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Receptor-based antibodies generated by templated insertions in the switch region

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“If you want something you’ve never had

You must be willing to do something you’ve never done ”

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Abstract

Summary

We recently isolated, from malaria-exposed individuals, antibodies that carried a large DNA fragment encoding the LAIR1 extracellular domain inserted in between the V_H and DJ_H or in the switch regions of the immunoglobulin gene locus. These genomic configurations result in the production of antibodies that display an LAIR1 extracellular domain at the tip of the HCDR3 or at the VH-CH1 junction. The LAIR1-containing antibodies recognize certain *Plasmodium falciparum* (*P. falciparum*) antigens, named RIFINs, which are expressed on the surface of infected erythrocytes. To investigate the general relevance of such receptor-based antibodies, we searched for antibodies containing LILRB1, an MHC-I binding inhibitory receptor that was suggested to bind to certain RIFINs. By screening the sera of malaria-exposed individuals, I discovered natural antibodies containing LILRB1 domains that bind to various malaria isolates. Analysis of the cDNA and gDNA revealed LILRB1 inserts in the switch region that, after appropriate splicing, led to the expression of domain 3 (D3) and D4 on the VH-CH1 elbow. Moreover, I found that these LILRB1-containing antibodies recognize a handful of RIFINs on *P. falciparum*-infected erythrocytes, which may serve as the potential candidates for the development of a malaria vaccine. The cryo-EM structure of RIFIN-V2 domain in complex with the LILRB1-containing antibody showed that the inserted domains open the VH elbow without interfering with VH-VL and CH1-CL pairing. The discovery of receptor-based antibodies inspired us to use this molecular platform for antibody engineering. I generated bispecific antibodies by inserting VHH or single-chain Fv domains between VH and the CH1 region of specific antibodies. The recombinant antibodies are well expressed, and their dual antigen specificities were validated by antigen binding analyses. This finding served as a proof of principle that insertion of additional domains in the elbows of conventional antibodies could be used to generate novel bispecific molecules for diagnostic or therapeutic purposes. Collectively, my work illustrates a general mechanism for the generation and selection of receptor-based antibodies, i.e. antibodies that incorporate the pathogen receptor thus effectively hijacking pathogen immune evasion.

Riassunto

Recentemente abbiamo isolato, da soggetti esposti alla malaria, anticorpi generati dall'inserzione di un frammento di DNA codificante il dominio extracellulare di LAIR1. L'inserzione tra VH e DJ o nella regione "switch" risulta nella produzione di anticorpi che mostrano il dominio extracellulare LAIR1 sulla punta della CDRH3 o sulla giunzione VH-CH1. Gli anticorpi contenenti LAIR1 riconoscono alcuni antigeni del *P. falciparum*, detti RIFIN, che sono espressi sulla superficie degli eritrociti infetti. Per studiare la rilevanza generale di tali "anticorpi basati sul recettore" , abbiamo cercato anticorpi contenenti LILRB1, un recettore inibitorio del legame MHC-I che è stato suggerito legare certi RIFIN. Analizzando i sieri di soggetti esposti alla malaria, ho scoperto anticorpi naturali contenenti domini LILRB1 che si legano a vari isolati di malaria. L'analisi del cDNA e del gDNA ha rivelato inserti di LILRB1 nella regione switch che, in seguito a corretto splicing, portano all'espressione dei domini 3 (D3) e D4 sul gomito VH-CH1. Inoltre, ho scoperto che questi anticorpi contenenti LILRB1 riconoscono certi RIFIN sugli eritrociti infetti che possono servire da potenziali candidati per lo sviluppo di un vaccino contro la malaria. La struttura crio-EM del dominio RIFIN-V2 in complesso con un anticorpo contenente LILRB1 ha mostrato che i domini inseriti aprono il gomito VH-CH1 senza interferire con l'appaiamento VH-VL e CH1-CL. La scoperta di anticorpi basati sul recettore ci ha ispirato a utilizzare questa piattaforma molecolare per l'ingegnerizzazione degli anticorpi. Ho generato anticorpi bispecifici inserendo domini VHH o scFv nel gomito VH-CH1 di anticorpi specifici. Questi anticorpi ricombinanti sono ben espressi e le loro doppia specificità è stata validata dalla capacità di legare diversi antigeni. Questa scoperta è stata una prova del principio secondo cui l'inserimento di domini aggiuntivi nei gomiti degli anticorpi convenzionali potrebbe essere usato per generare nuove molecole bispecifiche a fini diagnostici o terapeutici. Collettivamente, il mio lavoro illustra un meccanismo generale per la generazione e la selezione di anticorpi basati sul recettore, ovvero anticorpi che incorporano il recettore dei patogeni, dirottando così efficacemente l'evasione immunitaria dei patogeni.

Abbreviations

a-EJ	Alternative non homologous end joining
AID	Activation-induced cytidine deaminase
AP	Alkaline phosphatase
APRIL	A proliferation-inducing ligand
BAFF	B-cell activating factor
BCL	B-cell lymphoma
BCR	B cell receptor
BSA	Bovine serum albumin
BsAb	Bispecific antibody
c-NHEJ	Classical non-homologous end joining
CD	Cluster of differentiation
CDR	Complementarity-determining region
CH	Heavy chain constant domain
CL	Light chain constant domain
CSA	Chondroitin sulfate A
CSP	Circumsporozoite protein
CSR	Class switch recombination
DBL	Duffy-binding like
DMSO	Dimethyl sulfoxide
DNA-PKcs	DNA-dependent protein kinase catalytic subunit DTT Dithiothreitol
DSB	Double strand break
EBV	Epstein-Barr virus
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Fab	Fragment, antigen binding
FBS	Foetal bovine serum
Fc	Fragment, crystallisable
FCS	Foetal calf serum
FDC	Follicular dendritic cell
FITC	Fluorescein isothiocyanate
FR	Framework
FSC	Forward scatter
gDNA	Genomic DNA
GPI	Glycophosphotidylinositol
HA	Haemagglutinin
HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
iBAQ	Intensity-based absolute quantification
ICAM-1	Intercellular adhesion molecule-1
IE	Infected erythrocyte
IL	Interleukin
ITAM	Immunoreceptor tyrosine-based activation motif
ITIM	Immunoreceptor tyrosine-based inhibitory motif
KIR	Killer Ig-Like Receptors
LAIR1	Leukocyte-associated immunoglobulin-like 1
LC-MS	Liquid chromatography-mass spectrometry
LFA	Lymphocyte function-associated antigen
LFQ	Label-free quantification
LILRB1	Leukocyte Immunoglobulin Like Receptor B1
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
NHEJ	Non-homologous end joining
PARP	Poly (ADP-ribose) polymerase
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PE	Phycoerythrin
PEI	Polyethylenimine
PEXEL	Plasmodium export element
PfEMP1	Plasmodium falciparum erythrocyte membrane protein-1
PfMC-2TM	Plasmodium falciparum Maurer's cleft two transmembrane
pfRH5	Reticulocyte-binding protein homologue 5
pir	Plasmodium interspersed repeat
RAG	Recombination-activating gene
RIFIN	Repetitive interspersed family
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 repea
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
Sμ	Switch region of IgM
TBS	Tris-buffered saline
TdT	Terminal deoxynucleotidyl transferase
TLR	Toll-like receptor
TM	Transmembrane region
TNF-α	Tumor necrosis factor alpha
UCA	Unmutated common ancestor
uE	Uninfected erythrocyte
UNG	Uracil-DNA glycosylase
VCAM-1	Vascular cell adhesion molecule-1
VH	Heavy chain variable domain/region
VL	Light chain variable domain/region
VSA	Variant surface antigen
XLF	XRCC4-like factor
XRCC	X-ray repair cross-complementing protein

Contents

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Introduction

1.1 B cells and antibodies

1.1.1 Generation of diversity by V(D)J recombination

Introduction to V(D)J recombination

Adaptive immunity in vertebrates is highly dependent on the generation of a vast diversity of antigen receptors on lymphocytes. During the development of lymphocytes, antigen receptor genes are assembled via a process called V(D)J recombination (Bassing, Swat, & Alt, 2002). Particularly, Immunoglobulin (Ig) genes are assembled from gene segments during the development of B cell. The coding sequence for the heavy chain IgH variable region (VH) is assembled from V_H, D, and J_H gene segments, while the light chain IgL variable region is assembled between V_L and J_L segments, both assemblies mediated by V(D)J recombination (Fig. 1.1). The gene segments at Ig locus have multiple copies: the human heavy chain locus contains about 65 V genes, 27 D genes and 6 J genes, the κ locus contains 40 V genes and 5 J genes, and the λ locus contains 30 V genes and 4 J genes (Fig. 1.1). The joining of different V_H, D, and J_H gene segments generates diverse VH variable regions. A random combination of varying V_H, D, J_H segments is capable of producing roughly 65 x 27 x 6 = 10,530 different VH regions in humans. Similarly, the recombination in light chain genes could also give rise to a certain degree of diversity. Further diversifications in variable domains are generated due to the imprecise joining events of the gene segments during V(D)J recombination, which I will go through in the section below.

