell proliferation (Greenwood and Vick 1975; Greenwood, Oduloju and Plattsmills 1979). This polyclonal stimulation also includes responses against self antigens (Fesel *et al.* 2005; Mannoer *et al.* 2013), although whether these autoantibodies are pathogenic or are involved in protection from malaria remains an open question (Daniel-Ribeiro and Zanini 2000). Two mechanisms of polyclonal stimulation have been proposed. First, the PfEMP1 CIDR1 α domain of the *P. falciparum* strain FCR3S1.2var1 has been shown to bind directly to B cells to induce cellular proliferation and activation (Donati *et al.* 2004). Second, *P. falciparum* infection appears to stimulate the production of the B cell activating factor BAFF, based on observations that *P. falciparum*-infected individuals have higher levels of BAFF in serum (Nduati *et al.* 2011) and *P. falciparum* schizont extracts or haemozoin can stimulate the secretion of BAFF by monocytes *in vitro* (Kumsiri *et al.* 2010). It is currently unclear whether polyclonal B cell activation aids protection against malaria (Fesel *et al.* 2005; Mannoer *et al.* 2013) or leads to B cell exhaustion, impairing the anti-parasitic response (Simone *et al.* 2011).

Atypical memory B cells in malaria-infected individuals

The dysfunctional B cell response in some malaria-exposed individuals may also be associated with the high prevalence of CD19⁺CD20⁺CD21⁻CD27⁻ atypical memory B cells, which have recently been characterized to have elevated FcRL5 expression (Sullivan *et al.* 2015). Several lines of evidence suggest that *P. falciparum* infection drives the generation of these cells. First, several studies have reported that the prevalence of these cells is positively correlated with the intensity of malaria transmission (Weiss *et al.* 2009; 2011; Illingworth *et al.* 2013; Sullivan *et al.* 2015). Second, experimental challenge with *P. falciparum* in malaria-naïve individuals results in an expansion of this B cell population (Scholzen *et al.* 2014). Third, children who live in an area with malaria transmission have higher levels of atypical memory B cells than children living in a nearby village with reduced transmission but otherwise similar conditions (Illingworth *et al.* 2013).

Atypical memory B cells are also known as exhausted B cells in HIV-infected individuals due to their reduced ability to proliferate and differentiate into antibody-secreting cells (Moir *et al.* 2008). However, the role of these cells in malaria-exposed individuals has not always been clear. A study comparing classical and atypical memory B cells in immune adults from Gabon found peptide sequences in the serum that corresponded with the BCRs of atypical memory B cells, suggesting that these cells may contribute directly to the anti-*Plasmodium* immune response (Muellenbeck *et al.* 2013). However, several recent studies have reported that atypical memory B cells are hyporesponsive and do not differentiate efficiently into antibody-secreting cells as compared to

classical memory B cells (Portugal *et al.* 2015; Sullivan *et al.* 2015), suggesting that the expansion of these cells may be detrimental to the anti-parasite immune response.

1.5. Rationale and aims of project

Many open questions remain about the nature of the antibody response to *P. falciparum* IEs, particularly at the monoclonal antibody level. The difficulty in characterizing this response is largely due to clonal antigenic variation on the IE surface, as well as to limitations in technology that would allow large-scale analysis of *P. falciparum*-specific human monoclonal antibodies. Indeed, despite some evidence suggesting that broadly reactive antibodies against VSAs may exist, this possibility has yet to be fully investigated in a large-scale screen. In addition, a recent study has suggested that previously undiscovered conserved antigens may exist on the IE surface (Raj *et al.* 2014). Given that antibodies targeting antigens on the IE surface are associated with protection against malaria, a conserved VSA or yet unknown antigen may be a powerful malaria vaccine candidate. This study thus aims to investigate the existence of broadly reactive antibodies against the IE surface by screening plasma from adults who live in malaria-endemic regions in Kilifi County, Kenya. To achieve this goal, we plan to adapt the mixed agglutination technique to allow the detection of triple agglutinates by including a third parasite isolate stained with a distinct DNA dye. We will identify donors with cross-reactive plasma, immortalize memory B cells from these donors using EBV and screen the immortalized B cell supernatants by high-throughput flow cytometry against whole *P. falciparum* IEs to identify broadly reactive monoclonal antibodies that target the IE surface. As previously discussed, this antigen-agnostic approach has the advantage of allowing the identification of antibodies that are induced by natural infection without limiting the scope of the study to specific pre-determined antigens. We also plan to use

the broadly reactive antibodies to determine corresponding conserved (and potentially novel) antigens on the IE surface, which may be candidates for malaria vaccine development.

Chapter 2. Materials and Methods

2.1. Serum and plasma samples

Kenyan plasma

Plasma samples were obtained with informed consent from Kenyan adults during two separate periods; initial samples (n = 31) were acquired in 1995 from blood transfusion donors within Kilifi County on the Kenyan coast, and more recent samples (n = 557) were obtained between 2007 and 2014 from adults living in a malaria-endemic region within Kilifi County.

Tanzanian sera and plasma

The Tanzanian samples were obtained from three separate cohorts. The first two cohorts consisted of healthy adult volunteers who were recruited from Dar es Salaam for a controlled human malaria infection trial with cryopreserved *P. falciparum* sporozoites (Shekalaghe *et al.* 2014). In the first cohort, the volunteers were only challenged with live sporozoites, but in the second cohort, the volunteers were vaccinated with irradiated sporozoites prior to live sporozoite challenge. The third cohort contained healthy adults who were enrolled in a clinical trial testing P27A peptides, which are derived from the blood stage antigen trophozoite exported protein 1 (TEX1).

Malian sera

The majority of the Malian sera were obtained from a cohort in Kalifabougou, Mali (Tran *et al.* 2014), which has high levels of malaria transmission from June to December. This cohort consists of both children and adults, with an age range from 3 months to 25 years. The samples that were tested were obtained just after the malaria transmission season. Smaller numbers of sera were also obtained from adults in the Fulani and Dogon ethnic groups in Mantéourou, Mali, who were initially enrolled in a study testing the immunological basis of enhanced protection of members of the Fulani ethnic group to malaria (Arama *et al.* 2015).

Ethics approvals

In all cases, written informed consent was obtained from the participants (or guardians of participating children) before inclusion in the study. The acquisition and use of the Kenyan plasma samples were approved by the KEMRI-Wellcome Trust Research Programme Scientific Steering Committee and the KEMRI Ethics Review Committee (protocol numbers SSC1131 and SSC2565), as well as the Oxford Tropical Research Ethics Committee (541-14). The Tanzanian samples were obtained with informed consent from the participants of the different studies. The clinical trials from the first cohort were conducted according to Good Clinical Practices and with authorization from the institutional review boards of the Ifakara Health Institute ((IHI/IRB/No25), the National Institute for Medical Research Tanzania (NIMR/HQ/R.8a/Vol.IX/1217), and the Ethikkommission beider Basel (EKBB), Basel, Switzerland (EKBB 319/11). The trial was also authorized by the Tanzanian Food and Drugs Authority (TFDA) (Ref. No. CE.57/180/04A/50) and was registered at ClinicalTrials.gov (NCT01540903) (Shekalaghe *et al.* 2014). The second and third cohorts were also approved by the relevant institutional review boards (IHI/IRB/No02-2014, NIMR/HQ/R.8a/Vol.IX/1691, and TFDA13/CTR/0003 for the second; IHI/IRB/No04-2014, NIMR/HQ/R.8a/Vol.IX/1742 and TFDA13/CTR/004/03 for the third) and were registered at ClinicalTrials.gov (NCT02132299 and NCT01949909, respectively).

The study in Mali was authorized by the Ethics Committee of the Faculty of Medicine, Pharmacy, and Dentistry at the University of Sciences, Techniques, and Technologies of Bamako, and the Institutional Review Board of the National

Institute of Allergy and Infectious Diseases, National Institutes of Health (NIAID protocol number 11-I-N126; ClinicalTrials.gov Identifier: NCT01322581).

2.2. Donors

The three Kenyan donors with LAIR1-containing antibodies (donors B, C and D) are adults who are residents of an area in Kilifi County with moderate malaria transmission intensity (i.e. with an Entomological Inoculation Rate of 21.7 infective bites per person per year) (Midega *et al.* 2012). Adults in this area are clinically immune from febrile malaria, having acquired immunity during childhood. The three donors were *P. falciparum*-negative during sample collection (Tan *et al.* 2016). The two Tanzanian donors who were confirmed to possess LAIR1-containing antibodies (donors E and F) are male adults who were recruited from institutes of higher learning in Bagamoyo, Tanzania (Shekalaghe *et al.* 2014).

2.3. *Plasmodium falciparum* parasites

Parasite culture

P. falciparum parasites were initially obtained from children (n = 10) who were diagnosed with malaria in Kilifi County, Kenya (Table 1). The parasites were adapted to *in vitro* culture and cryopreserved at the ring stage for subsequent use. For the purposes of this study, these culture-adapted parasites were thawed and cultured to $\geq 5\%$ parasitaemia in blood group O erythrocytes using RPMI complete medium (RPMI-C; RPMI 1640 medium with 10% heat-inactivated serum from malaria-naïve donors and supplemented with 0.2% glucose, 2 mM L-glutamine, 25 $\mu\text{g/mL}$ gentamicin, 37.5 mM HEPES and 0.05 mg/mL hypoxanthine). The *P. falciparum* laboratory line 3D7 was also cultured under the same conditions. Parasites were cryopreserved at the late trophozoite stage in small aliquots for subsequent use in agglutination assays. They were tested negative for *Mycoplasma* and genotyped with the following primers to confirm their identities:

MSP-1 *fwd*: 5'-TTGTCAAAAACTAGAAGCTTTA-3',
rev: 5'-ATCAATTAAATATTTGAAACC-3';
MSP-2 *outer fwd*: 5'-ATGAAGGTAATTAAAACATTGTCTATT-3'
outer rev: 5'-CTTTGTTACCATCGGTACATT-3'
nested FC27 fwd: 5'-AATACTAAGAGTGTAGGTGCGATGCTCCA-3'
nested FC27 rev: 5'-TTTATTTGGTGCATTGCCAGAACTTGAAC-3',
nested IC fwd: 5'-AGAAGTATGGCAGAAAGTAAKCCTYCTACT-3',
nested IC rev: 5'-GATTGTAATTCGGGGGATTCAGTTTGTTCG-3';
GLURP *fwd*: 5'-TGAATTCGAAGATGTTCACTGAAC-3',
rev: 5'-TGTAGGTACCACGGGTTCTTGTGG-3'

Table 1. Details of parasite isolates

Parasite	Year put into culture	Malaria syndrome
6816	2008	Severe (impaired consciousness)
9106	2009	Severe (impaired consciousness)
9215	2009	Severe (respiratory distress)
9605	2009	Severe (impaired consciousness)
9775	2009	Non-severe
10668	2010	Severe (impaired consciousness and respiratory distress)
10936	2010	Non-severe
10975	2010	Severe (severe malarial anaemia)
11014	2010	Severe (severe malaria anaemia)
11019	2010	Severe (impaired consciousness)

Parasite selection by magnetic beads

To select for MGD21-reactive IEs, cultured IEs were incubated with MGD21 for 20 min at room temperature, washed, and rotated with Protein G-coated magnetic beads (Life Technologies) for 30 min at room temperature. Following magnetic sorting, enriched (MGD21⁺) and depleted (MGD21⁻) fractions were returned to *in vitro* culture. The following day, the magnetic beads were removed from the parasite culture medium and discarded (Tan *et al.* 2016).

2.4. Agglutination assays

Individual agglutination assays

Parasites were diluted to a 5% haematocrit in 10 µg/mL ethidium bromide in RPMI incomplete medium (RPMI-I; RPMI-C without serum) (Tan and Bull 2015). For each agglutination reaction, 10 µL of the erythrocyte suspension was mixed with 2.5 µL of adult plasma in a 96-well round-bottom plate. The plate was rotated at 45° for 1.5 h at room temperature. Each reaction mixture was transferred onto a microscope slide and covered with a coverslip with its edges previously lined with petroleum jelly. The slides were examined under a fluorescence microscope and agglutinates were scored based on a system previously described (Bull *et al.* 1999) as follows:

Size	Number
A = 3 to 5 cells	1 = 10 size A or 5 to 10 size A and 1 size B
B = 6 to 20 cells	2 = 10 size A and 1 size B or 2 to 5 size B
C = 20 to 100 cells	3 = 5 size B or 2 to 5 C
D = >100 cells	4 = 5 size C or 1 size D

Mixed agglutination assays

In the double mixed agglutination assay (Newbold *et al.* 1992), two different isolates were separately stained with a 100× pellet volume of 200 µg/mL ethidium bromide (in RPMI-I) or 2 µg/mL DAPI (in PBS). After staining for 1 h at room temperature, the parasites were washed five times and mixed in a ratio to give an equal final parasitaemia. The mixture was diluted to a 5% haematocrit in RPMI-I, and rotation with plasma and microscopy were conducted as in the individual agglutination assay. For assays investigating antibody isotypes, after rotation, plates containing agglutinates were centrifuged at $72 \times g$ for 3 min at room temperature and washed twice with 2% FBS in PBS. Agglutinates were then labelled with 40 µg/mL of FITC-conjugated mouse anti-human IgM (Beckman Coulter) or 80 µg/mL of FITC-conjugated sheep anti-human IgG (Binding Site) for 30 min at room temperature, washed, and analysed by fluorescence microscopy. In the triple mixed agglutination assay, IEs from three parasite isolates were separately stained with 10 µg/mL DAPI, 200 µg/mL ethidium bromide or 6.7× SYBR Green I for one hour at room temperature. The subsequent steps were conducted as described for the double mixed agglutination assay. For each mixed agglutination reaction, both individual agglutinates and mixed agglutinates were scored according to the following system:

<u>Size</u>	<u>Score</u>
None or <10	0
10-20	1
21-50	2
51-100	3
>100	4

In the primary screen, pools of five adult plasma were tested against six Kenyan isolates by triple mixed agglutination (in two separate reactions). Pools that formed mixed-color agglutinates were identified and individual plasma within these pools were tested against nine isolates using the same assay (all the parasite isolates in Table 1 except for 6816, which did not grow well in *in vitro* culture). A single isolate (10668) was not detected in mixed agglutinates formed by any of the plasma and was therefore excluded from the study. Three adults (donors B, C and D) with plasma that formed mixed agglutinates with at least six parasite isolates were selected for monoclonal antibody isolation and, following further informed consent, an additional blood sample was taken from each individual (Tan *et al.* 2016).

Trypsin sensitivity assay

IEs were stained with different dyes as in the triple agglutination assay and treated with the following concentrations of trypsin (Sigma): 1 µg/mL, 10 µg/mL and 100 µg/mL. Following incubation for 15 min at 37°C, the reactions were terminated by the addition of 100× trypsin inhibitor (Sigma) in RPMI-C. The parasites were rinsed three times with RPMI-I, rotated with plasma and examined by fluorescence microscopy for scoring of agglutinates.

2.5. IgG purification

IgG from adult plasma was purified on a Protein G-agarose column (Thermo Scientific) using the recommended protocol. Adsorbed IgG was eluted with acidic buffer (pH 2.8) (Thermo Scientific) and immediately neutralized with 1 M Tris pH 9. Purified IgG was buffer-exchanged into PBS using Ultracel 50K ultracentrifuge tubes (Millipore) and reconstituted into the same volume as the plasma used for purification. Verification of the purification process was carried out by running the original plasma, flowthrough and purified IgG fractions on polyacrylamide gel electrophoresis (SDS-PAGE). Purified IgG and flowthrough were stored long-term at -70°C or kept at 4°C with 0.02% w/v sodium azide for short-term use.

2.6. Peripheral blood mononuclear cell (PBMC) extraction from adult blood

Whole blood was obtained from adults and centrifuged at $850 \times g$ for 10 min at room temperature. The plasma fraction was collected and stored at -70°C , and the cells were resuspended in an equal volume of R10 (RPMI 1640 supplemented with 10% FCS, 100 units of penicillin, 0.1 mg/mL streptomycin and 2 mM glutamine). The cells were gently layered onto Lymphoprep (Axis-Shield) and centrifuged at $850 \times g$ (minimum acceleration, no brakes) for 20 min at room temperature. The PBMC layer was collected in a 50 mL tube and rinsed twice with R10 ($416 \times g$ for 7 min at 4°C , full acceleration and brakes). 1 mL of FCS was added to each PBMC sample and the PBMCs were put on ice for 30 min. Next, 1 mL of 20% DMSO in FCS was added to the PBMCs, which were transferred in 1 mL aliquots to Cryotube vials (Thermo Scientific) for storage in liquid nitrogen.

2.7. B cell immortalization and isolation of monoclonal antibodies

IgG⁺ memory B cells were isolated from cryopreserved PBMCs by magnetic cell sorting with mouse anti-CD19-PECy7 antibodies (BD Pharmingen) and mouse anti-PE microbeads (Miltenyi Biotec), followed by flow cytometry sorting for IgG⁺ IgM⁻ IgD⁻ cells. The B cells were immortalized with supernatants from B95-8 cells infected with Epstein–Barr virus (EBV) (added to a final 25% of the total volume) for 3 h at 37°C and cultured in the presence of the TLR agonist CL264 (2.5 µg/mL) and irradiated feeder cells as described previously (Traggiai *et al.* 2004). Two weeks post-immortalization, culture supernatants were tested for the ability to stain IEs from a mixture of up to four parasite isolates (3D7-MGD21⁺, 9106, 9605 and 11019) by flow cytometry. Positive supernatants were rescreened against IEs from each of eight parasite isolates in individual reactions (Figure 2.1). Cryopreserved IEs were thawed, stained with 10× SYBR Green I, and incubated with the B cell supernatants for 1 h at 4°C. Antibody binding was detected using 2.5 µg/mL of goat Alexa Fluor 647-conjugated anti-human IgG (Jackson ImmunoResearch). Reactivity was calculated based on the percentage of late-stage parasites (high SYBR Green) recognized by each antibody (Tan *et al.* 2016).

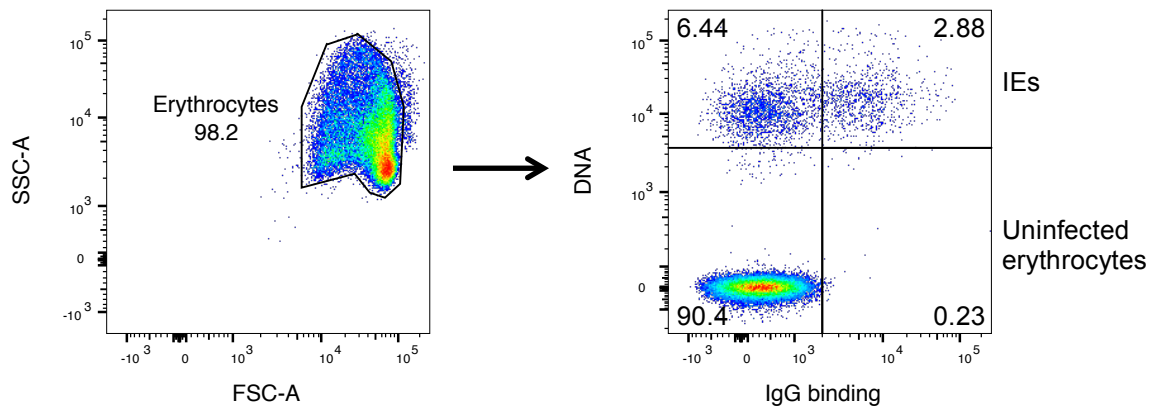


Figure 2.1. Gating strategy for detection of IE-reactive monoclonal antibodies. Erythrocytes were gated based on side and forward scatter. IEs could be separated from uninfected erythrocytes by DNA staining, allowing the calculation of the percentage of IEs that was stained by a monoclonal antibody of interest.

2.8. Analysis of monoclonal antibodies

Sequence analysis of antibody cDNA and genomic DNA

cDNA was synthesized from selected B cell cultures and both heavy chain and light chain variable regions (V_H and V_L) were sequenced as previously described (Tiller *et al.* 2008). The usage of V_H and V_L genes and the number of somatic mutations were determined by analyzing the homology of V_H and V_L sequences of the monoclonal antibodies to known human V, D and J genes in the IMGT database (Lefranc *et al.* 2009). Genomic DNA was isolated from selected B cell lines with a commercial kit (QIAGEN), and antibody-encoding sequences were amplified and sequenced with primers specific for the V and J regions of the given antibody. The sequences were aligned using ClustalW2 (Larkin *et al.* 2007). Potential cryptic RSS sites were identified using the RSSsite web server (Merelli *et al.* 2010). To determine the heterozygosity of LAIR1 on Chr19, the following primers were used to perform PCRs on genomic DNA (LAIR1_INTR_FW1 GGCGGTGGGCACTCAGGTTC; LAIR1_INTR_REV1 CACAGGCAGTCACCGGGTCTAGG; LAIR1_INTR_FW2 GGATGCACCATGTCACCCAGTCCTGG). Genomic DNA isolated from PHA- and IL-2-stimulated T cells from donor C was used as a control for sequence analysis (Tan *et al.* 2016).

Immunoglobulin lineage and genealogy analysis

Unmutated common ancestor (UCA) sequences of the VL were inferred with Antigen Receptor Probabilistic Parser (ARPP) UA Inference software, as previously described (Kepler 2013). UCA sequences of the V_H were constructed using IMGT/V-QUEST (Lefranc *et al.* 2009) and the genomic insert sequences. Nucleotide sequences of the mutated antibodies and the UCA were aligned using ClustalW2 (Larkin *et al.* 2007), and phylogenetic trees were generated with the DNA Maximum Likelihood program (Dnaml) of the PHYLIP package, version 3.69 (Liao *et al.* 2013; Pappas *et al.* 2014; Tan *et al.* 2016).

Production of recombinant antibodies, antibody variants and fusion proteins.

Antibody heavy and light chains were cloned into human IgG1, Ig κ and Ig λ expression vectors (Tiller *et al.* 2008) and expressed by transient transfection of Expi293F Cells (ThermoFisher Scientific) using polyethylenimine (PEI). Cell lines were routinely tested for *Mycoplasma* contamination. The antibodies were affinity purified by protein A chromatography (GE Healthcare). Variants of the MGD21 antibody were produced by i) exchanging heavy chain V, D, J elements or the light chain with the corresponding sequences of an irrelevant antibody (FI499, reactive to influenza virus (Pappas *et al.* 2014)), ii) deleting selected segments, or iii) reverting somatic mutations to the germline configuration with reference to the IMGT database and the original LAIR1 genomic sequence (NCBI Reference Sequence: NC_018930.2). In addition, LAIR1-Fc fusion proteins were produced recombinantly by cloning the mutated or unmutated LAIR1 fragment into a plasmid designed for expression of human IgG1 fusion proteins (pINFUSE-hIgG1-Fc2, Invivogen). Based on an alignment of the most potent LAIR1-containing antibodies with the unmutated LAIR1 sequence, five key residues that could contribute to gain of binding to IEs and loss of binding to collagen were identified and added alone or in various combinations to the unmutated LAIR1-Fc fusion protein. The MGD21 constructs and LAIR1 exon mutants were tested for staining of 3D7 IEs that were enriched for MGD21 recognition (3D7-MGD21⁺). For the LAIR1 exon mutants, MFI values at 1 μ g/mL antibody concentration were calculated by interpolation of binding curves fitted to a linear regression model (Graphpad Prism 6) (Tan *et al.* 2016).

ELISA

Total IgGs were quantified using 96-well MaxiSorp plates (Nunc) coated with goat anti-human IgG (Southern Biotech) using Certified Reference Material 470 (ERMs-DA470, Sigma-Aldrich) as a standard. To test binding to human collagen type I, ELISA plates were coated with 5 µg/mL of type I recombinant human collagen (Millipore), blocked with 1% BSA and incubated with titrated antibodies, followed by AP-conjugated goat anti-human IgG, Fcγ fragment specific (Jackson Immuno Research). The plates were then washed, substrate (p-NPP, Sigma) was added and the plates were read at 405 nm. To detect the presence of LAIR1-containing antibodies in B cell supernatants, ELISA plates were coated with 5 µg/mL polyclonal goat anti-human LAIR1 (R&D Systems) or goat serum as a negative control and incubated with these supernatants, followed by AP-conjugated goat anti-human IgG, Fcγ fragment specific (Jackson Immuno Research) (Tan *et al.* 2016).

2.9. Identification of target antigens

Immunoprecipitation and LC-MS

Erythrocyte ghosts were prepared by hypotonic lysis with 1× PBS diluted 15-fold in water, and ghost membranes were dissolved in a reducing lysis buffer containing 2% SDS, 10 mM DTT, 10 mM HEPES pH 8, sonicated and boiled. Solubilized proteins were alkylated with iodoacetamide (final concentration 55 µM) for 30 min at room temperature and precipitated with 80% acetone overnight at 4°C. The precipitates were resuspended in urea and digested with trypsin. For immunoprecipitation experiments, IEs were sonicated and dissolved in 7.2 M urea in RIPA buffer (1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate in HBS pH 7.4). The samples were centrifuged and supernatants were diluted 6.7-fold with RIPA buffer containing a protease inhibitor cocktail (Sigma-Aldrich) and incubated with 10 µg of MGD21 or BKC3 overnight at 4°C. Next, Protein G-Sepharose beads (GE Healthcare) were added and samples were incubated for 1 h at 4°C. The beads were washed four times and immunoprecipitates were digested directly on the beads with trypsin. After trypsin digestion, peptides were analyzed on a Q-Exactive instrument at the Functional Genomics Center in Zürich. Raw files were analyzed using the MaxQuant software (Cox and Mann 2008; Schwanhäusser *et al.* 2011) and MS/MS spectra were searched against human and *P. falciparum* 3D7 UniProt FASTA databases (UP000005640 and UP000001450). Peptide identifications were matched across several replicates. Subsequent data analysis was performed in the R statistical computing environment. Missing values were imputed with a normal distribution around an LFQ value of 21. Statistical

significance was evaluated by Welch tests (Tan *et al.* 2016).

Western blots

Ghosts and immunoprecipitates were dissolved in 2× SDS sample buffer (Bio-Rad) and run on a 12% polyacrylamide gel under non-reducing conditions. The proteins on the gel were transferred onto a PVDF membrane, which was blocked with 5% milk in TBS with 0.1% Tween (TBST) for 1 h at room temperature. The membrane was incubated with 5 µg/mL MGD21 overnight at 4°C, washed with TBST, and developed with HRP-conjugated sheep anti-human IgG (GE Healthcare) used in combination with a chemiluminescent substrate (Tan *et al.* 2016).

Expression of RIFINs

Genes encoding the A-RIFINs PF3D7_1400600, PF3D7_1040300, PF3D7_0100400, PF3D7_0100200, and PF3D7_1100500 were produced by gene synthesis (Genscript) and cloned into the pDisplay vector (Invitrogen), which contains an HA tag, as previously described (Goel *et al.* 2015). RIFIN chimeras containing the constant region of PF3D7_1400600 (residues 38-146) and the variable region of PF3D7_0100200 (residues 151-288) [PF3D7_1400600c_0100200v], or containing the constant region of PF3D7_0100200 (residues 42-150) and the variable region of PF3D7_1400600 (residues 147-325) [PF3D7_0100200c_1400600v], were generated. The pDisplay constructs were transiently transfected into CHOK1-SV cells (GS-System, Lonza) using PEI. Cell lines were routinely tested for *Mycoplasma* contamination. One day before transfection, CHOK1-SV cells were seeded at 0.5×10^6 cells/mL in 30 mL CD-CHO medium (Invitrogen) supplemented with 2 mM L-glutamine in 125 mL Erlenmeyer flasks (Corning). On the day of transfection, 20 µg DNA was diluted in OPTI-PRO SFM Medium (Invitrogen) and mixed with 200 µg PEI for 20 min at room temperature. The DNA-PEI complexes were added to the cells, which were cultured in a CO₂ shaker incubator at 37°C, 135 rpm. After 72 hours, the expression of RIFINs and their recognition by the LAIR1-containing antibodies were tested by flow cytometry. Briefly, 5 µg/mL of rabbit anti-HA tag and 2 µg/mL of MGC or MGD antibodies were added to the transfected cells. Antibody binding was detected by 5 µg/mL of Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies) and 2.5 µg/mL of Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch). Dead cells were excluded by staining with 7-AAD (BD Biosciences) (Tan *et al.* 2016).

2.10. Antibody functional assays

Inhibition of parasite growth

3D7-MGD21⁺ (5% parasitaemia, ring stage) was cultured with various concentrations of MGD21 or BKC3 for 2 d. After 2 d, 10× SYBR Green I was added to aliquots of each culture and parasitaemia was quantified by flow cytometry. The remaining parasites in each culture were washed to remove the antibodies and incubated for 1 d to allow the parasites to reach the late trophozoite/schizont stage. MGD21 recognition of these cultures was detected using 2.5 µg/mL of Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch) (Tan *et al.* 2016).

Inhibition of rosetting

9605-MGD21⁺ IEs at the late trophozoite/schizont stage were purified from uninfected erythrocytes and ring-stage parasites using a magnetic column (Miltenyi Biotec) and were resuspended in culture medium with 10% heat-inactivated human serum. The purified IEs were incubated with 10 µg/mL of MGD21 or BKC3 for 1 h at 4°C, mixed with O⁺ erythrocytes or A⁺ erythrocytes in a 1:20 ratio, and incubated for 30 min at room temperature to allow rosetting to occur. The IEs were stained with 10× SYBR Green I, and the number of rosettes formed by at least 200 IEs was counted by fluorescence microscopy to calculate the rosetting frequency. The Wilcoxon signed-rank test was used for statistical comparisons of pairs of data groups in the rosetting experiments (Tan *et al.* 2016).

Agglutination with monoclonal antibodies

3D7-MGD21⁺ and 11019-MGD21⁺ IEs (4-5% parasitaemia) were diluted to a 3% haematocrit in a 5× SYBR Green I solution containing 5 µg/mL of the test monoclonal antibody. Each sample was rotated for 1 h at room temperature and subsequently examined by fluorescence microscopy (Tan *et al.* 2016). The mixed agglutination reactions with monoclonal antibodies were done as previously described for the plasma, except that 10 µM SYTO 61 was used to replace ethidium bromide and a 5× concentration of SYBR Green I was used.

Indirect immunofluorescence assay with monoclonal antibodies

3D7-MGD21⁺ IEs were stained at a 5% haematocrit in a 5× SYBR Green I solution containing 5 µg/mL of the test monoclonal antibody for 1 h at room temperature. Antibody recognition was detected using 2.5 µg/mL of Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch). Each reaction mixture was transferred onto a microscope slide and covered with a coverslip with its edges previously lined with petroleum jelly. The coverslip was sealed with nail varnish and the samples were examined by fluorescence microscopy.

Opsonic phagocytosis by monocytes

IEs were stained with 10 µg/mL DAPI for 30 min at room temperature, washed four times and run on a magnetic column (Miltenyi Biotec) to purify late stage parasites. The purified parasites were opsonized with serially diluted antibodies for 1 h at 4°C. Monocytes were isolated from fresh PBMCs of healthy donors using mouse anti-CD14 microbeads (Miltenyi Biotec) and mixed with the opsonized parasites in a 1:2 ratio for 1 h at 37°C. Extracellularly bound, non-internalized IEs were lysed by treatment with red blood cell lysis solution (Miltenyi Biotec) for 10 min at room temperature. The cells were stained with mouse anti-CD14-PECy5 (Beckman Coulter) and analyzed by flow cytometry. The mean fluorescence intensity (MFI) of DAPI in CD14⁺ cells was used as a measure of phagocytosis of IEs by monocytes (Figure 2.2) (Tan *et al.* 2016).

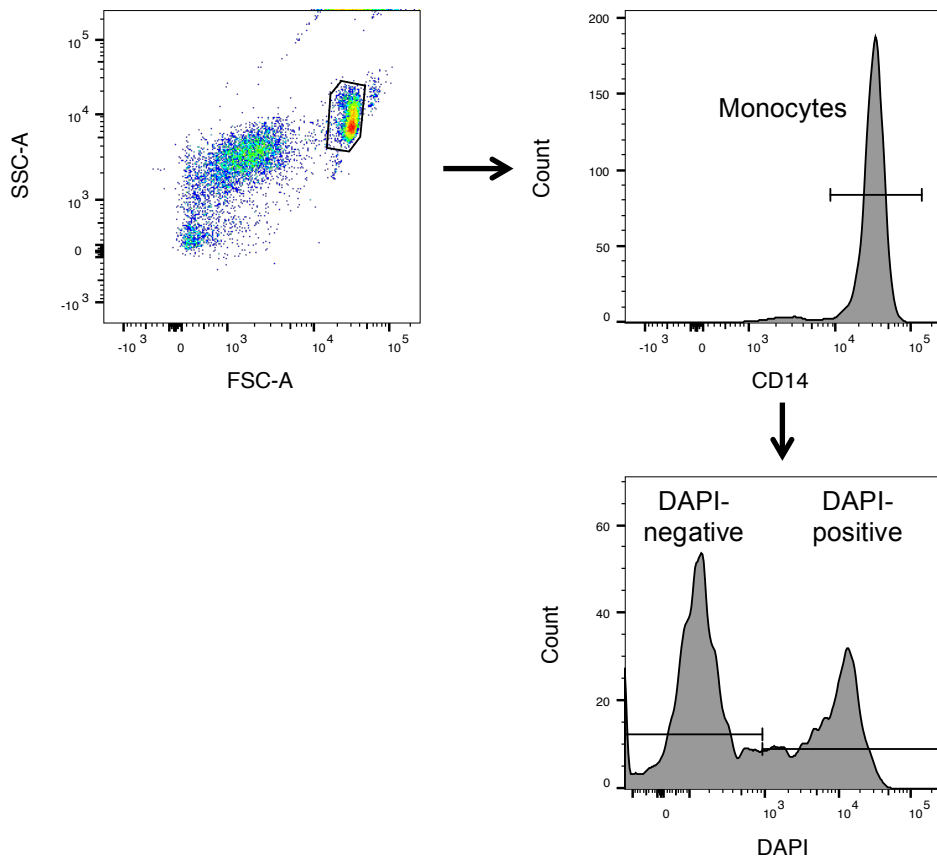


Figure 2.2. Gating strategy for detection of phagocytosis of IEs by monocytes. Monocytes were gated based on side and forward scatter, as well as CD14 staining. Monocytes that phagocytosed IEs were DAPI-positive and could clearly be separated from negative monocytes. The MFI of DAPI in all CD14⁺ monocytes was used to quantify phagocytosis.

2.11. Direct screening for LAIR1-containing antibodies

Selection of LAIR1-positive sera with IEs

IEs were stained with 10 µg/mL DAPI for 30 min at room temperature and subsequently incubated with serum or plasma samples at a 1/30 dilution for 20 min at room temperature. The labeled IEs were washed twice and stained with one of two different antibody combinations. In initial experiments on Tanzanian sera, the IEs were stained with 1/20 PE-conjugated mouse monoclonal anti-human LAIR1 (BD Biosciences, clone DX26) and 2.5 µg/mL of Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch). In subsequent experiments on Malian sera, the IEs were stained with 5 µg/mL of polyclonal goat anti-human LAIR1 (R&D Systems) for 20 min at room temperature, washed twice, and then stained with 2.5 µg/mL of Alexa Fluor 488-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) and 2.5 µg/mL of Alexa Fluor 647-conjugated donkey anti-human IgG (Jackson ImmunoResearch). After the secondary staining, the IEs were washed twice and analyzed by flow cytometry. Sera that displayed both IgG and LAIR1 binding to IEs were considered to be positive candidates with LAIR1-containing antibodies.

Screen of LAIR1-containing monoclonal antibodies with anti-human IgG microbeads

Anti-human IgG microbeads (Bangs Laboratories) were incubated with supernatants from immortalized B cell clones for 20 min at room temperature under shaking conditions. The beads were washed twice and stained with 5 µg/mL of polyclonal goat anti-human LAIR1 (R&D Systems) in 1% human serum for 20 min at room temperature. After two rounds of washing, the beads were stained with 2.5 µg/mL of Alexa Fluor 488-conjugated donkey anti-goat IgG (Jackson ImmunoResearch) and analyzed by flow cytometry.

Screen of LAIR1-containing sera and monoclonal antibodies with anti-goat IgG microbeads

Anti-goat IgG microbeads (Spherotech) were divided into two portions. The negative control portion was stained with 5 µg/mL polyclonal goat anti-human EGF (R&D Systems) for 20 min at room temperature. The test portion was stained with 5 µg/mL polyclonal goat anti-human LAIR1 (R&D Systems) in 40× SYBR Green I for 20 min at room temperature. The two bead portions were washed three times separately and mixed. The bead mixture was incubated with test sera at a 1/30 dilution for 1 h at room temperature under shaking conditions. The beads were washed twice, stained with 2.5 µg/mL of Alexa Fluor 647-conjugated donkey anti-human IgG (Jackson ImmunoResearch), and analyzed by flow cytometry (Figure 2.3). Screening of monoclonal antibodies was conducted as described, except that the beads were incubated with immortalized B cell supernatants instead of test sera, and no washing step was conducted after incubation with the B cell supernatants and the secondary antibody.

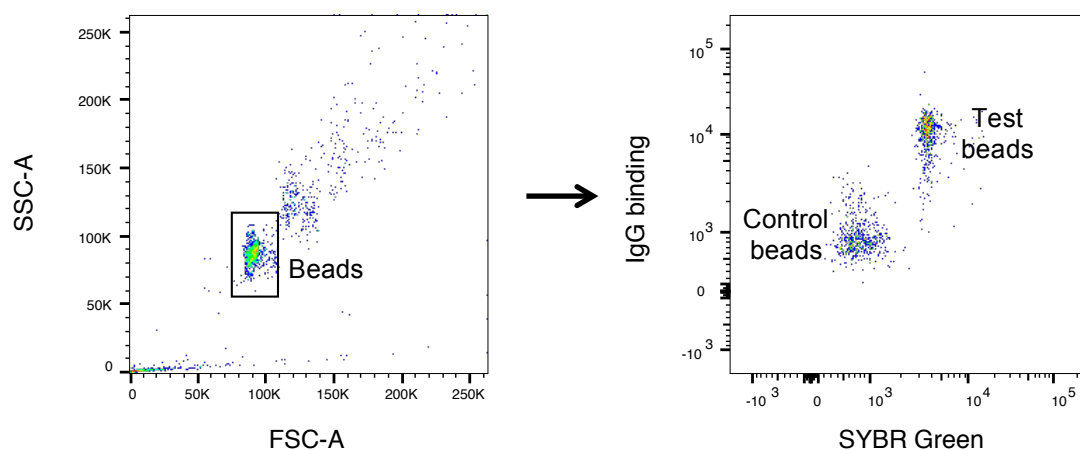


Figure 2.3. Gating strategy for detection of binding of LAIR1-containing antibodies to microbeads. Microbeads were gated based on side and forward scatter. Test and control beads could be distinguished by SYBR Green staining. Sera that displayed higher IgG staining of test beads relative to control beads were considered to be potential positives for the presence of LAIR1-containing antibodies.

Competition assay with recombinant LAIR1

The anti-goat IgG microbeads were stained with anti-human EGF or anti-human LAIR1, washed and mixed as described. The bead mixture was incubated with 5 µg/mL recombinant LAIR1 (R&D Systems) for 20 min at room temperature under shaking conditions. Without washing, test serum was added to a final concentration of 1/30 and the beads were incubated for 1 h at room temperature under shaking conditions. After washing twice, IgG binding was detected with 2.5 µg/mL Alexa Fluor 647-conjugated donkey anti-human IgG (Jackson ImmunoResearch) and the beads were analysed by flow cytometry.

Fluorescence resonance energy transfer (FRET)-based assay for the detection of B cells with LAIR1-containing BCRs

Immortalized B cells or primary B cells purified from fresh PBMCs using CD22⁺ magnetic beads were incubated with 5 µg/mL biotinylated goat anti-human LAIR1 (R&D Systems) for 20 min on ice. After washing twice, the B cells were co-stained with 2.5 µg/mL Alexa Fluor 647-conjugated goat anti-human IgG (Jackson ImmunoResearch) and 2 µg/mL PE-conjugated streptavidin for 20 min on ice. The cells were analysed by flow cytometry with only the 488 nm laser being utilized. Cells that emitted fluorescence at the Alexa Fluor 647 wavelength (~660 nm) after excitation by the 488 nm laser were considered to be FRET-positive.

Chapter 3. Identification of broadly reactive plasma against *P. falciparum* IEs

3.1. Introduction

Many open questions remain regarding the nature of the antibody response to the *P. falciparum* IE surface. One key question that has remained unresolved is whether broadly reactive antibodies are generated against this surface as part of the immune response to malaria infection. Serological studies on populations living in malaria-endemic areas have shown that adult sera display broad recognition of different isolates (Marsh and Howard 1986; Forsyth *et al.* 1989; Iqbal, Perlmann and Berzins 1993), but this is thought to be mainly due to gradual accumulation of a large repertoire of variant-specific antibodies rather than to cross-reactive antibodies (Newbold *et al.* 1992). Nevertheless, this hypothesis has not been rigorously tested with a large-scale screen of sera from malaria-exposed adults, and several factors suggest that this line is worth pursuing. First, several studies suggest that some adult sera have at least partial cross-reactivity to IEs from different *P. falciparum* isolates (Marsh and Howard 1986; Newbold *et al.* 1992; Chattopadhyay *et al.* 2003). These findings have been supported by preliminary agglutination experiments done in our laboratory, which have indicated that a few plasma could form mixed agglutinates between two parasite isolates (data not shown)^a. Second, antibodies against the IE surface have been associated with protection against malaria (Marsh *et al.* 1989; Bull *et al.* 1998; Dodo *et al.* 2001). Therefore, if they exist, broadly reactive antibodies targeting this surface may be protective against many different *P. falciparum*

^aDone by Dr Claire Tully, who performed these experiments during a short rotation project. 112

isolates, and the conserved antigens recognized by such antibodies may be promising malaria vaccine candidates.