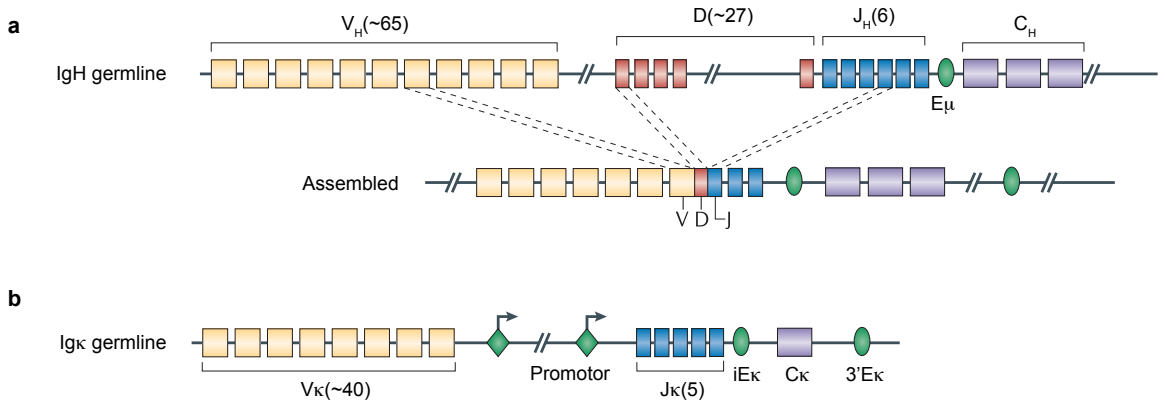

Figure 1.1. **Human immunoglobulin (Ig) loci and V(D)J recombination.** Human immunoglobulin (Ig) loci and V(D)J recombination. a. Human immunoglobulin heavy chain assembled via V(D)J recombination. Specifically, during the B cell development, D-to-J_H recombination at the antibody heavy chain locus occurs first, followed by V_H to DJ_H recombination. Rearrangement at the light chain then occurs to allow the formation of a complete BCR (not shown). b. Human immunoglobulin kappa light chain locus. Variable (V), diversity (D) and joining (J) gene segments are represented as yellow, brown and blue rectangles, respectively. Constant (C) regions are shown as grey rectangles, enhancer elements as green ovals and germline promoters are shown as green diamonds with arrows (Figure adapted from (Schatz & Ji, 2011)).

Mechanisms of V(D)J recombination

The following are the detailed underlying mechanisms of V(D)J recombination (Bassing et al., 2002). V, D, J gene segments are flanked by conserved recombination signal sequence (RSS), which served as cleavage sites for RAG1 and RAG2 recombinase which are the key enzymes for this process (McBlane et al., 1995; Zhang et al., 2019). V(D)J recombination can only occur between a 12-bp RSS and a 23-bp RSS nucleotide spacer, this is known as the 12-23 rule (Hiom & Gellert, 1998). Initially, RAG1 and RAG2 proteins bind 12-RSS and 23-RSS then form 12-RSS and 23-RSS complex, respectively (Fig. 1.2 a). Subsequently, RAG1 and RAG2 synapse a pair of 12 and 23-RSS result in the formation of paired complex, within which RAG proteins generate double strand breaks in between the RSSs and the gene segments (Fig. 1.2 a). Such restricted rule is essential for avoiding non-specific recombination between V_H and J_H gene segments in the heavy chain as they both have 12-bp RSS. Moreover, the 12-23 rule confers an ordered rearrangement event: D gene (flanking with 23bp-RSS) recombines with J_H first, followed by V_H-DJ_H joining

(Eastman, Leu, & Schatz, 1996).

So how exactly are the breaks made? RAG recombinases attack one of the DNA strand adjacent to the RSS, leading to the releasing of 3' hydroxyl (OH) that can attack the second DNA strand, resulting in a double strand break of the DNA and generating a hairpin structure at the coding end (Fig. 1.2 b). The processing of hairpin can introduce additional diversity in the variable region. Basically, Hairpin can be cut at different locations by Artemis (a hairpin cutting nuclease) and DNA-dependent protein kinase (DNA-PK) such that introducing so called p-nucleotides (Boboila, Alt, & Schwer, 2012). Following the double strand break, a number of DNA repair proteins are recruited to rejoin the DNA ends of gene segments which are mediated by non-homologous ending joining (NHEJ) pathway (Fig. 1.2 a). During the repair, the ends of the gene segment undergo non-templated nucleotide addition by terminal deoxynucleotidyl transferase (TdT) and usually cause addition of nucleotides known as N-nucleotides (Fig. 1.2 a). The gain of nucleotides (P- and N-nucleotides) at the coding joint allows the variable region to obtain further diversity, which conferred the variable chain a hypervariable region: CDR3 (Complementarity-determining region 3) which is also the junction of V(D)J. Such imprecise coding joints coincide with CDR3 in the variable region and diversify antigen binding specificity, creating the core of antibody repertoires.

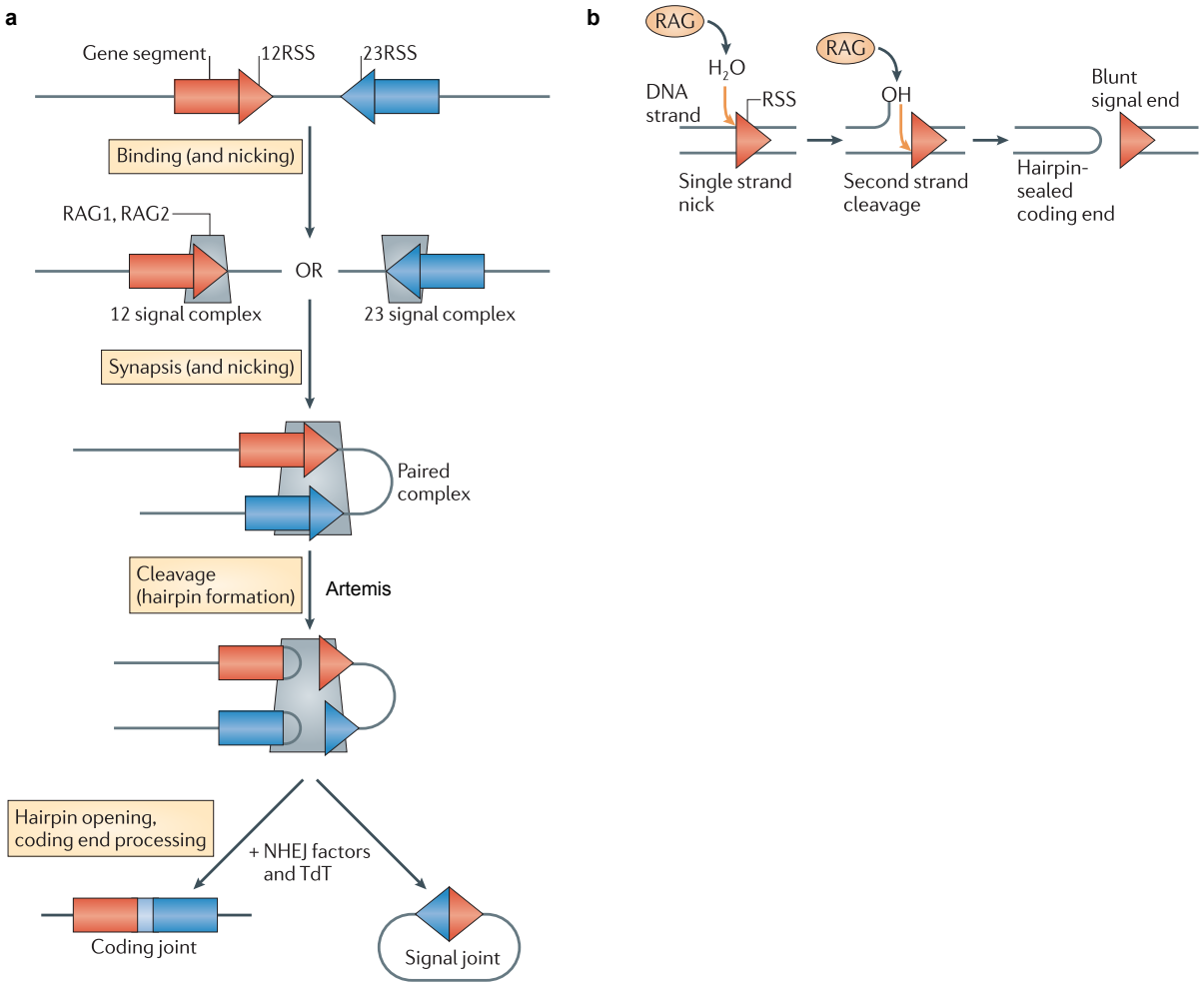

Figure 1.2. **Mechanism of V(D)J recombination.** a. Steps in V(D)J recombination. V, D or J gene segments are flanked by a 12RSS or by a 23RSS. Initially, the RAG1/2 complex aligns the RSS heptamers and cleaves the DNA, which results in hairpin formation. The hairpins are then cleaved by Artemis. Subsequently, the RAG proteins cooperate with 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 modified by exonucleases and terminal deoxynucleotidyl transferase (TdT). b. Mechanisms of RAG mediated DSB. The RAG protein first introduces a nick on one DNA strand; the 3 hydroxyl (OH) group is free from the nick and then attacks the opposite DNA strand. The DSB is generated as well as a hairpin coding end and a blunt signal end (Figure adapted from (Schatz & Ji, 2011)).

1.1.2 B cell development

Introduction

The generation of new B lymphocytes or B lymphopoiesis occurs in the bone marrow for most B cells (Hardy & Hayakawa, 2001). The primary goal of B lymphopoiesis is to produce a wide variety of B cell receptors so that an individual will be able to acquire adaptive immunity to a wide spectrum of pathogens during the lifetime. In this section, I will briefly introduce how B cells are developed from uncommitted progenitors to functional mature B lymphocytes.

B lymphocytes life history

The B cell life history comprises several stages all the way from uncommitted stem cell to specialized lymphocytes with its unique antigen receptor and ultimately become an antigen experienced effector or memory B cell.

In the first stage (pro-B cell), bone marrow progenitor B cells receive signals from bone marrow stromal cells and start to rearrange their heavy chain immunoglobulin genes (Hardy, Carmack, Shinton, Kemp, & Hayakawa, 1991). Specifically, in early pro-B cells, rearrangement of a D exon to a J_H exon occurred and followed by V_H to DJ_H rearrangement, which resulted in the formation of late pro-B cells. If the $V_H(D)J_H$ rearrangement leading to a productive heavy chain expression, the heavy chain will pair with a surrogate light chain and serve as a pre-B cell receptor to receive signals at the B cell surface and become a large pre-B cell (Fig. 1.3). The roles of pre-B cell receptor are in two folds, one is to check whether the heavy chain is productive, and another function is to stimulate the proliferative expansion of the B cell precursor, both of which depend on the signing from ITAM-containing singling chains Igα and Igβ (Herzog, Reth, & Jumaa, 2009). After several rounds of proliferation, the large pre-B cell progress to small pre-B cells that start to express heavy chain (μ chain) in the cytoplasm. At this point, the light chain Ig genes start to rearrange while the μ chain stays intracellularly. A successful light chain V-J rearrangement will lead to a complete IgM molecule expression at the cell membrane so that the small pre-B cell becomes immature B cell (Fig. 1.3).