I decided to explore this prospect by screening plasma from a large cohort of adults who live in a malaria-endemic region in Kilifi County, Kenya. Flow cytometry was considered to be unsuitable for this task, as it cannot distinguish between the presence of many different variant-specific antibodies and a limited number of cross-reactive antibodies in a given plasma sample. Therefore, I used the mixed agglutination assay, which is based on the principle that broadly reactive antibodies can cross-link IEs from several parasite isolates to form multi-coloured agglutinates that can be observed by fluorescence microscopy (Newbold *et al.* 1992). The initial agglutination experiments were conducted with a small number of plasma to establish proof of principle. Once the experimental approach was validated, I screened a large number of adult plasma for the presence of rare cross-reactive antibodies against the IE surface. Here, I report the results of this screen and describe some initial characterization of the broadly reactive plasma response.

3.2. Information on selected parasite isolates

Throughout this study, experiments were conducted on up to ten culture-adapted *P. falciparum* parasite isolates (Table 1 in Methods). The *P. falciparum* isolates were obtained from malaria patients in Kilifi County, Kenya, with differing levels of disease severity and severe malaria symptoms. The isolates were adapted to *in vitro* culture for several months or years. Parasites causing severe and non-severe malaria are thought to express different subsets of VSAs (Bull *et al.* 1999; Nielsen *et al.* 2002; Warimwe *et al.* 2009), and it was hoped that the selection of parasites linked to a wide range of malaria syndromes would allow us to examine diverse VSA variants on the IE surface. These specific isolates were also selected as their genomes were being sequenced at the Wellcome Trust Sanger Institute, facilitating the potential discovery of antigens expressed by these parasites. For the most part, I cultured large quantities of these parasites and cryopreserved them at the late trophozoite stage (when VSA expression is generally high) for use in agglutination assays. I observed no clear difference in agglutination patterns between cryopreserved and freshly cultured parasites (data not shown).

3.3. Pilot individual agglutination assays

To investigate the ability of plasma from malaria-exposed adults to recognize different *P. falciparum* isolates, I conducted a pilot individual agglutination assay on 31 Kenyan adult plasma (described in Methods section 2.1) and eight culture-adapted parasite isolates. For each isolate, parasites that were selected for the ability to bind human brain endothelial cells (HBECs) were tested alongside unselected counterparts. The reason for this approach is that IEs that bind to HBECs express a subset of PfEMP1 variants that are linked to cerebral malaria and may be more commonly recognized than unselected IEs (Bull *et al.* 1999; Avril *et al.* 2012; Claessens *et al.* 2012; Lavstsen *et al.* 2012). Thus, I wanted to investigate whether HBEC-selected IEs would form agglutinates with more adult plasma than their unselected counterparts.

The results of the individual agglutination screen are shown in Figure 3.1. Large agglutinates (>100 IEs) were commonly observed with many plasma, and bright-field images confirmed that the erythrocyte surface was intact and that the plasma were not agglutinating internal parasites from lysed IEs (Figure 3.1b). Overall, a diverse pattern of parasite recognition by the panel of plasma was observed. Several isolates, such as 9106 and 11019, could form agglutinates with almost every plasma tested, whereas others such as 9215 and 9775 were almost unrecognized. Considering the parasites associated with impaired consciousness, 9106, 9605 and 11019 were well recognized, but isolate 6816 was poorly recognized. Parasites causing non-severe malaria (9775, 10936) or respiratory distress (9215) were generally poorly agglutinated. As expected, HBEC-selected parasites were generally better recognized than their unselected counterparts.

Taken together, these results point towards trends in antibody recognition that are linked to disease severity and severe malaria syndromes but suggest that exceptions occur and that individual parasite isolates have unique agglutination phenotypes.

While most of the plasma displayed broad recognition of different parasite isolates as reported in previous studies, plasma 6 and 27 were exceptional in the sense that they were able to recognize every isolate tested. To test whether this was due to the presence of broadly reactive antibodies, I tested these plasma in the mixed agglutination assay, as described in the following section.

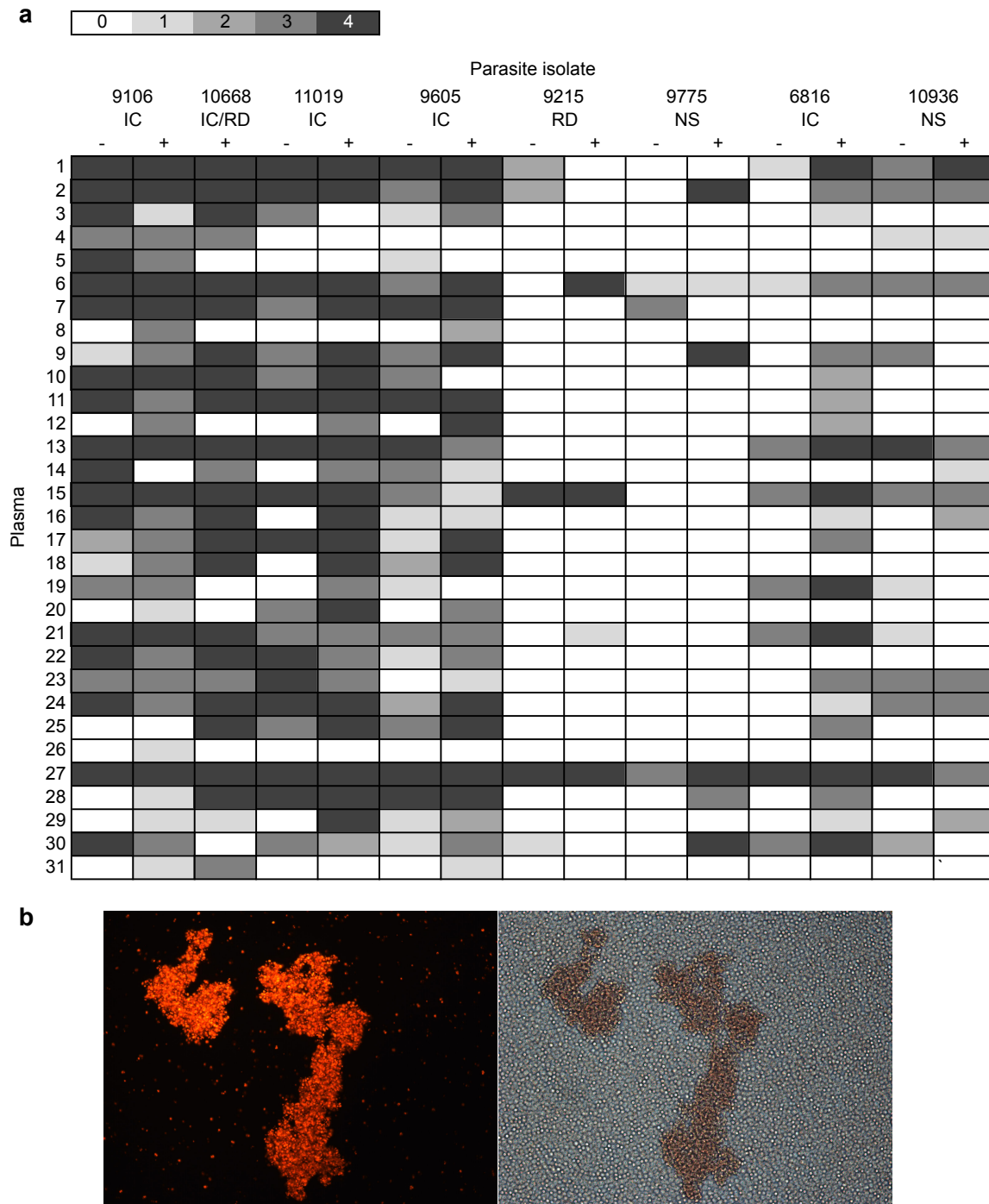
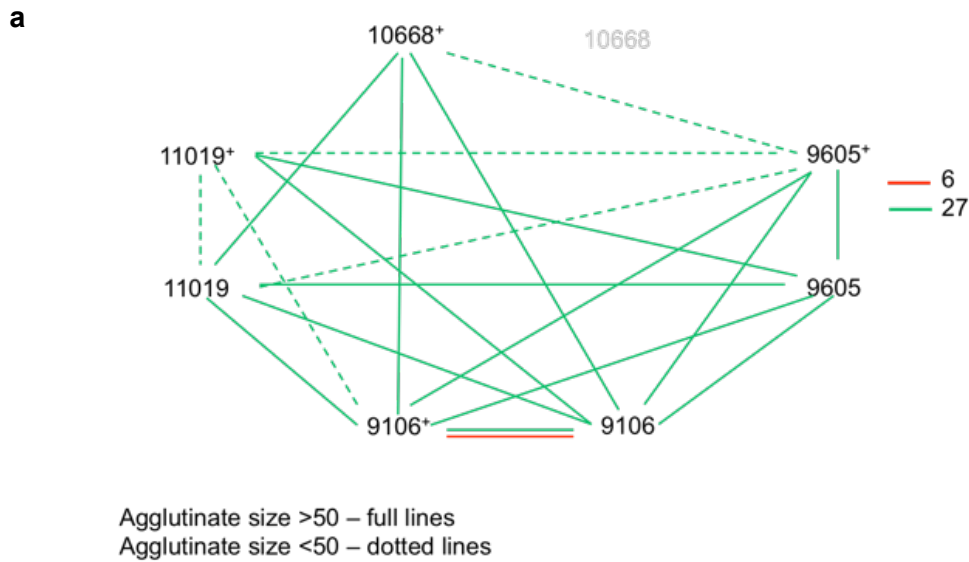


Figure 3.1. Pilot individual agglutination assays. a, Individual agglutination of eight parasite isolates by 31 plasma from adults living in malaria-endemic areas. Parasites labelled “-” are unselected isolates whereas “+” refers to HBEC-selected isolates. The symptoms of the malaria patients from whom these parasite isolates were obtained are shown below the isolate names: IC – severe, impaired consciousness; RD – severe, respiratory distress; NS – non-severe. Results involving the unselected 10668 isolate were omitted as subsequent genotyping indicated that it was mixed up with another isolate prior to the start of this study. Refer to the Methods section for the scoring system. b, Representative fluorescence (left) and bright field (right) images of agglutinates obtained in the individual agglutination assay.

3.4. Double mixed agglutination assays

I conducted double mixed agglutination assays to test whether the broad recognition of plasma 6 and 27 was mediated by variant-specific or broadly reactive antibodies. These assays were performed with matching pairs of unselected and HBEC-selected IEs from four parasite isolates (9106, 10668, 11019 and 9605) that were completely recognized by both plasma, with every possible pairwise parasite combination tested (Figure 3.2). As expected, plasma 6 could not form mixed agglutinates between parasites of different genotypes. This plasma formed mixed agglutinates between unselected and HBEC-selected 9106 IEs but failed to do so for IEs from 9605 and 11019, suggesting that for the latter two parasites, HBEC selection had altered the expression patterns of the relevant antigens on the IE surface. In contrast, plasma 27 formed mixed agglutinates with almost every parasite combination tested. This is consistent with the previous finding (by Dr Claire Tully) that plasma 27 could form mixed agglutinates with two parasite isolates. The formation of mixed agglutinates appeared to be unaffected (or even slightly reduced) by HBEC selection of the parasite isolates. Therefore, unselected parasite isolates were used exclusively in subsequent experiments. To further characterize the cross-reactive antibody response of plasma 27, this plasma was tested in a novel triple mixed agglutination assay.



b Plasma 27: **9106** + **9605**

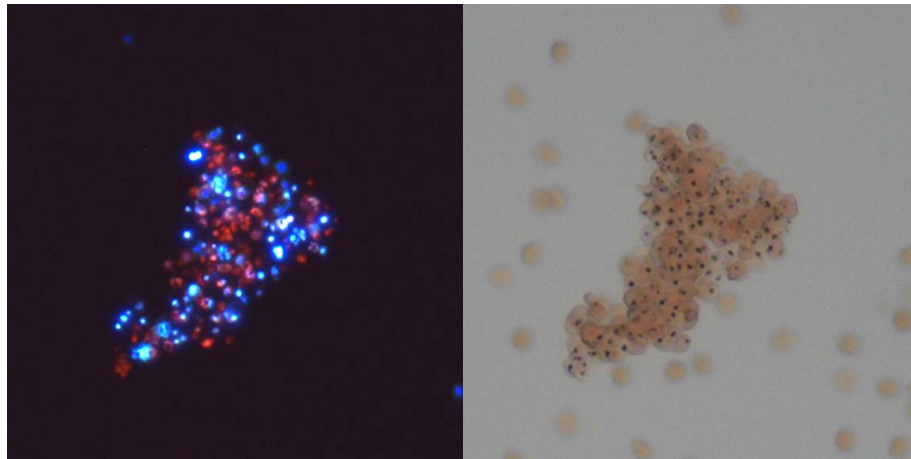


Figure 3.2. Double mixed agglutination assay with four parasite isolates. a, A line between two parasites indicates cross-agglutination by a specific plasma, with the two plasma (6 and 27) assigned different colours. Parasites labelled with a “+” are HBEC-selected whereas those without the symbol are unselected (this nomenclature will be used henceforth). Results involving 10668 were omitted as we subsequently discovered that it was mixed up with another isolate prior to the start of this study. Excluding 10668, the absence of a line between two isolates indicates lack of agglutination. b, Representative fluorescence (left) and bright field (right) images of a double mixed agglutinate. The isolate names are coloured according to the colour that was observed by fluorescence microscopy.

3.5. Development of triple mixed agglutination assay

I developed a novel triple mixed agglutination assay to reduce the number of assays required to test many different parasite isolates and to enable the detection of antibody binding to three parasite isolates simultaneously (Figure 3.3). In this assay, I included SYBR Green I as a third DNA-binding dye that contains a different fluorescence emission spectrum from that of the two dyes already being used (DAPI and ethidium bromide), allowing the staining of a third parasite isolate.

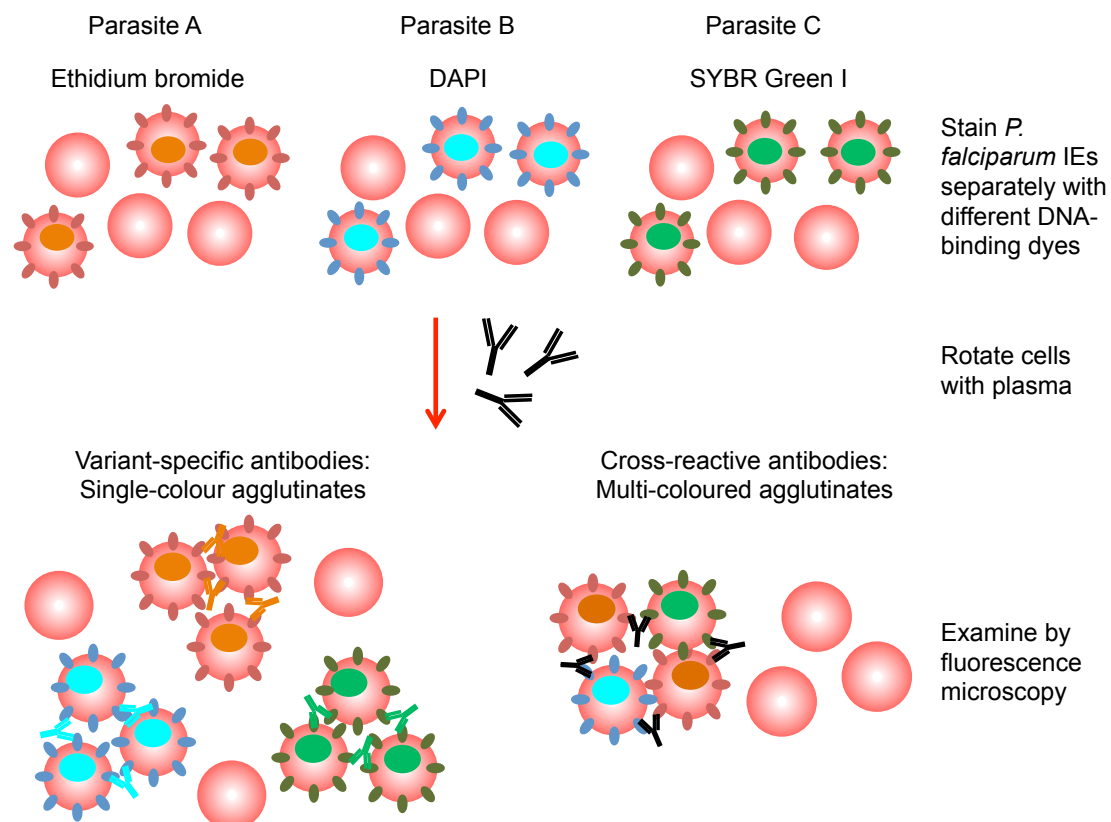


Figure 3.3. Outline of triple mixed agglutination assay. In this assay, IEs from three different parasite isolates are stained with distinct DNA-binding dyes, washed thoroughly to remove excess dye, and mixed with a plasma of interest. Plasma containing variant-specific antibodies would be expected to form single-colour agglutinates, while plasma with cross-reactive antibodies would be expected to form multi-coloured agglutinates.

3.6. Triple mixed agglutination assays on broadly reactive plasma

I tested the ability of plasma 6 and 27 to form triple agglutinates with the parasite isolates 9106, 9605 and 11019. As expected, plasma 6 could individually agglutinate each of the three parasite isolates but could not form mixed agglutinates (Figure 3.4). In contrast, plasma 27 could form large triple agglutinates, indicating that this plasma contained broadly reactive antibodies that recognized a common antigen in all three isolates (Figure 3.5a). This plasma also formed single-colour agglutinates with 11019 that were often joined to the mixed agglutinates, suggesting that this plasma also contained parasite-specific antibodies (Figure 3.5b). Plasma 27 was also tested against six other parasite isolates in two separate triple mixed agglutination reactions (10668+10936+11014, 9215+9775+10975). Double or triple mixed agglutinates were seen in both reactions (Figure 3.5c,d), suggesting that plasma 27 may contain broadly reactive antibodies that target a common IE surface antigen in most of the nine parasite isolates. Nevertheless, these agglutinates were smaller in size, indicating that a lower proportion of IEs from these isolates expressed the relevant antigen. The results of the mixed agglutination reactions are summarized in Figure 3.12.

9106 + 9605 + 11019

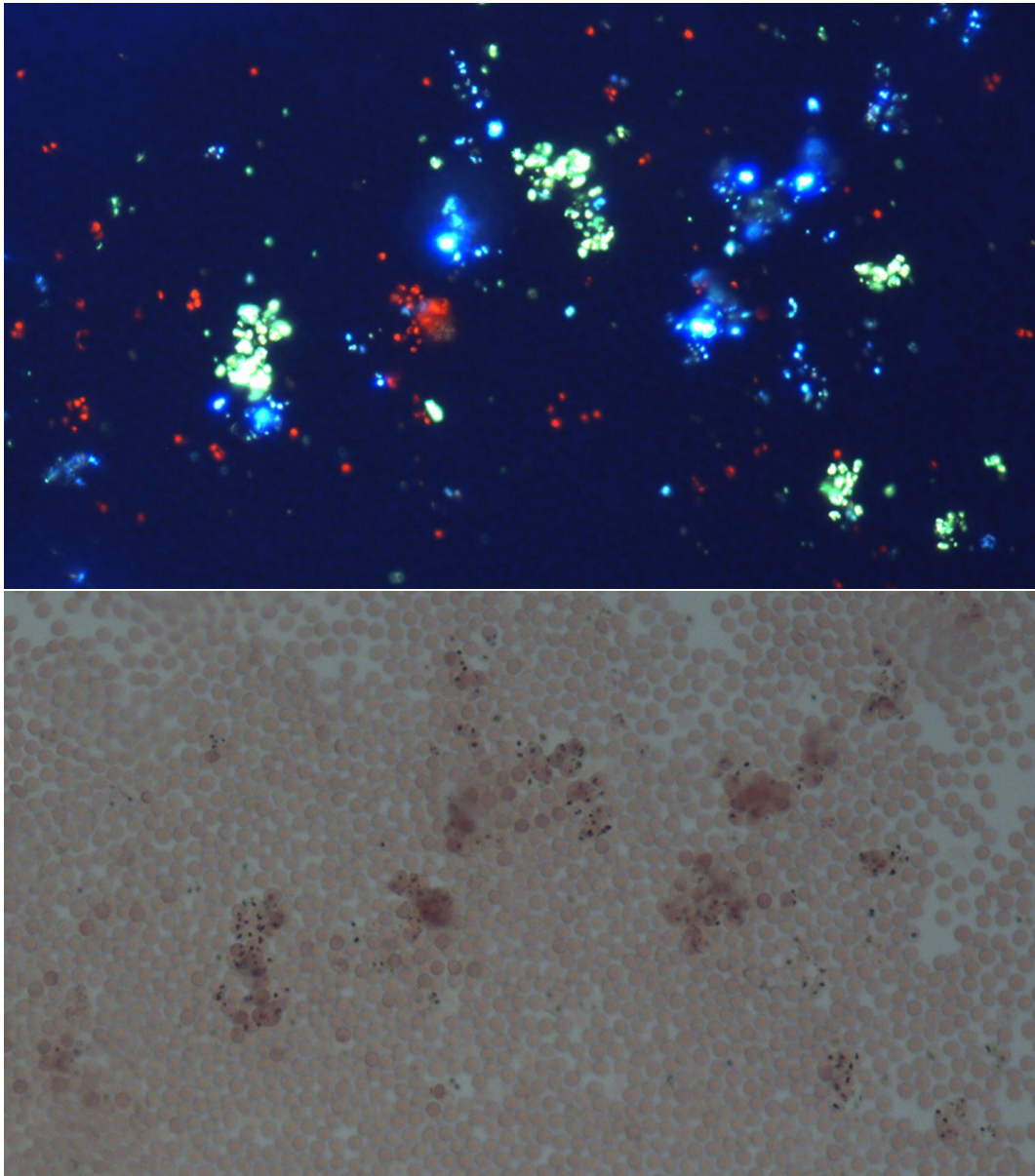


Figure 3.4. Triple agglutination assay with plasma 6 and parasite isolates 9106, 9605 and 11019. The corresponding bright field image is shown below the fluorescence image. The isolate names are coloured according to the colour that was observed by fluorescence microscopy. Adapted with permission from (Tan *et al.* 2016).

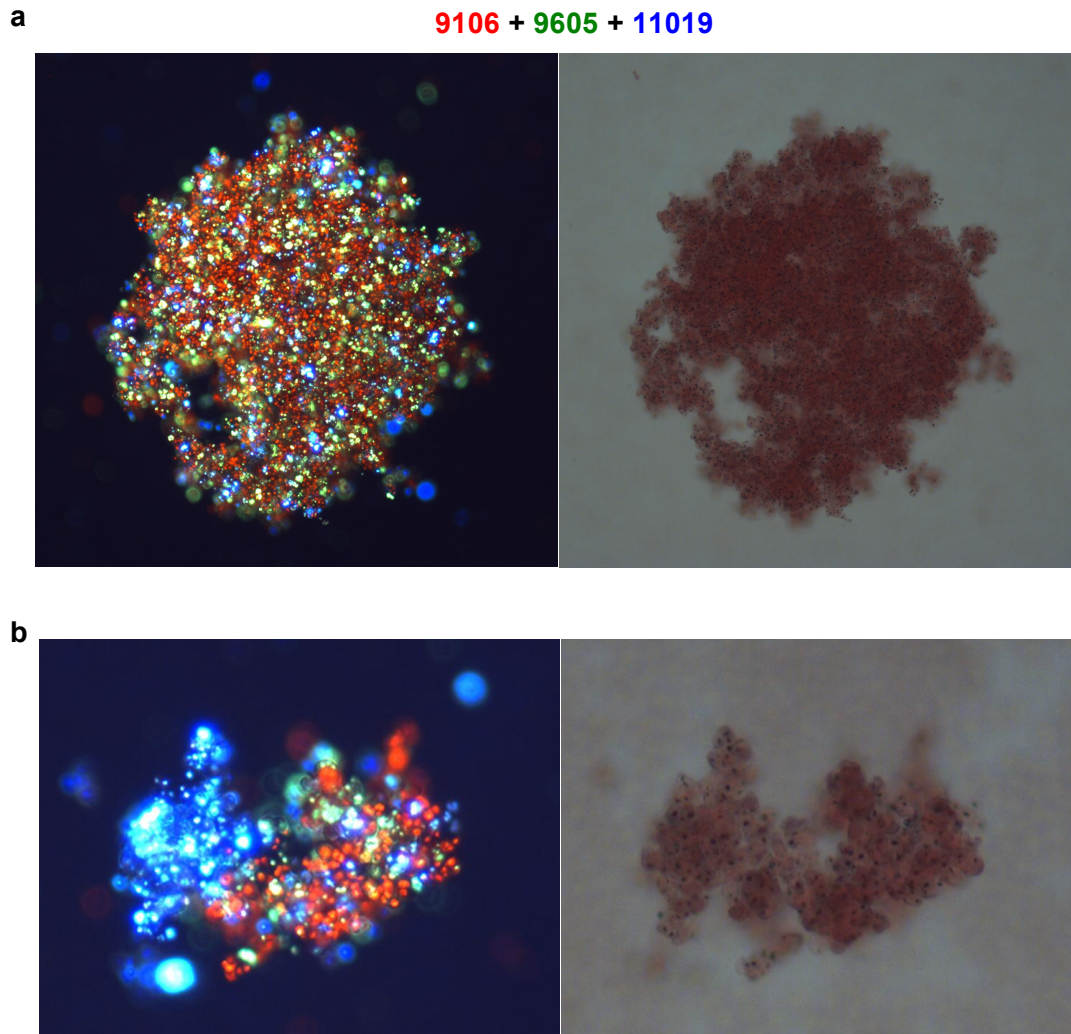


Figure 3.5. Triple agglutination assays with plasma 27 and nine parasite isolates. Shown are mixed agglutinates formed by plasma 27 with the 9106+9605+11019 parasite isolate combination (a-b), the 10668+10936+11014 combination (c), and the 9215+9775+10975 combination (d). Bright field images are shown alongside the fluorescence images. The isolate names are coloured according to the colour that was observed by fluorescence microscopy. Adapted with permission from (Tan *et al.* 2016)

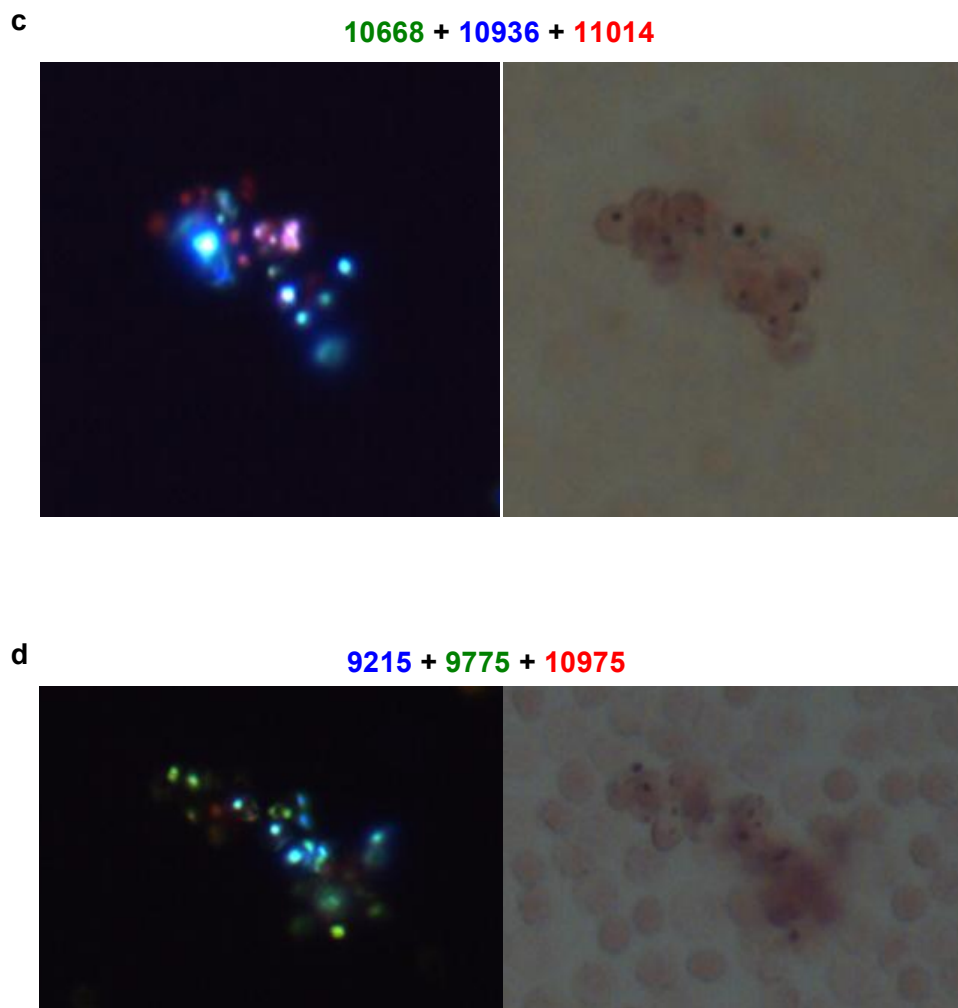


Figure 3.5. Triple agglutination assays with plasma 27 and nine parasite isolates.
(continued, see the previous page for complete legend).

3.7. Large-scale screening of plasma from Kenyan adults

The results of the triple agglutination assays suggested that plasma 27 contains broadly reactive antibodies to antigens on the *P. falciparum* IE surface. We would have liked to proceed by isolating and characterizing cross-reactive monoclonal antibodies from this plasma (if monoclonal antibodies were indeed mediating the mixed agglutination phenotype). To do so, I would have needed to obtain PBMCs from this donor for immortalization of memory B cells. Unfortunately, plasma 27 was donated anonymously in 1995, so its donor could not be identified, and we did not have any PBMCs from this donor already stored.

Therefore, I decided to screen a large number of plasma from adults (n = 557) living in Junju, a malaria-endemic area in Kilifi County, in the hope of finding more individuals with cross-reactive plasma. These plasma were obtained during yearly cross-sectional bleeds between 2007 and 2014 from donors whose details were recorded, allowing follow-up of these adults if necessary. Due to the large number of plasma tested, the plasma were pooled in groups of five and screened only using the triple mixed agglutination assay, which can also detect single-colour agglutinates. Two sets of three parasites were screened; 9106+9605+11019 and 10668+10936+11014.

The screening results of the first 45 plasma pools are shown in Figure 3.6 and the complete results of the screen are shown in the Supplementary Information section (Figure S1). In general, the 9106, 9605 and 11019 isolates were much better recognized than the 10668, 10936 and 11014 isolates. Most of the plasma pools that could agglutinate the second parasite combination did so by forming mixed agglutinates rather than single agglutinates. Also, mixed agglutination

mostly occurred in an all-or-nothing fashion, as plasma pools generally formed mixed agglutinates with most isolates tested, or did not form mixed agglutinates at all. I identified only three pools of plasma (M56-60, M151-155 and M156-160) that formed mixed agglutinates with at least five parasites and proceeded to screen individual plasma within these pools using the panel of nine parasite isolates previously used to screen plasma 27. These plasma were tested in three separate triple agglutination reactions to allow coverage of all nine isolates. Four plasma (M58, M151, M152 and M158) that could form triple agglutinates were identified (Figures 3.7 and 3.12). As seen previously for plasma 27, M58 and M151 could also form single-colour 11019 agglutinates. PBMCs were obtained from three of these adults during a cross-sectional bleed conducted in early 2014 (the donor of plasma M152 could not be located), as well as from one adult (M56) whose plasma formed variant-specific agglutinates with three parasite isolates. Plasma obtained during this bleed maintained their agglutinating phenotype.

Plasma	Individual			Mixed	Individual			Mixed
	9106	9605	11019		10668	10936	11014	
M1-5								
M6-10								
M11-15								
M16-20								
M21-25								
M26-30								
M31-35								
M36-40								
M41-45								
M46-50								
M51-55								
M56-60				9106 + 9605 + 11019				10668 + 10936 + 11014
M61-65								
M66-70								
M71-75								
M76-80								
M81-85								
M86-90								
M91-95								
M96-100								
M101-105								
M106-110								
M111-115								
M116-120								
M121-125								
M126-130								
M131-135								
M136-140								
M141-145								
M146-150				9605 + 11019				10936 + 11014
M151-155	nd	nd	nd	9106 + 9605 + 11019				10668 + 10936 + 11014
M156-160	nd	nd	nd	9106 + 9605 + 11019				10936 + 11014
M161-165								
M166-167								
JA001-005								
JA006-010								
JA011-015								
JA016-020								
JA021-025								
JA026-030								
JA031-035								
JA036-040								
JA041-045								
JA046-050								
JA051-055								10936 + 11014

Figure 3.6. Mixed agglutination screens of plasma from adults living in a malaria-endemic region in Kilifi County, Kenya. The plasma were pooled in groups of five and tested in two separate triple agglutination reactions. Single-colour agglutinates were scored alongside mixed agglutinates in the mixed agglutination reactions. Agglutinates were scored on a scale of 0-4 based on size (see Methods for scoring system). nd, not determined. Only the results of the first 45 plasma pools are shown here. The results of the entire screen are shown in Figure S1.

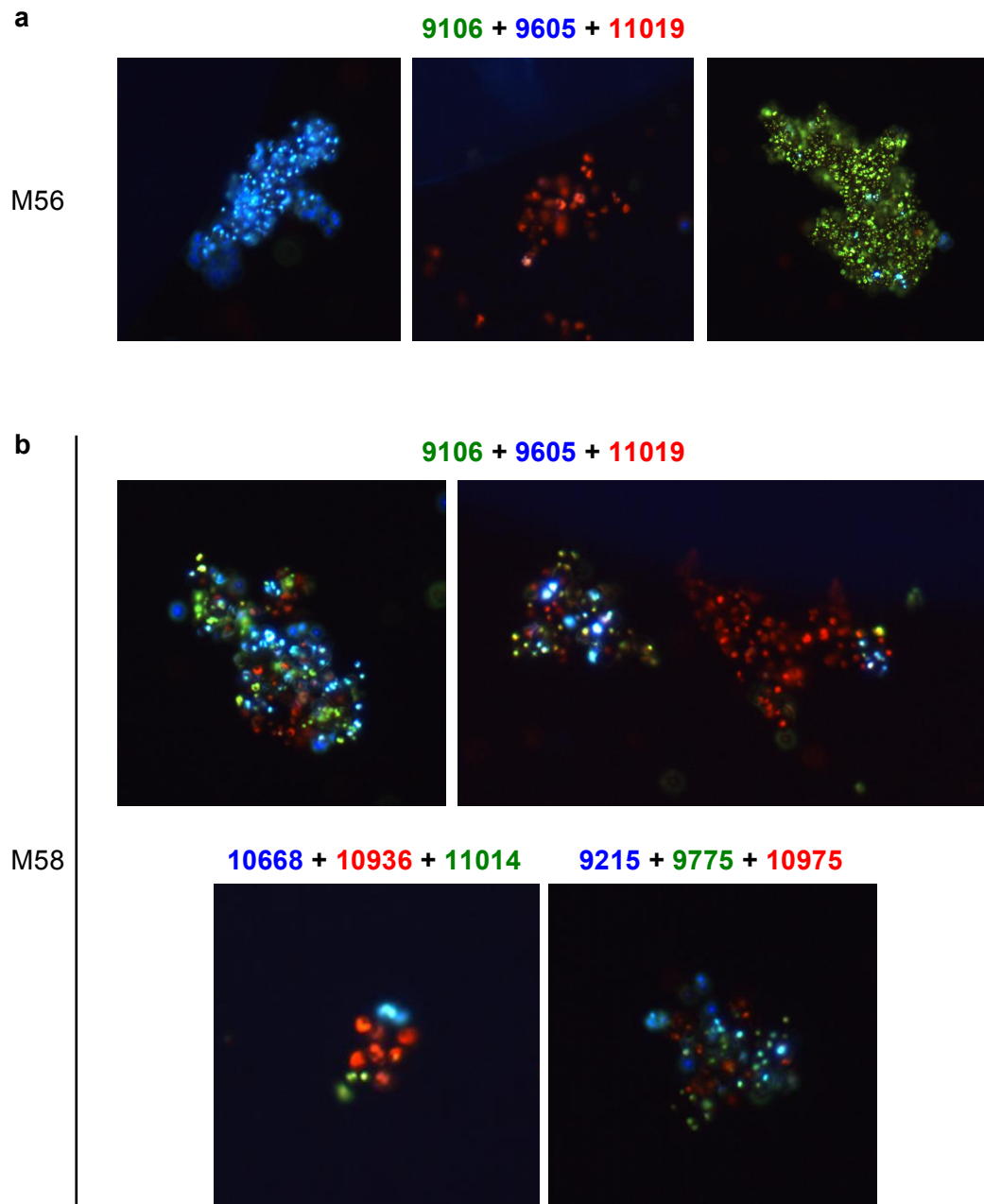


Figure 3.7. Agglutination of IEs by plasma from selected Junju adults. a, Mixed agglutination assay with plasma M56 and the parasite isolates 9106, 9605 and 11019. Only single-colour agglutinates were observed. b, Mixed agglutination assay with plasma M58 and nine parasite isolates (in three separate triple agglutination reactions). c, Mixed agglutination assay with plasma M151 and nine parasite isolates (in three separate triple agglutination reactions). d, Mixed agglutination reaction of plasma M158 with six parasite isolates (in two separate triple agglutination reactions). No mixed agglutinates were seen with the 10668+10936+11014 parasite combination. The isolate names are coloured according to the colour that was observed by fluorescence microscopy.

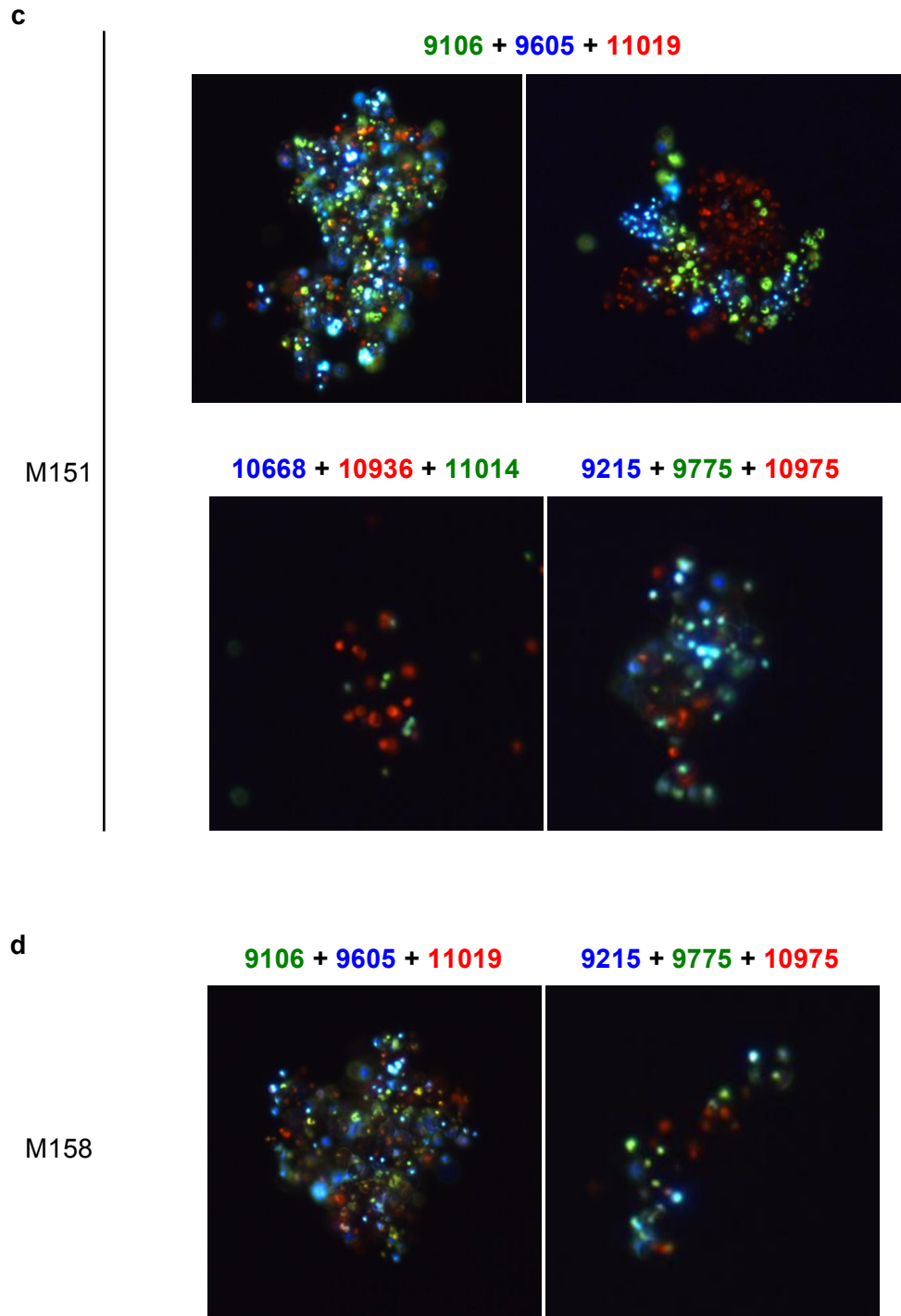


Figure 3.7. Agglutination of IEs by plasma from selected Junju adults (continued, see the previous page for complete legend).

3.8. Agglutination of circulating isolates

The *P. falciparum* isolates used thus far were culture-adapted to allow large-scale production of IEs for multiple agglutination assays. Nevertheless, previous studies have suggested that *in vitro* culture may result in the alteration of antigens expressed on the IE surface (Bachmann *et al.* 2012). For instance, PfEMP1 expression may be downregulated during *in vitro* culture (Peters *et al.* 2007). Therefore, I was interested in determining whether the broadly reactive plasma could also form mixed agglutinates with *ex vivo* isolates obtained directly from patients. Triple mixed agglutination experiments were performed on three parasite isolates (15003, 15184 and 15385) that were obtained from patients at the ring stage and cultured for half a cycle until the trophozoite stage. These isolates were chosen based on the blood type of the patients (O blood group) and a sufficient level of parasitaemia to allow detection by the agglutination assay. All three plasma could form double or triple agglutinates, with M58 forming the largest triple agglutinates (Figure 3.8).

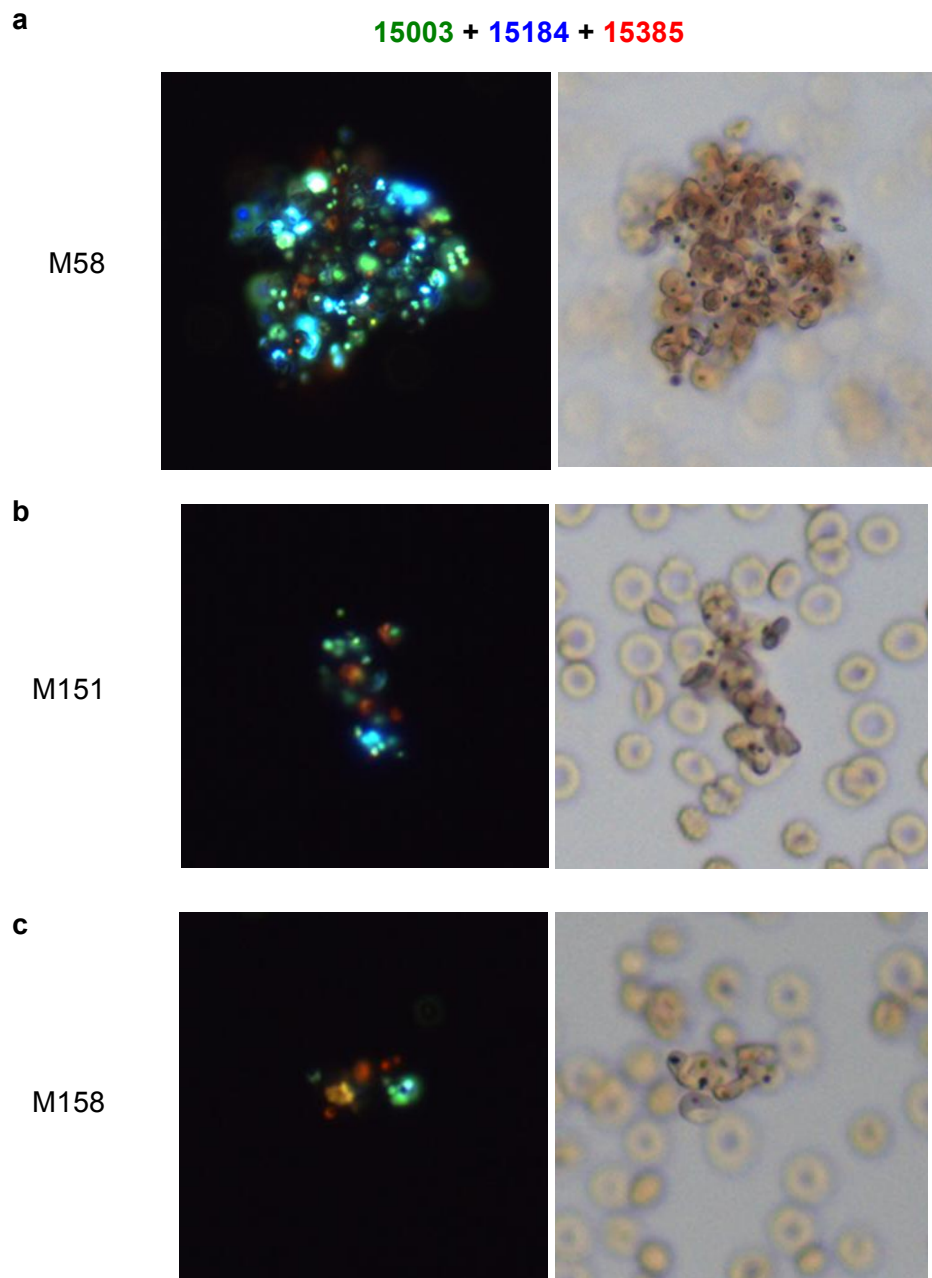


Figure 3.8. Agglutination of IEs from *ex vivo* isolates by broadly reactive plasma from selected Junju adults. M58 (a), M151 (b) and M158 (c) plasma were individually tested against three parasite isolates. The isolate names are coloured according to the colour that was observed by fluorescence microscopy. Bright field images are shown alongside the fluorescence images.

3.9. Investigation of antibody isotype mediating agglutination

Next, I proceeded to determine the antibody isotype that was causing mixed agglutination, as this would affect the subset of memory B cells that would be chosen for immortalization. It was suspected that IgG was the main isotype responsible, as this isotype was previously linked to agglutination and IE recognition (Piper, Roberts and Day 1999). Furthermore, the exposure of these adults to *P. falciparum* parasites over many years was likely to drive secondary immune responses, which are generally dominated by IgG antibodies. IgG from each adult plasma of interest was purified on protein G columns (Figure 3.9a) and retested in the agglutination assay along with the flowthrough from IgG purification (IgG-depleted plasma). The purified IgG fractions were able to form mixed agglutinates, although these were relatively small in the case of M58 IgG (Figures 3.9b and 3.12). Surprisingly, IgG-depleted M58, M151 and 27 fractions formed large mixed agglutinates, suggesting that the agglutination was also mediated by other factors in plasma such as IgM. In line with these findings, when I stained double mixed agglutinates with anti-human IgG or anti-human IgM, agglutinates formed by all tested plasma could be recognized by anti-IgG, but those formed by plasma M58 and M151 (and perhaps 27) were also recognized by anti-IgM (Figure 3.10).

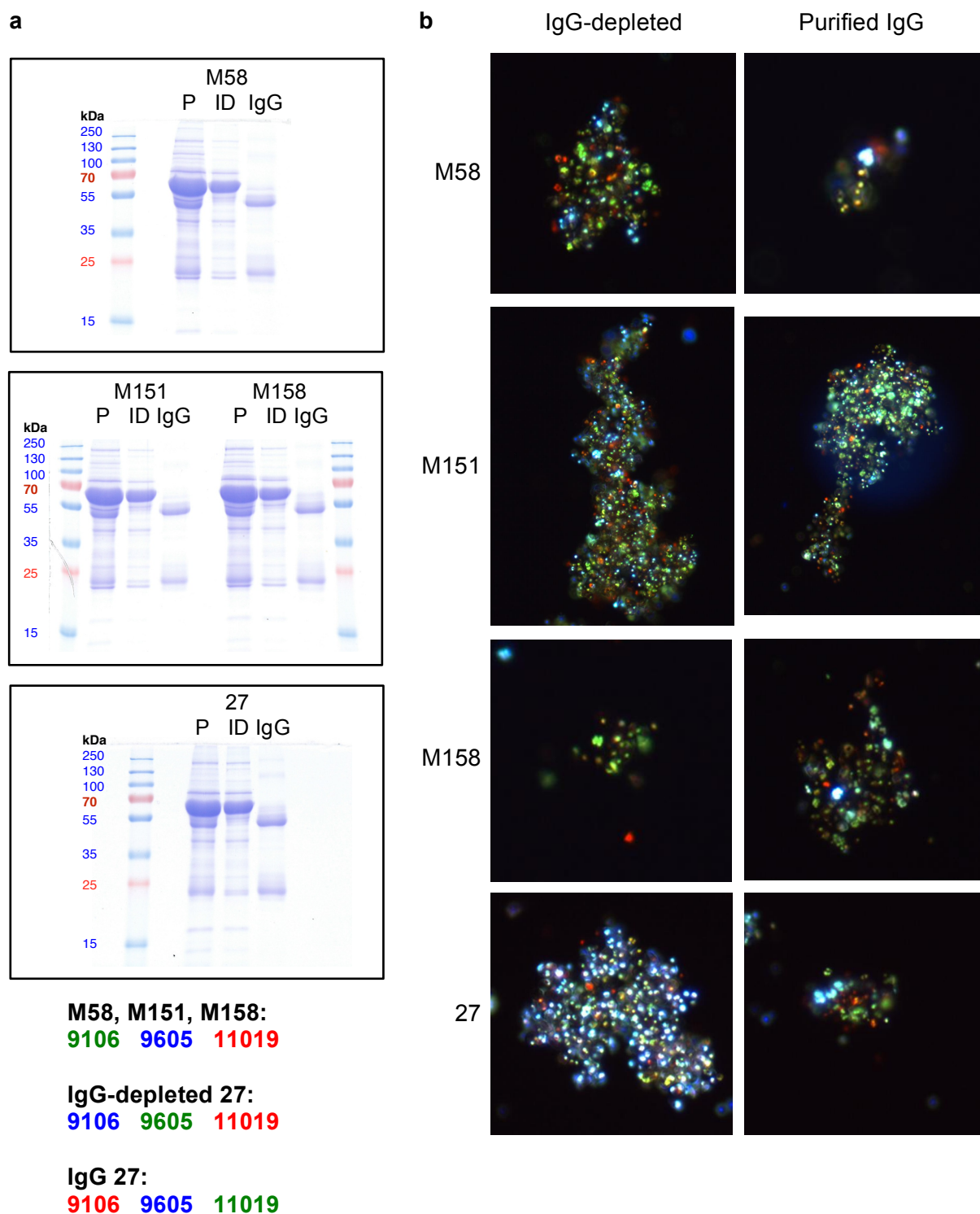


Figure 3.9. Triple mixed agglutination by IgG-depleted and purified IgG fractions from protein G purification of plasma. a, SDS-PAGE gel images showing original plasma (P) as well as IgG-depleted (ID) and purified IgG fractions from purification of M58, M151, M158 and 27. b, Images of mixed agglutinates formed by IgG-depleted and purified IgG fractions of M58, M151, M158 and 27.

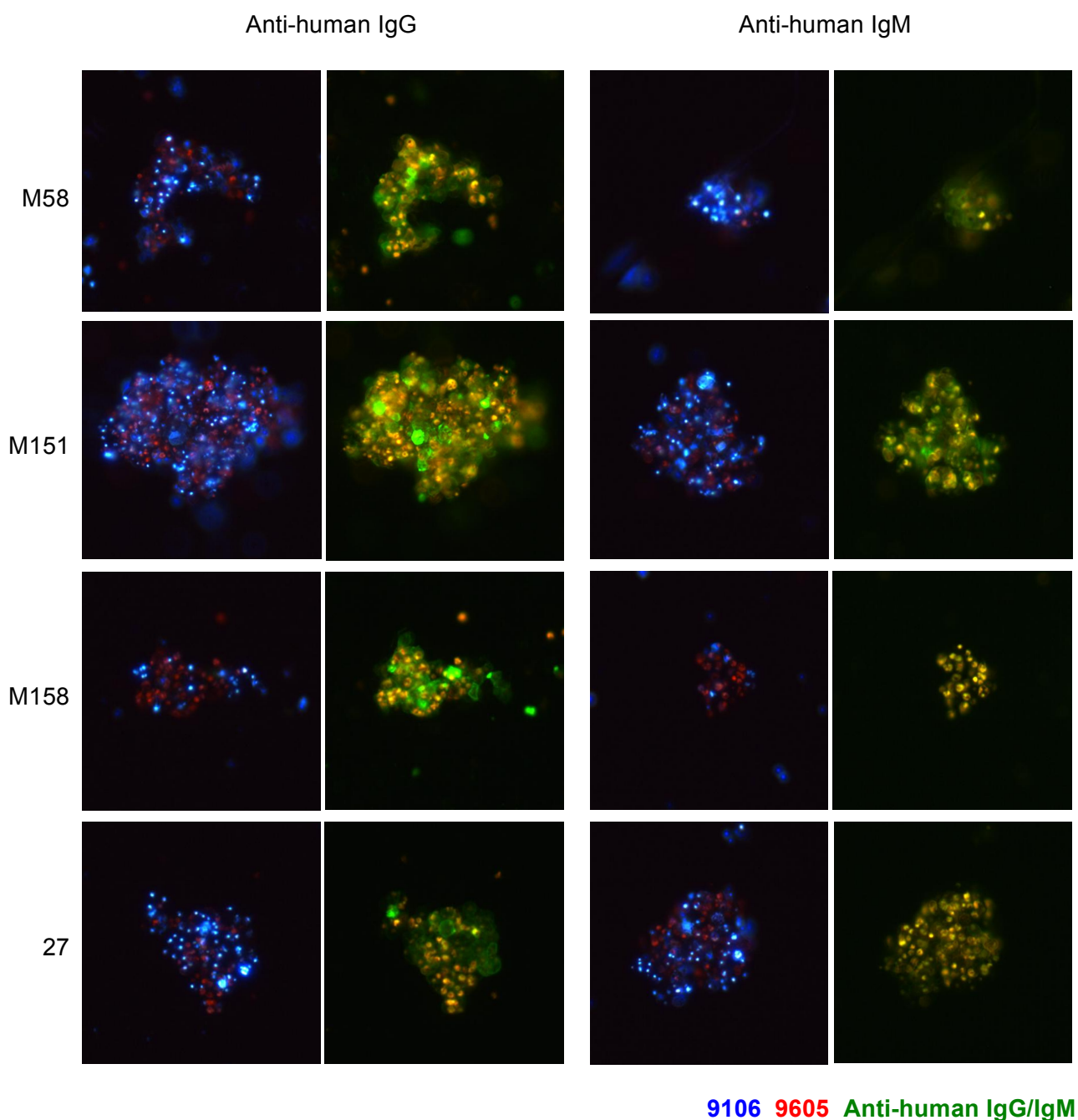


Figure 3.10. Anti-human IgG and anti-human IgM recognition of double mixed agglutinates formed by plasma M58, M151, M158 and 27. The parasite isolates 9106 and 9605 were labelled with DAPI or ethidium bromide, respectively, and stained with anti-human IgG or anti-human IgM conjugated to FITC after the formation of mixed agglutinates with the selected plasma. Two fluorescence filters are shown for each image: the first highlights the blue and red staining of the parasite isolates and the second shows the green anti-IgG/IgM staining.

3.10. Trypsin sensitivity assay

To conduct a preliminary investigation into the identity of the antigens recognized by the cross-reactive plasma, I treated IEs with various concentrations of trypsin prior to incubation with plasma for formation of mixed agglutinates. IEs treated with 1 µg/mL trypsin could still form mixed agglutinates with M58 plasma and M151 IgG, suggesting that PfEMP1, which is generally sensitive to trypsin at this concentration, may not be the antigen responsible for agglutination (Figure 3.11). Agglutinates formed by M56 IgG were unaffected even by 100 µg/mL trypsin, indicating that these antibodies do not recognize PfEMP1. I decided to postpone further experiments of this nature until we obtained monoclonal antibodies, which would allow us to conduct more precise antigen identification experiments.

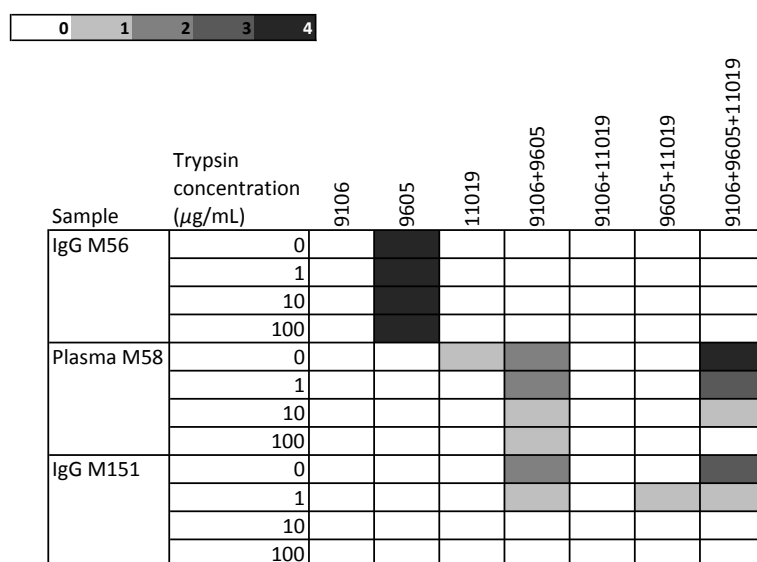


Figure 3.11. Triple mixed agglutination of isolates 9106, 9605 and 11019 by selected plasma or purified IgG following treatment of IEs with a range of trypsin concentrations. Agglutinates were scored on a scale of 0-4 based on size (see Methods for scoring system).

0	1	2	3	4
---	---	---	---	---

		9106 + 9605 + 11019			10668 + 10936 + 11014			9215 + 9775 + 10975		
		Individual			Individual			Individual		
		9106	9605	11019	Mixed	10668	10936	11014	Mixed	Mixed
Plasma	6									
	27				9106 + 9605 + 11019				10668 + 10936 + 11014	9215 + 9775 + 10975
	M56								nd	nd
	M58				9106 + 9605 + 11019				10668 + 10936 + 11014	9215 + 9775 + 10975
	M151				9106 + 9605 + 11019				10936 + 11014	9215 + 9775 + 10975
	M158				9106 + 9605 + 11019					9215 + 9775 + 10975
IgG-depleted	27				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M56					nd	nd	nd	nd	nd
	M58				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M151				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M158					nd	nd	nd	nd	nd
						nd	nd	nd	nd	nd
Purified IgG	27				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M56					nd	nd	nd	nd	nd
	M58				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M151				9106 + 9605 + 11019	nd	nd	nd	nd	nd
	M158				9106 + 9605 + 11019	nd	nd	nd	nd	nd
						nd	nd	nd	nd	nd

Figure 3.12. Summary of agglutination of various parasite isolates by selected plasma, as well as by IgG-depleted and purified IgG fractions of these plasma. Single-colour agglutinates were scored alongside mixed agglutinates in the mixed agglutination reactions. Agglutinates were scored on a scale of 0-4 based on size (see Methods for scoring system). nd, not determined.

3.11. Summary

In this chapter, I have reported the identification of broadly reactive plasma that target *P. falciparum* IEs from different parasite isolates. These plasma are relatively rare, even in a malaria-exposed adult population (4 out of 557, not considering the pilot experiments), but are able to bind to at least a proportion of IEs from most isolates tested, including from circulating isolates, suggesting that they recognize commonly expressed antigens. Surprisingly, the mixed agglutination phenotype was mediated both by IgG and a non-IgG factor (presumably IgM). I went on to obtain PBMCs from three donors of interest (M58, M151 and M158), and in the following chapter, I will describe the steps taken to obtain broadly reactive monoclonal antibodies through the immortalization of memory B cells.

Chapter 4. Discovery and characterization of broadly reactive LAIR1-containing antibodies

4.1. Introduction

In the previous chapter, I described the identification of broadly reactive plasma in several adults living in a malaria-endemic region in Kilifi County, Kenya. To characterize the antibody response against the *P. falciparum* IE surface in more detail, we decided to isolate monoclonal antibodies from these individuals. We chose to isolate these antibodies by immortalizing memory B cells, as these cells are likely to capture the history of each individual's antibody response to the relevant antigens. This chapter begins with an introduction of the immortalization platform, followed by a description of the broadly reactive monoclonal antibodies isolated, including the surprising finding that these antibodies contain an unusual insert from a different gene. In this chapter, I will rename M58 as donor D, M151 as donor C and M158 as donor B to fit into the existing nomenclature used for immortalization of *P. falciparum*-specific memory B cells at the Lanzavecchia laboratory. As a side note, donor A was a Tanzanian donor from whom I used B cells to set up the immortalization assay. No IE-specific antibodies were obtained from this donor. IgG and IgM antibodies will be given distinct names: for instance, IgG antibodies from donor D will be given a **Malaria IgG donor D** or MGD prefix while IgM antibodies from donor C will be given a **Malaria IgM donor C** or MMC prefix.

4.2. Platform for immortalization of memory B cells and production of monoclonal antibodies

Our approach for the immortalization of memory B cells allows us to obtain monoclonal antibody sequences from a large proportion (>30%) of the memory B cell compartment in selected individuals. Using this approach, memory B cells are first isolated from PBMC samples by cell sorting (Figure 4.1) and immortalized with EBV and CL264, a TLR7 agonist that increases the efficiency of immortalization to >30% (Figure 4.2) (Traggiai *et al.* 2004). The cells are then distributed into multiple 384-well plates at a density of 3 cells/well in the presence of irradiated PBMCs as feeder cells, such that each well contains one immortalized B cell on average when the efficiency of immortalization is taken into account. The immortalized B cell clones are then cultured for two to three weeks to allow B cell proliferation and the secretion of monoclonal antibodies. Next, the supernatants of the B cell clones from each well are screened by high-throughput flow cytometry for binding to *P. falciparum* IEs. Positive B cell clones are brought along two parallel paths. First, mRNA is extracted from the B cells and reverse-transcribed into antibody variable region cDNA using primers specific for the antibody heavy and light chain constant regions. The cDNA is then cloned into expression vectors containing the IgG1 constant regions and transiently transfected into a mammalian cell line for production of recombinant monoclonal antibodies. Second, the B cell clones are cultured and frozen for later use, allowing us to study the cells and extract genomic DNA if necessary. The supernatants of these cultures are also harvested as a source of monoclonal antibodies.

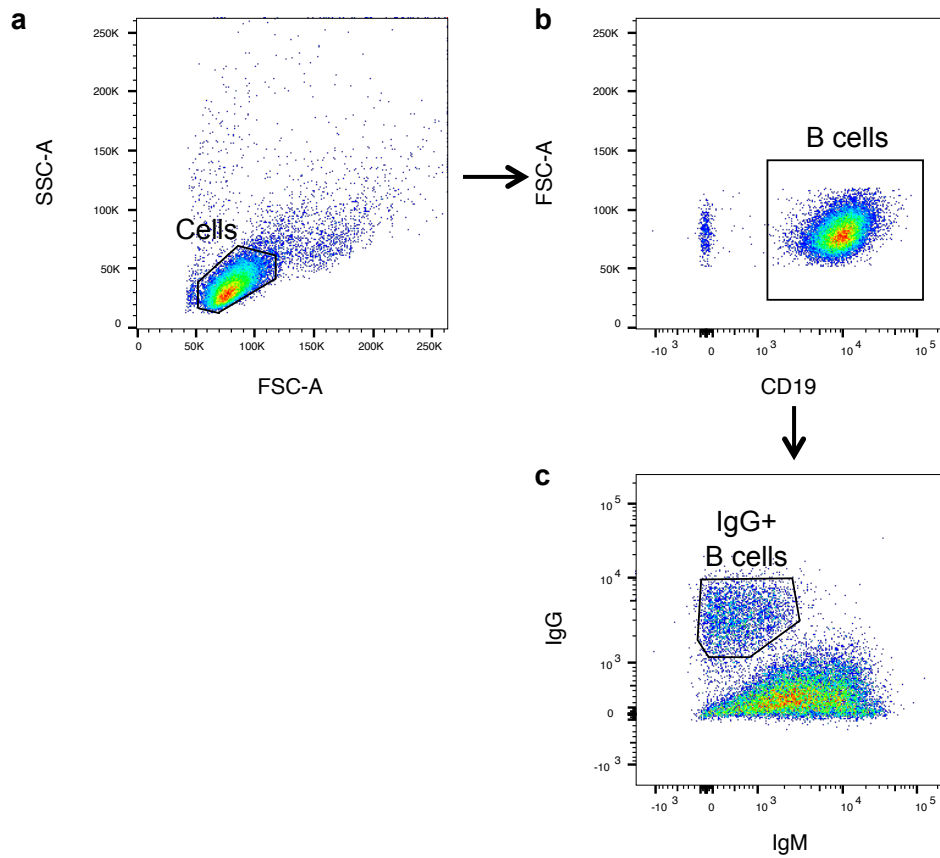


Figure 4.1. Gating strategy for memory B cells. B cells are enriched by magnetic selection (not shown). IgG memory B cells are sorted based on side and forward scatter (a), as well as expression of CD19 (b) and IgG (c). Alternatively, for IgM memory B cells, CD19⁺IgM⁺CD27⁺ or CD19⁺IgM^{hi}IgD^{lo} B cells are sorted.

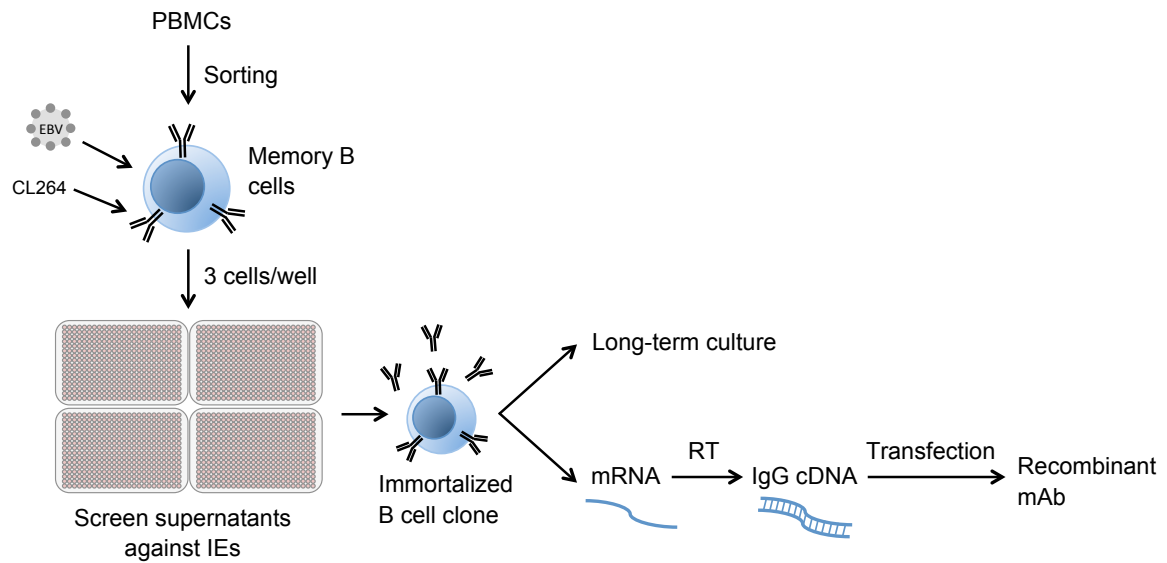


Figure 4.2. Platform for immortalization of memory B cells and production of monoclonal antibodies. Memory B cells are sorted from PBMCs, immortalized by EBV and a TLR agonist, and cultured at a frequency of 3 cells/well in multiple 384-well plates. The supernatants of these cells are then screened for binding to *P. falciparum* IEs. Positive B cell clones are kept in long-term culture or used for mRNA extraction, reverse transcription (RT) of mRNA to antibody cDNA, and recombinant monoclonal antibody (mAb) production.

4.3. Development of B cell supernatant screen against *P. falciparum* IEs

To identify potentially rare broadly reactive monoclonal antibodies targeting the *P. falciparum* IE surface, I developed a novel high-throughput system based on flow cytometry to screen for these antibodies. This antigen-agnostic approach involves the screening of B cell supernatants against whole, intact IEs rather than against selected antigens, allowing the detection of antibodies with many different specificities, including to previously uncharacterized antigens. The preliminary screening of B cell supernatants was conducted using a mixture of IEs from three Kilifi isolates stained with the same DNA-binding dye, such that any supernatant that stains at least one of the three isolates would be considered positive. I used IEs that were cryopreserved at the late trophozoite stage to facilitate large-scale screening at the time of our choice.

The primary screen of B cell supernatants was conducted using a high-throughput flow cytometer that uses a robotic system to screen multiple 384-well plates in a single run. The use of 384-well plates necessitated a no-wash approach, in which the plates were not washed after the addition of the B cell supernatants to the IEs or after the addition of the secondary anti-human IgG/IgM antibody. To avoid the selection of non-specific or “sticky” antibodies that bind more easily in this no-wash protocol, I did not purify IEs from uninfected erythrocytes, as the latter serve as excellent internal negative controls that can indicate non-specific binding (Figure 4.3).

4.4. Primary screen of B cell supernatants against *P. falciparum* IEs

Using the described approach, I screened the supernatants of ~30000 IgG⁺ B cells from donor B, ~90000 IgG⁺ B cells and ~28000 IgM⁺ memory B cells from donor C and ~130000 IgG⁺ B cells from donor D against a mixture of IEs from the Kenyan parasite isolates 9106, 9605 and 11019 (as well as the enriched 3D7 isolate 3D7-MGD21⁺, described later, for donor B), all stained with the DNA-binding dye SYBR Green I. An example of a typical image generated from the high-throughput flow cytometer is shown in Figure 4.3. In this figure, an example of a positive well is boxed in green, a negative well is boxed in red, and a non-specific well is boxed in purple. Only B cell supernatants that selectively stained IEs without staining uninfected erythrocytes were chosen as positive candidates for subsequent testing in a secondary screen against a larger panel of parasite isolates.

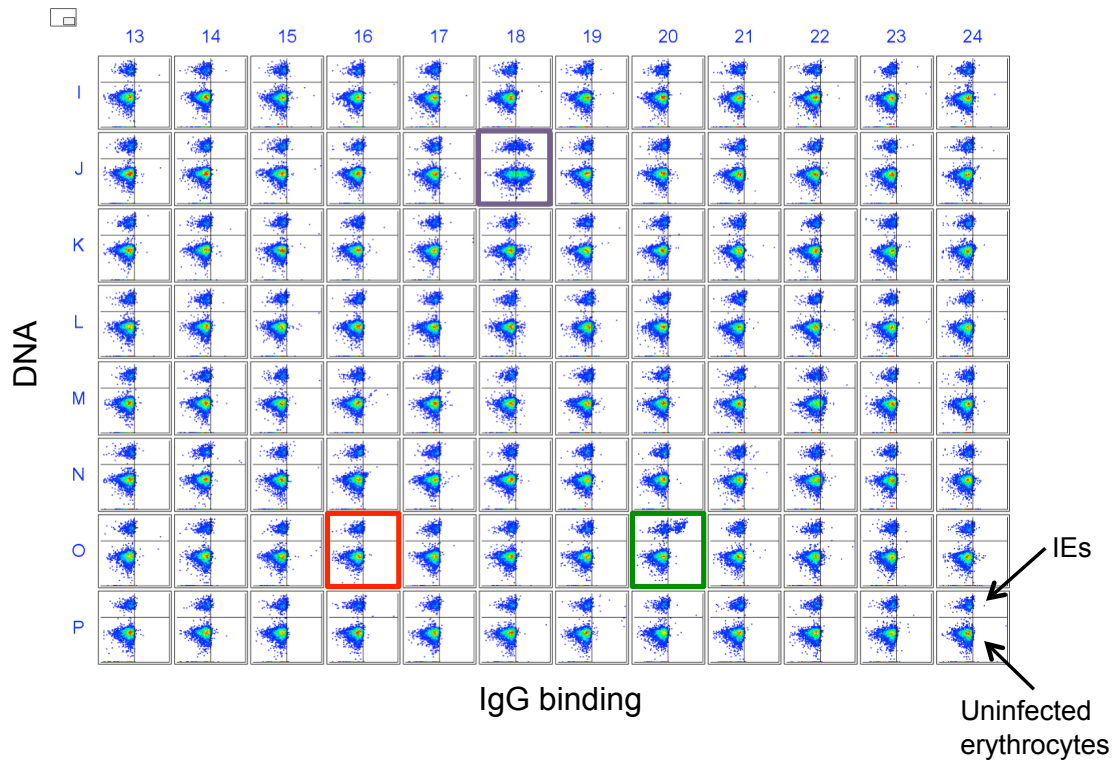


Figure 4.3. Example of an output image from the high-throughput flow cytometer. Only a quarter of a 384-well plate is shown. In each plot, the top population is the IE population, while the cells at the bottom are uninfected erythrocytes. An example of a positive well is boxed in green, a negative well is boxed in red, and a non-specific well is boxed in purple. Only positive wells were chosen for subsequent testing in a secondary screen.

4.5. Secondary screen of B cell supernatants against eight parasite isolates

In the secondary screen, I tested positive B cell supernatants for binding to IEs from up to eight Kenyan parasite isolates (9106, 9605, 11019, 9215, 9775, 10975, 10936, 11014), with staining of each isolate tested individually. An example of staining by a single monoclonal antibody is shown in Figure 4.4, and the results of this screen for B cell supernatants from donors B, C and D are shown in Figures 4.5, 4.6 and 4.7, respectively.

Most of the antibodies isolated from these donors were broadly reactive, with some antibodies binding to a percentage of IEs from all eight parasite isolates tested. Nevertheless, none of the antibodies from any donor could stain 100% of IEs from any given parasite isolate, suggesting that the target antigen is not completely conserved, but is likely to belong to a VSA family. In line with this observation, different antibodies showed distinct patterns of staining of the parasite panel, indicating that they were recognizing different antigens or VSA variants. While the percentage of IE recognition never reached 100%, it was sometimes quite high (>40%) in specific parasite-antibody combinations, suggesting that some target VSAs were commonly expressed. As seen in the agglutination experiments, certain parasite isolates (e.g. 9605, 11019) were better recognized than others (e.g. 10936, 11014) (Tan *et al.* 2016).

Regarding the specific donors, all of the IgG and IgM antibodies isolated from donor C reacted with at least three parasite isolates (Figure 4.6). The IgM antibodies shared similar binding patterns with their IgG counterparts, suggesting that they recognize similar antigens or VSA variants. For donor D, while most of the antibodies were broadly reactive, I also isolated a few

antibodies that only recognized a single parasite isolate, 11019, in agreement with the agglutination experiments (Figure 4.7). While this donor was also likely to have broadly reactive IgM antibodies based on the agglutination experiments, we decided to focus on the IgG response for the purposes of this project and did not attempt to isolate IgM antibodies from this donor. We are now planning a follow-up study investigating the IgM response in the different donors.

The antibodies from donor B are the most recent to be isolated and have only been tested against three Kenyan parasite isolates (Figure 4.5). Based on these results, it appears that the MGB antibodies do not bind to as high a percentage of IEs as the antibodies from the other two donors.

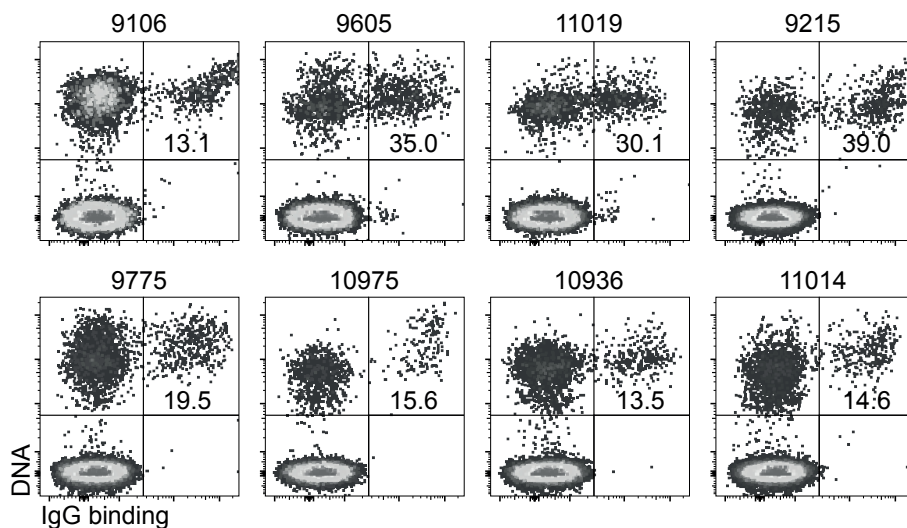


Figure 4.4. Example of staining of eight parasite isolates by the monoclonal antibody MGD55. In each plot, the top population is the IE population, while the cells at the bottom are uninfected erythrocytes. Reprinted with permission from (Tan *et al.* 2016).

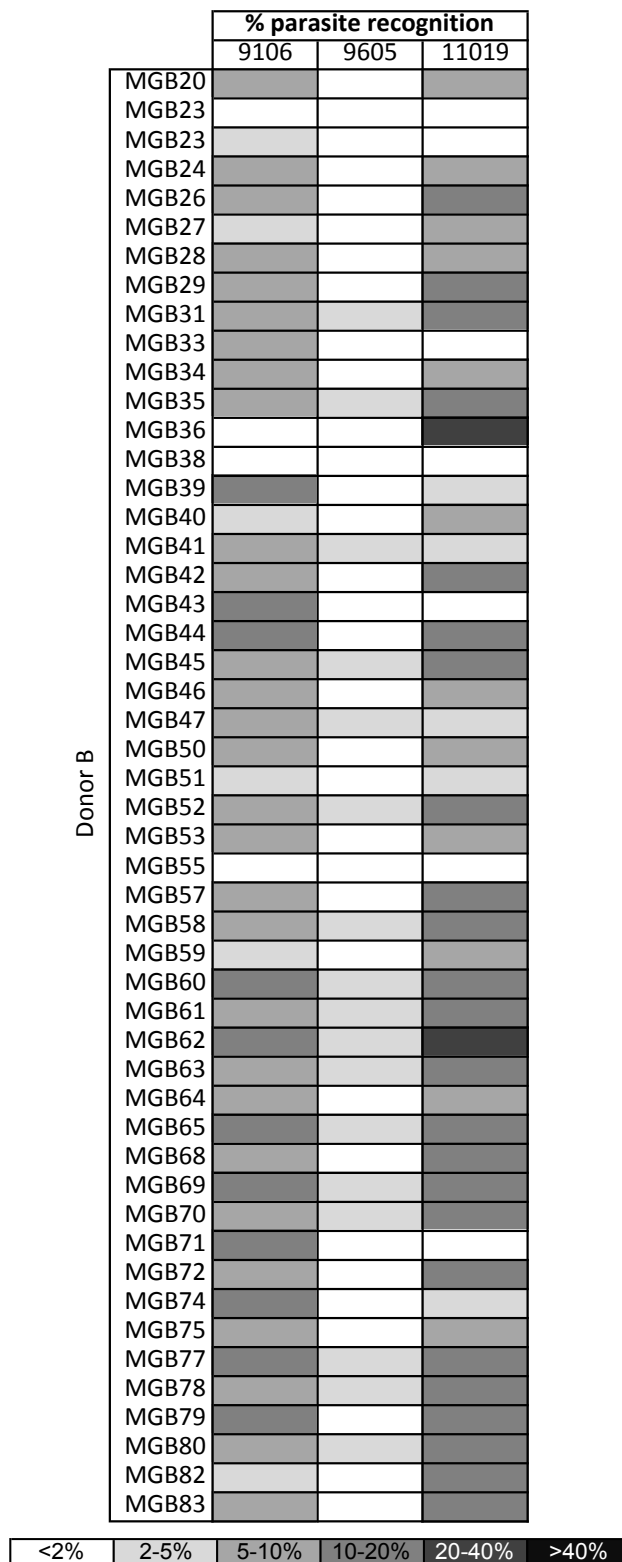


Figure 4.5. Recognition of three Kenyan parasite isolates by antibodies from donor B. The scale shows the percentage of IEs from each isolate recognized by the antibodies. Of the three donors, donor B has been the most recently studied, and the MGB antibodies have only been tested against three Kenyan isolates. Only IgG antibodies were isolated from donor B.