Immature B cell bears surface antigen receptors in the form of IgM will be utilized to interact with surrounding self-antigens (Levine et al., 2000). In this stage, strongly stimulated B cells

will either die or are inactivated via a process called negative selection. This selection step is crucial for removing potential self-reactive immature B cells from the B cell reservoir (Melchers et al., 1995). Interestingly, before B cells undergo apoptosis due to strong ligation of surface IgM to self-antigens, the B cell will have the second opportunity to rearrange its light chain, and if the new antibody is able to pass the signaling checkpoint, such immature B cell can still migrate to the periphery and further matures. This mechanism known as receptor editing is essential for eliminating autoreactive receptors, while defects in this process may lead to autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis (Ching, Nagy, Luning Prak, & Weigert, 1995). This checkpoint for an immature B cell to leave the bone marrow is known as central tolerance (Melchers et al., 1995). It is crucial to bear in mind that central tolerance is not perfect as an individual B cell cannot be exposed to all possibilities of self-antigens. A benefit of this inefficiency is that receptors might become less limited, allowing them to recognize a wide variety of pathogens, although some time may result in autoimmunity.

Subsequent to the negative selection, B cells that survived emigrate from the bone marrow to the peripheral and express IgM and IgD at the cell surface by a mechanism of alternative mRNA splicing. The production of IgM and IgD on the one hand allows the cells to be activated by specific foreign antigens in the peripheral lymphoid organs, which result in activated B cells (mature B cell) that will then proliferate and differentiate into plasma cells or long-lived memory cells (Fig. 1.3). On the other hand, immature B cells or at this point, also called transitional B cells are subjected to a second round of auto-reactive checkpoint which the cells that recognize self-antigens will experience peripheral tolerance (Melchers et al., 1995). Unlike bone marrow immature B cells that have the second chance to edit their receptor if they are self-reactive, transitional B cells that encounter strong cross-linking antigens in the periphery will directly undergo apoptosis, and only the ones with no self-reaction will continue their maturation process (Melchers et al., 1995).

Although large amounts of B cells are being generated everyday, most immature B cells will not survive to become fully functional mature B cells. This is due to the limited number of lymphoid follicles, and only the B cell that receives signals from the follicle will survive. In particular, the most important surviving factor is BAFF (B-cell activating factor), which is primarily produced by follicular dendritic cells (Mackay & Browning, 2002). Moreover, the B cell receptor will continue providing survival and proliferation signals required for maturation. Transitional B cells that receive maturation and survival signals in the follicle will then differentiate into follicular B cells

or marginal zone B cells, which will eventually differentiate into plasma cells secreting antibody or memory cells (Marie-Cardine et al., 2008). For more details of this process, please check the following section.

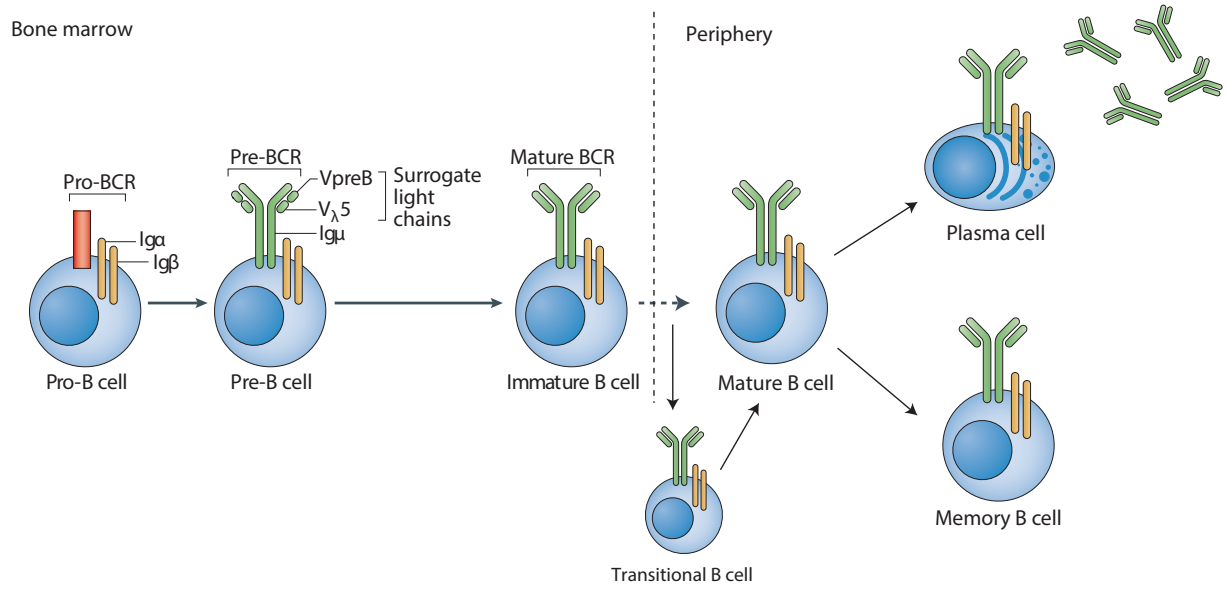

Figure 1.3. **B cell development stages.** In the bone marrow, the development of B cell progresses through the pro-B-cell, pre-B-cell, and immature-B-cell stages. In pre-B cell, rearrangements of Ig genes result in the expression of the pre-B-cell receptor, which is comprised of an Igμ and surrogate light chains (VpreB or Vλ 5). After light chain rearrangement, a mature BCR is formed. B cells undergo a negative selection to prevent any development of self-reactive cells before B cells enter the periphery. In the periphery, B cells become transitional B cells, eventually mature in the germinal center. Following an immune response, antigen-specific B cells differentiate into either plasma antibody-secreting plasma cells or memory B cells. Figure adapted from (Cambier, Gauld, Merrell, & Vilen, 2007).

1.1.3 B cell activation in the germinal center

In order to obtain a robust B cell response to certain antigen, B cells need to be polished in a process called germinal center reaction (Fig. 1.4). In particular, germinal centers that formed within the B cell follicle in secondary lymphoid organ are the place where B cells proliferate and undergo both somatic hypermutation and class switching and eventually differentiate into memory cells and long-lived plasma cells. The formation of the germinal centers begins when follicular dendritic cells (FDCs) present antigens on their surface, activate antigen specific CD4+ T cells which then proliferate and mature into effector cells capable of activating antigen specific B cells (Gatto

& Brink, 2010). Specifically, B cells need to receive several signals to be fully activated (Lanzavecchia, 1985). The first signal is delivered through B cell receptor (BCR) by its cognate antigen. Once IgM+ naive B cells migrate from bone morrow to follicles of the secondary lymphoid organ, they encounter antigens that can be recognized by its BCR and the antigen is processed and presented on the surface MHCII. Follicular helper CD4+ T cells (T_{FH}) which have the same pathogenic specificity will recognize the peptide:MHCII on B cell surface though TCR:peptide:MHC class II complex and together with the co-stimulatory molecule interactions, delivering additional signals to B cells for supporting their proliferation and differentiation (Gatto & Brink, 2010). In the meantime, T_{FH} cells are able to secrete cytokines that regulate class switching, which allow B cells to secrete different isotypes of antibody with same antigen specificity (Cerutti et al., 1998). For the detail of class switch recombination (CSR), I will discuss in the upcoming section.

Upon activation, antigen specific B cells start to proliferate to form primary focus (Fig. 1.4). Some B cells from the primary focus migrate to nearby follicles and proliferate, and other B cells from the primary focus, persist in the T cell area for a short while secreting the antibody but eventually die. B cells that enter the follicle, begin to proliferate rapidly and form a structure called dark zone, during this time, they undergo somatic hypermutation to introduce new variations into the BCR and obtain antigen binding sites with different affinity (Fig. 1.4). Subsequently, B cells undergo a process of selection, in which the receptors are tested for their ability to bind antigen on FDCs, those the fail to bind, or the failed to compete efficiently against other B cell receptors undergo apoptosis (Gatto & Brink, 2010). This selection event is established in a structure called light zone within the germinal center. In order to generate the best possible antibody, B cells is capable of shuttling back and forth between the dark zone and the light zone for additional rounds of mutation and selection: BCRs accumulate mutations in the dark zone and get tested in the light zone (Fig. 1.4). Some antibodies require tremendous mutations to acquire high affinity to a specific antigen, while others only need one of few to reach the same goal. This is mainly depending on the antigen. For example, broadly neutralizing HIV antibodies often require enormous somatic mutations while a specific influenza antibody just need one to influenza H1 antigen (Briney et al., 2016; Pappas et al., 2014; Zhou et al., 2010). Sometimes, B cell may contain a BCR that is slightly cross-reactive to the host antigen. Such cross-reactivity maybe resolved through SHM and the BCR will only retain the specificity to the pathogen. Eventually, the B cells that obtain high affinity BCR interact with antigens on FDC will differentiate into antibody-secreting plasma cell or memory cells that survive long-term in the body (Fig. 1.4).