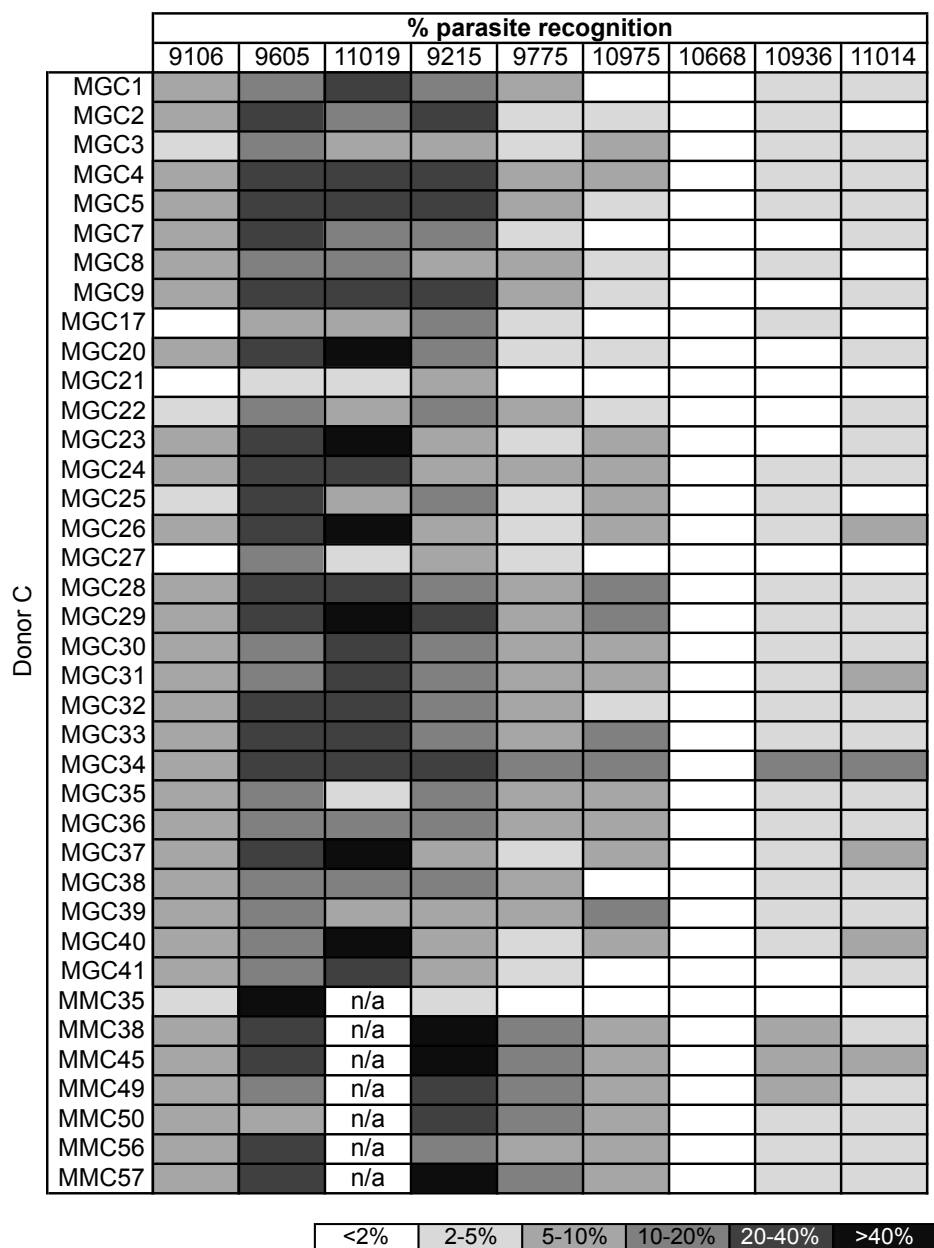


Figure 4.6. Recognition of eight Kenyan parasite isolates by antibodies from donor C. The scale shows the percentage of IEs from each isolate recognized by the antibodies. Antibodies with an MGC prefix are IgG antibodies while those with an MMC prefix are IgM antibodies. The MMC antibodies were not tested against 11019 as this parasite is a rosetting isolate that can bind non-specifically to IgM.

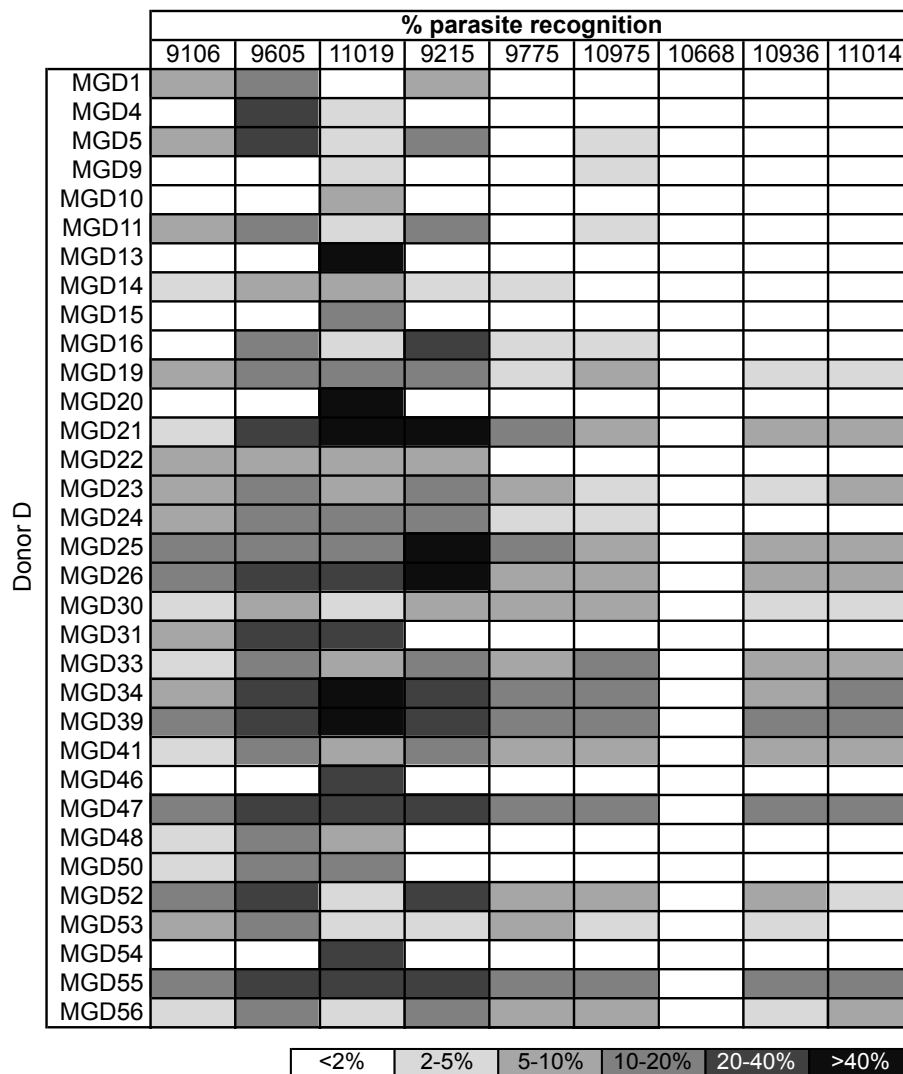


Figure 4.7. Recognition of eight Kenyan parasite isolates by antibodies from donor D. The scale shows the percentage of IEs from each isolate recognized by the antibodies. Only IgG antibodies were isolated from donor D.

4.6. Analysis of antibody cDNA sequences

We investigated the molecular basis of the broad reactivity of these antibodies by sequencing the antibody heavy and light chain cDNA^b. While the antibodies with narrow reactivity had the expected heavy chain size and showed classical VDJ organization (Figures 4.8^c, 4.9), the broadly reactive antibodies from donors C and D had heavy chains that were ~300 bp larger than expected (Figure 4.8) due to the insertion of a large DNA fragment between the V and D/J segments. In both donors, the core of the inserts encoded an amino acid sequence that was 85–96% identical to the extracellular domain of LAIR1, a collagen-binding inhibitory receptor encoded in the leukocyte receptor locus on chromosome 19 (Figure 4.10). LAIR1 intronic sequences were found adjacent to the LAIR1 exon in antibodies from both donors. Moreover, in the MGD antibodies, we identified a second insert upstream of LAIR1 that belonged to an intergenic region of chromosome 13. The inserts were separated from the V and D/J segments by sequences that was not derived from the insert or antibody (labelled “junction” in the figures), suggesting that non-templated addition of nucleotides had occurred, presumably during insertion. Interestingly, the inserted LAIR1 exon and intron had slightly different sequences from the chromosome 19 LAIR1 gene, indicating that these regions had undergone somatic hypermutation alongside the V and D/J segments during the germinal centre reaction (Tan *et al.* 2016).

^bThe molecular cloning work was done by laboratory technicians and the DNA sequencing was conducted by a private sequencing company (Microsynth AG). The analysis of the sequences was done by Dr Kathrin Pieper, Dr Luca Piccoli and me.

^cGel prepared by Dr Sonia Barbieri.

Thus far, we have only successfully obtained and verified a single complete cDNA sequence from antibodies from donor B (Figure 4.11). These antibodies appear to have a more complicated structure, as they do have the LAIR1 insert but appear to have lost a large portion of the variable region and the whole C_H1 domain (unlike the antibodies from donors C and D, which have normal constant regions). We are currently working on characterising these antibodies, and I will focus exclusively on the description of the antibodies from donors C and D for the remainder of this thesis.

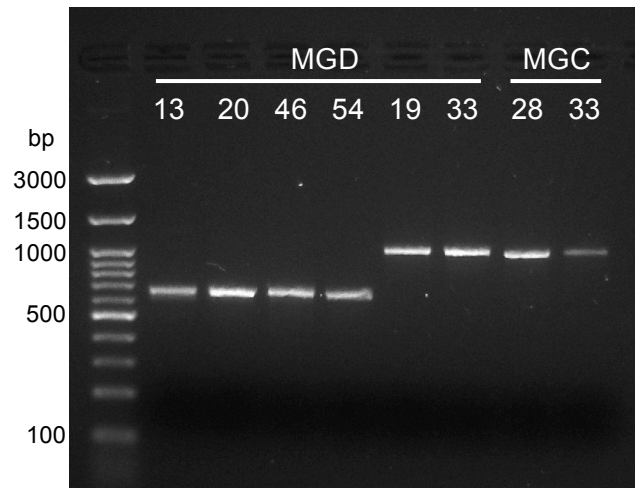


Figure 4.8. Broadly reactive antibodies have larger heavy chains than parasite-specific antibodies. The gel shows the PCR products of the antibody heavy chain after cloning into an IgG1 expression vector. MGD13, MGD20, MGD46 and MGD54 are parasite-specific antibodies, while MGD19, MGD33, MGC28 and MGC33 are broadly reactive antibodies.

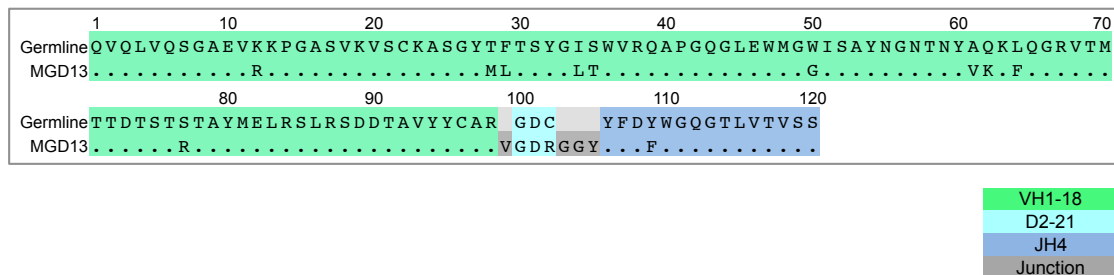


Figure 4.9. The isolate-specific antibody MGD13 has a conventional heavy chain sequence. Shown is the protein sequence alignment of the isolate-specific MGD13 antibody with germline heavy chain V (green), D (cyan) and J (blue) sequences. Grey areas show conventional junctional sequences for which no homology was found. Dots indicate positions where the amino acid of MGD13 is identical to that of the germline.



Figure 4.11. Protein sequence of MGB47. Shown is the protein sequence alignment of MGB47 to germline LAIR1 (red), IgG3 hinge (lilac) and IgG3 C_H2 and C_H3 (beige) regions. The antibody sequence starts with a small fragment that is very similar to the start of the variable domain (green) and a junctional sequence for which no homology was found (grey). Dots indicate positions where the amino acids of MGB47 are identical to those of the germline sequences.

4.7. Global alignment of MGC and MGD cDNA sequences

To determine whether the unusual characteristics of the MGC and MGD antibodies described so far are shared by all of the LAIR1-containing antibodies from the two donors, we sequenced the heavy and light chain cDNA of a larger number of broadly reactive antibodies (14 from donor C and 13 from donor D) and performed sequence alignments on all of the antibodies from each donor (heavy chain DNA alignments in Figures 4.12 and 4.13; light chain DNA alignments in Figure 4.14; protein alignments in Figure 4.15). These alignments revealed that the antibodies from the two donors have distinct architectures, as they use different heavy chain V genes (VH3-7 in MGC antibodies and VH4-4 in MGD antibodies) and have junctions of different lengths and sequences. Moreover, the MGC and MGD antibodies have different insert structures, as the MGC antibodies have an upstream LAIR1 intron while the MGD antibodies have a short upstream insert derived from chromosome 13 and a longer downstream LAIR1 intron. Furthermore, the broadly reactive antibodies from donor D shared a single (conventional) light chain (VK1-8/JK5), while the antibodies from donor C had one of three different (conventional) light chains (VK1-5/JK2, VK4-1/JK2, VL7-43/JL3). In contrast, the LAIR1-containing antibodies within each donor are remarkably similar, as they use the same V and J segments, have very similar junction structures and do not differ greatly in their LAIR1 insert sequences. Based on these observations, we concluded that within each individual, a single B cell clone carrying a LAIR1 insert expanded after stimulation by malaria antigens and progressively accrued mutations in the LAIR1, V_H and V_L regions. The clonality of the LAIR1-containing antibodies from each donor allowed us to plot genealogy trees showing a developmental pathway with progressive acquisition

of somatic mutations from the unmutated common ancestors (UCAs) to the mature antibodies (Figure 4.16)^d. Notably, the trees generated with the V_H sequence were consistent with those generated using only the LAIR1 insert or the V_L sequence, suggesting that the mutations in the different antibody sections occurred in tandem (Tan *et al.* 2016).

^dThe genealogy trees were constructed by Ms Mathilde Foglierini.

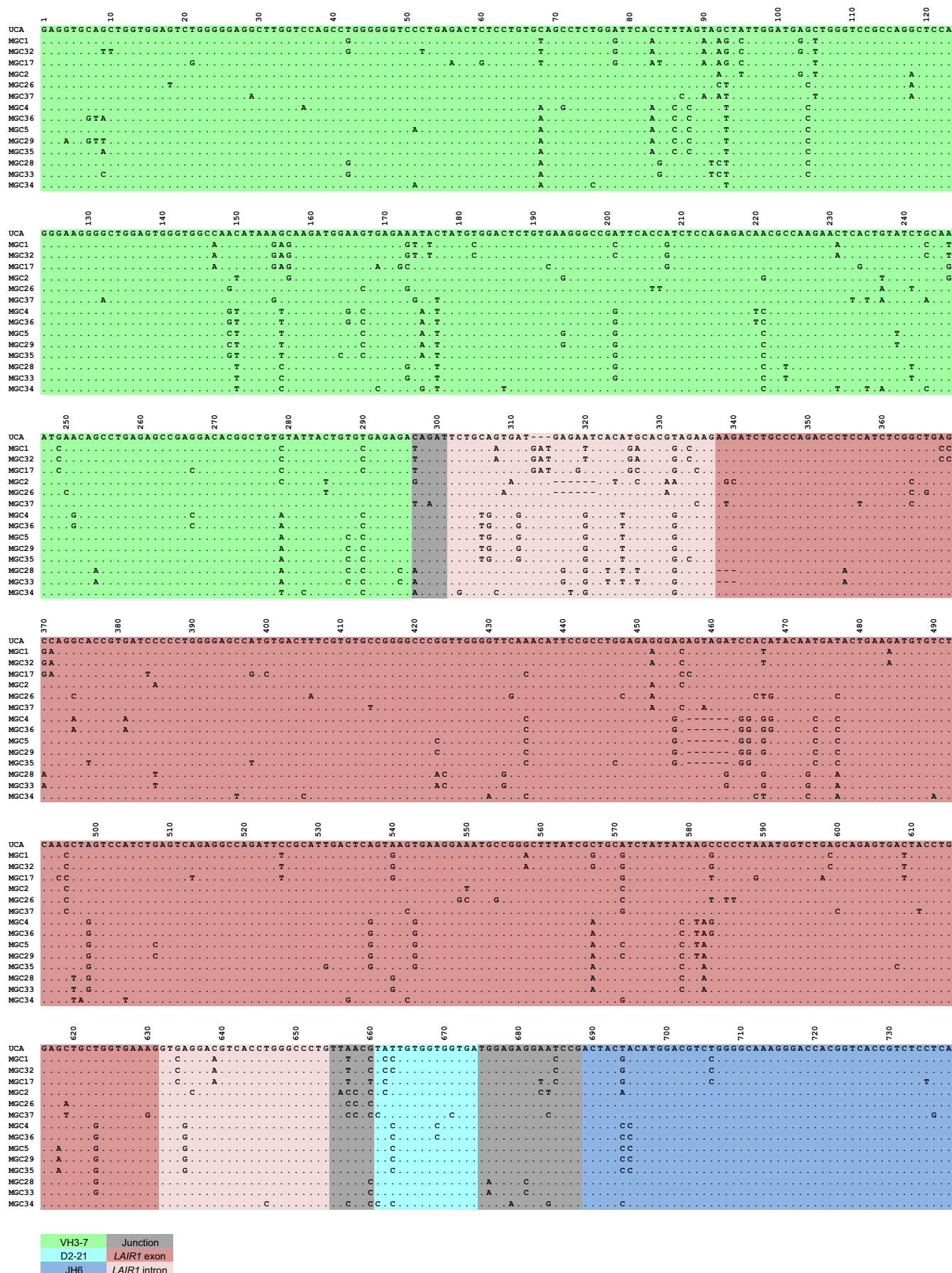


Figure 4.12. Nucleotide sequence alignments of V_H regions of antibodies isolated from donor C. Dots indicate positions where the nucleotide of a mature antibody is identical to that of the UCA. Reprinted with permission from (Tan *et al.* 2016).

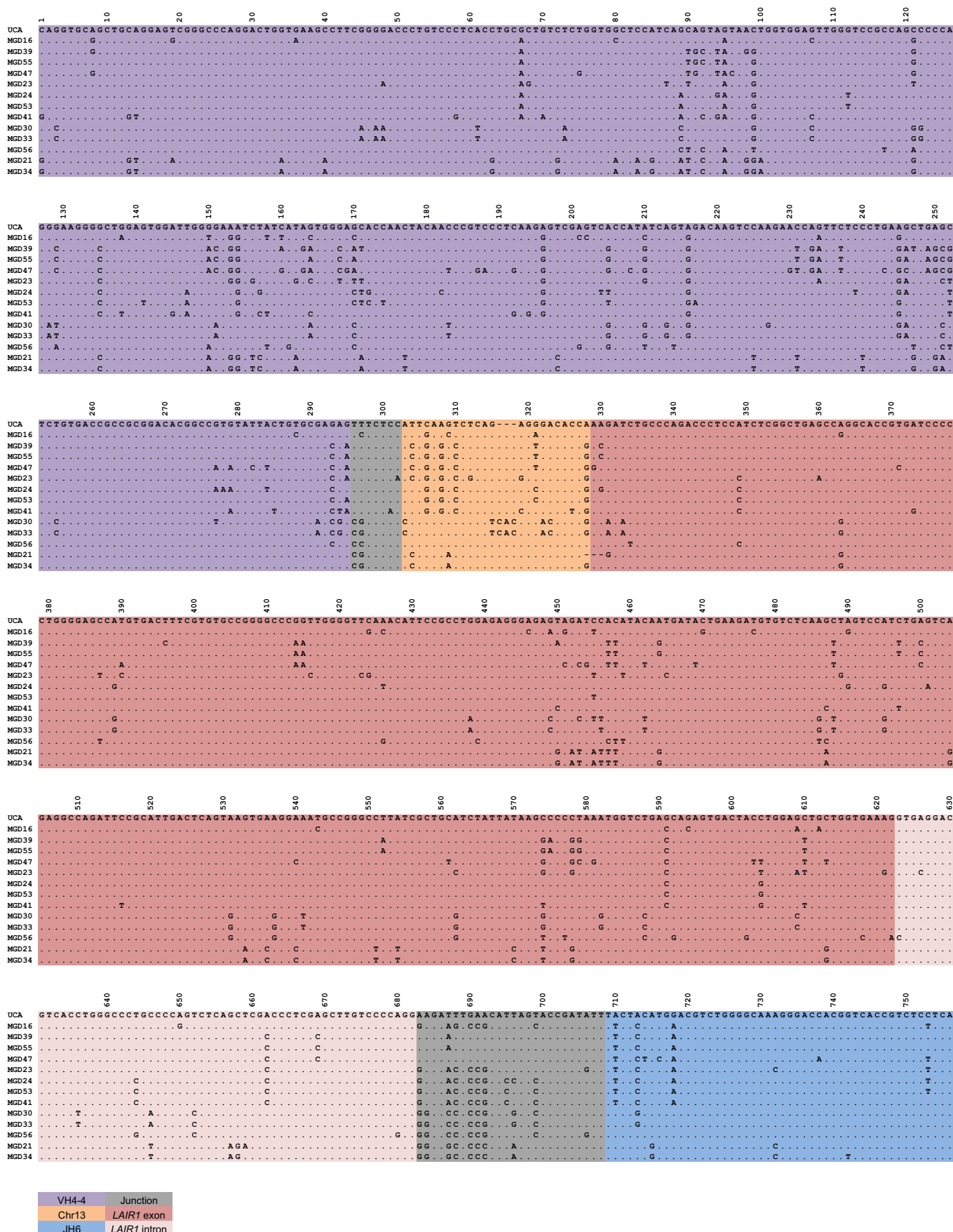


Figure 4.13. Nucleotide sequence alignments of V_H regions of antibodies isolated from donor D. Dots indicate positions where the nucleotide of a mature antibody is identical to that of the UCA. Reprinted with permission from (Tan *et al.* 2016).

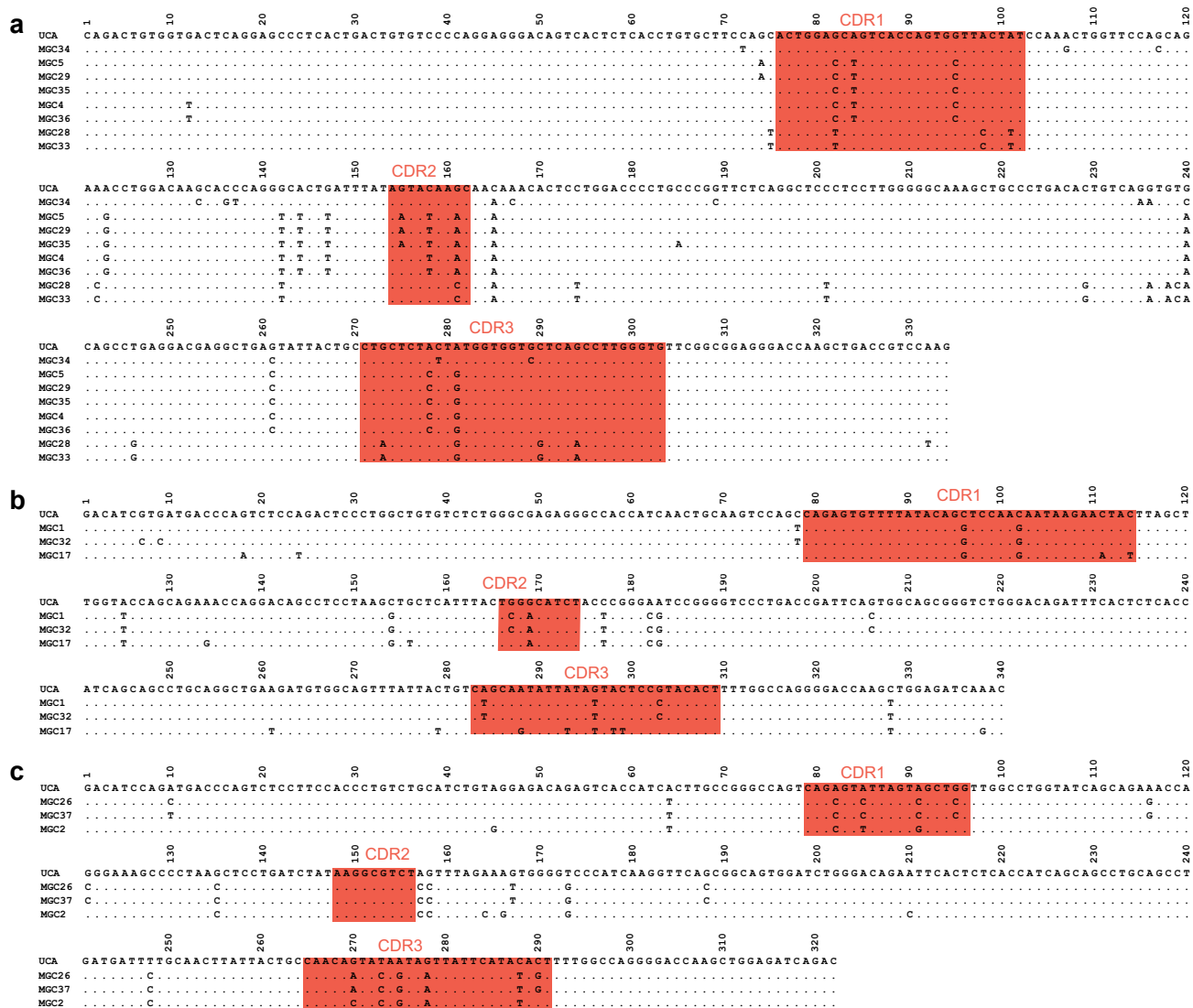


Figure 4.14. Nucleotide sequence alignments of V_L regions of antibodies isolated from donors C and D. a–d, Antibodies from donor C use VL7-43/JL3 (a), VK4-1/JK2 (b), or VK1-5/JK2 (c), while antibodies from donor D use VK1-8/JK5 (d). CDRs are highlighted in red. Dots indicate positions where the nucleotide of a mature antibody is identical to that of the UCA. Reprinted with permission from (Tan *et al.* 2016).

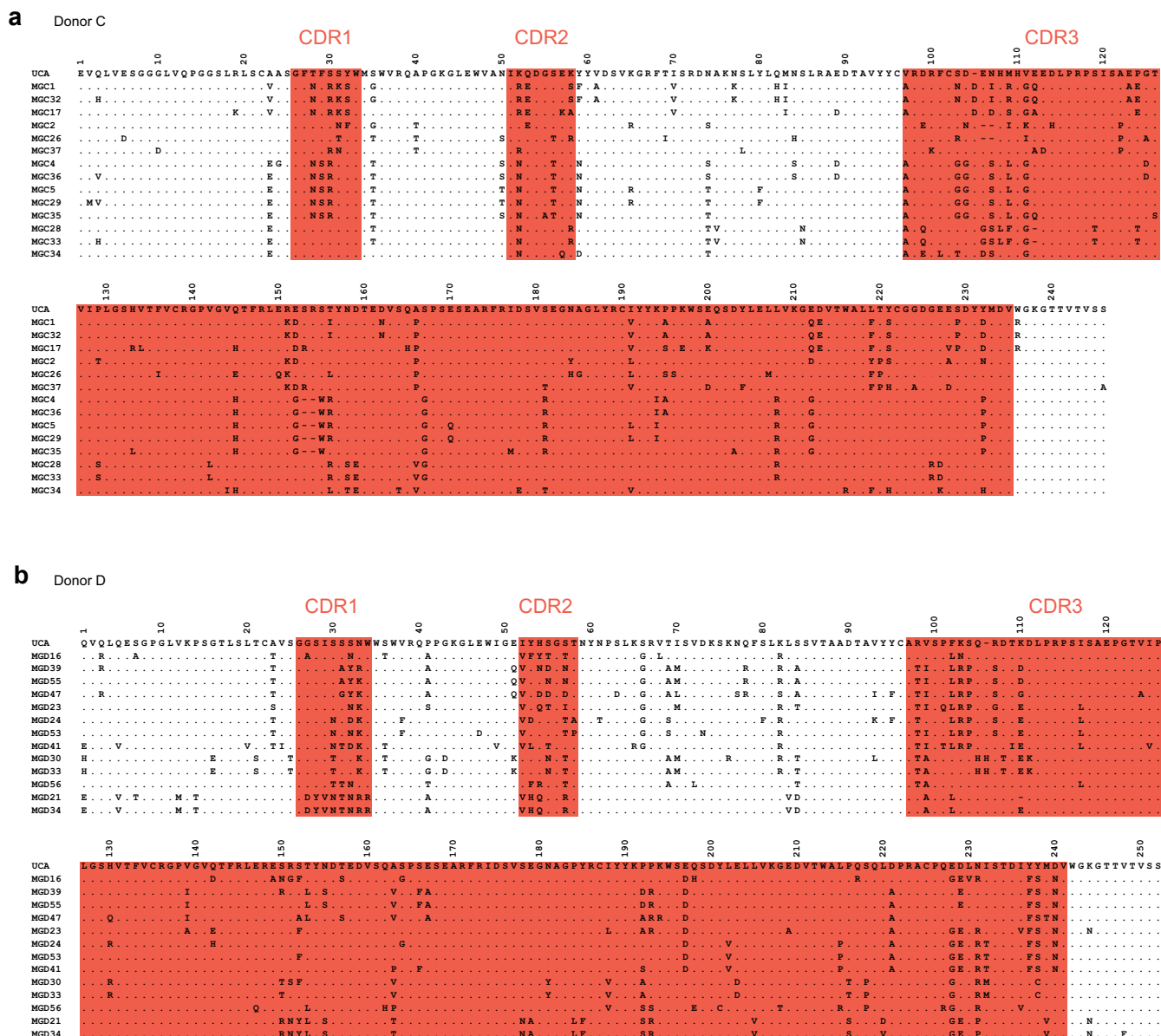


Figure 4.15. Protein sequence alignments of V_H regions of antibodies isolated from donors C and D. a, Donor C. b, Donor D. Putative CDRs are highlighted in red. Dots indicate positions where the amino acid of a mature antibody is identical to that of the UCA. Reprinted with permission from (Tan *et al.* 2016).

4.8. Splicing in the variable regions of LAIR1-containing antibodies

While analyzing the heavy chain cDNA sequences of the MGC antibodies, I observed that the upstream LAIR1 intron in these antibodies is not actually contiguous to the LAIR1 exon in the original chromosome 19 LAIR1 sequence, but is separated by a longer segment of the same intron (marked by asterisks in Figure 4.17). The boundaries of this segment are marked by consensus splice donor (GT) and acceptor sites (AG), suggesting that this segment was spliced out during mRNA processing. To test this hypothesis, we decided to investigate the genomic DNA sequences of the B cell clones producing LAIR1-containing antibodies, as detailed in the next section.

LAIR1	1	TCTGCAGTGATGAGAATCACATGCACGTAGAAG [*] GCAGGAGGCTTGGGAATGTTCTAAG	60
MGC37	1	TCTGCAGTGATGAGAATCACATGCACGTAGCAG-----	30
LAIR1	61	GTTGGGCTGTGGTCATGGCTGCATAACTCTATAAAATTGCTAAATCCCTGAATTGTGAT	120
MGC37	31	-----	30
LAIR1	121	GCTAAATGACGTGTGTGGCATGGTGACTTCCTACAGTGACGCTGAGATCCTGCTCTGC	180
MGC37	31	-----	35
LAIR1	181	TTCCCTCCT [*] AGAAGATCTGCCCAGACCCTCCATCTCGGCTGAGCCAGGCACCGTGATCCC	240
MGC37	36	-----ATGATCTGCCCAGACCCTCTATCTCGCCTGAGCCAGGCACCGTGATCCC	82
LAIR1	241	CCTGGGGAGCCATGTGACTTTCGTGTGCGGGGCCCGGTTGGGGTTCAAACATTCCGCCT	300
MGC37	83	CCTGGGGAGCCATGTGACTTTCGTGTGTGCGGGGCCCGGTTGGGGTTCAAACATTCCGCCT	142

Figure 4.17. Alignment of the LAIR1-containing antibody MGC37 with the germline chromosome 19 LAIR1 sequence. MGC37 has an upstream LAIR1 intron (light red) that is not contiguous to the LAIR1 exon (red) in the original chromosome 19 sequence, but is separated by a longer piece of the same intron that is flanked by consensus splice donor and acceptor sites (highlighted yellow) The start and end of this piece of intron are marked by asterisks.

4.9. Comparison of cDNA and genomic DNA sequences of LAIR1-containing antibodies

We extracted genomic DNA from the immortalized B cell clones producing LAIR1-containing antibodies and sequenced the rearranged antibody heavy chain sequences on chromosome 14^e. In both donors, the genomic DNA contained a LAIR1 insert that was larger than that found in the corresponding cDNA, presumably due to mRNA splicing (Figure 4.18). In particular, in donor C, the insert comprised not only the 294 bp exon encoding the extracellular LAIR1 domain, but also a 190 bp upstream LAIR1 intron that was partially spliced out in the mRNA, and a shorter 23 bp 3' intronic region that was maintained in the mRNA (Figure 4.19). Donor D had a somewhat different genomic insertion, with larger 5' (378 bp) and 3' (60 bp) LAIR1 intronic sequences, and, upstream of the LAIR1 insertion, an additional sequence of 135 bp that belonged to an intergenic region of chromosome 13 (Figure 4.20a-b). In this donor, the entire LAIR1 5' intronic sequence and much of the 5' Chr13 sequence were spliced out in the mRNA (Figure 4.20c). Interestingly, the spliced intron in donor D had duplicated 135 bp elements that shared many, but not all, of their somatic mutations (Figure 4.20d). The finding that the inserts were located exactly between the heavy chain V and D/J segments in the antibody sequences and were joined to these segments by non-templated nucleotides suggested that RAG1/2 might be involved in the transposition process. Indeed, cryptic recombination signal sequences (RSSs) that followed the 12/23 rule were found flanking both the LAIR1 and the Chr13 inserts, although their RSS prediction scores were low and the positioning of the RSSs at the ends of the inserts was not precise (Figure 4.21). To investigate whether the insertion of LAIR1 into the antibody heavy

chain locus was a germline or somatic event, we extracted genomic DNA from T cells from donor C and attempted to amplify LAIR1-containing sequences using multiple PCR reactions across all of the relevant antibody segments. We did not find any evidence for a LAIR1 insertion in these cells. This finding, together with the observation that all of the LAIR1-containing antibodies from each donor were derived from a single B cell clone, suggested that the LAIR1 insertion was a somatic event that occurred in a single B cell clone in each donor (Tan *et al.* 2016).

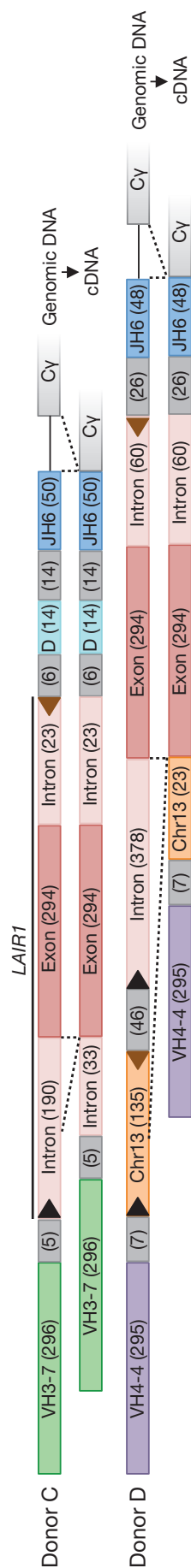


Figure 4.18. Scheme showing genomic DNA and cDNA of LAIR1-containing antibodies from donors C and D. Shown are the lengths of the fragments (bp in parentheses), cryptic 12 and 23 RSS sites (black and brown triangles, respectively) and splicing positions (dashed lines). Reprinted with permission from Tan *et al.* (2016).

DonC_AB_cDNA GAGACACCGTCAAGAAGTCACTGTATTGCAAAATGAACAACCTGAGAGCCGAGGACACGGCTGTATATTACTGCGCGAGACAAGATTCTGCAGTGATGGGAGTCTCTTTCACGGAGAAG 120
 DonC_Chrl4_gDNA GAGACACCGTCAAGAAGTCACTGTATTGCAAAATGAACAACCTGAGAGCCGAGGACACGGCTGTATATTACTGCGCGAGACAAGATTCTGCAGTGATGGGAGTCTCTTTCACGGAGAAG 120
 DonC_AB_cDNA ----- 120
 DonC_Chrl4_gDNA GTCCGGGGGGCTTGGGTTTTCTCACGTTGGGCTGTGGTCAAGGCTGCACAGCTCTAGAAAATCGCTAAATCCCTGAATTGTGCCGTATAATGACGTGTGTGCCATGGTGATCTCTCA 240
 DonC_AB_cDNA -----ATCTGCCAGACCCACCATCTCGGCTGAGACAGGCACCGTGATCTCCCTGGGAGCCATGTGACTTTTCGTGTGCCGGGGC 200
 DonC_Chrl4_gDNA CAGTGGACACTTAGATCTCTGCTGCTTCCCTCCTAGCAGATCTGCCAGACCCACCATCTCGGCTGAGACAGGCACCGTGATCTCCCTGGGAGCCATGTGACTTTTCGTGTGCCGGGGC 360
 DonC_AB_cDNA CCACTTGGGGTGCAAACATTCCGCCTGGAGAGGGAGAGTAGGTCCAGATACAGTGAAACTGAAGATGTGTCTCAAGTTGGTCCATCTGAGTCAGAGGCCAGATTCCGCATTGACTCAGTG 320
 DonC_Chrl4_gDNA CCACTTGGGGTGCAAACATTCCGCCTGGAGAGGGAGAGTAGGTCCAGATACAGTGAAACTGAAGATGTGTCTCAAGTTGGTCCATCTGAGTCAGAGGCCAGATTCCGCATTGACTCAGTG 480
 DonC_AB_cDNA AGTGAAGGAAATGCCGGGCTTTATCGATGCATCTATTACAAACCCCTAAATGGTCTGAGCAGAGTGACTACCTGGAGCTGCGGGTGAAAGTGAGGACGTCACCTGGGCCCTGTTAACC 440
 DonC_Chrl4_gDNA AGTGAAGGAAATGCCGGGCTTTATCGATGCATCTATTACAAACCCCTAAATGGTCTGAGCAGAGTGACTACCTGGAGCTGCGGGTGAAAGTGAGGACGTCACCTGGGCCCTGTTAACC 600
 DonC_AB_cDNA TATTCTGGTGGTGATAGAGACGAATCCGACTACTACATGGACGCTCTGGGCAAGGGAC 499
 DonC_Chrl4_gDNA TATTCTGGTGGTGATAGAGACGAATCCGACTACTACATGGACGCTCTGGGCAAGGGAC 659

VH3-7	Junction
D2-21	LAIR1 exon
JH6	LAIR1 intron

Figure 4.19. Genomic DNA analysis of LAIR1-containing antibodies of donor C. The sequence alignment of genomic DNA (gDNA) and cDNA of a LAIR1-containing antibody (AB) from donor C (DonC) reveals a 507 bp LAIR1 insert in chromosome 14 (Chr14) and the removal of a 160 bp fragment by RNA splicing. Splice donor and acceptor sites are highlighted in yellow. Reprinted with permission from (Tan *et al.* 2016).

	(Score)	12-cryptic-RSS	23-cryptic-RSS-antiparallel	(Score)
MGC_Ch19	(-57.29)	TCTG <u>CAGTGATGAGAATCACATGCACGTAGAA</u>	GAGCTGCTGGTGAAAGGTGAGGACGTACCTGGGCCCTG	(-79.68)
MGD_Ch19	(-62.84)	TTGTGAG <u>CAAGTCTCAGGGTCCTCACTGTCAACTG</u>	CTGGGCCCTGCCCCAGTCTCAGCTCGACCTCGAGCTTGTCCCCAGG	(-77.42)
MGD_Ch13	(-64.12)	ATT <u>CAAGTCTCAGAGGGACACCAGTGTGTTT</u>	TGACAAGTGGGGTCTTGGAGTTCTTTAATTTCCCATTTGA	(-75.63)

Figure 4.21. The LAIR1 and chromosome 13 inserts are flanked by 12/23 cryptic RSS sites. The regions on chromosome 19 (Chr19) and chromosome 13 (Chr13) of donor-derived gDNA corresponding to the ends of the inserts were sequenced and RSS sites were identified using the RSSsite web server. The sequences shown begin from the ends of the inserts. Cryptic RSS sites are highlighted in grey, with complementary ends underlined and prediction scores shown in parentheses. Reprinted with permission from (Tan *et al.* 2016).

4.10. Investigation of original LAIR1 locus on chromosome 19

To further investigate the mechanism of LAIR1 insertion into the antibody heavy chain locus, we analysed the chromosome 19 LAIR1 sequence in B and T cells from donor Cf. Specifically, we attempted to determine whether one LAIR1 allele was missing or truncated due to insertion into the immunoglobulin locus. We selectively amplified the chromosome 19 LAIR1 gene and avoided the LAIR1 insert in chromosome 14 by choosing PCR primers that were specific for LAIR1 intronic sequences that were beyond the insert in the antibody heavy chain locus (Figure 4.22a) (see Methods section 2.8 for the sequence of the primers). Sequencing of the chromosome 19 LAIR1 gene in T cell genomic DNA revealed that the original LAIR1 exon contained a heterozygous nucleotide site that allowed us to distinguish between the two alleles that were present in these cells (Figure 4.22b). As expected, similar results were obtained with a B cell clone from donor C that did not produce LAIR1-containing antibodies. Surprisingly, the heterozygous site was also present in a B cell clone producing LAIR1-containing antibodies (MGC33), suggesting that both chromosome 19 LAIR1 alleles were present in this clone. These results suggest that a “copy-and-paste” mechanism was used for LAIR1 transposition into rearranging immunoglobulin genes and is inconsistent with RAG1/2 being responsible for the insertion, as this enzyme complex acts by excising a target sequence (Tan *et al.* 2016).