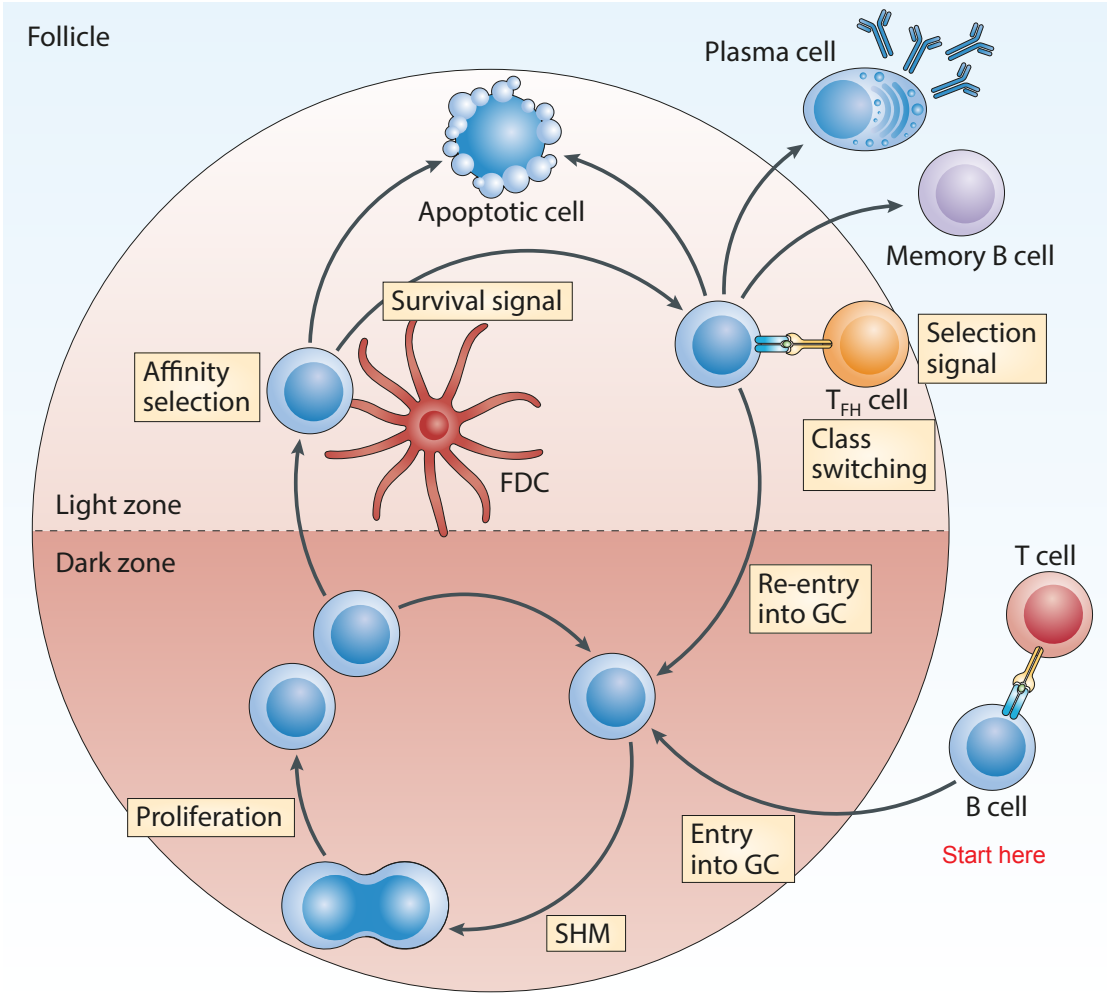

Figure 1.4. **B cell activation and Germinal center reaction.** B cells that are activated by antigen experienced T cells enter the dark zone of the germinal center. In the dark zone, they start to proliferate and undergo SHM to modify their BCR. B cells acquired new affinities for the target antigen enter the light zone, where they undergo affinity selection. In the light zone, the B cells are competing for binding to the antigens presented by FDCs. B cells that bind strongly to the antigen will receive survival signals, while the ones do not bind will undergo apoptosis. B cells can also re-enter the dark zone for further rounds of proliferation and modification or differentiate into plasma cells that secrete antibodies or memory B cells. Reprinted with permission from (Heesters, Myers, & Carroll, 2014).

1.1.4 Somatic mutations

Somatic hypermutation (SHM) in germinal center B cells results in various binding affinity of the BCRs, through positive selection, only the high affinity B cells get selected and leading to affinity maturation (Di Noia & Neuberger, 2007). How exactly are the mutations introduced and accumulated? In this section, I will delve into the molecular details of SHM.

Upon B cell activation, germinal center B cells undergo SHM, which exploits a mutation rate at $1x10^{-3}$/bp per generation that is 10^6 folds higher than spontaneous mutation. SHM is initiated by activation-induced cytidine deaminase (AID), an enzyme that deaminates cytosines (C) to uridines (U) in single-stranded DNA in the germinal center B cells (Odegard & Schatz, 2006). As AID can only act on single-strand DNA, the targeted gene has to be under transcribed such that the DNA double helix becomes temporarily unwound. V exon that is actively transcribed upon B cell activation permits AID accessible to the DNA, thus introduces point mutations at and around the site of original C:G pair in the V exon. AID preferentially deaminates C in WRCY (W=A or T, R=A or G, Y=C or T), a mutation hotspot for AID targeting. Basically, AID converts C to U and introduces dual lesions that bring foreign bases into the DNA and lead to mismatch of the guanosine nucleotide (Fig. 1.5). To repair these two lesions, different repair strategies are utilized and lead to district mutational outcomes (Fig. 1.5). If the mismatch is ignored and the DNA goes through normal DNA replication, which will result in a C to T mutation. If the two major DNA repair pathways are engaged, namely mismatch repair and base-excision repair pathway, which will generate alternative mutational outcomes (Boboila et al., 2012). In the mismatch repair pathway, the lesion is detected by mismatch repair proteins MSH2 and MSH6 and resolved by recruiting nucleases that remove the lesion and followed by filling the "patch" using error-prone DNA polymerase η (Polη), which result in mutations at nearby A:T base pairs (Roa et al., 2010). In the base-excision repair (UNG, uracil-DNA glycosylase) pathway, the uracil lesion is removed to create an abasic site in the DNA and following a further round of DNA replication, random insertion of a nucleotide takes place leading to a mutation at the AID acted C:G residues (Fig. 1.5).

In summary, AID initiates DNA lesions in V exon during SHM, and B cells are able to employ several types of machinery to repair these lesions. The mutational outcomes are depending on how the lesions are repaired. Of note, the mismatch repair pathway in other cell types usually employs more accurate polymerases that faithfully copy the undamaged template strand. In B cell, however, DNA is repaired by error-prone DNA polymerases Polη to facilitates V exon variation.

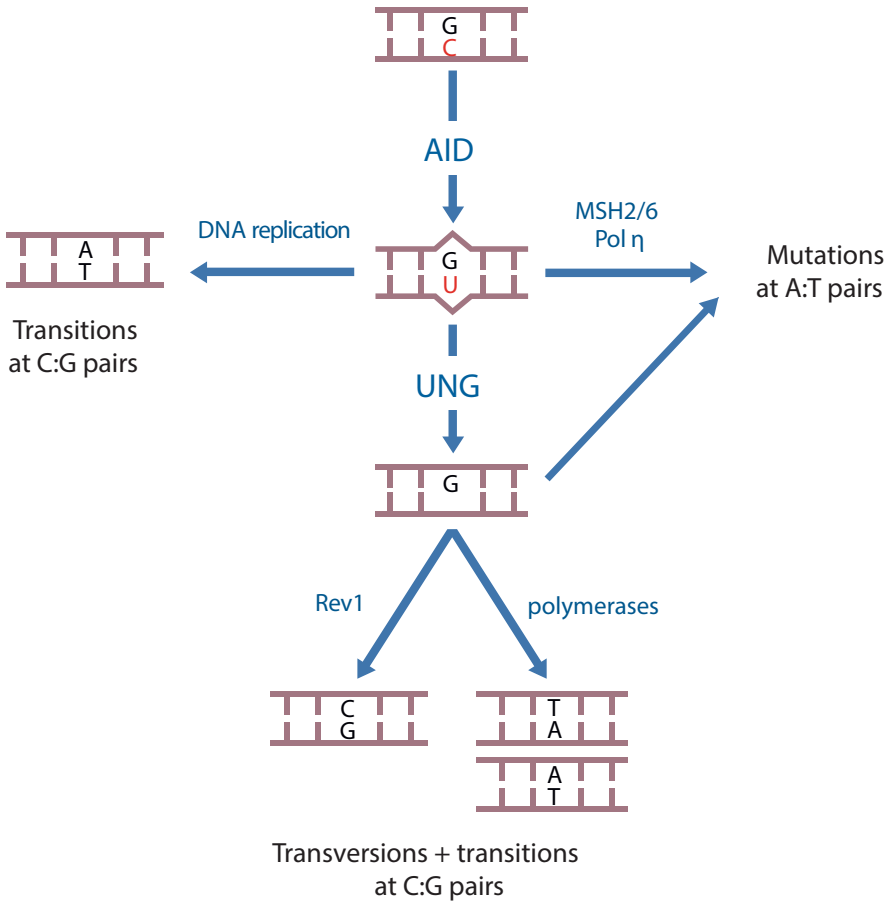

Figure 1.5. **Mechanisms of somatic hypermutation (SHM).** AID targets DNA by deamination of C to U, resulting in a G:U mismatch. If the lesion is ignored and goes through conventional DNA replication, it will result in a C to T transition at the original C:G pair. Alternatively, U can be removed by UNG glycosylase, resulting in an abasic site. There are two outcomes following this route, the site can either be filled in by deoxycytidyl transferase Rev 1, resulting in a G to C transversion, or by unknown polymerase that gives rise to further mutations. The mismatch repair enzyme complex MSH2/6 associated with other enzymes such as Polη may creates mutations at the A:T pair. Figure adapted with permission from (Di Noia & Neuberger, 2007).

1.1.5 Class switching

For class switching to occur, two signals are required: germline transcription of the switch region. And crosslinking of CD40 on B cells with CD40 ligand on activated T cells (Xu, Zan, Pone, Mai, & Casali, 2012). A particular B cell activated in immune response will give rise to progenies with the same VH genes. Although the VH gene can be modified by SHM as described above, these B cells are considered as B cell clones. Within the B cell clones, they may express distinct constant

region (C region gene or CH gene).

Initially, B cells express IgM and IgD as their antigen receptor and IgM as a secreted antibody during the primary immune response. In the following development, antibodies that assembled the same VH exon may be expressed in different isoform: IgA, IgG, or IgE. This process is known as class switching, which provides another level of functional diversity in antibody/BCR besides V(D)J recombination and SHM. Specifically, different classes of antibodies have distinct roles when defending diverse pathogens. For example, IgA is important for targeting pathogens in intestinal and respiratory tracts (Macpherson, McCoy, Johansen, & Brandtzaeg, 2008). IgG can efficiently facilitate the opsonization of pathogens by phagocytes and induce activation of the complement system (Unkeless, Scigliano, & Freedman, 1988). The main function of IgE is immunity to parasites such as immunity to protozoan parasites *Plasmodium falciparum* (Gurish et al., 2004; Shreffler, Beyer, Chu, Burks, & Sampson, 2004).

Class switching involves replacing the μ chain CH gene with a new downstream CH gene via a DNA recombination event, namely CSR. The basis of CSR is DNA double strand break (DSB) and rejoining that allow recombination of the new downstream CH gene to previously assembled VH exon (Fig. 1.6). The DNA DSBs are induced by AID, a critical enzyme not only for SHM but also for CSR (Muramatsu et al., 2000). And as a consequence, an individual who lacks AID results in the production of primarily IgM antibodies and the absence of affinity maturation (Revy et al., 2000).

CSR begins when AID starts deaminating cytosines in the switch regions (S region) that are located at the intron between J_H gene segments and the Cμ gene, and at equivalent sites upstream of each heavy chain CH exon, except for the d gene (Fig. 1.6). The DNA recombination takes place between donor switch μ (Sμ) and acceptor S region upstream of the targeted C region gene. Specifically, AID generates breaks in the Sμ and a second S region, during the recombination, the constant μ (Cμ) coding region and upstream of targeted S regions are deleted (Fig. 1.6). Whereas the upstream of Sm is joining with downstream of the acceptor S region mediated by non-homologous end joining (NHEJ) repair pathway. As I mentioned in the previous section, AID can only target single stranded DNA (ssDNA) and typically act on an actively transcribed region. So how intronic S region meets the requirement for AID targeting?

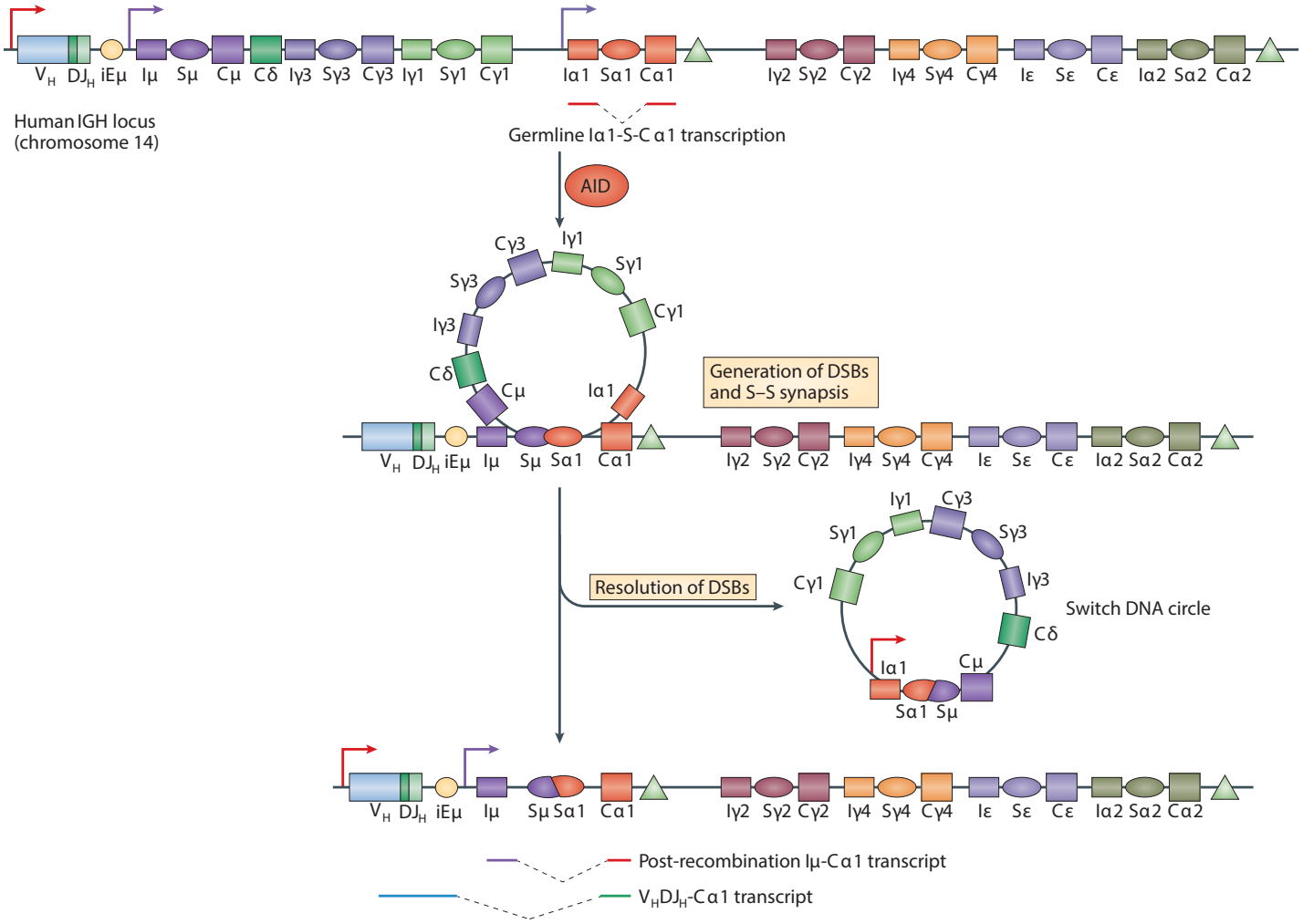

Figure 1.6. **Mechanism of class switch recombination (CSR).** CSR exchanges the constant region (CH) of Ig heavy chain with one of the downstream CH gene. In this example, a CSR occurred between Sμ and Sα 1 (IgM to IgA1). The transcription derived by Iμ and Iα1 promotors opens up the DNA helixes which allow AID to act and induce double strand breaks (DSBs) in two switch sites. The DSBs in the Sμ and Sα1 are resolved by DNA repair pathway and lead to the rejoining of two broken ends and deletion of the switch DNA circle. The replacement of the new CH gene is brought in close proximity to the V_HDJ_H DNA, can transcript new antibody transcript. Alternatively, since the new switch junction (Sμ - Sα 1) is formed, it can be derived by Iμ for the next round of CSR. iEμ , IGH intronic enhancer; I, promotor; S, switch region; C, constant. Figure adapted with permission from (Xu, Zan, Pone, Mai, & Casali, 2012).

S regions are repetitive DNA sequences that consist of many repeats of a G-rich sequence element and high density of AID hotspots WGCW (A/T-G-C-A/T) (Zheng Z et al., 2014). A promoter located upstream of each S region can be triggered by surrounding cytokines (typically produced by activated TFH) and initiates transcription mediated by RNA polymerase. Due to the repetitive sequence nature of S region, RNA polymerase is constantly stalled, which results in the exposure of non-templated DNA strand as ssDNA (Zheng Z et al., 2014). This event prepared S region as

the substrate for AID as well as for the subsequent enzymes that involved in inducing DSB. AID, UNG, and APE1 are important enzymes for generating nicks on both strands of DNA, which then are converted into DBSs by an unknown mechanism (Stavnezer & Schrader, 2014). Similar to SHM, once the break is generated, the DNA repair machinery of cells is activated and, in this case, NHEJ pathway is activated and enzyme such DNA-PKcs, Ku proteins, two-protein ligase complex XRCC4-ligase IV and other repair proteins are involved (Fig. 1.7). The two broken ends (>100kb distance) of participating S regions are brought together by repair proteins, and the CSR is completed when joining the donor S region with the acceptor S region and excision of the intervening region of DNA containing the previous CH gene (Fig. 1.7). In general, the ligation of two broken DNA ends does not precisely preserve the original sequence and may sometimes lead to mutations or even insertions in repaired sequences (Yan et al., 2007).

B cells that undergo a successful CSR may leave the secondary lymphoid organ to the peripheral circulation and start making antibodies or function as antigen presenting cells. Theoretically, these B cells are able to undergo several rounds of CSR as long as they receive two signals required for CSR to occur. However, CSR is an irreversible event as the deletion of the previous CH gene can no longer be reverted. Based on the genomic organization of human Ig S region loci (Fig. 1.6), the S region can only undergo a limited number of sequential switching. For example, once Sμ-Sγ1 junction is formed, the new S region can be triggered for the next round of switching, forming Sμ-Sγ1-Sγ4 junction. However, this B cell will never be able to switch to Sγ2 that was deleted during the formation of Sμ-Sγ1. Sequential switching was first demonstrated in mice when switch circle fragments showed that in some cases, the Sϵ and Sμ were flanked by Sγ1 sequence (Yoshida et al., 1990). This evidence was later confirmed in human B cells by demonstrating Sμ-Sγ1-Sϵ junctions on the chromosome (Cameron et al., 2003; Jabara, Loh, Ramesh, Vercelli, & Geha, 1993).

In conclusion, deletional recombination brings the new CH gene in close proximity to previously assembled VH exon allowing the B cell to express a new functional receptor/antibody with the same antigen specificity.