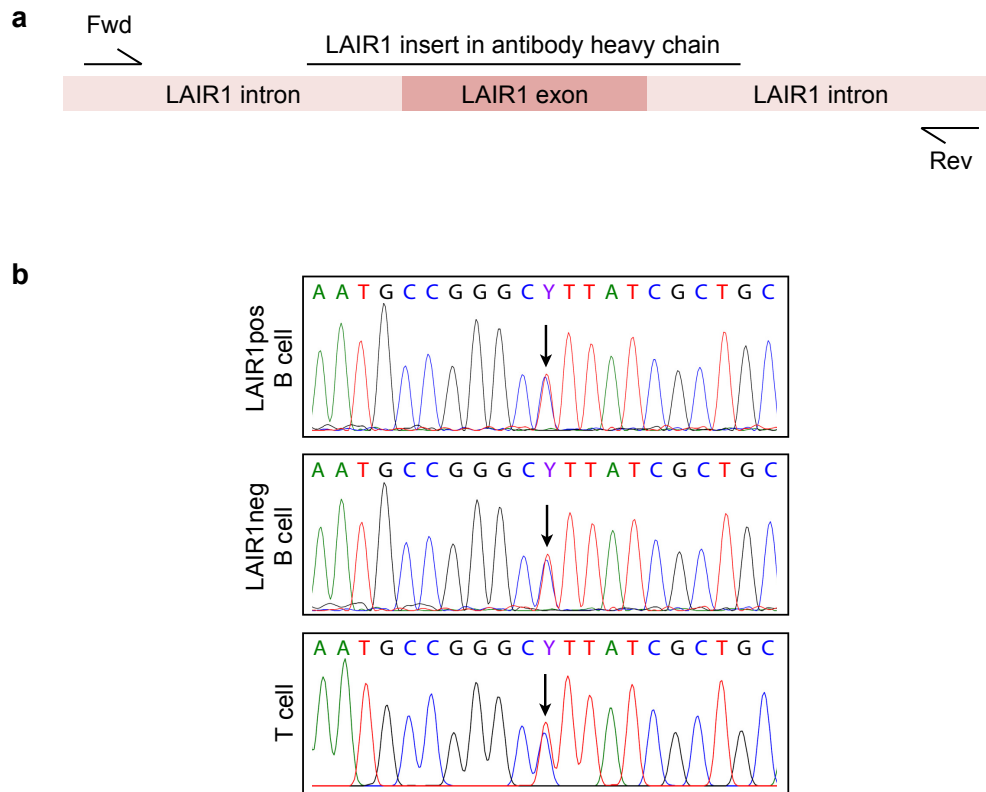


Figure 4.22. Both LAIR1 alleles on chromosome 19 are intact in B cells producing LAIR1-containing antibodies. a, Scheme showing design of forward (Fwd) and reverse (Rev) primers that are specific for LAIR1 intronic sequences beyond the insert to enable the selective amplification of the chromosome 19 LAIR1 sequence. See Methods section 2.8 for the sequence of the primers. Not to scale. b, Heterozygosity of the chromosome 19 LAIR1 exon in cells from donor C showing that both LAIR1 alleles are intact in B cells producing LAIR1-containing antibodies. Displayed are the chromatograms obtained for B cell clones with (MGC33) or without a LAIR1 insertion (LAIR1pos or LAIR1neg B cell) and for polyclonal T cells. Y = C/T. Reprinted with permission from (Tan *et al.* 2016).

4.11. Elements required for binding of LAIR1-containing antibodies to IEs and collagen

The presence of the LAIR1 insert in all of the broadly reactive antibodies from donors C and D and its corresponding absence in the isolate-specific antibodies from donor D led us to suspect that this insert was responsible for broad reactivity to IEs. We also wanted to determine if the presence of the LAIR1 insert in these antibodies would confer the ability to bind to collagen, which is the natural ligand of LAIR1. To investigate the contributions of this insert to antibody specificity relative to the V_H and V_L regions, we designed a series of constructs based on the broadly reactive antibody MGD21^g. These constructs were made by exchanging heavy chain V, D, J elements or the light chain with the corresponding sequences of an irrelevant antibody (FI499, reactive to influenza virus) (C1, C2, C9), deleting selected segments (C3-C6), or reverting somatic mutations to the germline configuration (C7, C8) (Figure 4.23a). In addition, IgG Fc fusion proteins containing the MGD21 LAIR1 domain were produced recombinantly to test the ability of the LAIR1 domain alone to bind to IEs. I found that substitution of the V, J or L chain of MGD21 with that of FI499 did not affect binding to IEs, suggesting that these elements are dispensable for binding (Figure 4.23b). In contrast, deletion of the LAIR1 exon alone, or its reversion to the unmutated genomic sequence, led to a complete loss of binding. Furthermore, fusion proteins displaying only the mutated LAIR1 exon with or without flanking sequences showed specific binding to IEs, although with lower affinity. This reduced affinity may simply reflect the different structure of these fusion proteins compared to that of full antibodies. When we tested selected constructs for the ability to bind to collagen^h, we found that wild type MGD21

^gDesigned by Dr Luca Piccoli.

^hAll collagen-binding ELISAs were done by Dr Luca Piccoli.

bound poorly to collagen, whereas a construct with the germline LAIR1 sequence (C8) bound well to the protein (Figure 4.24). As expected, a construct without the LAIR1 exon (C3) did not bind to collagen. Taken together, these findings indicate that the binding of the broadly reactive antibodies to IEs relies mainly on the mutated LAIR1 domain, which evolves under selective pressure to lose collagen binding and gain binding to IEs (Tan *et al.* 2016).

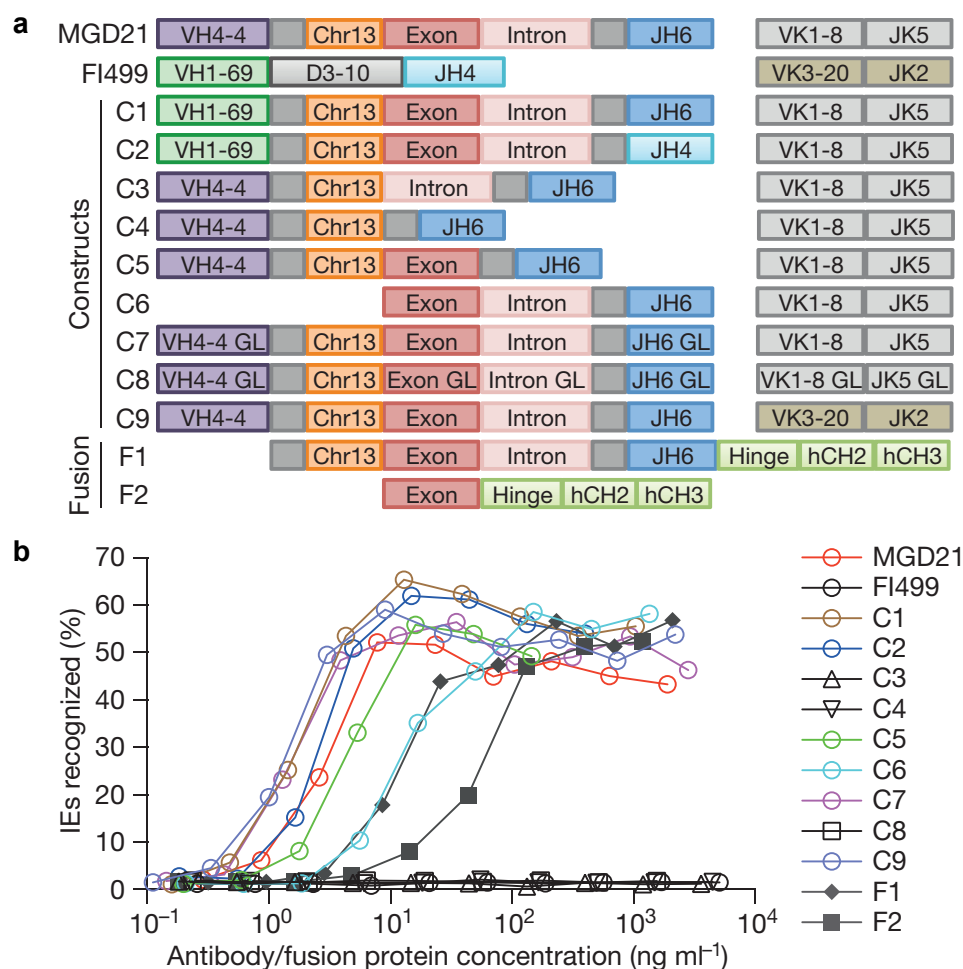


Figure 4.23. The mutated LAIR1 insert is necessary and sufficient for binding to IEs. a, Design of modified MGD21 antibody constructs with selected regions replaced with counterparts from an unrelated antibody (FI499) (C1–C2, C9), deleted (C3–C6), or reverted to germline (GL) (C7–C8). Fc fusion proteins that incorporated the LAIR1 insert, junction and downstream sequences (F1), as well as the LAIR1 domain alone (F2), were also designed. b, Binding of MGD21 constructs and Fc fusion proteins to IEs. Reprinted with permission from (Tan *et al.* 2016).

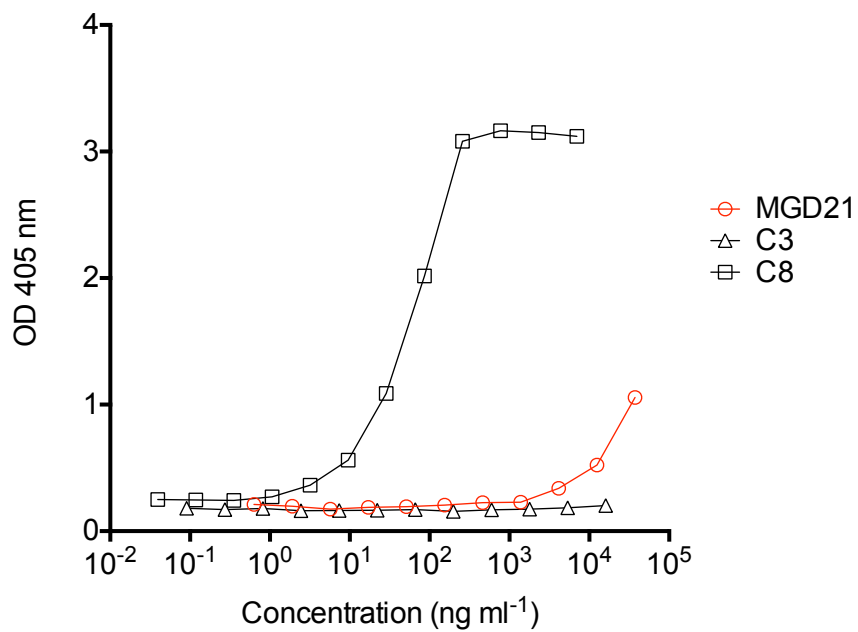


Figure 4.24. Binding of selected constructs to collagen. The constructs were designed as shown in Figure 4.22. Wild type MGD21 bound poorly to collagen, while a construct with the antibody sequence reverted to germline (C8) bound well to collagen. An MGD21 construct with a missing LAIR1 exon (C3) did not bind to collagen.

4.12. Effects of mutations in the LAIR1 domain on binding to IEs and collagen

To understand the contribution of the individual somatic mutations of the LAIR1 insert to collagen and IE recognition, we created a set of germline LAIR1-Fc fusion proteins carrying, either singly or in various combinations, the mutations shared by MGD21 with other antibodies of the same clonal familyⁱ. We tested the mutants for binding to IEs and to collagen. Interestingly, two different sets of mutations were identified: those that reduced collagen binding and those that increased binding to IEs (Figure 4.25). In particular, a single mutation (P107R) was sufficient to abrogate collagen binding, while three mutations (T67L, N69S and A77T) synergized in promoting binding to IEs. These findings suggested that distinct mutations in the LAIR1 domain were responsible for the increase in binding to IEs and the loss of binding to collagen (Tan *et al.* 2016).

ⁱThe mutations were chosen by Dr Luca Piccoli and me.

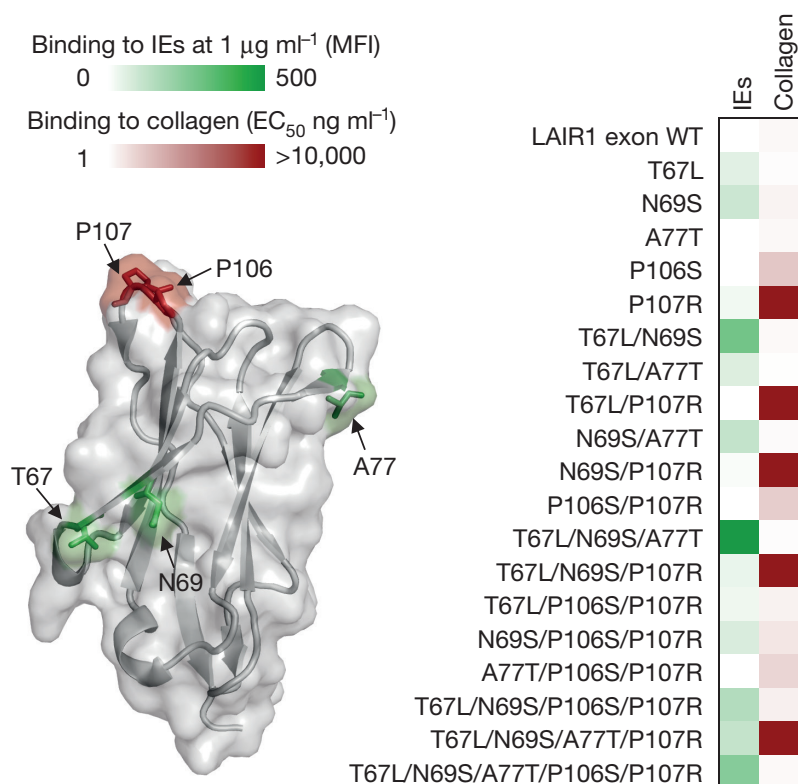


Figure 4.25. Distinct mutations in the LAIR1 domain are required for gain of binding to IEs and loss of binding to collagen. Selected amino acid substitutions found in MGD21 were added individually or in different combinations to the germline LAIR1-Fc fusion protein. These mutants were tested for binding to collagen and to IEs. Shown are the effects of the mutations on binding to IEs or collagen and their location on the LAIR1 structure (Protein Data Bank accession 3kgr) (Brondijk *et al.* 2010). Gain of IE binding is shown in green (background mean fluorescence intensity (MFI) values subtracted). Loss of collagen binding (half-maximum effective concentration (EC_{50}) enzyme-linked immunosorbent assay (ELISA) values) is shown in red. WT, wild type. Reprinted with permission from (Tan *et al.* 2016).

4.13. Summary

In this chapter, I have reported the discovery of LAIR1-containing antibodies that are broadly reactive to IEs from different *P. falciparum* isolates. These antibodies are unprecedented in terms of the number of different parasite isolates recognized, but the most unusual feature of these antibodies is the insertion of a large sequence derived from the inhibitory receptor LAIR1 between the V and DJ segments of the heavy chain. The LAIR1 insert is the primary element required for binding to IEs, with distinct mutations in this domain required for gain of binding to IEs and loss of binding to collagen. In each of the donors studied, the LAIR1-containing antibodies are derived from a single, expanded B cell clone. Having analysed the sequences of these unusual LAIR1-containing antibodies, we turned our attention to the identification of the antigens on the IE surface recognized by these antibodies, as will be described in the following chapter.

Chapter 5. Identification of RIFINs as target antigens and investigation of function of LAIR1-containing antibodies

5.1. Introduction

In this chapter, I will describe the identification of the *P. falciparum* IE surface antigens that are recognized by the LAIR1-containing antibodies. The finding that these antibodies show distinct binding patterns to different parasite isolates suggests that the antigens are not completely conserved but belong to a VSA family. PfEMP1 is thought to be the main surface antigen that is recognized by malaria-exposed individuals (Chan *et al.* 2012; 2016), but preliminary trypsin digestion experiments suggest that the antigen might belong to a different VSA family.

To facilitate the identification of the target antigens, I decided to use a *P. falciparum* line that has a well-characterized genome and proteome. While the genomes of the Kilifi isolates used so far are largely sequenced, they have not been completely annotated and their proteomes are not completely characterized. Therefore, I used the well-characterized *P. falciparum* laboratory line 3D7 for the antigen identification experiments. *In vitro* *P. falciparum* 3D7 lines that were either enriched or depleted for recognition by the LAIR1-containing monoclonal antibody MGD21 were created and used to identify the antigen through a combination of various biochemical techniques. After identifying the targets of the LAIR1-containing antibodies, I investigated the potential role of these antibodies in mediating destruction of IEs through a series of *in vitro* functional assays.

5.2. Magnetic selection of MGD21⁺ and MGD21⁻ lines

To determine whether *P. falciparum* 3D7 was suitable for antigen identification, I tested whether 3D7 IEs could be stained by the LAIR1-containing antibody MGD21. This antibody stained about 12% of wild type 3D7 IEs (Figure 5.1a). Next, I used magnetic protein G beads to generate 3D7 lines that were enriched (3D7-MGD21⁺) or depleted (3D7-MGD21⁻) of MGD21 reactivity. One or two magnetic selections were sufficient to create a depleted line that was ~2% stained by MGD21 (Figure 5.1b) and an enriched line that was >70% stained by the antibody (Figure 5.1c). The enriched line contained a mixture of IEs with intermediate and high staining, suggesting that MGD21 may recognize several antigens that are heterogeneously expressed in different IEs in the enriched parasite population. The amount of antigen expression by the 3D7-MGD21⁺ line was relatively stable and only decreased slightly over extended periods in culture (Figure 5.1d).

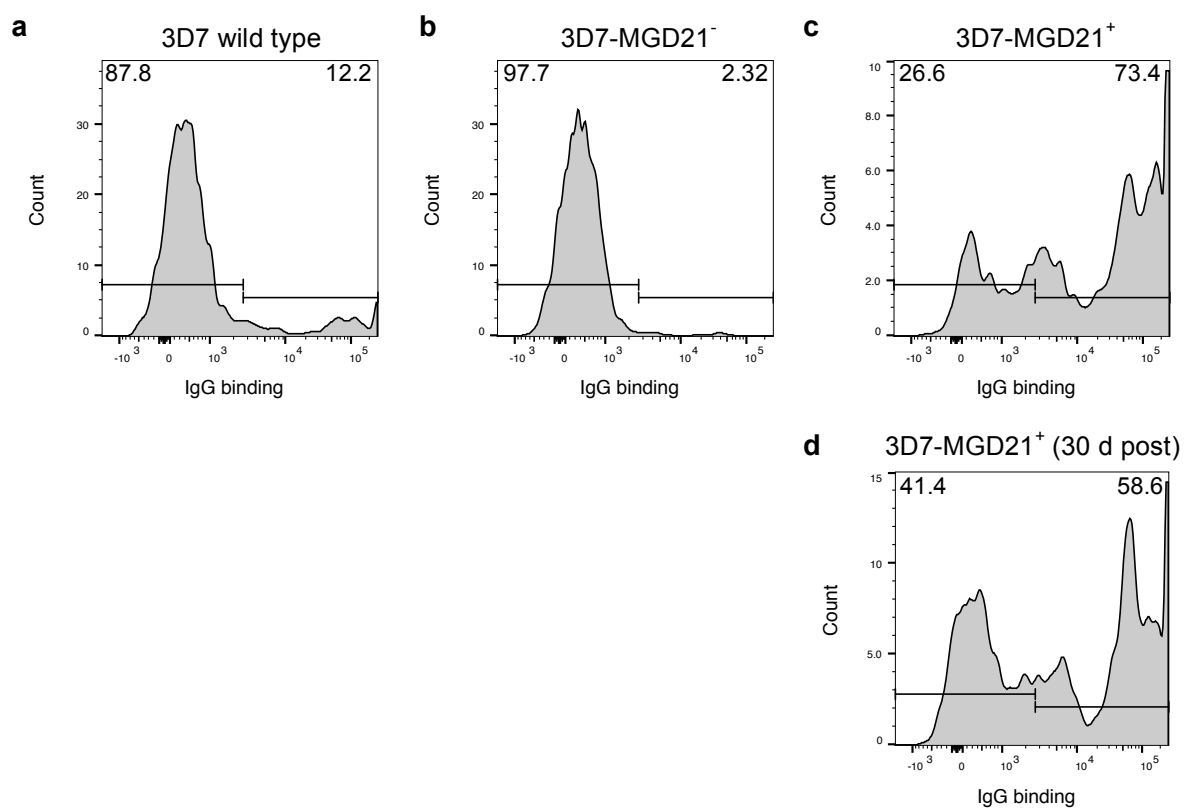


Figure 5.1. Magnetic selection of 3D7 lines that were depleted (3D7-MGD21⁻) or enriched (3D7-MGD21⁺) for recognition by the LAIR1-containing antibody MGD21. a, Staining of wild type 3D7 IEs by MGD21. b-c, Staining of depleted (b, 3D7-MGD21⁻) or enriched (c, 3D7-MGD21⁺) parasite lines. d, Antigen expression on the surface of 3D7-MGD21⁺ IEs after 30 days in culture.

5.3. Recognition of 3D7-MGD21⁺ and 3D7-MGD21⁻ by LAIR1-containing antibodies

The differing abilities of the LAIR1-containing antibodies to bind to the various Kenyan parasite isolates suggest that different antibodies may recognize distinct antigens on the IE surface. To investigate whether the other LAIR1-containing antibodies recognize the same antigens as MGD21, I tested the ability of these antibodies to stain 3D7-MGD21⁻ and 3D7-MGD21⁺ IEs. I found that enrichment by MGD21 greatly increased recognition by all of the other broadly reactive antibodies from donor D tested and, notably, by two broadly reactive antibodies from donor C, suggesting that these antibodies recognize the same antigens as MGD21 (Figure 5.2). Nevertheless, some LAIR1-containing antibodies from donor C did not bind to 3D7-MGD21⁺ IEs, suggesting that these antibodies do not recognize the same antigens or recognize different variants of these antigens (Tan *et al.* 2016).

	IEs	
	3D7-MGD21 ⁻	3D7-MGD21 ⁺
MGD21	3.4	54.2
MGD39	2.8	58.0
MGD47	3.5	55.3
MGD55	4.2	61.3
MGC1	0.9	7.1
MGC2	0.3	0.9
MGC4	0.6	0.8
MGC5	0.8	0.9
MGC17	0.4	0.7
MGC26	0.9	0.5
MGC28	1.8	42.4
MGC29	0.3	0.8
MGC34	1.9	48.8
BKC3	0.7	0.3

IEs recognized (%)
0 50

Figure 5.2. Recognition of 3D7-MGD21⁻ and 3D7-MGD21⁺ IEs by LAIR1-containing antibodies from donors C and D. BKC3 is a negative control antibody. Reprinted with permission from (Tan *et al.* 2016).

5.4. Immunoprecipitation and Western blot with MGD21

Since many of the LAIR1-containing antibodies appeared to recognize the same antigens as MGD21, I focused my efforts on identifying the antigens targeted by this antibody. Erythrocyte membrane ghosts were created from 3D7-MGD21⁺ and 3D7-MGD21⁻ IEs by hypotonic lysis. These ghosts should contain the IE membrane and associated proteins, including parasite-expressed surface antigens. I analyzed the erythrocyte ghosts by a Western blot and identified two MGD21-specific bands at about 40 kDa that were present in 3D7-MGD21⁺ but were absent in 3D7-MGD21⁻ or uninfected erythrocyte ghosts (Figure 5.3). The molecular weights of these bands would be consistent with the antigens being either RIFINs or STEVORs. To allow more specific determination of the MGD21-targeted antigens, I conducted immunoprecipitation experiments on protein extracts from intact 3D7-MGD21⁺ and 3D7-MGD21⁻ IEs (not from erythrocyte ghosts) with MGD21 or with an irrelevant antibody BKC3. Analysis of the samples by a Western blot revealed that MGD21 immunoprecipitates of 3D7-MGD21⁺ IEs had two MGD21-specific bands at the same molecular weights as the bands obtained from the 3D7-MGD21⁺ IE ghosts (Figure 5.3). These bands were absent in control samples prepared from 3D7-MGD21⁻ IE ghosts or from samples immunoprecipitated with BKC3 (Tan *et al.* 2016).

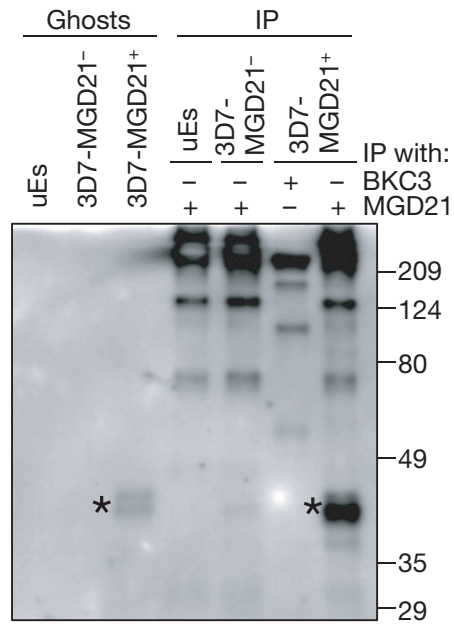


Figure 5.3. Western blot showing MGD21 binding to erythrocyte ghosts and MGD21 immunoprecipitates (IP) prepared from intact 3D7-MGD21⁺ and 3D7-MGD21⁻ IEs. Controls include uninfected erythrocytes (uEs) and immunoprecipitates with an irrelevant antibody (BKC3). Specific bands are marked with asterisks. Anti-human IgG was used as the secondary antibody, resulting in detection of antibodies used for immunoprecipitation alongside antigens of interest. Numbers on the right indicate kDa. Reprinted with permission from (Tan *et al.* 2016).

5.5. Identification of *P. falciparum* 3D7 antigens by mass spectrometry

To identify the proteins detected on the Western blot, we sent the immunoprecipitates and IE ghost samples for analysis by liquid chromatography-coupled mass spectrometry (LC-MS)ⁱ. The protein samples were analysed by intensity-based absolute quantification (iBAQ), which normalizes the quantity of each protein of interest to the total protein quantity of the sample (Schwanhäusser *et al.* 2011). Analysis of the immunoprecipitates revealed that a member of the A-RIFIN family (PF3D7_1400600) was significantly enriched in 3D7-MGD21⁺ immunoprecipitates as compared to 3D7-MGD21⁻ immunoprecipitates (Log₂ fold change >2; P < 0.01) (Figure 5.4a). PF3D7_1400600, as well as a second A-RIFIN (PF3D7_1040300), was also identified by LC-MS in 3D7-MGD21⁺ but not in 3D7-MGD21⁻ ghosts in the absence of immunoprecipitation (Figure 5.4b). The molecular weights of these two RIFINs (41 and 42 kDa) and their degrees of enrichment were consistent with the two bands observed in the Western blot. Notably, four other A-RIFINs, including one recently characterized for its capacity to induce rosetting (PF3D7_0100400) (Goel *et al.* 2015), were detected in similar amounts by LC-MS in both 3D7-MGD21⁺ and 3D7-MGD21⁻ ghosts, indicating that MGD21 is not pan-reactive towards A-RIFINs (Tan *et al.* 2016).

ⁱLC-MS was done by the Functional Genomics Center in Zurich and the analysis of the MS data was done by Dr Roger Geiger.

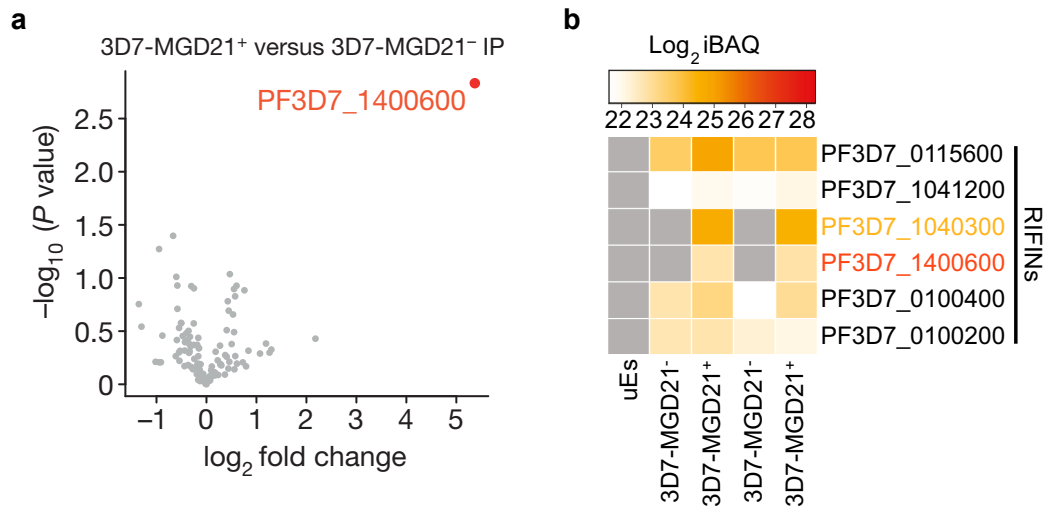


Figure 5.4. Analysis of immunoprecipitates and IE ghost samples by mass spectrometry. a, Volcano plot from LC-MS analysis of MGD21 immunoprecipitates prepared from 3D7-MGD21⁺ IEs versus from 3D7-MGD21⁻ IEs (from n = 4 independent experiments). Statistical significance was evaluated by Welch tests ($P < 0.01$ for PF3D7_1400600). LAIR1 and antibody sequences were excluded from this plot. b, Heat map from LC-MS analysis showing RIFIN expression levels (calculated as intensity-based absolute quantification (iBAQ) scores) in erythrocyte ghosts prepared from 3D7-MGD21⁺ and 3D7-MGD21⁻ IEs (two experiments shown). Analysis of ghosts from uninfected erythrocytes (uEs) is also shown. Grey boxes indicate that expression levels are below the detection limit. Reprinted with permission from (Tan *et al.* 2016).

5.6. Binding of LAIR1-containing antibodies to recombinant 3D7 RIFINs

To confirm the mass spectrometric analysis, we cloned the soluble extracellular portions of the candidate positive RIFINs (PF3D7_1400600 and PF3D7_1040300), as well as negative control RIFINs that were similarly expressed (PF3D7_0100200 and PF3D7_0100400) or not present (PF3D7_1100500) in 3D7-MGD21⁺ and 3D7-MGD21⁻ ghosts, into a pDisplay expression vector as previously described (Goel *et al.* 2015)^k. This vector contains an N-terminal haemagglutinin (HA) tag that allows detection of protein expression and a C-terminal platelet-derived growth factor receptor (PDGFR) transmembrane domain, allowing the expression of the RIFINs on the surface of transfected cells (Figure 5.5a). Constructs containing each of the RIFINs were transiently transfected into Chinese hamster ovary (CHO) cells, which were then surface-stained with the LAIR1-containing antibodies without fixation or permeabilization. Anti-HA antibodies were able to stain CHO cells transfected with all of the different RIFIN constructs, suggesting that the RIFINs were expressed on the surface and that the N-termini of these proteins were surface-exposed (Figure 5.5b). These results support previous findings indicating that RIFINs only have a single C-terminal transmembrane domain (Bultrini *et al.* 2009; Bachmann *et al.* 2015; Goel *et al.* 2015). In contrast, MGD21 stained the cells transfected with the candidate antigens (PF3D7_1400600 and PF3D7_1040300), but not the cells transfected with any of the negative control RIFINs (Figure 5.5b-d). CHO cells transfected with PF3D7_1400600 or PF3D7_1040300 were also stained by most of the antibodies that stained 3D7-MGD21⁺ IEs (Figure 5.5d, also see Figure 5.2), but not by those that failed to stain

^kRIFIN expression was done by Dr Luca Piccoli. I tested the binding of the different LAIR1-containing antibodies to the RIFIN-transfected cells.

this parasite line. These findings suggest that the LAIR1-containing antibodies recognize different members of the RIFIN VSA family (Tan *et al.* 2016).

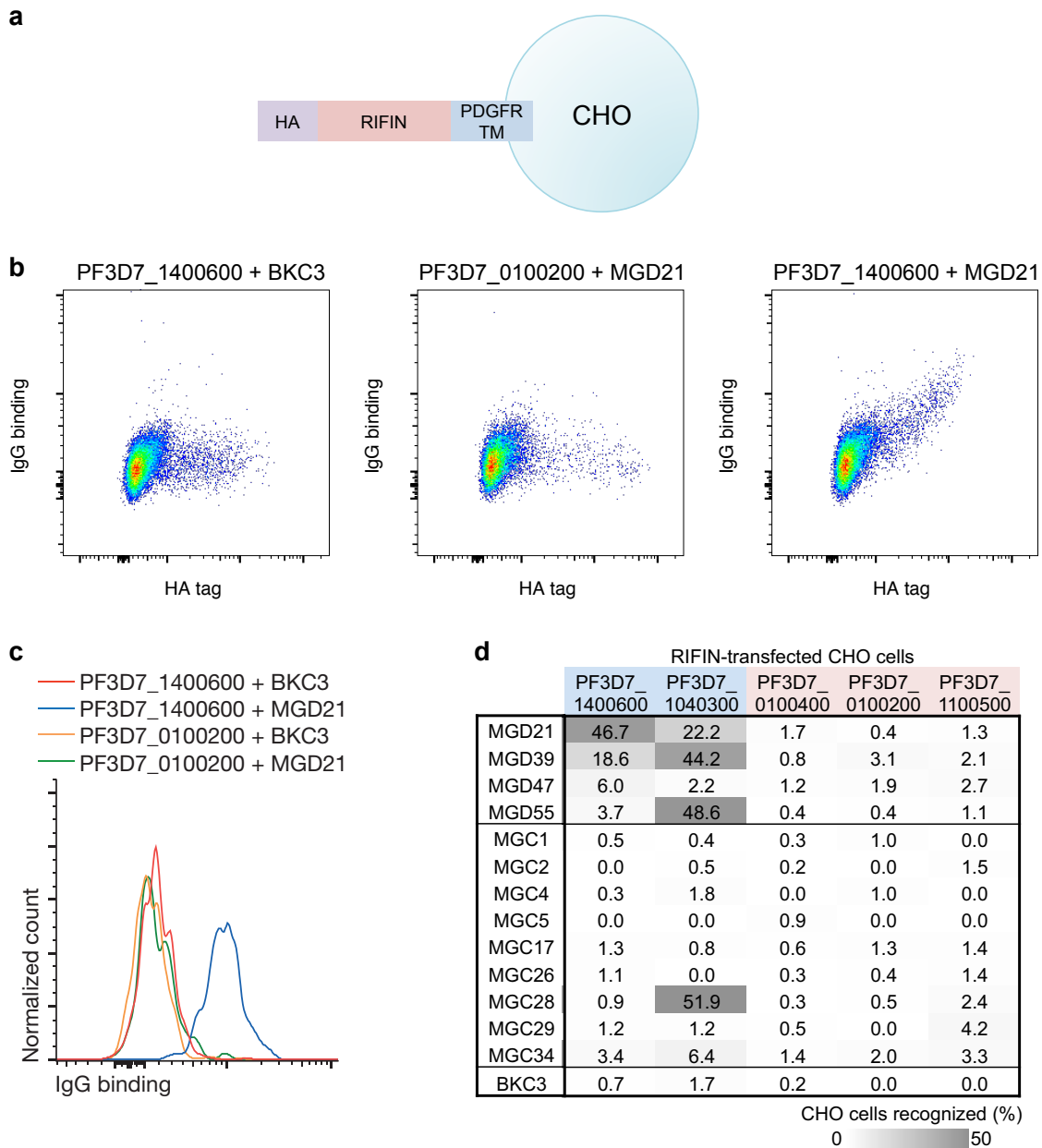


Figure 5.5. Staining of CHO cells by LAIR1-containing antibodies. a, Scheme of RIFIN construct. This construct contains an N-terminal HA tag, followed by the extracellular RIFIN sequence and a PDGFR transmembrane (TM) domain that allows expression of the RIFIN on the CHO cell surface. b, Example of staining of CHO cells transfected with a candidate positive RIFIN (PF3D7_1400600) or a negative control RIFIN (PF3D7_0100200) by anti-HA, a negative control antibody BKC3 and/or MGD21. c, Comparison of staining of PF3D7_1400600- or PF3D7_0100200-transfected CHO cells by MGD21 and BKC3. d, Percentage of RIFIN-transfected CHO cells stained by different LAIR1-containing antibodies. RIFINs that were enriched in 3D7-MGD21⁺ ghosts are highlighted blue, while RIFINs that were similarly expressed or not detected in 3D7-MGD21⁻ and 3D7-MGD21⁺ ghosts are shown in red. Adapted with permission from (Tan *et al.* 2016).

5.7. Investigation of the RIFIN epitope recognized by LAIR1-containing antibodies

RIFINs contain a relatively conserved N-terminal region and a hypervariable central region that differs substantially between different variants (Figure 1.4). To determine whether the LAIR1-containing antibodies recognize the conserved or variable RIFIN region, we designed RIFIN chimaeras containing shuffled constant and variable regions and transfected these constructs into CHO cells¹ (Figure 5.6a). I found that MGD21 and an Fc fusion protein containing the MGD21 LAIR1 exon stained CHO cells transfected with a RIFIN chimaera containing the constant region of PF3D7_0100200 and the variable region of PF3D7_1400600 (PF3D7_0100200c_1400600v), but not cells transfected with the inverse chimaera (PF3D7_1400600c_0100200v), suggesting that MGD21 targets the RIFIN variable region (Figure 5.6b-c) (Tan *et al.* 2016). These findings are consistent with our observations that MGD21 does not recognize all A-RIFINs (Figure 5.4b). Furthermore, these results confirm that the mutated LAIR1 exon alone is capable of binding to recombinant RIFINs. I am planning to conduct further experiments investigating the exact RIFIN epitope recognized by the LAIR1-containing antibodies, but given the extremely diverse nature of the variable region and the lack of a solved RIFIN structure, it is likely that this line of research will be challenging.

¹Design of RIFIN chimaeras was done by Dr Luca Piccoli. I expressed and tested the chimaeras.

a Original RIFINs

PF3D7_1400600

LCCELYSPNTYDSPEMKRVMQQFVDRRTQRFHEYDESLQSKRKQCKDQCDKEIQKIILKDKIEKEFTEKLSTLQT
DITTKDIPTCVCEKSLADKMEKVKLCQAQNLGGIVAPSTGVLGEIAALAVNAWKTALKNAIAAAQKAGDAAGKIAG
ESKGVETIIGILEQYYSIYELKGTPLKSFATTHYTDISNIATVIDTELNTSCGLNSLANQAICGLRTRLGLVAKPG
QVMVTQKEAITKMITNVVHKSEITAEAAKTEVAATKTAAAIKMNTAEIAAATTPYYT

PF3D7_0100200

LCCELYSPANNDNDAEMKRVMMQQFEDRTTQRFHEYDERMKTTRQKCKEQCDKEIQKIILKDKLEKELMDKFATLQT
DIQSDSIPTCICEKSLADKMEKVKLCQAQNLGGIAPGWSLVSLGYAVWTNYVTQTALQKGEIAGVKAGIEGLRDF
SGLGKLIPISVIQNLINHTNYDIAKTYITFVKSVNSTKCAVKEHSFCFSTYISNENALSKRAAGIAEYAADMAKITE
RGVLDAATPGLTYSN

Chimaeras

PF3D7_1400600c_0100200v

LCCELYSPNTYDSPEMKRVMQQFVDRRTQRFHEYDESLQSKRKQCKDQCDKEIQKIILKDKIEKEFTEKLSTLQT
DIQSDSIPTCICEKSLADKMEKVKLCQAQNLGGIAPGWSLVSLGYAVWTNYVTQTALQKGEIAGVKAGIEGLRDF
SGLGKLIPISVIQNLINHTNYDIAKTYITFVKSVNSTKCAVKEHSFCFSTYISNENALSKRAAGIAEYAADMAKITE
RGVLDAATPGLTYSN

PF3D7_0100200c_1400600v

LCCELYSPANNDNDAEMKRVMMQQFEDRTTQRFHEYDERMKTTRQKCKEQCDKEIQKIILKDKLEKELMDKFATLQT
DIQSDSIPTCICEKSLADKMEKVKLCQAQNLGGIAPGWSLVSLGYAVWTNYVTQTALQKGEIAGVKAGIEGLRDF
ESKGVETIIGILEQYYSIYELKGTPLKSFATTHYTDISNIATVIDTELNTSCGLNSLANQAICGLRTRLGLVAKPG
QVMVTQKEAITKMITNVVHKSEITAEAAKTEVAATKTAAAIKMNTAEIAAATTPYYT

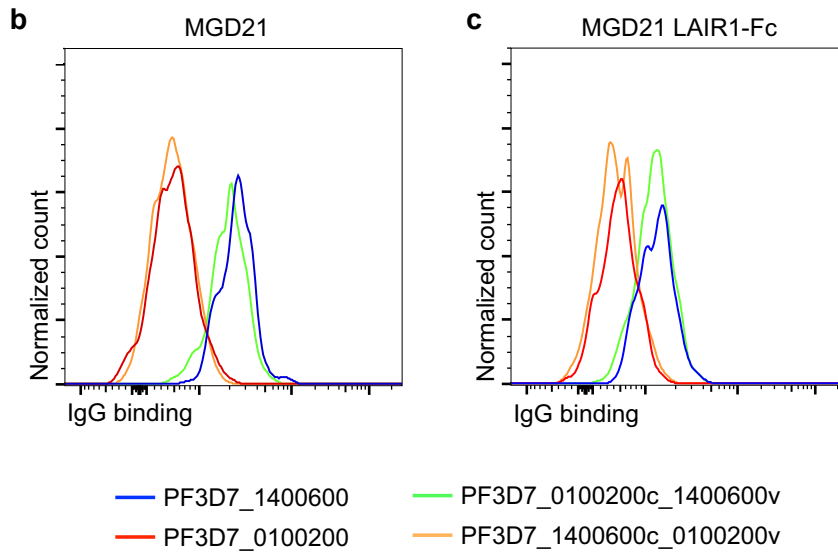


Figure 5.6. Sequences of RIFIN chimaeras (a) and binding of MGD21 (b) or an Fc fusion protein containing the mutated MGD21 LAIR1 domain (c) to CHO cells transfected with RIFINs or RIFIN chimaeras. The cells were either transfected with a candidate positive RIFIN (PF3D7_1400600), a negative control RIFIN (PF3D7_0100200), a RIFIN chimaera containing the constant region of PF3D7_0100200 and the variable region of PF3D7_1400600 (PF3D7_0100200c_1400600v), or the inverse chimaera (PF3D7_1400600c_0100200v). Reprinted with permission from (Tan *et al.* 2016).