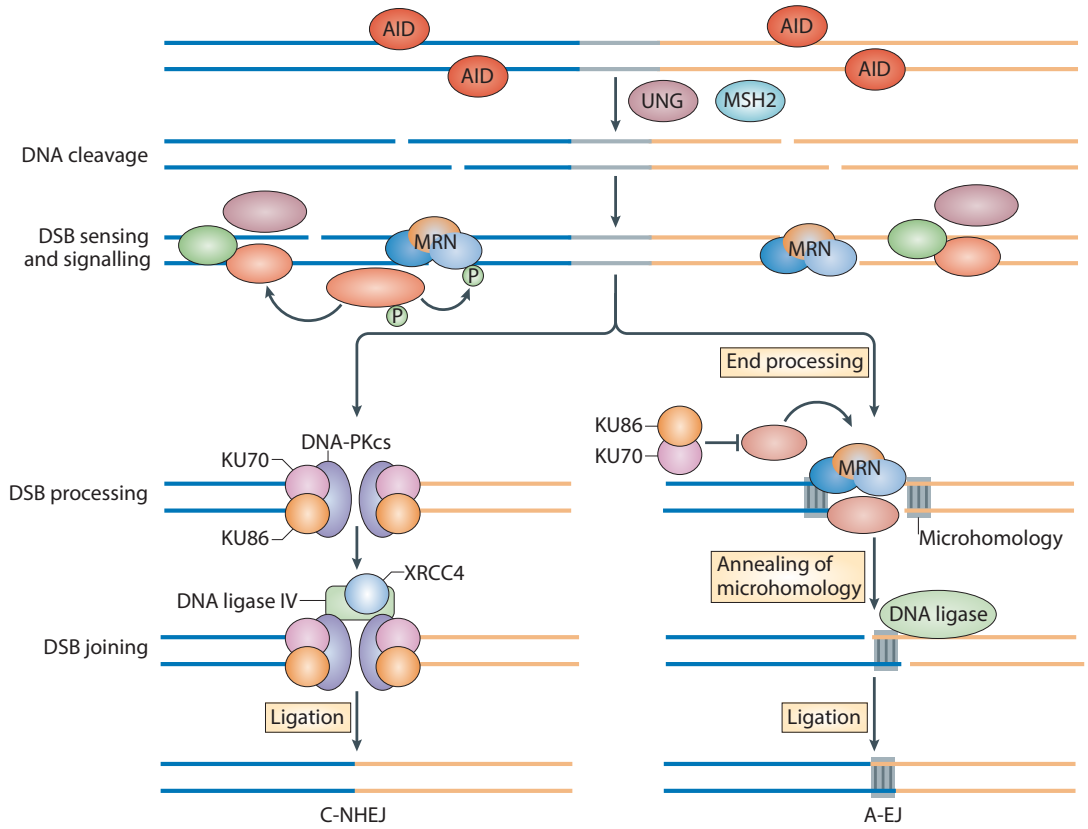

Figure 1.7. **Repair of the double strand break (DSB) in the CSR.** AID induced DSBs can be resolved in two different pathways: classic-NHEJ (C-NHEJ) or alternative-NHEJ (A-EJ). Initially, AID acts on ssDNA and associates with enzymes such as UNG and MSH2, DSBs are generated. In the C-NHEJ pathway, DSBs are sensed by a DNA damage sensor MRN complex, which recruits a wide variety of enzymes to further recruit protein complex KU. DSBs are bound by KU70 and KU86, which form complexes with the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and recruit other essential factors, such as XRCC4 and DNA ligase IV, to recombining the two broken ends. In the A-EJ pathway, the DSBs are processed by MRN as well as other important sensors (not shown), thereby generating microhomologies between the two broken ends. The junction is formed by microhomology and repaired by unknown DNA ligase. By contrast, C-NHEJ showed less or no microhomology. Figure adapted with permission from (Xu, Zan, Pone, Mai, & Casali, 2012).

1.1.6 Antibody structure and engineering

Introduction

Antibody, also known as the immunoglobulin, is the secreted form of B cell receptors and is produced by terminally differentiated B cells: plasmablasts and plasma cells. Because monoclonal antibody can be highly specific to its target, the use of antibodies is prevalent in research diagnostic and therapeutic applications. For example, antibodies can be fluorescently-conjugated or enzyme conjugated to allow detection of cells or proteins through flow cytometry or ELISA. Moreover, antibodies such as anti-PD1 has been used to treat colorectal cancer. To fulfill the needs of antibody applications, researchers have developed engineered antibodies or antibody derivatives. The innovation has advanced dramatically during the past century owing to the rapid evolving genetic engineering approaches (Weiner, 2015). One of the rising stars in antibody engineering is bispecific antibody (bsAb) which combines two antigen binding specificity into one molecule (Labrijn, Janmaat, Reichert, & Parren, 2019). A solid background knowledge of the antibody structure will help us facilitate these advances to develop next-generation antibodies and antibody-derived molecules further. In this section, I will first introduce the basis of antibody structure and followed by current knowledge of engineering of bsAbs or antibody derivatives.

Antibody structure

The structure of an antibody is roughly Y-shaped, as shown in Fig. 1.8 a, which corresponds to its dual functions: antigen recognition and effector recruitment (Schroeder Jr. & Cavacini, 2010). The two antigen binding sites are at the tip of the Y known as the variable region (V region), while the trunk of the Y or C region is critical for the effector function. The V region is very diverse owing to the somatic recombination and hypermutation, as I described in the previous sections. Although C region is less variable as compared to the V region, they still have subtle structure and property variations which determine the five different classes of antibody: immunoglobulin M (IgM), immunoglobulin D (IgD), immunoglobulin G (IgG), immunoglobulin A (IgA), and immunoglobulin E (IgE) (Schroeder Jr. & Cavacini, 2010). Different antibody isotypes are constructed in a similar way as a monomer, which is paired from two identical heavy chains and two identical light chains. Antibody molecules are highly modular proteins, which

composed of immunoglobulin domains (Ig domains). The heavy chain contains four or five Ig domains, while the light chain has two Ig domains. The N-terminal domains of the heavy and light chain are the V region and the C-terminal of the heavy chain is the C region. In both the V and C regions, a conserved folding pattern is observed, known as the immunoglobulin fold (Berg, Tymoczko, & Stryer, 2002). In general, the Ig domain is a barrel-shared structure and contains several b strands running in opposite directions packing together to form two β sheets (Clarke, Cota, Fowler, & Hamill, 1999). The two β sheets are connected through a disulfide bond and forming a b sandwich structure. Prior studies have shown that the antibody variable and constant domains are slightly different based on how many b-strands they bear (Bork, Holm, & Sander, 1994). The variable domain has 9 β stands while the constant domain has 7. The ends of the β -sheets are lined by flexible loops that are critical for interaction with other molecules. Interestingly, such distinct structures were also found in other proteins whose domains have sequence similarity to the immunoglobulins, namely immunoglobulin-like domains (Ig-like domains) (Adjadj, Quiniou, Mispelter, Favoudon, & Lhoste, 1992). For instance, T cell receptor and coreceptors (CD3, CD4, CD8), intracellular adhesion molecules 1 (ICAM1) (Yang et al., 2004), hemolymph protein hemolin, and inhibitory receptor Leukocyte Associated Immunoglobulin Like Receptor 1 (LAIR1), all of which consisting of Ig-like domains (Brondijk et al., 2010). Although the structures are similar in molecules that composed of Ig-like domains, based on the number of strands in the b-sheets as well as in their sequence pattern, Ig-like domains can be further classified into four different sets: V set domains (variable domain-like), C1 set domains (constant domain-like), C2 set domains and I set domains (antibody intermediated domain-like). Proteins may contain different domains within one molecule. For example, the human CD2, has both V set and C2 set domain (Anton van der Merwe, McNamee, Davies, Barclay, & Davis, 1995). In contrast, inhibitory receptor LAIR1 and LILBR1 (Leukocyte Immunoglobulin Like Receptor B1) only contain V set domains (Brondijk et al., 2010; Qihui et al., 2019). The composition of Ig and proteins that consist of Ig-like domains suggested a structural building block that can be used to generate molecules with similar functions. For example, nanobody or so called VHH (Bannas, Hambach, & Koch-Nolte, 2017) which only has one Ig domain was engineered as a representative of antibody-derivatives for various purposes including therapeutic and diagnostic applications (Fig. 1.8 a). By connecting a heavy chain V region with a light chain V region, an single chain variable fragment (scFv) (Weidle, Tiefenthaler, Weiss, Georges, & Brinkmann, 2013) is generated and could also be used in different applications (Fig. 1.8 a).

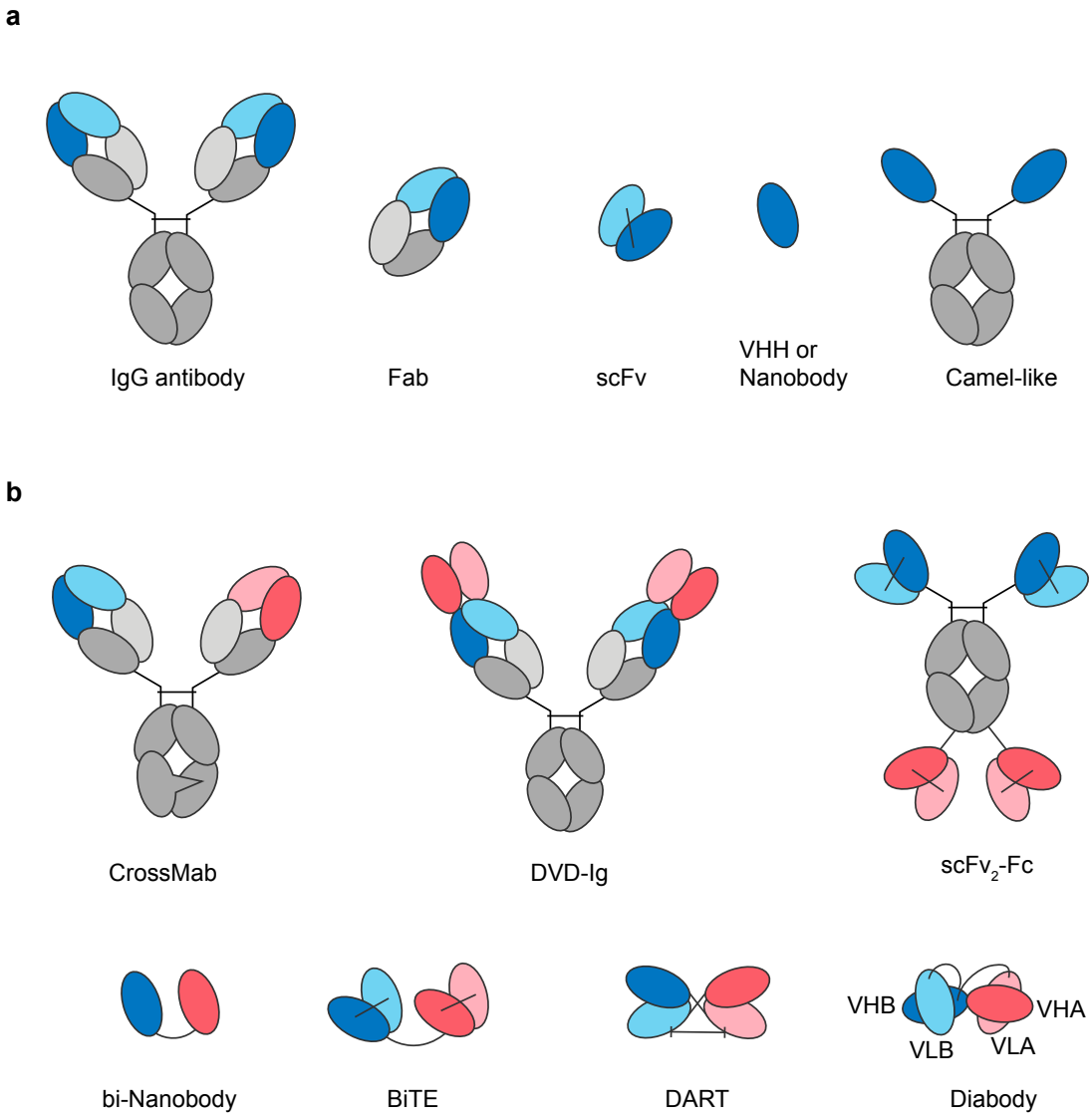

Figure 1.8. **Antibody structure and examples of bispecific antibodies (bsAbs).** a. scheme of antibody IgG, Fab fragment, scFv (Single-chain variable fragment), VHH single domain antibody and camel-like antibody. b. Currently available bsAbs used in the clinic or research. BiTE, bispecific T cell engager; DART, Dual affinity retargeting; DVD-Ig, dual variable domain immunoglobulins (Kontermann & Brinkmann, 2015).