5.8. Investigation of target antigens expressed by the Kenyan isolate 9605

The data described so far demonstrate that RIFINs on *P. falciparum* 3D7 IEs are the targets of the LAIR1-containing antibodies. To determine whether these antibodies also recognize RIFINs on IEs from the Kenyan parasite isolates, I performed magnetic selection on the Kenyan isolate 9605 to generate stable 9605-MGD21⁺ and 9605-MGD21⁻ lines and repeated the immunoprecipitation and Western blot experiments on these parasite lines. I identified two specific ~35 kDa bands in the 9605-MGD21⁺ sample that were absent in the negative control samples (Figure 5.7a). The molecular weights of these bands would be consistent with the antigens being RIFINs, and I plan to investigate this further when we obtain the proteome of this parasite isolate. Enrichment of 9605 IEs for MGD21 binding also enhanced recognition by all of the LAIR1-containing antibodies from donors C and D tested, suggesting that these antibodies recognize the same antigens expressed by this parasite isolate (Figure 5.7b) (Tan *et al.* 2016). This finding differs from that obtained with 3D7, as a number of the MGC antibodies did not display enhanced binding to the 3D7-MGD21⁺ parasite line (Figure 5.2). Overall, these results point to a diverse, parasite-dependent pattern of RIFIN recognition by different LAIR1-containing antibodies.

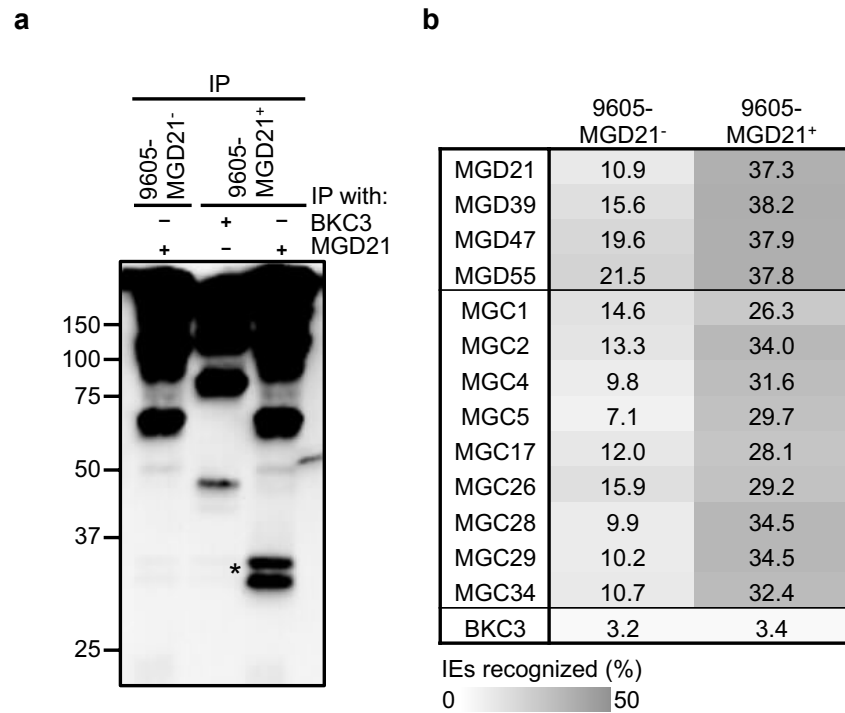


Figure 5.7. Preliminary experiments to identify antigens on 9605 IEs that are recognized by LAIR1-containing antibodies. a, Western blot showing MGD21 binding to immunoprecipitates (IP) prepared from 9605-MGD21⁻ and 9605-MGD21⁺ IEs. Specific bands are marked with an asterisk. Anti-human IgG was used as the secondary antibody, resulting in detection of antibodies used for immunoprecipitation alongside antigens of interest. Numbers on the left indicate kDa. b, Percentage of 9605-MGD21⁻ and 9605-MGD21⁺ IEs recognized by a panel of MGC and MGD antibodies. Reprinted with permission from (Tan *et al.* 2016).

5.9. Investigation of parasite growth inhibition and antigen downregulation by LAIR1-containing antibodies

Having identified the antigens targeted by the LAIR1-containing antibodies, I next investigated whether the LAIR1-containing antibodies could cause the destruction of IEs through various *in vitro* functional assays. First, I tested whether these antibodies inhibit *P. falciparum* growth in *in vitro* culture. Different concentrations of MGD21 or the negative control antibody BKC3 were added to the 3D7-MGD21⁺ culture at the ring stage, and parasitaemia was counted a full cycle (48 h) later (Figure 5.8a). MGD21 did not inhibit *in vitro* parasite growth. These results are in agreement with previous observations that the parasites were able to grow robustly after magnetic selection with MGD21. To test if MGD21 would result in the downregulation of the recognized RIFINs on the IE surface, the MGD21 antibodies were removed from culture after 48 h and the parasites were allowed to develop for another 24 h into the late trophozoite/schizont stage. These IEs were then stained with different concentrations of MGD21 or BKC3. I found no evidence that MGD21 induced the downregulation of RIFIN expression (Figure 5.8b) (Tan *et al.* 2016).

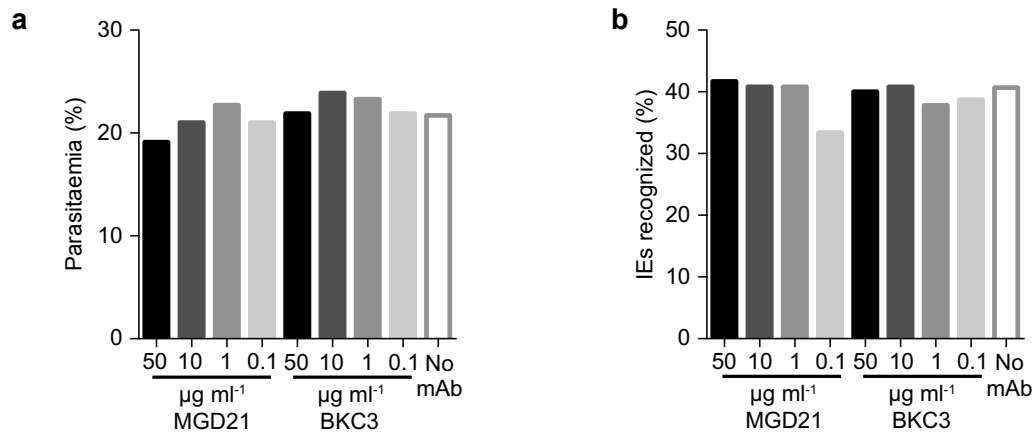


Figure 5.8. Investigation of growth inhibition and antigen downregulation by the LAIR1-containing antibody MGD21. a, Parasitaemia of 3D7-MGD21⁺ *in vitro* culture after 2 days of incubation with various concentrations of MGD21 or the irrelevant antibody BKC3. mAb, monoclonal antibody. b, Percentage of 3D7-MGD21⁺ IEs recognized by MGD21 after 2 days of incubation with various concentrations of MGD21 or BKC3. The antibodies were removed after 2 days (during the ring stage of the life cycle) and the parasites were allowed to grow for 24 h to the late trophozoite/schizont stage before staining with various concentrations of MGD21 or BKC3. Reprinted with permission from (Tan *et al.* 2016).

5.10. Investigation of rosette inhibition by LAIR1-containing antibodies

A recent study demonstrated that the RIFIN variant PF3D7_0100400 is able to mediate rosetting of IEs with blood group A erythrocytes (Goel *et al.* 2015). I tested if MGD21 could inhibit rosetting of 9605 IEs with blood group O⁺ or A⁺ erythrocytes by binding to target RIFINs, but did not find evidence for rosette inhibition by this antibody (Figure 5.9).

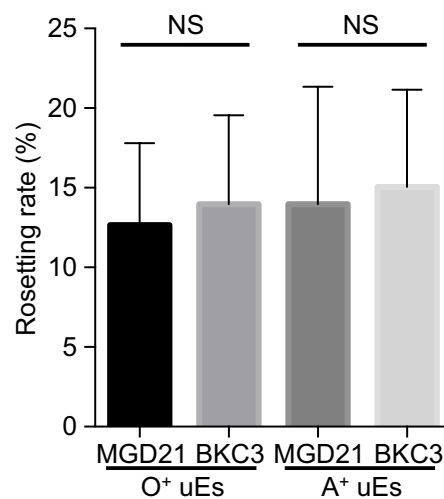


Figure 5.9. Rosetting of 9605-MGD21⁺ IEs with blood group O⁺ or A⁺ uninfected erythrocytes (uEs) after incubation with MGD21 or BKC3. Shown is the mean $\pm$ s.d. from $n = 4$ independent experiments. Statistical significance was evaluated by the Wilcoxon signed-rank test ($P > 0.1$ for both blood groups). NS, not significant. Reprinted with permission from (Tan *et al.* 2016).

5.11. Agglutination of IEs by LAIR1-containing antibodies

The plasma with LAIR1-containing antibodies were initially identified by mixed agglutination of IEs. I investigated whether this phenotype was mediated by LAIR1-containing monoclonal antibodies by testing these antibodies in individual and mixed agglutination assays. The monoclonal antibodies tested could form individual and triple mixed agglutinates, matching the serum phenotype (Figure 5.10).

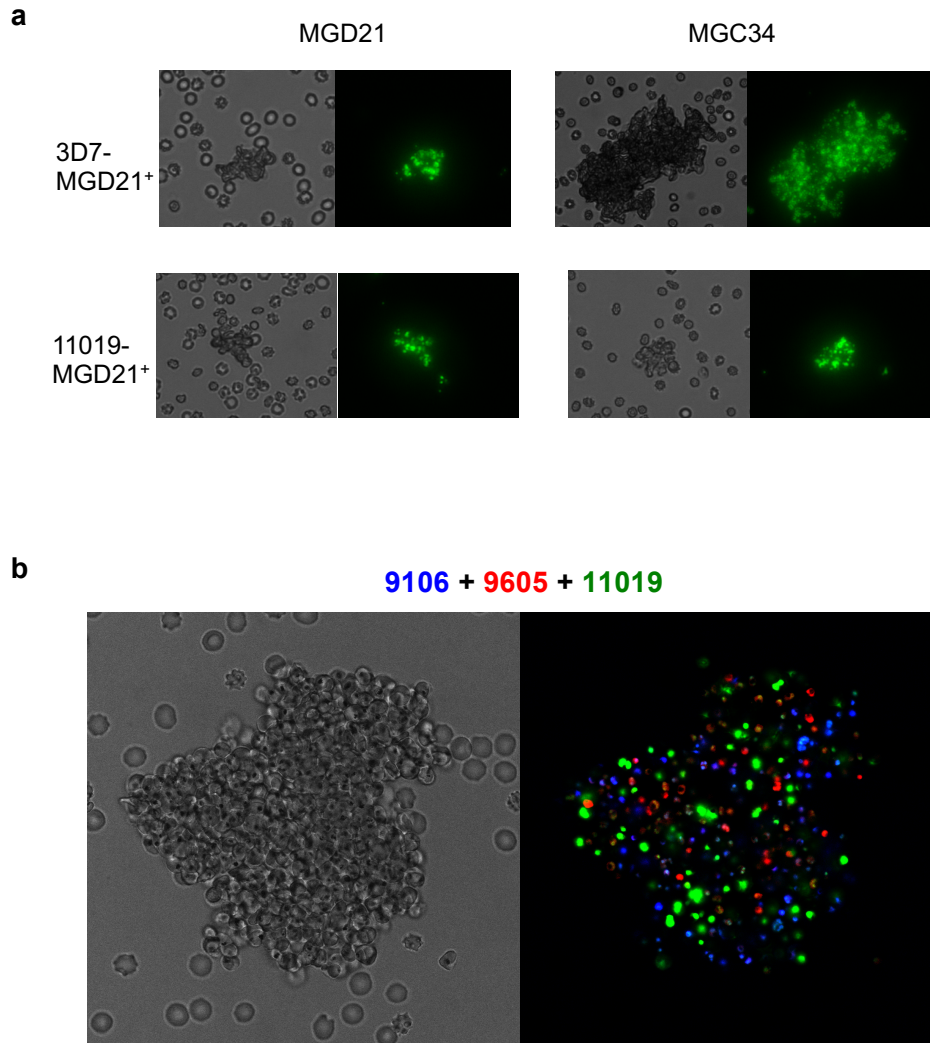


Figure 5.10. Agglutination of IEs from different parasite isolates by LAIR1-containing monoclonal antibodies. a, Single agglutinates of 3D7-MGD21⁺ or 11019-MGD21⁺ IEs formed by MGD21 or MGC34. b, Mixed agglutination of the Kenyan parasite isolates 9106, 9605 and 11019 by MGD21. No agglutinates were seen with the negative control antibody BKC3. The isolate names are coloured according to the colour that was observed by fluorescence microscopy. Adapted with permission from (Tan *et al.* 2016).

5.12. Surface staining of IEs by LAIR1-containing antibodies

Next, I tested the pattern of IE surface staining by the LAIR1-containing antibodies. MGD21 and MGC34 could stain the surface of 3D7-MGD21⁺ and 11019 IEs in an apparently uniform manner (Figure 5.11). As expected, not all IEs were stained by these antibodies.

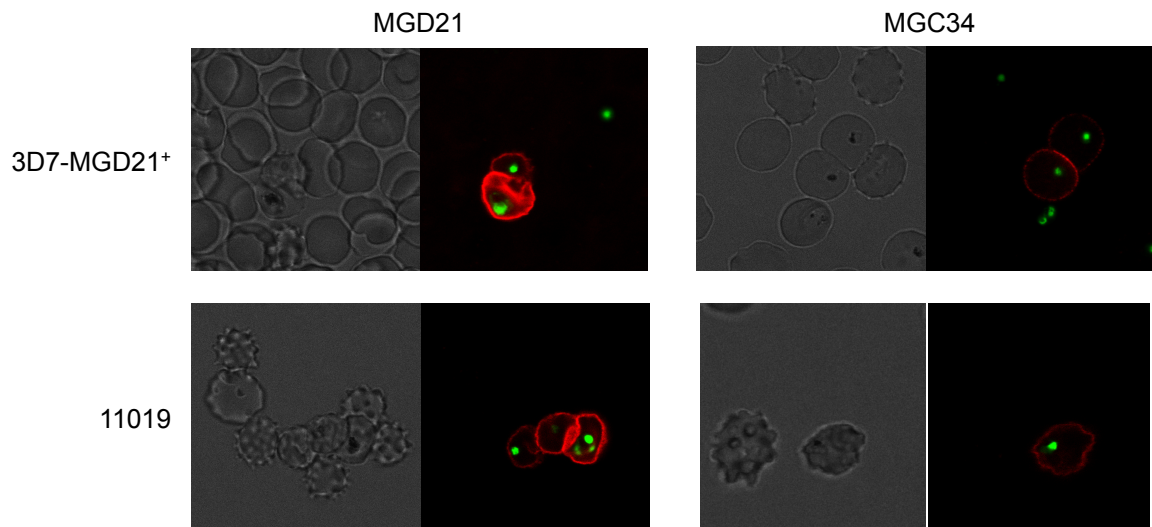


Figure 5.11. Surface staining of 3D7-MGD21⁺ and 11019 IEs by MGD21 and MGC34.
The nuclei of the parasites were stained with SYBR Green.

5.13. Opsonic phagocytosis of IEs by LAIR1-containing antibodies

Antibodies may contribute to the *in vivo* destruction of IEs through the opsonisation of these cells for phagocytosis by monocytes or macrophages. I tested whether the LAIR1-containing antibodies could opsonize IEs by incubating 3D7-MGD21⁺ IEs with human CD14⁺ monocytes (isolated from a buffy coat from a healthy donor) in the presence of MGD21. MGD21 showed a strong capacity to opsonize these IEs for phagocytosis (Figure 5.12a). Opsonization was dependent on an intact Fc, as an MGD21 mutant with reduced Fc receptor binding (MGD21 LALA) did not induce substantial phagocytosis. Similar results were obtained with other broadly reactive antibodies isolated from both donors and with a different parasite isolate (11019) (Figure 5.12b), suggesting that these broadly reactive antibodies could be effective in promoting phagocytosis and destruction of IEs *in vivo* (Tan *et al.* 2016).

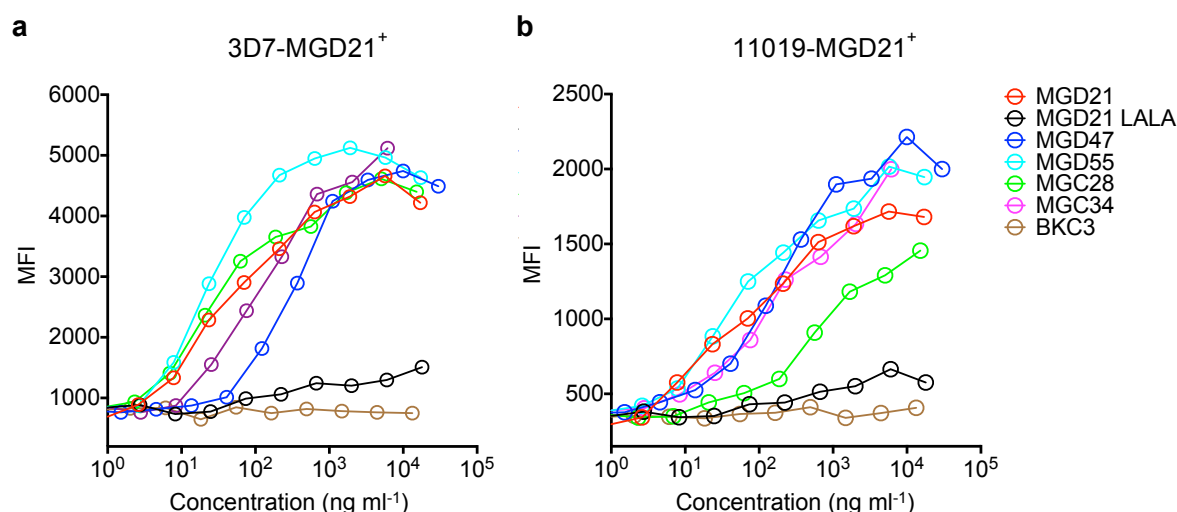


Figure 5.12. Opsonic phagocytosis of 3D7-MGD21⁺ (a) or 11019-MGD21⁺ (b) IEs by monocytes. The IEs were stained with DAPI, which was quantified in monocytes as a measure of phagocytosis. MGD21 LALA is a mutant of MGD21 with reduced Fc receptor binding, while BKC3 is a negative control antibody with an irrelevant specificity. Reprinted with permission from (Tan *et al.* 2016).

5.14. Summary

In this section, we have identified RIFINs as the targets of the broadly reactive LAIR1-containing antibodies. Different LAIR1-containing antibodies appear to recognize different RIFIN variants, probably because of distinct mutations in the LAIR1 domain of each antibody. These antibodies do not inhibit the growth of parasites *in vitro* and do not inhibit rosetting with blood group O or A uninfected erythrocytes. However, the LAIR1-containing antibodies can agglutinate IEs and can opsonize these cells for phagocytosis by monocytes, suggesting that they may aid *in vivo* clearance of parasites. While these assays may offer hints as to potential protective roles of these antibodies, a more direct way to test this is to conduct immunoepidemiological studies on populations in malaria-endemic areas to determine if individuals possessing these antibodies are protected from clinical malaria. To this end, I am currently developing novel assays to screen sera specifically for LAIR1-containing antibodies, as will be described in the following chapter.

Chapter 6. Discovery of new LAIR1-containing antibodies using novel techniques

6.1. Introduction

In the previous chapters, I described the identification and characterisation of unusual LAIR1-containing antibodies from three malaria-exposed adults living in a village in Kilifi County, Kenya. We wanted to find out whether the insertion of LAIR1 was an unusual occurrence limited to that particular village, or if the insertion can occur more generally, particularly in malaria-endemic areas. Therefore, we decided to search for more individuals with LAIR1-containing antibodies from different malaria-endemic regions in Tanzania and Mali. By screening large cohorts, we hoped not only to find more of these unusual antibodies, but also to estimate the frequency of LAIR1 insertion in malaria-endemic populations.

In order to conduct these experiments, we needed to develop a high-throughput assay that screens specifically for the presence of LAIR1-containing antibodies. While the mixed agglutination assay unexpectedly enabled us to find several plasma with LAIR1-containing antibodies, it searches for broad reactivity rather than for the presence of antibodies with a LAIR1 insert. Therefore, it is possible that some of the variant-specific plasma that were not chosen for isolation of monoclonal antibodies also possessed LAIR1-containing antibodies. Moreover, mixed agglutination is not high-throughput or rigorously quantitative, and is hence unsuitable for screening large numbers of sera. In this section, I will describe several new methods that I have developed to search specifically for

LAIR1-containing antibodies. These new methods exploit the finding that anti-LAIR1 antibodies that are commercially available can be used to detect LAIR1-containing antibodies. These techniques have enabled us to identify more individuals with LAIR1-containing antibodies from different malaria-endemic regions, demonstrating that the phenomenon of LAIR1 insertion is not limited to a particular area or population.

6.2. Development of IE dual-staining assay

The first assay that I developed is based on the fact that LAIR1-containing antibodies have both a LAIR1 and a normal Fc component. Therefore, if LAIR1-containing antibodies are bound to IEs, double-positive staining of IEs after the addition of anti-LAIR1 antibodies and anti-IgG/IgM Fc antibodies conjugated to different fluorophores can be used to detect the presence of the LAIR1-containing antibodies. A scheme of the assay is shown in Figure 6.1.

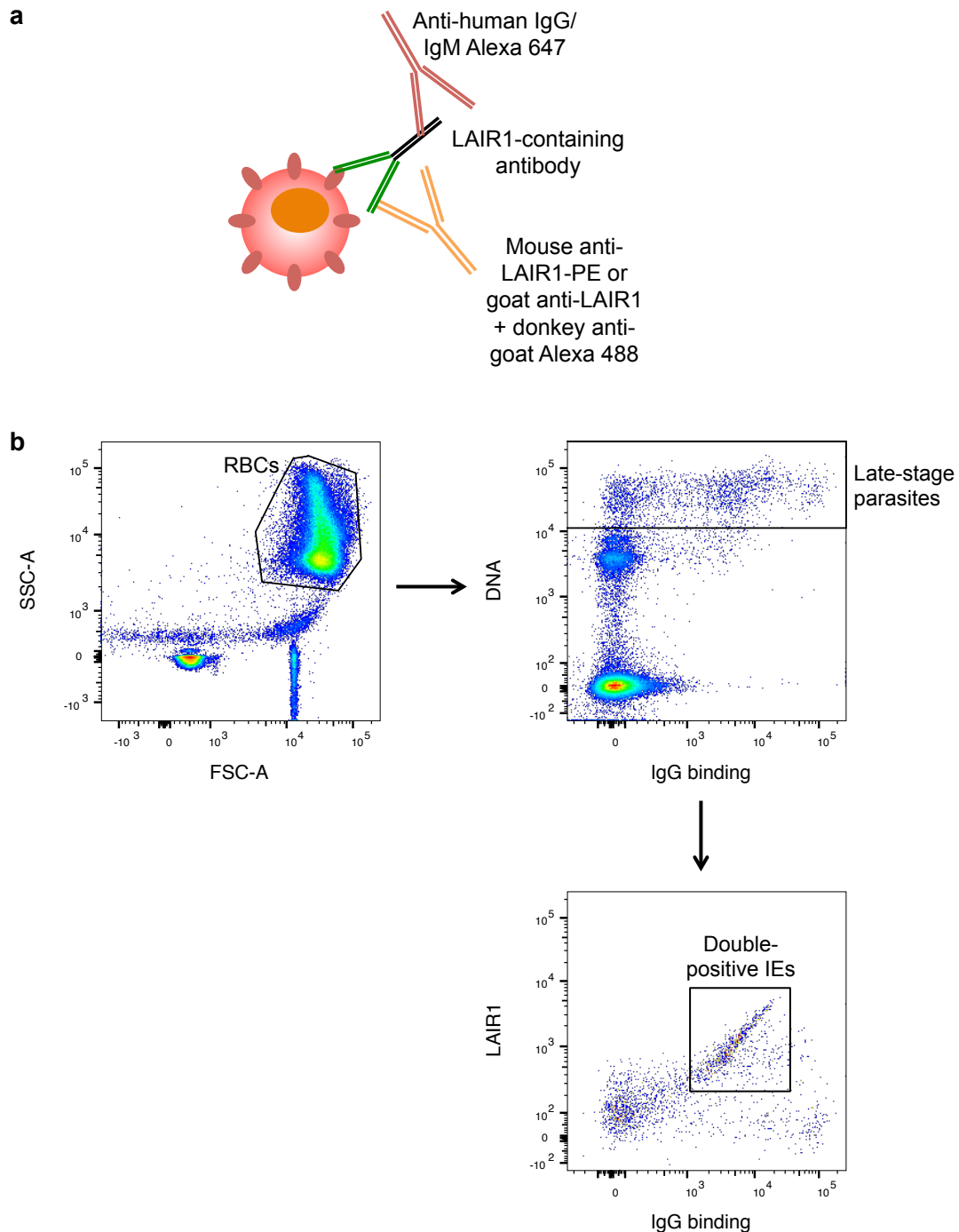


Figure 6.1. Scheme of IE dual-staining assay. a, In this assay, IEs are stained with a serum with LAIR1-containing antibodies. The presence of these antibodies is detected by dual staining with anti-human IgG/IgM and anti-LAIR1 antibodies conjugated to different fluorophores. b, Gating strategy to identify LAIR1-containing antibodies. Erythrocytes are gated based on side and forward scatter. Late-stage IEs can be separated from uninfected erythrocytes and ring-stage parasites by the level of DNA staining. IEs bound by LAIR1-containing antibodies would be double-positive for LAIR1 and IgG binding (see Methods section 2.11 for details).

6.3. Screen of Tanzanian sera or plasma with IE dual-staining assay

Using the IE dual-staining assay, I screened 84 sera or plasma from Tanzanian adults. Most of the samples (82 out of 84) only contained conventional IgG antibodies that stained IEs (an example is shown in Figure 6.2a), but I identified sera from two donors (named E and F) that had LAIR1-containing antibodies. The LAIR1-containing antibodies from donor E appeared to be mainly of the IgM isotype with a small IgG (and possibly IgA) component (Figure 6.2b), while the LAIR1-containing antibodies from donor F were exclusively IgG (Figure 6.2c). To obtain LAIR1-containing monoclonal antibodies from these individuals, we immortalized IgG⁺ and IgM⁺ memory B cells from donor E and IgG⁺ memory B cells from donor F. I screened the B cell supernatants for binding to a mixture of IEs from four different parasite isolates (3D7-MGD21⁺, 9106, 9605 and 11019). In parallel, the IgG supernatants from donor F (MGF) were also screened directly for the presence of LAIR1-containing monoclonal antibodies by an ELISA using polyclonal goat anti-LAIR1 as the coating antibody and anti-human IgG as the detecting antibody^m, as well as by a novel microbead-based assay (described later). Using these approaches, we obtained two LAIR1-containing IgG antibodies and three LAIR1-containing IgM antibodies from donor E, and 11 LAIR1-containing IgGs from donor F (based on the microbead-based assay, see section 6.5). These results are still preliminary, as these antibodies are in the process of being sequenced and are thus incompletely characterized. Nevertheless, preliminary sequencing results indicate that the MGE and MGF antibodies have similar architectures to the MGC and MGD antibodies (Figure 6.3).

^mThe ELISA was conducted by Dr Kathrin Pieper.

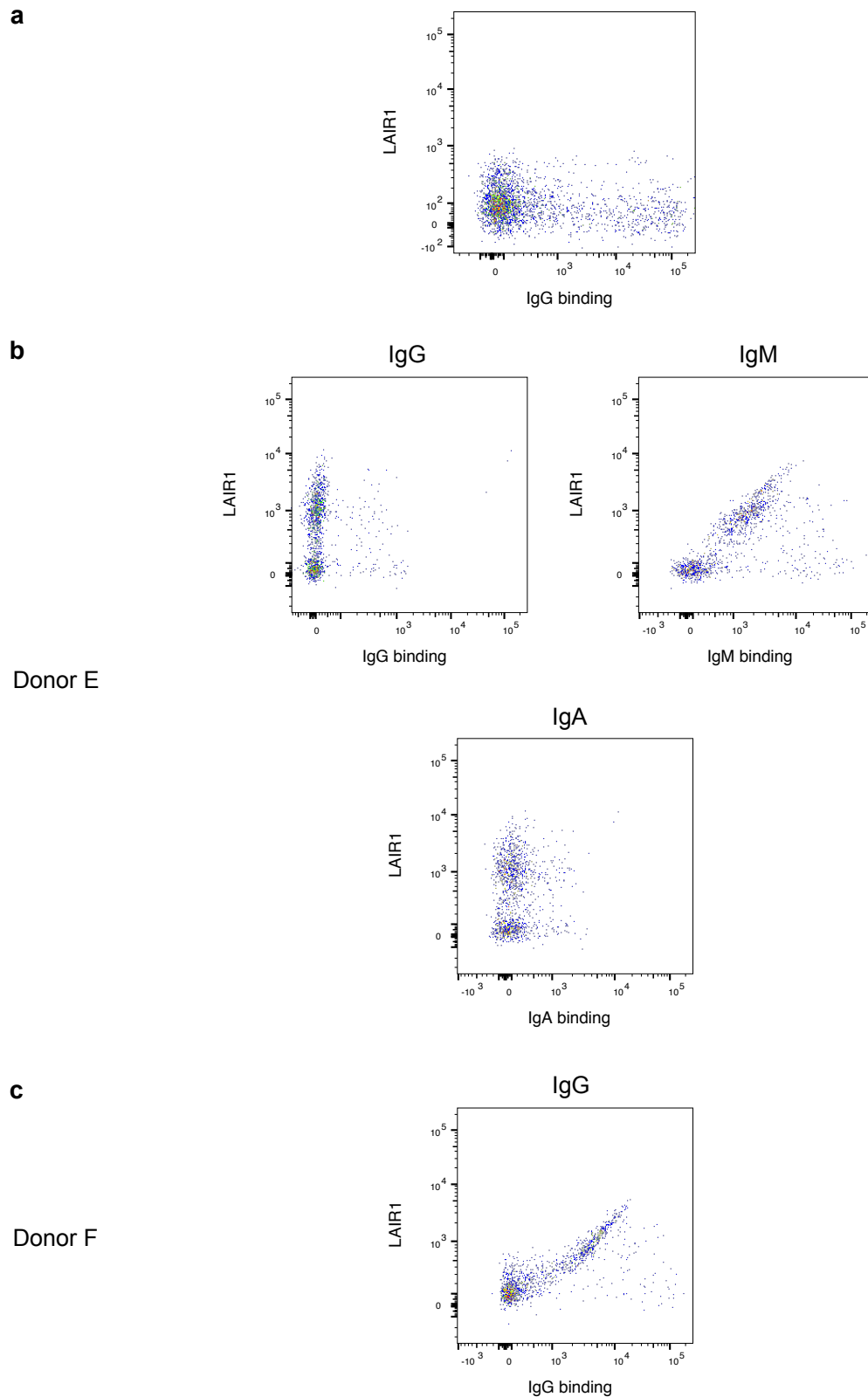


Figure 6.2. Tanzanian sera with LAIR1-containing antibodies identified by the IE dual-staining assay. a, Example of staining of IEs by conventional IgG antibodies found in most serum or plasma samples. b-c, The serum of donor E (b) had mostly LAIR1-containing IgM antibodies, while the serum of donor F (c) had LAIR1-containing IgG antibodies.

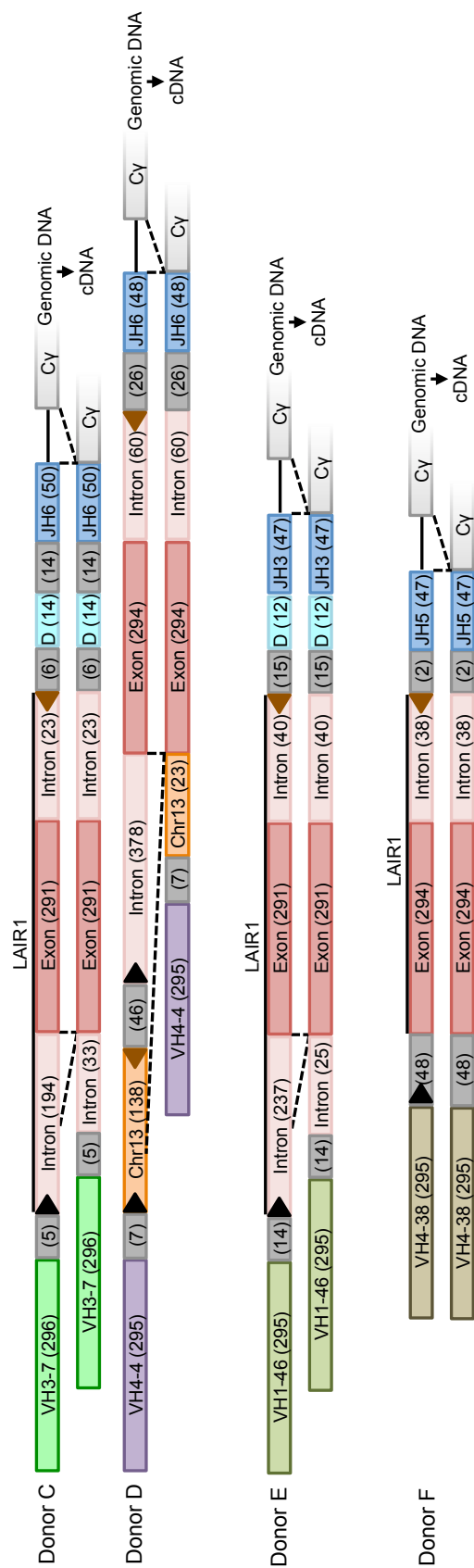


Figure 6.3. Genomic DNA and cDNA structures of LAIR1-containing antibodies from donors C, D, E and F. Preliminary sequencing results indicate that the LAIR1-containing antibodies from donors E and F have similar cDNA and genomic DNA architectures to those isolated from donors C and D. Adapted with permission from Tan *et al.* (2016).

6.4. Anti-human IgG microbeads assay

While the IE dual-staining assay enabled the identification of two new donors with LAIR1-containing antibodies, it is unlikely that this assay is able to detect all sera with LAIR1-containing antibodies as it relies on the ability of these antibodies to bind to the specific IEs chosen for the assay. LAIR1-containing antibodies that do not bind to RIFINs (or other antigens) that are expressed on those specific IEs or that do not bind to IEs at all will not be detected with this assay. This is a major drawback, especially considering the diverse pattern of binding of different LAIR1-containing monoclonal antibodies to the various parasite isolates tested. Furthermore, at this point we were interested in the frequency of LAIR1 insertion into the antibody locus, even if the resulting antibodies failed to gain specificity for RIFINs. Therefore, I developed several assays based on microbeads that should detect all LAIR1-containing antibodies regardless of binding specificity. The first variation of this technique utilized anti-human IgG microbeads to capture LAIR1-containing monoclonal antibodies, followed by detection of these antibodies with anti-LAIR1 antibodies (Figure 6.4a). To allow the detection of non-specific binding to these microbeads, I included uncoated beads as internal negative controls alongside the anti-human IgG microbeads. This technique allowed me to clearly distinguish between LAIR1-containing monoclonal antibodies and conventional monoclonal antibodies (Figure 6.4b).

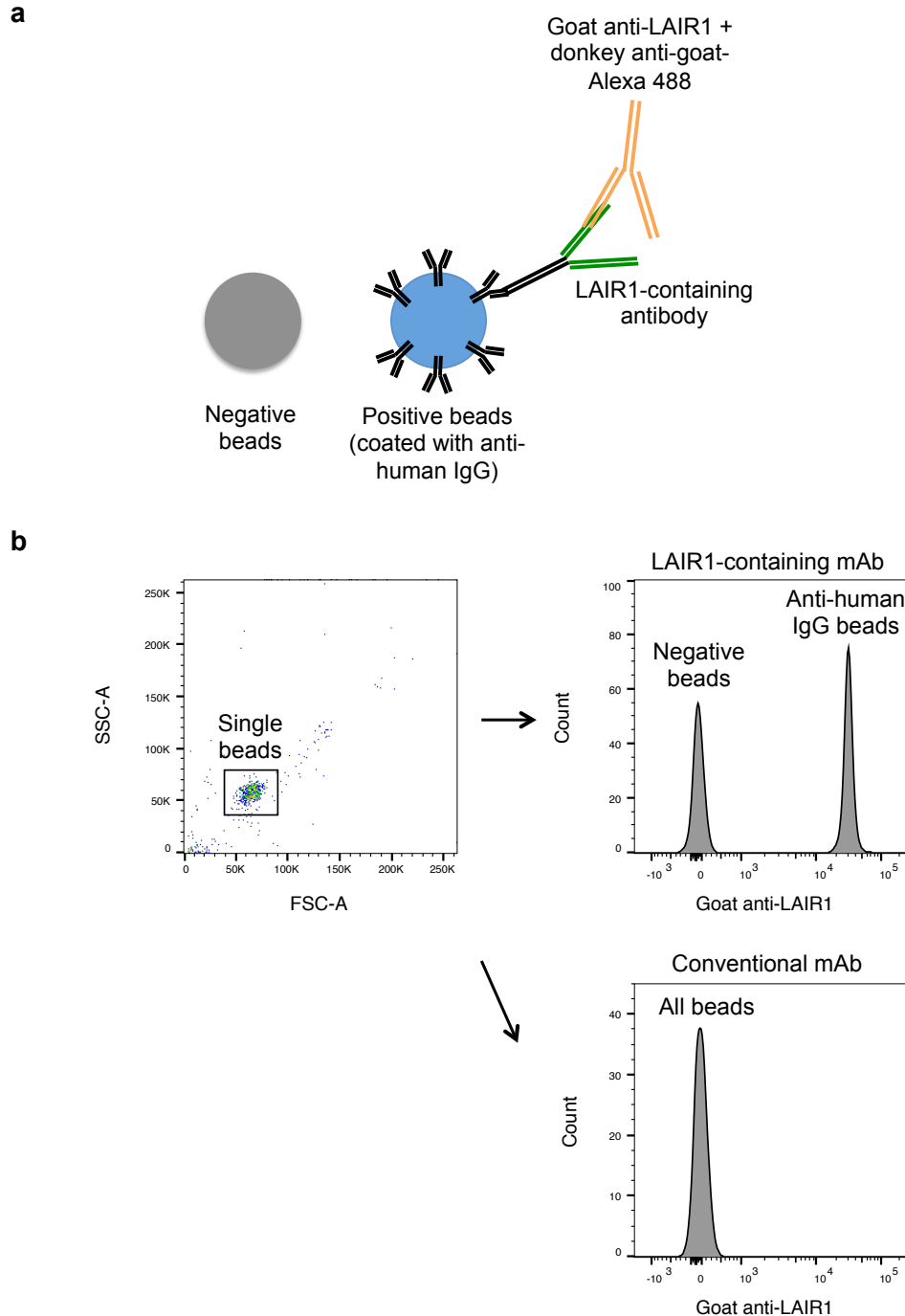


Figure 6.4. Scheme of anti-human IgG microbeads assay. a, In this assay, a mixture of negative microbeads and mouse anti-human IgG microbeads is stained with monoclonal IgG antibodies. The presence of LAIR1-containing monoclonal antibodies is detected by anti-LAIR1 secondary antibodies. b, Gating strategy to identify LAIR1-containing antibodies. Single beads are gated based on side and forward scatter. Beads bound by LAIR1-containing antibodies would form two clearly distinct peaks, while conventional antibodies would only form a single peak.

6.5. Validation of anti-human IgG microbeads assay with MGE and MGF monoclonal antibodies

To validate the novel microbead-based assay, I used the assay to examine the MGE and MGF antibodies that were previously identified by ELISA and/or the IE dual-staining assay as LAIR1-positive. Surprisingly, 28 out of 39 of the MGF monoclonal antibodies that were identified as LAIR1-positive by ELISA were LAIR1-negative using this assay, indicating that either the ELISA resulted in false positives or the microbeads assay resulted in false negatives (Figure 6.5). To distinguish between the two possibilities, we performed PCRs on cDNA from 13 MGF antibodies using a forward primer specific for the LAIR1 region and a reverse primer that targets the IgG constant regionⁿ. We found that six out of seven of the MGF monoclonal antibodies (except for MGF63, with the weakest microbead signal) that were positive by the beads assay were also LAIR1-positive by PCR, while all six of the monoclonal antibodies that were negative based on the beads assay but were apparently LAIR1-positive based on the ELISA were LAIR1-negative by PCR (Figure 6.6). These results strongly suggest that the microbeads assay is more specific than the ELISA in identifying true LAIR1-containing antibodies. Based on the microbeads assay, 11 of the MGF antibodies that we have obtained are LAIR1-positive. Out of these 11, only six (MGF8, MGF21, MGF33, MGF39, MGF45 and MGF67) were identified as positive for binding to IEs during the primary screen, suggesting that the microbeads assay can identify more LAIR1-containing antibodies than the IE dual-staining assay.

ⁿPCRs performed by Ms Yiwei Chen.

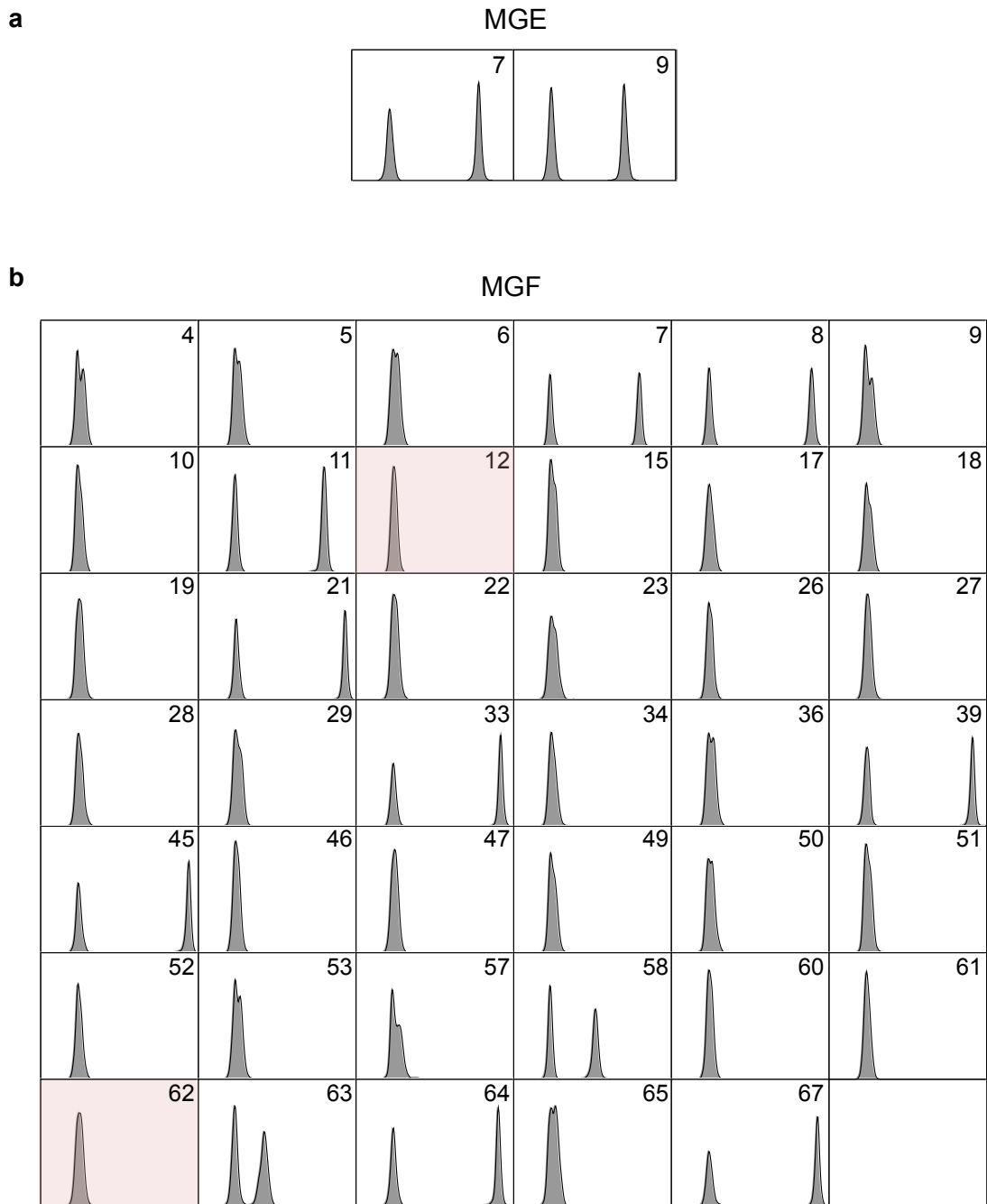


Figure 6.5. Screen of MGE (a) and MGF (b) monoclonal antibodies using the anti-human IgG microbeads assay. The MGE antibodies were not tested by ELISA, and all of the MGF antibodies were identified as LAIR1-positive by ELISA except MGF12 and MGF62 (highlighted red). The microbeads assay only identified a fraction of these antibodies as LAIR1-positive.

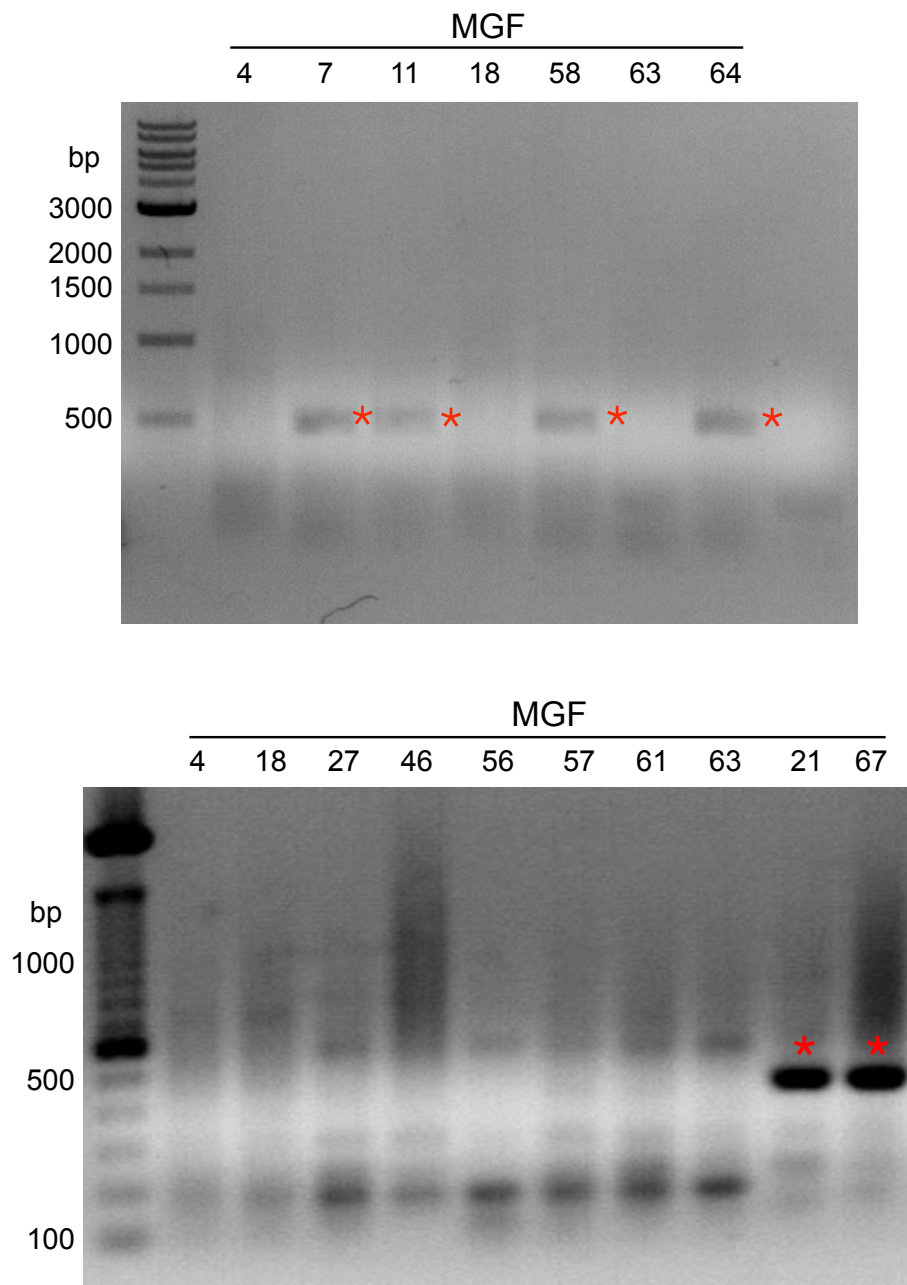


Figure 6.6. PCR amplification of MGF cDNA to detect the presence of LAIR1 inserts.

The PCRs were done with a forward primer that is specific for the LAIR1 exon and a reverse primer that binds to the IgG constant region. The expected size of a positive band is approximately 500 bp. MGF7, MGF11, MGF21, MGF58, MGF63, MGF64 and MGF67 were LAIR1-positive based on the microbeads assay and the ELISA. The rest of the MGFs were LAIR1-negative by the microbeads assay and LAIR1-positive by ELISA, except for MGF56, which was not tested by the microbeads assay.

6.6. Anti-goat IgG microbeads assay

While the anti-human IgG microbeads assay appears to be a selective and sensitive approach that is useful to screen monoclonal antibodies for the presence of LAIR1 inserts, it is unsuitable for the detection of LAIR1-containing antibodies in serum, as these beads would be rapidly saturated by conventional IgG antibodies. Therefore, I developed a microbead-based assay that specifically captures LAIR1-containing antibodies in serum (Figure 6.7). In this assay, donkey anti-goat IgG microbeads are divided into two equal portions and one portion is stained with SYBR Green I (which we fortuitously found to be able to label these microbeads). The stained test beads are incubated with goat anti-human LAIR1 antibodies, while the unstained control beads are incubated with an isotype control (goat anti-human epidermal growth factor (EGF)). The beads are then washed, mixed, and incubated with the serum of interest. Antibody binding is detected by adding secondary donkey anti-human IgG/IgM antibodies conjugated to a fluorescent dye. The mean fluorescence intensity (MFI) ratio of antibody binding to the test beads relative to the control beads is used to determine LAIR1 positivity.

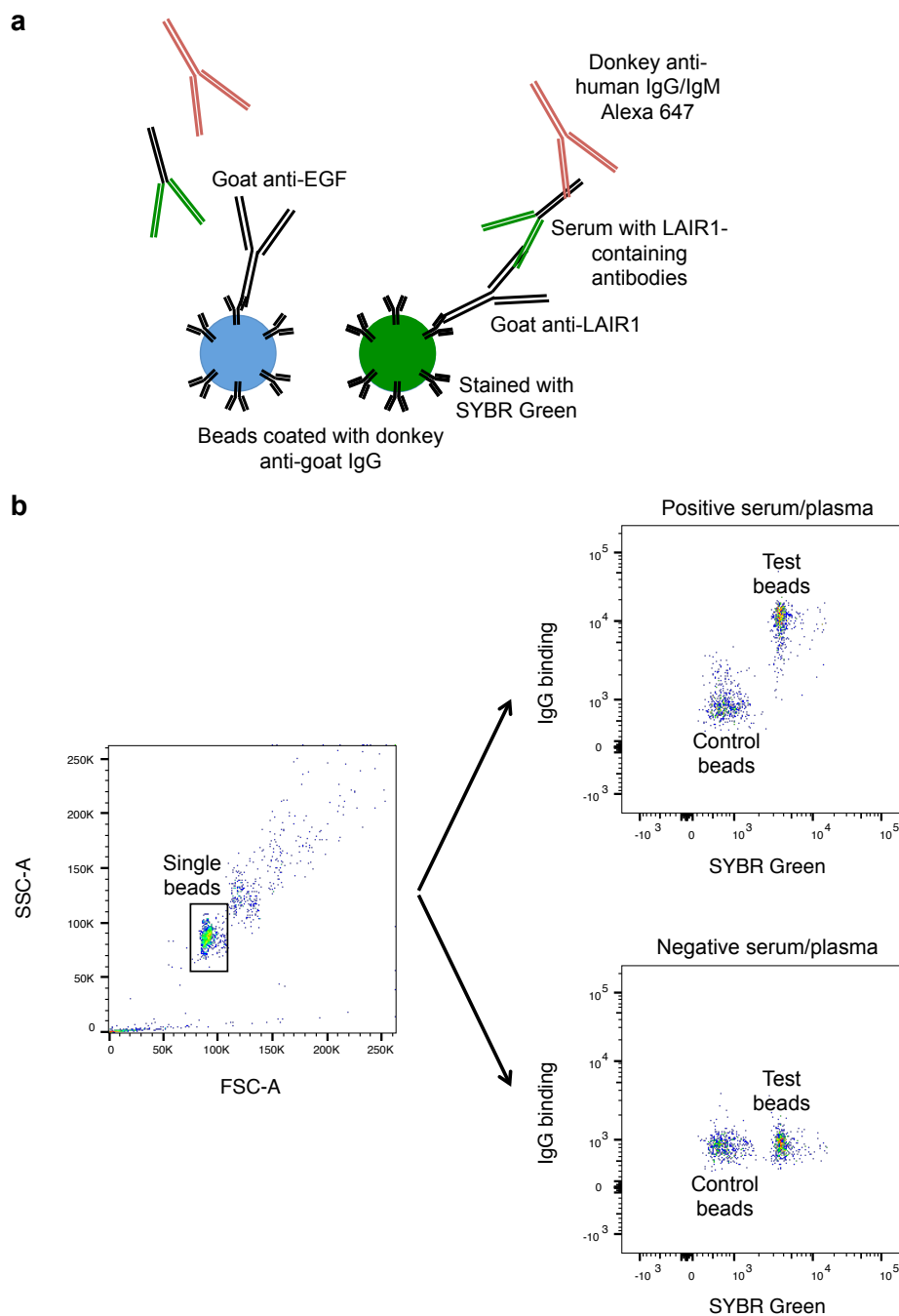


Figure 6.7. Anti-goat IgG microbeads assay. a, In this assay, donkey anti-goat IgG beads are divided into two portions. One portion (test beads) is stained with SYBR Green and goat anti-human LAIR1, while the other (control beads) is stained with an isotype control (goat anti-human EGF). The beads are mixed and incubated with a test serum or plasma, and antibody binding is detected with donkey anti-human IgG/IgM conjugated to a fluorophore. b, Gating strategy to identify sera with LAIR1-containing antibodies. Single beads are gated based on side and forward scatter. Sera with LAIR1-containing antibodies would stain test beads more strongly than control beads. The MFI ratio of antibody binding to the test beads relative to the control beads is used to determine LAIR1 positivity.

6.7. Screen of Tanzanian and Malian sera/plasma for LAIR1-containing antibodies using anti-goat IgG microbeads assay

I used the anti-goat IgG microbeads assay to screen 92 Tanzanian sera/plasma (consisting of 52 samples that were negative based on the IE dual-staining assay and 40 new samples) and 682 Malian plasma for the presence of LAIR1-containing antibodies. The results of the screen of the Tanzanian sera/plasma (Figure 6.8) and the top 94 Malian plasma based on the MFI ratio of test beads to control beads (Figure 6.9) are shown here, while the complete results of the Malian screen are shown in the Supplementary Information section (Figure S2). A number of sera/plasma from both cohorts appeared to have LAIR1-containing antibodies. If a threshold MFI ratio of 1.5 was used, there were five positive Tanzanian sera/plasma and 47 positive Malian plasma. To obtain more evidence to support these results, I also performed a competition assay with recombinant LAIR1. In this assay, a high concentration of recombinant LAIR1 was added prior to the addition of sera to potentially compete with the binding of LAIR1-containing antibodies. The results of the competition assay are shown in Figures 6.8, 6.9 and S2. In general, these results matched the findings of the anti-goat IgG microbeads assay. We are starting to immortalize memory B cells from donors who were identified as LAIR1-positive using these assays, and preliminary screens of B cell supernatants indicate that these individuals do indeed have LAIR1-containing monoclonal antibodies (Figure 6.10).

Cohort	Sample	Binding ratio	Competition ratio
Bagamoyo immunized and challenged	205G2	1.72	1.82
	117G2	1.19	
	179G2	2.42	1.48
	198G2	1.14	
	078G2	1.19	
	240G2	1.23	
	144G2	1.14	
	581G5	0.96	
	139G2	1.18	
	156G2	1.31	
	253G2	1.27	
	239G2	1.26	
	590G5	1.40	
	126G2	1.15	
	054G2	0.96	
	228G2	1.38	
	048G2	1.19	
	104G2	1.20	
	274G2	1.28	
	087G2	1.10	
	269G2	0.84	
	093G2	1.20	
	160G2	1.27	
	218G2	1.12	
	325G3	1.51	0.88
	290G3	1.23	
	351G3	1.06	
	377G3	1.16	
	445G3	1.03	
	383G3	1.12	
	067G2	1.02	
	430G3	1.25	
	067G2	1.09	
	310G3	1.13	
	281G3	1.21	
	489G3	1.24	
	473G3	1.06	
	345G3	1.29	
	401G3	1.30	
	397G3	0.99	
	411G3	1.26	
	616G5	1.23	
	469G3	1.32	
	495G3	1.12	
	360G3	1.25	
	512G3	1.01	
	335G3	1.17	
	500G3	1.19	
	603G5	0.93	
	306G3	1.19	
	453G3	1.11	
	420G3	1.32	
Cohort	Sample	Binding ratio	Competition ratio
P27	V16RN1	1.38	
	V16RN2	1.24	
	V16RN3	1.15	
	V16RN4	1.24	
	V16RN5	1.10	
	V16RN6	1.21	
	V16RN7	1.19	
	V16RN8	1.21	
	V16RN9	1.20	
	V16RN10	1.20	
	V16RN11	1.11	
	V16RN12	1.14	
	V16RN13	1.17	
	V16RN14	1.25	
	V16RN15	1.40	
	V16RN16	1.32	
	V16RN17	1.64	1.37
	V16RN18	1.15	
	V16RN19	1.21	
	V16RN20	1.22	
	V16RN21	1.27	
	V16RN22	1.20	
	V16RN23	1.22	
	V16RN24	1.27	
	V16RN25	3.28	1.78
	V16RN26	1.38	
	V16RN27	1.29	
	V16RN28	1.28	
	V16RN29	1.21	
	V16RN30	1.25	
	V16RN31	1.15	
	V16RN32	1.17	
	V16RN33	1.22	
	V16RN34	1.37	
	V16RN35	1.02	
	V16RN36	1.21	
	V16RN37	1.30	
	V16RN38	1.25	
	V16RN39	1.30	
	V16RN40	1.21	
	Naive	1.08	0.97

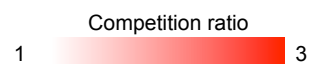


Figure 6.8. Results of screen of Tanzanian sera or plasma using anti-goat IgG microbeads and results of competition assay with recombinant LAIR1. The binding ratio is the MFI ratio of antibody binding to the test beads relative to the control beads. Sera or plasma that have a ratio of >1.5 are shown in red. Serum from a malaria-naïve donor is shown as a negative control. The competition ratio is the MFI ratio of antibody binding to the test beads in the absence of recombinant LAIR1 to antibody binding to the same beads in the presence of 10 µg/mL LAIR1. The colour scale of the competition ratio is as shown. Only the positive sera/plasma from the anti-goat IgG microbeads assay were tested in the competition assay.

Cohort	Sample name		Binding ratio	Competition ratio
Dogon/Fulani	Dog05		1.33	1.60
	Dog11		1.53	1.55
	Dog12		10.91	8.25
	Dog15		3.38	3.87
	Dog21		1.37	1.67
	Dog22		3.31	2.88
	Dog23		1.33	1.36
	Fini10		1.44	1.18
	Fini18		4.55	5.31
	Fini22		1.30	1.23
Kalifabougou	Kali	14 m07	1.39	1.98
	Kali	24 m07	5.90	5.52
	Kali	36 m07	1.33	1.18
	Kali	37 m07	1.31	1.69
	Kali	64 m07	3.27	4.96
	Kali	70 m07	10.68	10.82
	Kali	71 m07	1.29	1.43
	Kali	76 m07	4.19	5.04
	Kali	80 m07	5.80	8.75
	Kali	90 m07	1.30	1.21
	Kali	92 m07	1.76	1.90
	Kali	96 m07	1.64	1.19
	Kali	104 m07	1.31	1.56
	Kali	105 m07	1.31	1.21
	Kali	112 m07	1.43	1.35
	Kali	136 m07	1.31	0.77
	Kali	137 m07	4.02	6.26
	Kali	140 m07	1.36	1.21
	Kali	152 m07	1.39	1.34
	Kali	177 m07	7.51	9.54
	Kali	189 m07	1.32	1.27
	Kali	192 m07	4.58	6.11
	Kali	193 m07	4.64	4.95
	Kali	206 m07	1.31	1.13
	Kali	210 m07	1.36	1.50
	Kali	215 m07	10.08	6.98
	Kali	216 m07	3.43	4.80
	Kali	219 m07	1.29	1.33
	Kali	223 m07	1.29	1.25
	Kali	228 m07	6.51	7.75
	Kali	232 m07	1.31	1.27
	Kali	246 m07	1.29	1.07
	Kali	247 m07	6.88	6.38
	Kali	249 m07	5.23	6.74
	Kali	255 m07	1.46	1.27
	Kali	259 m07	1.30	1.15
	Kali	260 m07	3.20	2.92

Cohort	Sample name		Binding ratio	Competition ratio
Kalifabougou	Kali	261 m07	1.59	1.23
	Kali	264 m07	2.48	3.09
	Kali	266 m07	6.73	8.02
	Kali	271 m07	2.76	2.28
	Kali	274 m07	1.34	1.11
	Kali	277 m07	1.55	1.82
	Kali	288 m07	2.86	2.85
	Kali	290 m07	1.40	2.37
	Kali	297 m07	1.55	1.40
	Kali	301 m07	1.29	1.18
	Kali	312 m07	1.38	1.04
	Kali	318 m07	1.36	1.39
	Kali	321 m07	3.39	4.02
	Kali	325 m07	1.61	1.96
	Kali	326 m07	4.98	6.23
	Kali	328 m07	2.07	1.60
	Kali	375 m07	2.26	3.22
	Kali	382 m07	10.70	9.31
	Kali	403 m07	1.29	1.28
	Kali	415 m07	4.13	8.65
	Kali	424 m07	1.36	1.33
	Kali	438 m07	3.44	4.21
	Kali	452 m07	2.98	2.96
	Kali	454 m07	2.35	2.81
	Kali	458 m07	1.56	1.12
	Kali	470 m07	7.01	9.83
	Kali	475 m07	1.31	1.60
	Kali	486 m07	1.32	1.17
	Kali	495 m07	1.92	3.00
	Kali	496 m07	1.34	1.41
	Kali	521 m07	1.52	1.14
	Kali	523 m07	2.77	3.22
	Kali	529 m07	1.29	1.21
	Kali	543 m07	1.35	1.37
	Kali	545 m07	1.30	1.29
	Kali	555 m07	1.34	1.12
	Kali	565 m07	1.48	1.30
	Kali	622 m07	1.60	2.05
	Kali	645 m07	1.29	1.25
	Kali	648 m07	1.41	1.15
	Kali	707 Apr-13	1.33	1.04
	Kali	712 Apr-13	9.46	7.95
	Kali	718 Apr-13	1.29	1.24
	Kali	730 Apr-13	10.48	5.02
	Kali	751 Apr-13	1.33	1.86
	Kali	752 Apr-13	1.37	1.13
	Kali	755 Apr-13	1.29	1.68



Figure 6.9. Results of screen of Malian plasma using anti-goat IgG microbeads and results of competition assay with recombinant LAIR1. The binding ratio is the MFI ratio of antibody binding to the test beads relative to the control beads. Sera that have a ratio of >1.5 are shown in red. Only the 94 Malian plasma with the highest binding ratios are shown. The competition ratio is the MFI ratio of antibody binding to the test beads in the absence of recombinant LAIR1 to antibody binding to the same beads in the presence of 5 µg/mL LAIR1. The colour scale of the competition ratio is as shown.

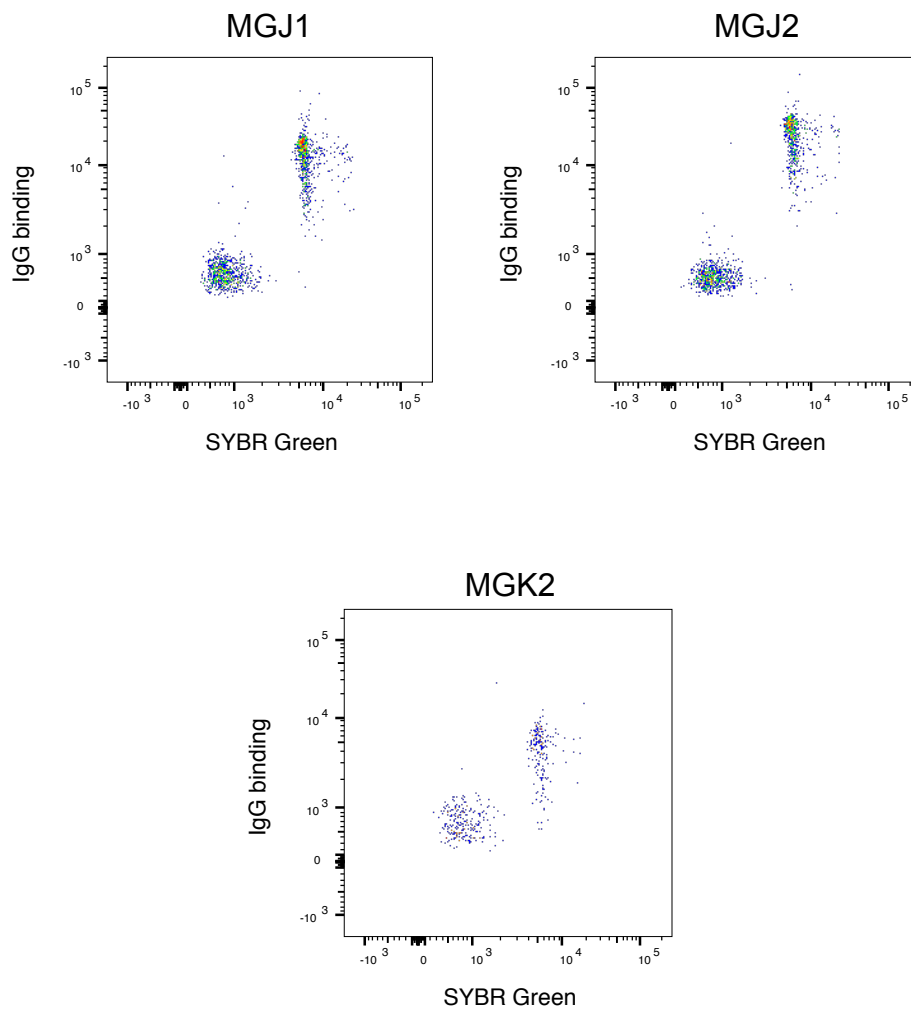


Figure 6.10. Preliminary examples of LAIR1-positive monoclonal antibodies from Malian and Tanzanian donors. Donor J is from Tanzania (205G2) and donor K is from Mali (Kali 382 m07). These antibodies were tested with the anti-goat IgG microbeads assay (see Figure 6.7 for a description of this assay).

6.8. Fluorescence resonance energy transfer (FRET)-based assay for the detection of B cells with LAIR1-containing BCRs

Using the anti-goat IgG microbeads assay, I have identified many donors that appear to possess LAIR1-containing antibodies. While it would be interesting to isolate and characterize the LAIR1-containing antibodies from many of these individuals, the process of B cell immortalization and supernatant screening is labourious and would limit the speed of this investigation. A simpler alternative would have been to sort B cells with LAIR1-containing BCRs based on the staining of LAIR1 on the surface of these cells, but memory B cells also express a heterogeneous amount of the endogenous LAIR1 receptor, complicating this approach. To overcome this problem, I am currently developing a FRET-based assay to specifically detect B cells with LAIR1-containing BCRs. This approach is based on the fact that B cells with LAIR1-containing BCRs have the LAIR1 domain in close proximity to the antibody constant domains in the BCRs. Therefore, if these B cells are stained with an anti-LAIR1 antibody conjugated to PE and an anti-human IgG antibody conjugated to Alexa Fluor 647 (A647), the excitation of PE may result in the transfer of energy to A647, resulting in a FRET fluorescence signal from A647 even without direct excitation of this fluorophore (Figure 6.11a). A FRET signal can be observed from an immortalized LAIR1-positive IgG⁺ B cell clone (MGB47) but not from negative control primary B cells from a malaria-naïve individual that express the endogenous LAIR1 receptor (Figure 6.11b). I am starting to test this approach on memory B cells from the Tanzanian and Malian donors.

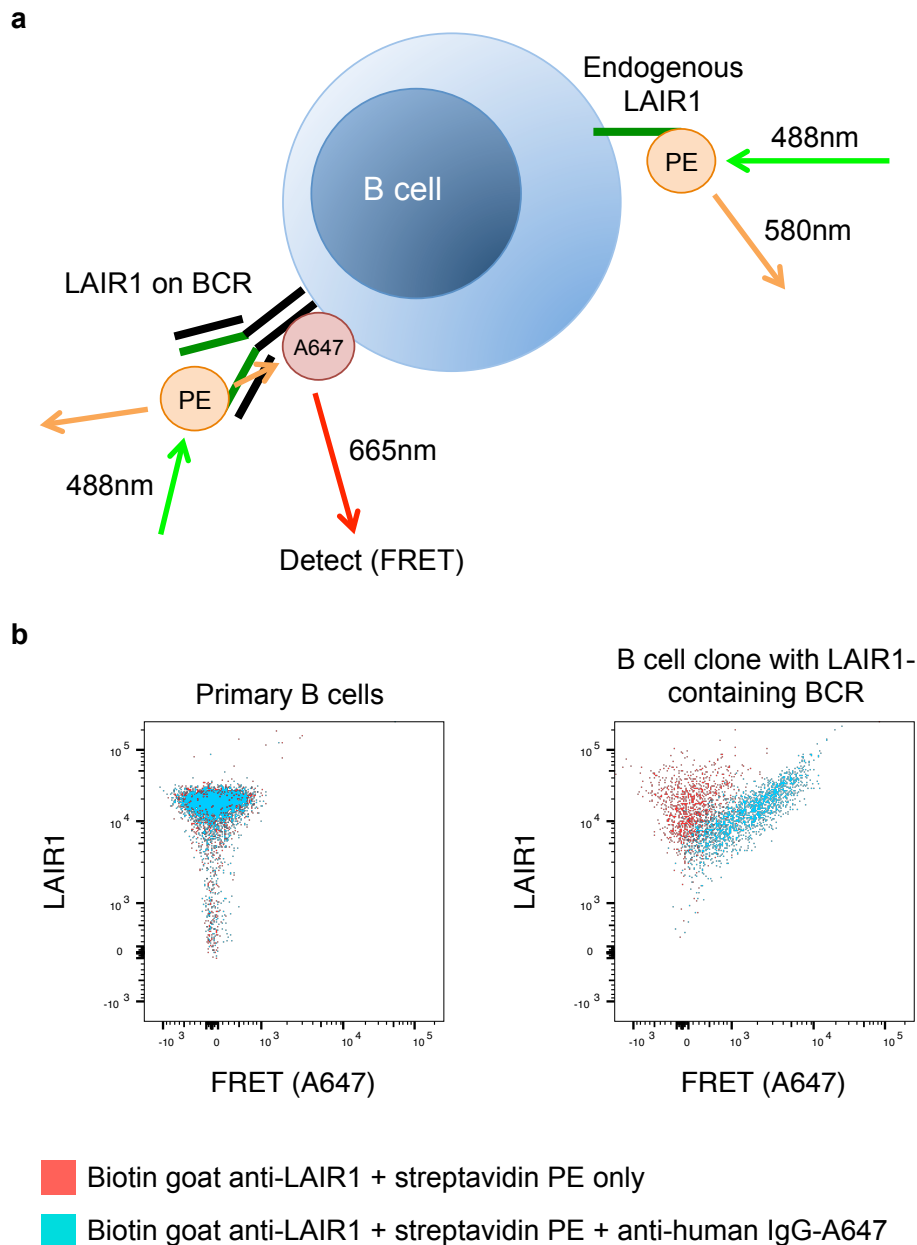


Figure 6.11. FRET for the detection of B cells with LAIR1-containing BCRs. a, B cells are stained with anti-LAIR1-PE (or biotin goat anti-LAIR1 + streptavidin-PE) and anti-human IgG-A647. PE molecules bound to endogenous LAIR1 will only emit at the regular wavelength (~ 580 nm), but PE on LAIR1-containing BCRs may transfer energy to A647 fluorophores, which then emit at ~ 665 nm without direct excitation. b, Preliminary results of the FRET-based assay on primary B cells and an immortalized LAIR1-positive IgG⁺ B cell clone (MGB47). The x-axis shows the fluorescence emission of A647 in the absence of regular excitation at ~ 633 nm. Primary B cells express endogenous LAIR1 but show no FRET signal, whereas MGB47 appears to show FRET. Note that immortalized B cell clones like MGB47 lose endogenous LAIR1 expression after several weeks in culture.

6.9. Summary

In this chapter, I have described the development of novel techniques to screen for the presence of LAIR1-containing antibodies. The preliminary findings demonstrate that these antibodies are not limited to a specific place or population, but can apparently be found at a frequency (5-10%) higher than we expected in different malaria-endemic regions. Nevertheless, the microbeads assays only detect cases in which B cells with LAIR1-containing BCRs have proliferated and secreted a sufficient level of antibodies to be detected in the serum. It is possible that these cases are only the tip of the iceberg, and that the event of LAIR1 insertion occurs at an even higher frequency than what we have observed. Thus far, the new LAIR1-containing antibodies appear to have similar architectures to those we isolated from the Kenyan cohort. Based on the MGF antibodies, about half (six out of 11) of these antibodies recognize IEs from the parasite isolates that we tested. I plan to investigate whether the remaining antibodies recognize IEs from other parasite isolates e.g. Malian or Tanzanian isolates, or if they do not recognize IEs at all. I also hope to further develop the FRET-based approach to enable more rapid isolation of B cells with LAIR1-containing BCRs, simplifying the characterization of new LAIR1-containing monoclonal antibodies.

Chapter 7. Discussion and Outlook

This study reveals a new mechanism of diversification that leads to the generation of potent and broadly reactive antibodies that bind to RIFINs on erythrocytes infected with different *P. falciparum* isolates. These findings open new questions on the potential use of RIFINs as targets for passive and active vaccination and on the general relevance of the mechanism of DNA transposition that generated these broadly reactive antibodies. This chapter aims to discuss the findings of this study in the context of the literature and to examine possible future paths that can be taken to elucidate the nature of the broadly reactive antibody response and the mechanism of LAIR1 insertion into the immunoglobulin locus (Tan *et al.* 2016).

7.1. Broadly reactive antibody response to RIFINs

To our knowledge, the LAIR1-containing antibodies that we describe in this study are the most cross-reactive antibodies against the *P. falciparum* IE surface discovered to date. The targets of these antibodies are RIFINs, which represent the largest family of VSAs in *P. falciparum*, with approximately 150 polymorphic genes (Kyes *et al.* 1999; Fernandez *et al.* 1999). The identity of the antigens is perhaps unexpected, as it was previously thought that PfEMP1 is the main antigen on the *P. falciparum* IE surface that is targeted by antibodies from malaria-exposed individuals (Chan *et al.* 2012; 2016). Nevertheless, antibodies against RIFINs have also been identified in individuals from malaria-endemic regions (Abdel-Latif *et al.* 2002; 2003; 2004) and the LAIR1-containing antibodies have only been found in several individuals who may have unusual antibody responses. For instance, it is striking that in these individuals, LAIR1-containing antibodies appear to dominate the antibody response to the *P. falciparum* IE surface. In spite of the use of an antigen-agnostic approach that selects any antibody that recognizes the IE surface, all of the antibodies isolated from donor C and most of the antibodies isolated from donor D contained a LAIR1 insert.

Given the high level of RIFIN diversity and the finding that the LAIR1-containing antibodies target the RIFIN variable region, it is surprising that almost every parasite isolate tested has a proportion of IEs that express the relevant antigens. This observation suggests that these antibodies recognize a common motif in the variable region of different RIFINs, or that the specific target epitope belongs to RIFIN variants that are preferentially expressed in most parasite isolates. It is

also possible that the LAIR1-containing antibodies bind to conserved structural folds that are derived from diverse RIFIN sequences, as is the case for the PfEMP1 DBL domain (Higgins and Carrington 2014). If either of these ideas is correct, an intriguing hypothesis is that the specific RIFINs targeted by the LAIR1-containing antibodies may play a yet unknown functional role apart from immune evasion, and these antibodies may be useful in disrupting this function. Furthermore, RIFINs are not only expressed during the IE stage of the *P. falciparum* life cycle, but are also expressed in other stages such as in sporozoites, merozoites and gametocytes (Florens *et al.* 2002; Le Roch *et al.* 2003). It would be interesting to determine whether the LAIR1-containing antibodies recognize the RIFINs that are expressed during these stages (Tan *et al.* 2016).

A possible limitation regarding the different parasite isolates tested in this study is that most of the isolates were obtained from malaria patients in Kilifi, Kenya. The only parasite tested that is probably not from this region is the long-term *P. falciparum* laboratory line 3D7, which is derived from an isolate that was discovered in Amsterdam but is thought to have an African origin (Ponnudurai, Leeuwenberg and Meuwissen 1981; Mu *et al.* 2005). The Kenyan parasite isolates were chosen as they were recently adapted to *in vitro* culture, are relatively well characterized and have genomes that have been sequenced. We tried to compensate for their restricted geographical profile by choosing parasite isolates from patients with differing malaria severities and syndromes. However, it is possible that these isolates share similar RIFIN expression profiles due to their geographical proximity, and it would be interesting to test the reactivity of

the LAIR1-containing antibodies to *P. falciparum* isolates from different parts of the world such as South America, Asia and West Africa. We are planning to acquire long-term laboratory isolates and recently cultured-adapted isolates from these regions to conduct these experiments. Furthermore, as RIFINs are encoded by *rif* genes that are thought to be part of the *pir* multigene family (Janssen 2004), it would also be interesting to test if the LAIR1-containing antibodies can also bind to IEs from other *Plasmodium* species, such as *P. berghei*, *P. vivax* and *P. knowlesi*, which also express members of the *pir* family.

While the LAIR1-containing antibodies are able to bind to most or all of the different *P. falciparum* isolates tested, they only recognize a percentage (<50%) of IEs from each isolate. This is consistent with the clonal expression of RIFINs (Kyes *et al.* 1999; Fernandez *et al.* 1999) and suggests that these antibodies may not be sufficient to terminate a given *P. falciparum* infection. Nevertheless, since *in vitro* cultured parasites are thought to downregulate VSA expression due to the lack of selection pressure (Bachmann *et al.* 2012), it is difficult to extrapolate these *in vitro* results to the *in vivo* setting. It is possible that *ex vivo* parasites that are freshly isolated from malaria patients and that have not been put through extensive *in vitro* culture would be better recognized by these antibodies. We have shown that plasma with LAIR1-containing antibodies are able to form mixed agglutinates with *ex vivo* isolates, but this experiment did not give any information on the percentage of IEs recognized by the LAIR1-containing antibodies. To this end, we are planning to use these antibodies to stain *ex vivo* Kenyan parasite isolates that were isolated from patients and cultured for half a cycle until the late trophozoite/early schizont stage.

The LAIR1-containing antibodies displayed differing abilities to bind to the panel of parasite isolates and to the recombinant 3D7 RIFINs, suggesting that the binding specificities of these antibodies do not completely overlap. This is supported by the finding that the enrichment of 3D7 IEs for recognition by MGD21 did not increase their capacity to be recognized by all of the MGC antibodies. In contrast, enrichment of 9605 IEs for MGD21 recognition did increase binding by all of the MGC and MGD antibodies tested, suggesting that these antibodies recognize the same antigens on the 9605 IE surface. Taken together, these results point to a complex, parasite-dependent pattern of RIFIN recognition by different LAIR1-containing antibodies. These differences are likely to be driven by distinct mutations in the LAIR1 domain of each antibody. For instance, we identified a few mutations in the MGD21 LAIR1 domain that increase binding to 3D7 IEs. However, many of the LAIR1-containing antibodies from the two donors do not have these mutations, indicating that there are many alternative pathways to acquire binding to RIFINs. Along a similar line, it is possible that the LAIR1-containing antibodies acquire specific mutations that allow them to bind to the circulating parasites in the region. To test this hypothesis, we will acquire Malian culture-adapted parasite isolates to compare the ability of the Kenyan and Malian LAIR1-containing antibodies to bind to the parasite isolates from their respective regions.

Given the fact that the individuals with LAIR1-containing antibodies have been exposed to malaria over many years, it is surprising that many of the LAIR1-containing antibodies are of the IgM isotype. Our preliminary sequencing data have suggested that these antibodies originate from the same B cell clone as the

IgG antibodies in each individual (data not shown – we need to obtain more IgM sequences in the future to confirm this), indicating that the IgM antibodies were not generated in a new primary response to a more recent infection. The presence of the IgM antibodies may reflect the key role of the spleen in retaining and clearing *P. falciparum* IEs and generating an immune response against these parasites (Chotivanich *et al.* 2002; Engwerda, Beattie and Amante 2005). It is possible that so-called marginal zone-like or IgM memory (IgM⁺CD27⁺) B cells that reside in the spleen, but that might be able to take part in the germinal centre reaction (Dunn-Walters, Isaacson and Spencer 1995; Klein, Rajewsky and Kuppers 1998; Weill, Weller and Reynaud 2009; Seifert and Küppers 2009), may be key players in the response to blood-stage *P. falciparum* parasites.