Bispecific antibody (bsAb)

The idea of combining building blocks of Ig-like domains has been expanded to develop bsAbs, which is a group of man-made recombinant molecules that have two different antigen specificities (Kufer, Lutterbüse, & Baeuerle, 2004). The concept of adding an additional antigen binding site to an antibody was first introduced by Alfred Nisonoff and co-workers 50 years ago (Nisonoff, Wissler, & Lipman, 1960). Ever since then, the design of recombinant bsAbs has been evolved ranging from relatively small proteins that only contain two linked antigen-binding epitopes to large Ig-like molecules with the insertion of additional antigen recognition domain/domains (Fig. 1.8 b) (Garber, 2014). In general, based on whether there is an Fc region (fragment crystallizable region), bsAbs can be classified into two classes (Fig. 1.8 b). Most Fc-based bsAbs use IgG as the backbone and building upon it. The benefit of using IgG rather than the other four antibody isotypes is due to its smaller size, longer half-life, and higher affinity. Furthermore, the Fc region in IgG facilitates bsAb purification and contributes to improved solubility and stability. The effector function such as antibody-dependent cellular cytotoxicity (ADCC), complement fixation (CDC) mediated by Fc is also retained, which might be desirable for therapeutic applications (T. H. Kang & Jung, 2019).

One example of Fc-based bsAb is the Double-variable domain (DVD)-Igs (Jakob et al., 2014) (Fig. 1.8 b). Its dual binding specificity is achieved by introducing a second VH domain to the VH of the backbone heavy chain and a second VL that is fused to the light chain. Such engineering results in a tetravalent bsAb and there are also similar formats shown in the (Fig. 1.8 b). A simplest bsAb can be produced by combining two different heavy and light chains expressed in the same producer cell and result in a bivalent Fc-based antibody. However, this process is highly reliant on random assembly and leading to a substantial number of non-functional antibodies. A breakthrough in the generation of bivalent bsAb was the development of the knobs-into-holes technology (Shatz et al., 2014). Here, different mutations are introduced in the heavy chain CH3 domain forcing heterodimerization between the two Fc regions, resulting in asymmetric antibodies (Fig. 1.8 b). To ensure the correct light chain pairing, CrossMab technology was developed (Klein et al., 2019). They swap the CH1 domain of one heavy chain with the constant domain of the corresponding light chain (Fig. 1.8 b).

Small bsAb lacks an Fc region, usually only comprised of VH and VL domains of the two antibodies. A successful example was created by Bispecific T cell Engager (BiTE) technology (Mølhøj

et al., 2007; Nagorsen & Baeuerle, 2011), which they fuse two scFv fragments generating tandem scFv molecules with a flexible peptide linker. Alternatively, the V region from two antibodies, A and B, are expressed as two molecules, VHA–VLB and VHB–VLA, with the domains connected by a short peptide linker, forcing heterodimerization of the two proteins (Holliger, Prospero, & Winter, 1993). Concurrently, a disulfide bond was introduced to stabilize the structure (Kontermann & Brinkmann, 2015).

Two attractive features of bsAb over a conventional antibody are that they are able to interfere with multiple ligands concurrently or bring targets in proximity such as linking an effector cell to a target cell. For example, BiTE recognizes tumor cells and at the same engage tumors specific effector to kill. Based on various physiological conditions, the form of bsAb used will vary. The innovation of novel formats of bsAb will fulfill the needs of all sorts of applications.

1.2 The immune response to *P. falciparum*

Malaria is a mosquito-borne disease prevails in Africa. There are six strains of parasite that can infect human and the most deadly of which is *P. falciparum* (Cowman, Healer, Marapana, & Marsh, 2016). Despite the fact that anti-malaria drugs are effective in malaria control, in 2018, there are still 230 million cases in African region, accounting for approximately 400,000 deaths every year (World Health Organization, 2019). The idea of malaria eradication has been brought for more than a decade, we have yet the strategy to combat such disease in terms of an effective vaccine or strong vector control approaches (Macdonald, 1956). A very interesting fact is that *P. falciparum* is a very old species, as old as human. This suggests that the human immune system and the parasite must have co-evolved. Indeed, malaria parasites have developed tons of evasion strategies to be shed from the host immune system, making the natural defense inefficient (Hisaeda, Yasutomo, & Himeno, 2005). An individual that develops protective immunity after recurrent exposures to parasite infection can still be reinfected (Cowman et al., 2016). Therefore, knowledge that underlying the mechanisms of acquisition of immunity to natural malaria infection will undoubtedly facilitate in the development of high efficacy vaccines that would reduce malaria disease and death.

1.2.1 *Plasmodium* life cycle

P. falciparum has a relatively complex life cycle involving two hosts: human and mosquito (Bousema & Drakeley, 2011) (Fig. 1.9). The cycle initiates with a female *Anopheles* mosquito bite on the human skin for a blood meal. The saliva of the carrier mosquito contains *P. falciparum* sporozoites and a small number of sporozoites are delivered into the skin where they can stay for hours or days. The sporozoites can cross the endothelium of the capillaries in the skin and enter the bloodstream toward the liver. The sporozoites can also traverse kupffer cells and hepatocytes then eventually infect hepatocytes. Following the replication within hepatocytes, the sporozoites multiplex up to 40 thousand folds and produce a large number of asexual blood stage parasites known as merozoites. Before merozoites enter the bloodstream and replicate in the red blood cells, infected individuals have no clinical symptoms. In the bloodstream, merozoites start 48h cycles of red blood cells (RBCs) invasion, replication, rupture, and the release of new progenies (Fig. 1.9). Upon rupture of the infected erythrocytes (IEs), a large number of parasites are produced and released which causing clinical symptoms such as headaches, fever and lethargy. During the development inside the RBCs, blood stage parasites dramatically remodel RBC and export proteins to the surface of the RBC membrane (Wahlgren, Goel, & Akhouri, 2017). These proteins are known as variant surface antigens which I will explain in more detail in the following section. The life cycle in the human host of *P. falciparum* is completed when the asexual blood stage parasites differentiate into female and male gametocytes following a mosquito's blood meal taken up. When the female and male gametes meet in the mosquito midgut, they form ookinetes that will ultimately differentiate into sporozoites and develop in mosquito's salivary glands prepared for the next round of infection.

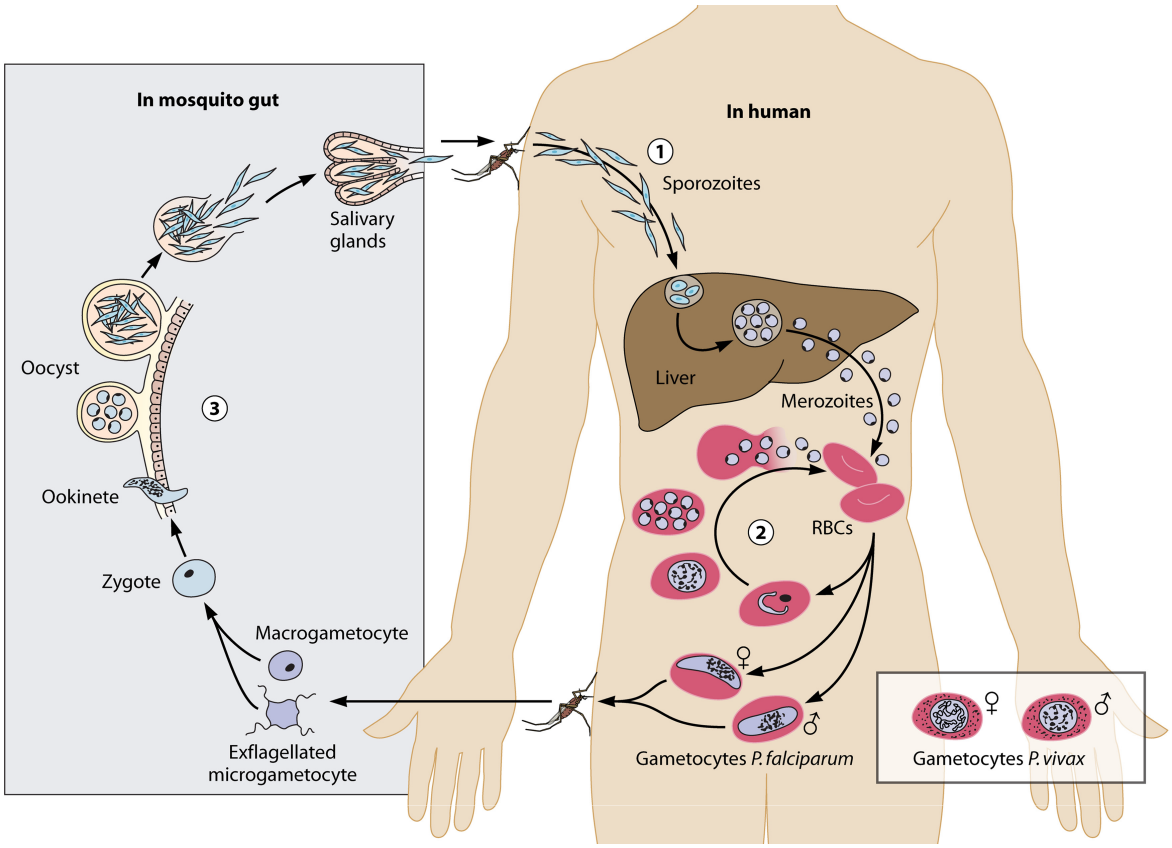

Figure 1.9. **Life cycle of the Plasmodium parasite in its mosquito vector and human host.** Malaria infection starts when female Anopheles mosquitoes inject sporozoites into the human skin. The sporozoites then migrate to the liver, where they infect hepatocytes and multiply to form eco-erythrocytic schizonts that rupture to release thousands of merozoites into the bold stream. These merozoites can invade healthy erythrocytes and start a 48-hour blood stage cycle. The merozoites are matured to become trophozoites and undergone nuclear division to form schizonts. The rupture of schizonts results in merozoites released in the bloodstream. Some blood stage parasites differentiate into sexual stage gametocytes that can be picked up by mosquitoes. In the mid-gut of mosquito, female and male gametes fuse to form ookinetes and differentiate into sporozoites, which can then invade mosquito's salivary glands for following rounds of infection. Figure adapted with permission from (Bousema & Drakeley, 2011).