We attempted to examine possible functions of the LAIR1-containing antibodies through various *in vitro* assays. RIFINs have recently been associated with severe malaria through rosetting of IEs with blood group A erythrocytes (Goel *et al.* 2015). Accordingly, we tested the ability of MGD21 to inhibit rosetting with blood group A and O erythrocytes. We did not find any evidence for rosette inhibition, which is consistent with the fact that MGD21 did not recognize the specific RIFIN variant implicated in the rosetting study (Goel *et al.* 2015). Nonetheless, the negative result may simply be due to the specific parasite isolate and LAIR1-containing antibody chosen for the assay. We also observed that the LAIR1-containing antibodies do not inhibit parasite growth in *in vitro* culture. In contrast, these antibodies have potent agglutinating and opsonizing activity, which would be consistent with their role in decreasing the burden of IEs *in vivo* by enhancing parasite clearance in the spleen (Tan *et al.* 2016).

Indeed, based on a series of *in vitro* assays conducted on blood stage parasites, the ability of antibodies to agglutinate IEs was the only factor associated with protection, suggesting that this feature may be particularly relevant to the *in vivo* situation (Marsh *et al.* 1989).

While the *in vitro* functional assays that we have conducted may offer hints as to the protective role of these antibodies against malaria, a more concrete way to investigate this role is through immunoepidemiological studies that assess whether individuals that have LAIR1-containing antibodies are protected from clinical malaria. The three individuals that we identified to possess LAIR1-containing antibodies were parasite-negative during sample collection, but these individuals were part of a cohort of adults who live in a malaria-endemic area and are thus expected to be generally clinically immune to malaria (Langhorne *et al.* 2008). To accurately assess protection, the immunoepidemiological studies would have to focus on children in malaria-endemic regions, as they are still susceptible to disease. The assays that we are currently developing to screen for LAIR1-containing antibodies in serum or plasma should aid in these studies. Alternative approaches to determine potential protective roles of the LAIR1-containing antibodies include animal trials and controlled human experimental infections, which may be useful at a more advanced stage.

In the context of vaccine development, several steps need to be taken to test the suitability of the RIFINs targeted by the LAIR1-containing antibodies as malaria vaccine candidates. As mentioned, we aim to determine the breadth of the LAIR1-containing antibodies in targeting *ex vivo* isolates and whether these antibodies are associated with protection against malaria. Additionally, we will

identify the specific epitope in the RIFIN variable region that is recognized by these antibodies and its level of conservation in RIFINs from different *P. falciparum* strains. As it is unlikely that every individual would be able to generate LAIR1-containing antibodies upon immunization with a RIFIN-based vaccine, it also remains to be established whether the conserved RIFIN epitopes identified by the LAIR1-containing antibodies could also be recognized by conventional antibodies and whether this antibody response would have sufficient breadth to control disease (Tan *et al.* 2016).

7.2. Mechanism of LAIR1 insertion

The insertion of LAIR1 into the antibody heavy chain locus is the first example of an insertion that gives rise to a functional antibody in which the insert represents the fundamental binding element (Tan *et al.* 2016). In this section, I will begin with a brief description of LAIR1 and will proceed by discussing this novel insertion mechanism.

LAIR1, or CD305, is an inhibitory receptor that is encoded in the leukocyte receptor complex on chromosome 19 (Meyaard *et al.* 1997). It is a 32 kDa glycoprotein that contains an extracellular immunoglobulin-like domain, a transmembrane region and an intracellular tail containing immunoreceptor tyrosine-based inhibitory motifs (ITIMs) (Lebbink *et al.* 2006; Jansen *et al.* 2007). The extracellular domain of LAIR1, which forms the core of the insert in the antibody heavy chain, binds with high affinity to transmembrane and extracellular forms of collagen (Lebbink *et al.* 2006), as well as to proteins with collagen-like folds, such as the complement protein C1q (Son *et al.* 2012). The crystal structure of the LAIR1 extracellular domain has been solved, revealing sites that are thought to be important for collagen binding (Brondijk *et al.* 2010). LAIR1 is widely expressed on the surface of most leukocytes including B cells, T cells, natural killer cells, dendritic cells, monocytes (Meyaard *et al.* 1997), basophils (Florian *et al.* 2006) and eosinophils (Verbrugge *et al.* 2006). In the B cell compartment, LAIR1 is expressed in pre-B cells in the human bone marrow and in naïve B cells in blood (de Vries *et al.* 1999). Activated B cells generally have lower expression of LAIR1, as approximately 50% of memory (CD27⁺) B cells express LAIR1 and plasma cells lack LAIR1 expression (de Vries *et al.* 1999).

We are confident that the LAIR1 insertion is not an artefact of EBV immortalization of memory B cells, as LAIR1-containing antibodies can be detected in the serum and these antibodies contain slightly different mutations that are characteristic of somatic hypermutation during the germinal centre reaction. One of the key questions regarding this insertion is whether it occurred in the germline and can be inherited, or whether it was a somatic event that occurred in a single B cell in each individual we studied. The absence of detectable LAIR1 insertions at the antibody heavy chain locus in T cells from these individuals, as well as the observation that all of the B cells producing LAIR1-containing antibodies in each individual were derived from a single B cell clone, suggests that the LAIR1 insertion was a somatic event that occurred in a single B cell in each individual. Moreover, the LAIR1-containing antibodies isolated from different individuals have distinct architectures, indicating that the LAIR1 insertion was an independent event in each of these individuals.

Another key area of interest concerns the timing of the LAIR1 insertion during B cell development and activation. As this process is likely to have required a DNA double-strand break, it probably occurred either during RAG-mediated V(D)J recombination during B cell development in the bone marrow, or during AID-mediated somatic hypermutation in the germinal centre reaction. The precise location of the LAIR1 and chromosome 13 inserts between the heavy chain V and D/J segments, as well as the presence of N-nucleotides and cryptic 12/23 RSSs at the ends of the inserts, suggests that RAG could be involved in the insertion of the DNA fragments. RAG has been implicated in interchromosomal genomic rearrangements at cryptic RSSs outside the immunoglobulin and T-cell antigen

receptor loci (Vaandrager *et al.* 2000; Messier 2003) and in the formation of chromosomal translocations found in human lymphomas (Kuppers *et al.* 1999; Kuppers and Dalla-Favera 2001). Furthermore, RAG binds to transcriptionally active sites at many locations in the genome, including at non-immunoglobulin loci (Teng *et al.* 2015), and LAIR1 is transcribed in developing B cells (de Vries *et al.* 1999). *In vitro* experiments have suggested that RAG is able to act in the reverse direction of its well-known excision function, as the enzyme complex was able to attach an excised fragment flanked by signal ends back to unresolved coding ends (Melek 1998; Agrawal, Eastman and Schatz 1998). However, RSSs are frequently found in the genome and are generally inactive, according to recent data (Lewis *et al.* 1997; Teng *et al.* 2015; Hu *et al.* 2015). Therefore, the presence of cryptic RSSs near the ends of the inserts do not necessarily suggest the involvement of RAG. Furthermore, the conservation of the two LAIR1 alleles in B cells producing LAIR1-containing antibodies is inconsistent with a RAG-mediated 'cut-and-paste' pathway and suggests a new mechanism by which LAIR1 DNA is duplicated (Tan *et al.* 2016).

Alternative pathways that could have contributed to the production of LAIR1-containing antibodies include gene conversion (Reynaud *et al.* 2003) or AID-dependent genomic instability caused by chronic *Plasmodium* infection (Robbiani *et al.* 2015). AID can lead to insertions and deletions of multiple codons in the V genes that contribute to the specificity of the antibody in the context of the whole V gene (Wilson *et al.* 1998; Kepler *et al.* 2014). Nevertheless, to the best of our knowledge, these insertions are distributed over the whole V-gene sequence, are of smaller size and cannot be traced back to a particular

genomic sequence as is the case for LAIR1 (Tan *et al.* 2016). Therefore, while AID activity can explain a double-strand break in the V_H region, like RAG, it cannot explain the duplication and insertion of the foreign LAIR1 sequence.

A recent study has described a possible mechanism that may explain this process of duplication (Onozawa *et al.* 2014). This mechanism, which has been named templated sequence insertion (TSI), involves the reverse transcription of pre-mRNA or mRNA from distant parts of the genome and subsequent insertion of the duplicated fragment to repair a DNA double-strand break. Some of these insertions appear to occur relatively commonly in different individuals and have been named TSI polymorphisms (TSIPs) (Onozawa, Goldberg and Aplan 2015). Strikingly, individuals of African origin have higher levels of TSIPs than individuals from other parts of the world (Onozawa, Goldberg and Aplan 2015). TSIPs have been divided into two classes, and in particular, the so-called class 2 TSIPs show similarity to the LAIR1 inserts as they involve non-templated nucleotide addition at the site of insertion (Onozawa *et al.* 2014). Therefore, a possible hypothesis to explain the LAIR1 insertion is that RAG or AID activity generated a double-strand DNA break at the immunoglobulin heavy chain locus, following which the TSI pathway mediated the duplication of LAIR1 pre-mRNA and insertion of the resulting DNA fragment into the break region.

Another piece of the puzzle that remains to be answered is how the initial B cell clone expressing a LAIR1-containing BCR survived negative selection due to collagen autoreactivity and was activated through binding to RIFIN antigens. Our findings suggest that the germline version of the LAIR1-containing antibody MGD21 bound strongly to collagen but could not bind to IEs. Therefore, if the

LAIR1 insertion had occurred during B cell development, the resulting B cell would presumably have undergone negative selection, as its LAIR1-containing BCR would bind tightly to collagen. This may explain the presence of three different light chains in clones from donor C, which may have arisen from receptor editing during the negative selection process (Gay *et al.* 1993; Tiegs, Russell and Nemazee 1993). However, given that in this case the self-reactivity is uniquely encoded by the LAIR1 insert, the substitution of light chains would not mitigate self-reactivity. Therefore, such a B cell would be expected to be deleted or rendered anergic. As the former did not occur, it is possible that the B cell was made anergic but survived and entered the periphery, where it was activated strongly enough to overcome its anergic state. Alternatively, it is possible that the LAIR1 insertion occurred after the process of negative selection, such as during the germinal centre reaction. A second question is how this B cell was activated if its BCR cannot bind to RIFINs prior to somatic hypermutation. This is currently unclear, but a possible explanation is that the germline BCR can bind to IEs from parasite isolates that we did not test, or that the binding of the germline BCR to RIFINs occurs at an affinity or avidity that is too low to be detected in our assays but is strong enough to trigger B cell activation. As only a few mutations in the LAIR1 domain are required to confer binding to RIFINs, a B cell with a LAIR1-containing BCR would probably undergo robust activation and proliferation after passing this initial threshold. This B cell clone would have expanded under selective pressure to lose collagen reactivity and to increase binding to the RIFINs present in the parasite population. The large expansion of the clones and the extraordinary degree of intraclonal diversification that we

have observed could be the consequence of continuous stimulation by variable parasites encountered over a period of several years (Tan *et al.* 2016).

Several factors are likely to have facilitated the successful insertion of LAIR1 into the antibody sequence. LAIR1 is expressed during B cell development, providing mRNA as a template for duplication, and it has an immunoglobulin-like domain, which may fit into the antibody structure with minimal disruption. Furthermore, its insertion between the V and D/J segments results in its expression in the CDR3 loop, which is probably the most permissive part of the antibody structure to receive an insert. It remains to be determined how often sequences other than those belonging to LAIR1 are inserted into immunoglobulin genes. We do know that this is possible, as we have found an intergenic sequence derived from chromosome 13 in LAIR1-containing antibodies from donor D. We speculate that the insertion of LAIR1 is not necessarily much more common than the insertion of other foreign sequences, but were frequently detected in our assays simply because we were looking specifically for LAIR1-containing antibodies or for antibodies that bind to RIFINs.

We have shown that the LAIR1 insertion event is not limited to the single village in Kenya from which the initial samples were obtained, but appears to occur quite commonly in different malaria-endemic areas. Based on our newly developed serological assays, the frequency of individuals in these regions that possess LAIR1-containing antibodies appears to be reasonably high (i.e. 5-10%), but it is possible that the event occurs more frequently than is detected with our assays. Nonetheless, we do not expect this event to occur ubiquitously, as an extremely high frequency of LAIR1 insertion would probably have resulted in the

generation of multiple B cell clones with LAIR1-containing antibodies in each individual we studied. It is likely that this event also occurs in individuals from non-malaria-endemic areas, but without the stimulation provided by RIFINs, it may be difficult to detect these B cells in such individuals, although we speculate that such clones may be recruited in an anti-collagen response that is characteristic of certain rheumatic diseases (Holmdahl, Malmström and Burkhardt 2014; Tan *et al.* 2016).

To end this section on a more general note, if one considers the breadth of antibody diversity in the mammalian kingdom, the LAIR1-containing antibodies are similar in some aspects to immunoglobulins from other species. For instance, as is the case for the LAIR1-containing antibodies, cow antibodies have extra-long CDR3 regions that form an external domain that is used for binding to target antigens (Wang *et al.* 2013). This knob domain, like the LAIR1 immunoglobulin-like domain, is stabilized by intra-chain disulphide bonds. Additionally, the MGB47 LAIR1-containing antibody that we are in the process of characterizing appears to lack a C_H1 domain or a light chain, and is thus reminiscent of camelid HCAb or shark IgNAR antibodies (Hamers-Casterman *et al.* 1993; Greenberg *et al.* 1995). While these LAIR1-containing antibodies may not occur universally in the human population and thus may not represent true cases of convergent evolution, it is striking that radically different mechanisms working in different species can result in the generation of antibodies with similar features that may confer unique binding properties that are unavailable to their conventional counterparts.

7.3. Outlook

This study began as an investigation of the antibody response of malaria-immune individuals to *P. falciparum*-infected erythrocytes, but has resulted in an unexpected discovery of unusual LAIR1-containing antibodies that opens up new avenues of research in the fields of malaria biology and basic B cell immunology. Key malaria-related questions that should be addressed include whether these antibodies are protective against disease and broadly target circulating isolates, whether the targeted RIFINs are suitable malaria vaccine candidates, and how often the LAIR1 insertion occurs in malaria-endemic regions. Important questions related to B cell immunology that will be further investigated in the future include the precise mechanism of the LAIR1 insertion and the possibility that other sequences can be inserted into the immunoglobulin locus. In conclusion, I believe that the discovery of these broadly reactive LAIR1-containing antibodies is the proverbial tip of the iceberg, and that many exciting discoveries in the fields of malaria and B cell immunology lie ahead.

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Supplementary Information

0	1	2	3	4
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Plasma	Individual			Mixed	Individual			Mixed
	9106	9605	11019		10668	10936	11014	
M1-5								
M6-10								
M11-15								
M16-20								
M21-25								
M26-30								
M31-35								
M36-40								
M41-45								
M46-50								
M51-55								
M56-60				9106 + 9605 + 11019				10668 + 10936 + 11014
M61-65								
M66-70								
M71-75								
M76-80								
M81-85								
M86-90								
M91-95								
M96-100								
M101-105								
M106-110								
M111-115								
M116-120								
M121-125								
M126-130								
M131-135								
M136-140								
M141-145								
M146-150				9605 + 11019				10936 + 11014
M151-155	nd	nd	nd	9106 + 9605 + 11019				10668 + 10936 + 11014
M156-160	nd	nd	nd	9106 + 9605 + 11019				10936 + 11014
M161-165								
M166-167								
JA001-005								
JA006-010								
JA011-015								
JA016-020								
JA021-025								
JA026-030								
JA031-035								
JA036-040								
JA041-045								
JA046-050								
JA051-055								10936 + 11014

Figure S1. Mixed agglutination screens of plasma from adults living in a malaria-endemic region in Kilifi County, Kenya. The plasma were pooled in groups of five and tested in two separate triple agglutination reactions. Single-colour agglutinates were scored alongside mixed agglutinates in the mixed agglutination reactions. Agglutinates were scored on a scale of 0-4 based on size (see Methods for scoring system). nd, not determined. The Figure is continued on the next two pages.

0	1	2	3	4
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Plasma	Individual			Mixed	Individual			Mixed
	9106	9605	11019		10668	10936	11014	
JA056-060								
JA061-065								
JA066-070								
JA071-075								
JA076-080								
JA081-085								
JA086-090								
JA091-095								
JA096-100								
JA101-105								
JA106-110								
JA111-115								
JA116-120								
JA121-125								
JA126-130								
JA131-135								
JA136-140								
JA141-145								
JA146-150								
JA151-155								
JA156-160								
JA161-165								
JA186-190								
JA191-195								
JA196-200								
JA201-205								
JA206-210								
JA211-215								
JA216-220								
JA221-225								
JA226-230								
JA231-235								
JA236-240								
JA241-245								
JA246-250								
JA251-255								
JA256-260								
JA261-265								
JA266-270								
JA271-275								
E001-005								
E006-010								
E011-015								
E016-020				9106 + 11019				
E021-025								

Figure S1. Mixed agglutination screens of plasma from adults living in a malaria-endemic region in Kilifi County, Kenya (continued).

0	1	2	3	4
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Plasma	Individual			Mixed	Individual			Mixed
	9106	9605	11019		10668	10936	11014	
F01-05								
F06-10								
F11-15								
F16-20								
F21-25								
F26-30								
F31-35								
F36-40								
F41-45								
F46-50								
F51-55								
F56-60								
F61-65								
F66-70								
F71-75								
JA14_213-217								
JA14_218-223				9605 + 11019				
JA14_224-228								
JA14_229-233								
JA14_234-239								
JA14_240-244								
JA14_245-252								
JA14_253-257								
JA14_258-260								

Figure S1. Mixed agglutination screens of plasma from adults living in a malaria-endemic region in Kilifi County, Kenya (continued).

Cohort	Sample name	Binding ratio
Dogon/Fulani	Dog02	1.15
	Dog03	1.17
	Dog05	1.33
	Dog07	1.16
	Dog08	1.23
	Dog09	1.10
	Dog11	1.53
	Dog12	10.91
	Dog13	1.14
	Dog14	1.02
	Dog15	3.38
	Dog16	1.21
	Dog17	1.08
	Dog18	1.06
	Dog19	1.22
	Dog20	1.14
	Dog21	1.37
	Dog22	3.31
	Dog23	1.33
	Dog24	1.20
	Dog25	1.07
	Fini01	1.19
	Fini02	1.23
	Fini03	1.19
	Fini04	1.25
	Fini05	1.28
	Fini06	1.19
	Fini07	1.04
	Fini08	0.94
	Fini09	0.97
	Fini10	1.44
	Fini11	1.13
	Fini12	1.13
	Fini13	1.16
	Fini14	1.26
	Fini15	1.03
	Fini16	1.11
	Fini17	1.08
	Fini18	4.55
	Fini19	1.22
	Fini20	1.15
	Fini21	1.12
	Fini22	1.30
	Fini23	1.23
	Fini24	1.28
	Fini25	1.14
Kalifabougou	Kali 1 m07	1.07
	Kali 2 m07	1.17
	Kali 4 m07	0.99
	Kali 5 m07	1.05
	Kali 6 m07	1.17
	Kali 8 m07	1.10
	Kali 11 m07	1.12
	Kali 12 m07	1.03
	Kali 14 m07	1.39
	Kali 16 m07	1.14
	Kali 17 m07	1.09
	Kali 18 m07	1.27
	Kali 19 m07	1.19
	Kali 20 m07	1.15
	Kali 23 m07	1.23
	Kali 24 m07	5.90
	Kali 25 m07	1.21
	Kali 26 m07	1.07
	Kali 27 m07	1.24
	Kali 28 m07	1.07
	Kali 29 m07	1.18
	Kali 31 m07	1.17
	Kali 32 m07	1.19
	Kali 33 m07	1.06

Cohort	Sample name	Binding ratio
Kalifabougou	Kali 35 m07	1.06
	Kali 36 m07	1.33
	Kali 37 m07	1.31
	Kali 38 m07	1.19
	Kali 39 m07	1.00
	Kali 40 m07	1.11
	Kali 41 m07	0.98
	Kali 42 m07	1.14
	Kali 43 m07	1.19
	Kali 45 m07	1.23
	Kali 47 m07	0.96
	Kali 48 m07	0.72
	Kali 49 m07	1.22
	Kali 50 m07	0.93
	Kali 52 m07	0.97
	Kali 54 m07	1.23
	Kali 55 m07	1.11
	Kali 56 m07	1.14
	Kali 58 m07	1.21
	Kali 59 m07	1.17
	Kali 60 m07	1.04
	Kali 61 m07	0.93
	Kali 62 m07	1.23
	Kali 64 m07	3.27
	Kali 65 m07	0.97
	Kali 66 m07	1.17
	Kali 69 m07	1.10
	Kali 70 m07	10.68
	Kali 71 m07	1.29
	Kali 72 m07	1.16
	Kali 74 m07	1.11
	Kali 75 m07	1.10
	Kali 76 m07	4.19
	Kali 79 m07	1.22
	Kali 80 m07	5.80
	Kali 81 m07	1.18
	Kali 83 m07	1.09
	Kali 84 m07	1.01
	Kali 86 m07	1.12
	Kali 87 m07	1.15
	Kali 88 m07	1.20
	Kali 89 m07	1.13
	Kali 90 m07	1.30
	Kali 91 m07	1.11
	Kali 92 m07	1.76
	Kali 93 m07	1.27
	Kali 94 m07	1.07
	Kali 95 m07	1.27
	Kali 96 m07	1.64
	Kali 97 m07	1.20
	Kali 98 m07	1.20
	Kali 99 m07	1.09
	Kali 100 m07	1.12
	Kali 101 m07	1.07
	Kali 102 m07	0.88
	Kali 103 m07	1.25
	Kali 104 m07	1.31
	Kali 105 m07	1.31
	Kali 106 m07	1.09
	Kali 107 m07	1.12
	Kali 109 m07	1.13
	Kali 110 m07	1.15
	Kali 111 m07	1.10
	Kali 112 m07	1.43
	Kali 113 m07	1.20
	Kali 114 m07	1.17
	Kali 115 m07	1.26
	Kali 116 m07	1.06
	Kali 117 m07	1.13
	Kali 118 m07	1.17

Figure S2. Complete results of screen of Malian plasma using anti-goat IgG microbeads. The binding ratio is the MFI ratio of antibody binding to the test beads relative to the control beads. Plasma that have a ratio of >1.5 are shown in red. The Figure is continued on the next four pages.

Cohort	Sample name			Binding ratio
Kalifabougou	Kali	119	m07	1.23
	Kali	120	m07	1.22
	Kali	121	m07	1.19
	Kali	122	m07	1.25
	Kali	123	m07	1.15
	Kali	124	m07	1.10
	Kali	125	m07	1.06
	Kali	126	m07	1.23
	Kali	127	m07	1.09
	Kali	128	m07	1.20
	Kali	129	m07	1.17
	Kali	130	m07	1.17
	Kali	131	m07	1.19
	Kali	132	m07	1.07
	Kali	133	m07	1.03
	Kali	135	m07	1.27
	Kali	136	m07	1.31
	Kali	137	m07	4.02
	Kali	138	m07	1.23
	Kali	139	m07	1.19
	Kali	140	m07	1.36
	Kali	141	m07	1.15
	Kali	142	m07	1.14
	Kali	143	m07	1.10
	Kali	144	m07	1.25
	Kali	145	m07	1.26
	Kali	146	m07	1.10
	Kali	147	m07	1.26
	Kali	148	m07	1.24
	Kali	149	m07	0.82
	Kali	150	m07	1.13
	Kali	152	m07	1.39
	Kali	153	m07	1.17
	Kali	154	m07	1.13
	Kali	155	m07	1.10
	Kali	158	m07	1.22
	Kali	159	m07	1.28
	Kali	160	m07	1.18
	Kali	162	m07	1.14
	Kali	163	m07	0.87
	Kali	166	m07	1.25
	Kali	167	m07	1.21
	Kali	168	m07	1.28
	Kali	169	m07	1.21
	Kali	170	m07	1.15
	Kali	171	m07	1.12
	Kali	172	m07	1.16
	Kali	174	m07	1.20
	Kali	175	m07	1.16
	Kali	176	m07	1.23
	Kali	177	m07	7.51
	Kali	178	m07	1.13
	Kali	179	m07	1.18
	Kali	180	m07	1.21
	Kali	181	m07	1.25
	Kali	182	m07	1.05
	Kali	183	m07	1.14
	Kali	184	m07	1.17
	Kali	186	m07	1.17
	Kali	188	m07	1.13
	Kali	189	m07	1.32
	Kali	192	m07	4.58
	Kali	193	m07	4.64
	Kali	194	m07	1.27
	Kali	195	m07	1.13
	Kali	197	m07	0.80
	Kali	198	m07	1.14
	Kali	199	m07	1.24
	Kali	200	m07	1.11
	Kali	201	m07	1.13

Cohort	Sample name			Binding ratio
Kalifabougou	Kali	202	m07	1.27
	Kali	203	m07	1.28
	Kali	204	m07	1.17
	Kali	205	m07	1.11
	Kali	206	m07	1.31
	Kali	207	m07	1.12
	Kali	208	m07	1.21
	Kali	209	m07	1.16
	Kali	210	m07	1.36
	Kali	211	m07	1.21
	Kali	212	m07	1.12
	Kali	213	m07	0.99
	Kali	214	m07	1.25
	Kali	215	m07	10.08
	Kali	216	m07	3.43
	Kali	217	m07	1.22
	Kali	218	m07	1.23
	Kali	219	m07	1.29
	Kali	221	m07	1.16
	Kali	222	m07	1.16
	Kali	223	m07	1.29
	Kali	224	m07	1.07
	Kali	226	m07	1.07
	Kali	227	m07	1.17
	Kali	228	m07	6.51
	Kali	229	m07	1.11
	Kali	230	m07	0.90
	Kali	231	m07	1.07
	Kali	232	m07	1.31
	Kali	234	m07	1.13
	Kali	235	m07	1.17
	Kali	236	m07	1.10
	Kali	237	m07	1.23
	Kali	241	m07	0.92
	Kali	243	m07	1.19
	Kali	244	m07	1.28
	Kali	245	m07	1.03
	Kali	246	m07	1.29
	Kali	247	m07	6.88
	Kali	249	m07	5.23
	Kali	250	m07	1.17
	Kali	251	m07	1.05
	Kali	252	m07	1.18
	Kali	253	m07	1.10
	Kali	254	m07	1.15
	Kali	255	m07	1.46
	Kali	256	m07	1.09
	Kali	258	m07	1.14
	Kali	259	m07	1.30
	Kali	260	m07	3.20
	Kali	261	m07	1.59
	Kali	262	m07	1.21
	Kali	263	m07	1.23
	Kali	264	m07	2.48
	Kali	265	m07	1.08
	Kali	266	m07	6.73
	Kali	267	m07	1.14
	Kali	268	m07	1.13
	Kali	269	m07	1.14
	Kali	270	m07	1.14
	Kali	271	m07	2.76
	Kali	272	m07	1.08
	Kali	273	m07	1.06
	Kali	274	m07	1.34
	Kali	275	m07	1.19
	Kali	276	m07	1.25
	Kali	277	m07	1.55
	Kali	278	m07	1.13
	Kali	279	m07	1.10
	Kali	280	m07	1.10

Figure S2. Complete results of screen of Malian plasma using anti-goat IgG microbeads (continued).

Cohort	Sample name	Binding ratio
Kalifabougou	Kali 281 m07	1.11
	Kali 282 m07	1.12
	Kali 283 m07	1.21
	Kali 284 m07	1.19
	Kali 285 m07	1.09
	Kali 286 m07	1.15
	Kali 287 m07	1.25
	Kali 288 m07	2.86
	Kali 289 m07	1.10
	Kali 290 m07	1.40
	Kali 291 m07	1.26
	Kali 292 m07	1.25
	Kali 293 m07	1.00
	Kali 294 m07	1.22
	Kali 296 m07	1.16
	Kali 297 m07	1.55
	Kali 298 m07	1.19
	Kali 299 m07	1.21
	Kali 300 m07	1.17
	Kali 301 m07	1.29
	Kali 302 m07	1.13
	Kali 303 m07	1.13
	Kali 304 m07	1.18
	Kali 305 m07	1.12
	Kali 308 m07	1.15
	Kali 310 m07	1.04
	Kali 311 m07	1.12
	Kali 312 m07	1.38
	Kali 313 m07	1.13
	Kali 315 m07	1.12
	Kali 318 m07	1.36
	Kali 319 m07	1.13
	Kali 320 m07	1.13
	Kali 321 m07	3.39
	Kali 322 m07	1.19
	Kali 324 m07	1.20
	Kali 325 m07	1.61
	Kali 326 m07	4.98
	Kali 328 m07	2.07
	Kali 332 m07	1.18
	Kali 333 m07	1.05
	Kali 334 m07	1.04
	Kali 335 m07	1.07
	Kali 336 m07	1.09
	Kali 338 m07	1.27
	Kali 339 m07	1.15
	Kali 340 m07	1.13
	Kali 343 m07	1.20
	Kali 344 m07	1.07
	Kali 345 m07	1.02
	Kali 346 m07	1.09
	Kali 348 m07	1.14
	Kali 349 m07	1.21
	Kali 350 m07	0.99
	Kali 351 m07	1.25
	Kali 353 m07	1.13
	Kali 354 m07	1.11
	Kali 355 m07	1.19
	Kali 356 m07	1.07
	Kali 357 m07	1.17
	Kali 367 m07	1.12
	Kali 368 m07	1.22
	Kali 369 m07	1.24
	Kali 370 m07	1.26
	Kali 371 m07	0.87
	Kali 372 m07	1.23
	Kali 373 m07	1.08
	Kali 374 m07	1.07
	Kali 375 m07	2.26
	Kali 376 m07	1.08

Cohort	Sample name	Binding ratio
Kalifabougou	Kali 377 m07	1.07
	Kali 378 m07	1.21
	Kali 379 m07	1.01
	Kali 380 m07	1.12
	Kali 381 m07	1.08
	Kali 382 m07	10.70
	Kali 383 m07	0.99
	Kali 384 m07	1.18
	Kali 385 m07	1.18
	Kali 388 m07	1.08
	Kali 389 m07	1.26
	Kali 390 m07	1.01
	Kali 391 m07	1.21
	Kali 392 m07	1.21
	Kali 394 m07	0.93
	Kali 395 m07	1.22
	Kali 396 m07	1.14
	Kali 397 m07	1.26
	Kali 398 m07	1.18
	Kali 399 m07	1.20
	Kali 400 m07	1.19
	Kali 402 m07	1.16
	Kali 403 m07	1.29
	Kali 404 m07	0.95
	Kali 405 m07	1.17
	Kali 406 m07	1.07
	Kali 408 m07	1.22
	Kali 413 m07	1.23
	Kali 414 m07	1.09
	Kali 415 m07	4.13
	Kali 416 m07	1.06
	Kali 417 m07	1.18
	Kali 418 m07	1.24
	Kali 419 m07	1.22
	Kali 420 m07	1.21
	Kali 421 m07	1.15
	Kali 422 m07	1.19
	Kali 423 m07	1.13
	Kali 424 m07	1.36
	Kali 425 m07	1.02
	Kali 426 m07	1.10
	Kali 427 m07	1.00
	Kali 428 m07	1.05
	Kali 429 m07	1.08
	Kali 430 m07	1.18
	Kali 431 m07	1.28
	Kali 432 m07	1.09
	Kali 433 m07	1.14
	Kali 435 m07	1.20
	Kali 437 m07	1.09
	Kali 438 m07	3.44
	Kali 439 m07	1.19
	Kali 440 m07	1.14
	Kali 443 m07	1.05
	Kali 444 m07	1.10
	Kali 445 m07	1.01
	Kali 446 m07	1.22
	Kali 447 m07	1.12
	Kali 448 m07	1.10
	Kali 449 m07	1.21
	Kali 450 m07	1.08
	Kali 451 m07	0.97
	Kali 452 m07	2.98
	Kali 453 m07	1.09
	Kali 454 m07	2.35
	Kali 455 m07	1.25
	Kali 456 m07	1.08
	Kali 457 m07	1.20
	Kali 458 m07	1.56
	Kali 459 m07	1.12

Figure S2. Complete results of screen of Malian plasma using anti-goat IgG microbeads (continued).

Cohort	Sample name	Binding ratio
Kalifabougou	Kali 460 m07	1.17
	Kali 461 m07	1.20
	Kali 462 m07	1.17
	Kali 463 m07	1.20
	Kali 464 m07	0.83
	Kali 465 m07	0.92
	Kali 466 m07	1.10
	Kali 468 m07	1.19
	Kali 469 m07	1.18
	Kali 470 m07	7.01
	Kali 471 m07	1.12
	Kali 472 m07	1.17
	Kali 473 m07	1.21
	Kali 475 m07	1.31
	Kali 476 m07	1.16
	Kali 477 m07	1.17
	Kali 479 m07	1.22
	Kali 480 m07	1.27
	Kali 481 m07	0.99
	Kali 482 m07	1.18
	Kali 483 m07	1.25
	Kali 485 m07	1.05
	Kali 486 m07	1.32
	Kali 487 m07	1.05
	Kali 488 m07	1.22
	Kali 489 m07	1.00
	Kali 490 m07	1.09
	Kali 491 m07	1.04
	Kali 493 m07	0.96
	Kali 494 m07	1.06
	Kali 495 m07	1.92
	Kali 496 m07	1.34
	Kali 499 m07	1.18
	Kali 502 m07	1.25
	Kali 505 m07	1.13
	Kali 506 m07	1.17
	Kali 508 m07	1.17
	Kali 509 m07	1.25
	Kali 510 m07	1.28
	Kali 511 m07	1.13
	Kali 512 m07	1.15
	Kali 513 m07	1.14
	Kali 514 m07	1.14
	Kali 515 m07	1.21
	Kali 516 m07	1.18
	Kali 518 m07	1.16
	Kali 519 m07	1.21
	Kali 521 m07	1.52
	Kali 523 m07	2.77
	Kali 524 m07	1.28
	Kali 525 m07	1.21
	Kali 526 m07	1.16
	Kali 527 m07	1.15
	Kali 528 m07	1.10
	Kali 529 m07	1.29
	Kali 530 m07	1.25
	Kali 531 m07	1.15
	Kali 533 m07	1.21
	Kali 534 m07	1.17
	Kali 535 m07	0.97
	Kali 537 m07	1.17
	Kali 538 m07	1.23
	Kali 543 m07	1.35
	Kali 545 m07	1.30
	Kali 547 m07	1.06
	Kali 548 m07	1.23
	Kali 549 m07	1.11
	Kali 551 m07	1.06
	Kali 553 m07	1.09
	Kali 554 m07	1.16

Cohort	Sample name	Binding ratio
Kalifabougou	Kali 555 m07	1.34
	Kali 556 m07	1.19
	Kali 558 m07	1.21
	Kali 559 m07	1.27
	Kali 561 m07	1.22
	Kali 562 m07	1.12
	Kali 563 m07	1.18
	Kali 565 m07	1.48
	Kali 570 m07	1.19
	Kali 571 m07	1.05
	Kali 572 m07	1.16
	Kali 573 m07	0.99
	Kali 574 m07	1.15
	Kali 575 m07	1.02
	Kali 576 m07	1.26
	Kali 577 m07	1.18
	Kali 578 m07	1.27
	Kali 579 m07	1.18
	Kali 581 m07	1.24
	Kali 582 m07	1.07
	Kali 583 m07	1.25
	Kali 584 m07	1.20
	Kali 585 m07	1.17
	Kali 587 m07	1.14
	Kali 589 m07	1.01
	Kali 591 m07	0.99
	Kali 592 m07	1.02
	Kali 594 m07	1.11
	Kali 595 m07	1.28
	Kali 597 m07	1.19
	Kali 600 m07	1.12
	Kali 601 m07	1.15
	Kali 605 m07	1.20
	Kali 606 m07	1.18
	Kali 607 m07	1.13
	Kali 608 m07	1.04
	Kali 610 m07	1.04
	Kali 611 m07	1.08
	Kali 612 m07	1.07
	Kali 613 m07	1.07
	Kali 614 m07	1.13
	Kali 615 m07	0.99
	Kali 616 m07	1.16
	Kali 620 m07	1.07
	Kali 621 m07	0.94
	Kali 622 m07	1.60
	Kali 623 m07	0.98
	Kali 625 m07	1.13
	Kali 626 m07	0.89
	Kali 627 m07	1.00
	Kali 628 m07	1.08
	Kali 630 m07	1.01
	Kali 631 m07	1.07
	Kali 632 m07	0.96
	Kali 634 m07	1.18
	Kali 635 m07	1.09
	Kali 636 m07	1.16
	Kali 637 m07	1.01
	Kali 638 m07	1.15
	Kali 639 m07	0.93
	Kali 642 m07	1.07
	Kali 643 m07	1.12
	Kali 644 m07	1.03
	Kali 645 m07	1.29
	Kali 646 m07	0.99
	Kali 647 m07	1.01
	Kali 648 m07	1.41
	Kali 649 m07	1.06
	Kali 650 m07	0.90
	Kali 651 m07	1.09

Figure S2. Complete results of screen of Malian plasma using anti-goat IgG microbeads (continued).

Cohort	Sample name	Binding ratio
	Kali 652 m07	1.05
	Kali 653 m07	1.20
	Kali 654 m07	1.08
	Kali 655 m07	1.05
	Kali 656 m07	1.10
	Kali 658 m07	1.00
	Kali 659 m07	0.96
	Kali 660 m07	1.01
	Kali 661 m07	1.24
	Kali 662 m07	1.03
	Kali 663 m07	1.19
	Kali 664 m07	1.01
	Kali 665 m07	1.06
	Kali 666 m07	1.09
	Kali 667 m07	1.04
	Kali 668 m07	1.02
	Kali 669 m07	1.09
	Kali 670 m07	1.08
	Kali 671 m07	0.92
	Kali 672 m07	1.06
	Kali 673 m07	1.07
	Kali 674 m07	1.25
	Kali 677 m07	1.24
	Kali 678 m07	1.12
	Kali 681 m07	0.96
	Kali 682 m07	1.19
	Kali 684 m07	1.19
	Kali 685 m07	1.09
	Kali 687 m07	1.03
	Kali 688 m07	1.08
	Kali 689 m07	1.15
	Kali 690 m07	1.13
	Kali 692 m07	0.95
	Kali 693 m07	0.95
	Kali 694 m07	1.00
	Kali 697 m07	1.15
	Kali 698 m07	1.11
	Kali 699 Apr-13	1.09
	Kali 700 Apr-13	0.99
	Kali 701 Apr-13	1.08
	Kali 702 Apr-13	0.99
	Kali 703 Apr-13	1.04
	Kali 704 Apr-13	1.00
	Kali 705 Apr-13	1.10
	Kali 706 Apr-13	0.95
	Kali 707 Apr-13	1.33
	Kali 708 Apr-13	0.97
	Kali 709 Apr-13	1.24
	Kali 710 Apr-13	1.18
	Kali 711 Apr-13	1.00
	Kali 712 Apr-13	9.46
	Kali 713 Apr-13	1.13
	Kali 714 Apr-13	1.12
	Kali 715 Apr-13	0.96
	Kali 716 Apr-13	1.05
	Kali 717 Apr-13	1.06
	Kali 718 Apr-13	1.29
	Kali 719 Apr-13	1.03
	Kali 720 Apr-13	1.15
	Kali 721 Apr-13	1.05
	Kali 723 Apr-13	1.28
	Kali 724 Apr-13	1.07
	Kali 725 Apr-13	1.05
	Kali 726 Apr-13	0.99
	Kali 727 Apr-13	1.09
	Kali 728 Apr-13	1.26
	Kali 729 Apr-13	1.15
	Kali 730 Apr-13	10.48
	Kali 731 Apr-13	1.23
	Kali 732 Apr-13	1.07

Cohort	Sample name	Binding ratio
	Kali 733 Apr-13	1.12
	Kali 734 Apr-13	1.17
	Kali 735 Apr-13	1.11
	Kali 736 Apr-13	1.17
	Kali 737 Apr-13	1.16
	Kali 738 Apr-13	0.99
	Kali 739 Apr-13	1.12
	Kali 740 Apr-13	1.16
	Kali 741 Apr-13	0.97
	Kali 742 Apr-13	1.14
	Kali 743 Apr-13	1.13
	Kali 744 Apr-13	1.15
	Kali 745 Apr-13	1.08
	Kali 746 Apr-13	1.02
	Kali 747 Apr-13	1.09
	Kali 748 Apr-13	0.86
	Kali 749 Apr-13	0.98
	Kali 750 Apr-13	1.04
	Kali 751 Apr-13	1.33
	Kali 752 Apr-13	1.37
	Kali 753 Apr-13	0.94
	Kali 754 Apr-13	1.27
	Kali 755 Apr-13	1.29
	Kali 756 Apr-13	1.08
	Kali 757 Apr-13	1.04
	Kali 758 Apr-13	1.10
Kalifabougou	Kali 733 Apr-13	1.12
	Kali 734 Apr-13	1.17
	Kali 735 Apr-13	1.11
	Kali 736 Apr-13	1.17
	Kali 737 Apr-13	1.16
	Kali 738 Apr-13	0.99
	Kali 739 Apr-13	1.12
	Kali 740 Apr-13	1.16
	Kali 741 Apr-13	0.97
	Kali 742 Apr-13	1.14
	Kali 743 Apr-13	1.13
	Kali 744 Apr-13	1.15
	Kali 745 Apr-13	1.08
	Kali 746 Apr-13	1.02
	Kali 747 Apr-13	1.09
	Kali 748 Apr-13	0.86
	Kali 749 Apr-13	0.98
	Kali 750 Apr-13	1.04
	Kali 751 Apr-13	1.33
	Kali 752 Apr-13	1.37
	Kali 753 Apr-13	0.94
	Kali 754 Apr-13	1.27
	Kali 755 Apr-13	1.29
	Kali 756 Apr-13	1.08
	Kali 757 Apr-13	1.04
	Kali 758 Apr-13	1.10

Figure S2. Complete results of screen of Malian plasma using anti-goat IgG microbeads (continued).