1.2.2 Malaria variant surface antigens

Introduction

The blood stage is one of the most crucial periods in the life cycle of *P. falciparum*. In this stage, parasites live inside the IEs where they proliferate and differentiate. In order to acquire the maximum proliferative capacity and survival, parasites express proteins on the surface of IEs to import nutrients, bind to adjacent RBCs, and host microvasculature to avoid immune clearance (Goel et al., 2015; Maier, Cooke, Cowman, & Tilley, 2009; Tiller et al., 2008). The surface proteins that involved in adherence are encoded by three major multi-gene families: *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), repetitive interspersed families of polypeptides (RIFINs), and subtelomeric variant open reading frame (STEVOR). In each of the families, clonal variants of the same gene confer antigenic variation and evasion of host immunity (Chen et al., 1998). These surface molecules are known as variant surface antigens (VSAs) that are suggested to facilitate the sequestration of IEs in the microvasculature and block the blood flow (David, Hommel, Miller, Udeinya, & Oligino, 1983). VSA exposure to a host immune response triggers an immune selection that constantly induce switching of antigenic epitopes avoiding immune recognition while maintaining protein function to establish an infection (Roberts et al., 1992). This section will give a brief introduction to the variable gene families of *P. falciparum* and the with a focus of RIFINs.

PfEMP1

PfEMP1 family is the first and best characterized VSA in *P. falciparum* and is primarily expressed at the trophozoite and schizont stage of the parasite life cycle (Kraemer & Smith, 2006). The members of the PfEMP1 family are large transmembrane proteins (200-400kDa) encoded by var genes (Baruch et al., 1995). There are about 60 var genes per parasite genome, and they are expressing in a mutually exclusive fashion (Dzikowski, Frank, & Deitsch, 2006). Despite the variation within one particular parasite isolate, the repertoire of genes also differs substantially between the parasite strains, which confers the second level of diversity. Expression of diverse PfEMP1 allows binding of PfEMP1 to a range of receptors such as CD36, ICAM1 (intercellular cytoadhesion molecule 1), chondroitin sulphate A (CSA) (Reeder et al., 1999), complement receptor 1 (CR1) (Rowe, Moulds, Newbold, & Miller, 1997), heparan sulfate (HS) (Chen et al., 1998), thrombosondin and others on the vascular endothelial cells mediating a process called sequestra-

tion. Adhesion occurs via specialized PfEMP1 domains, known as Duffy binding like (DBL) and cysteine-rich interdomain region (CIDR). The consequence of PfEMP1 sequestration in the brain or placenta may lead to the pathogenesis of severe malaria in children or pregnant women (Andrews & Lanzer, 2002). PfEMP1 also mediates rosette, a process in which the uninfected RBCs aggregate around a central IEs, which also promotes RBC sequestration (Juillerat et al., 2011).

RIFIN

RIFIN, the largest family of antigenically variable molecules in *P. falciparum*, are small (28 - 45kDa) (Kyes, Rowe, Kriek, & Newbold, 1999), adhesive proteins that has 150-200 copies per haploid parasite genome based on genomic sequencing data (Fernandez, Hommel, Chen, Hagblom, & Wahlgren, 1999). The molecules are exported onto the membrane of IEs and contribute to the rosetting and sequestration of *P. falciparum* (Goel et al., 2015).

Gene and protein

RIFIN proteins are encoded by the rif genes in the subtelomeric region, and a clonal copy of the gene usually spans around 1000bp (Petter et al., 2007). The protein is constructed in an architecture composed of a signal peptide, a PEXEL motif, a conserved domain, a variable region, and following a conserved C-terminal domain (Andersson et al., 2019)(Fig. 2.8 b).

The N-termini of the RIFINs are exposed to the extracellular environment, leading to interactions with host ligands/receptors. The majority (>70%) of RIFINs has an insertion of 25 amino acids at the N-terminus, and these RIFINs are clustered as group A-RIFIN (Joannin, Abhiman, Sonnhammer, & Wahlgren, 2008). Conversely, around 30% of RIFINs that in the absence of 25 amino acid regions are named as group B-RIFIN (Joannin et al., 2008). RIFIN molecule has two predicted hydrophobic regions, one at C-terminal which is stably inserted in the membrane. The other is located at the N-terminal constant region, which has been suggested to function as a hydrophobic patch that dimerizes the RIFINs (Goel et al., 2015). A study using purified A-RIFIN revealed that RIFINs migrate as a dimer in size-exclusion chromatography experiment (Goel et al., 2015). RIFIN contains a PEXEL motif where the peptides can be cleaved and transported to various locations. It has been suggested that different number of cysteine residues within the PEXEL sequences have conferred A-RIFIN and B-RIFIN distinct destinations thereby possibly unique functions (Bultrini et al., 2009). For example, A-RIFINs are mainly expressed on the

surface of IEs responsible for host interaction, whereas B-RIFINs emerge to be mostly retained inside the parasite for some unknown functions (Mwakalinga et al., 2012). The variable region of RIFINs exhibits the most unique sequences across the family as well as in different parasite isolates and accounts for the main feature of antigenic variation (Joannin et al., 2008).

RIFIN Expression

In general, RIFINs are expressed on the surface of IEs during the late blood stage such as the trophozoite stage (Petter et al., 2007). However, recent evidence has shown that they can also be expressed at the apex of merozoites, in gametocytes and in sporozoites (Mwakalinga et al., 2012), which leads to the speculation of varying functions (Wahlgren et al., 2017). Unlike var gene expression that is being strictly regulated to allow only one particular PfEMP1 expressed at a given time, parasites are capable of transcribing multiple rif genes concurrently, but maybe expressing one at a relatively high level (Abdel-Latif, Khattab, Lindenthal, Kremsner, & Klinkert, 2002). RIFIN and PfEMP1 are usually co-expressed on the surface of a given IE and both involved in binding to host cell receptors or serum proteins via a complex mechanism. It was suggested that IEs can bind via multiple receptors (Ockenhouse et al., 1992) therefore creating a synergistic effect on adhesion of IE (McCormick, Craig, Roberts, Newbold, & Berendt, 1997). It is not clear how this interaction is regulated but it is likely that such complicity renders the parasite survival advantage within the host environment over the simpler adhesion pattern.

Function of RIFIN

Due to the large copy number, the role of RIFIN is not as well characterized as PfEMP1. Nonetheless, RIFINs have been shown to mediate the sequestration of IEs in animal models and human (Goel et al., 2015), a phenotype that is linked to severe malaria. Specifically, it has been shown that RIFIN also promotes rosette formation during malaria infection (Nunes-Alves, 2015). Immunize animals with purified RIFIN peptides can generate antibodies that disrupt rosettes (Ch'ng et al., 2017). Furthermore, naturally acquired antibodies against A-RIFINs showed that rosetting parasites might lose their ability to form rosettes (del Pilar Quintana et al., 2018). These evidences suggest that RIFIN function as rosetting mediator. Although both A-RIFIN and pfEMP1 mediate rosetting, it seems that A-RIFIN mediates both blood group A and blood group O, while PfEMP1

only mediates blood group O (Goel et al., 2015). This finding suggests an overlapping but not redundant function between PfEMP1 and RIFIN. In contrast, B-RIFINs are mainly detected in the late schizont and merozoite stages (Mwakalinga et al., 2012), which suggests that they may be involved in host cell invasion. Future studies are needed to understand the function of B-RIFIN.

Concurrently, some RIFINs may have other versatile functions considering the large copy number and various expression locations. Recently, we identified two RIFINs that are involved in binding to a mutated form of host inhibitory receptor LAIR1 (Tan et al., 2016). A following study has shown that a set of RIFINs are able to bind to another inhibitory receptor LILRB1 (Saito et al., 2017). These data suggest that RIFINs may have a role in targeting host inhibitory receptors to evade host immunity. However, there are only a few studies on RIFIN so far, future in-depth studies are required to resolve the mystery of their roles in the parasite pathogenesis.

STEVORs and other VSAs

Other VSAs expressed by *P. falciparum* include STEVOR (Niang, Yam, & Preiser, 2009), SURFIN (surface-associated interspersed protein), PHIST (Plasmodium helical interspersed subtelomeric) (V. Kumar, Behl, Sharma, Sharma, & Hora, 2019), CLAG (cytoadherence-linked asexual protein) (Ocampo et al., 2005), ETRAMP (early transcribed membrane protein), and PfMC-2TM (*P. falciparum* Maurer's cleft two transmembrane) (Tsarukyanova, Drazba, Fujioka, Yadav, & Sam-Yellowe, 2009).

STEVOR family which has 28 copies of the stevor gene in the reference strain is closely related to RIFIN regarding their gene structure and function. For example, stevor gene also composed of a signal peptide, a PEXEL motif, a variable region and a C-terminal transmembrane domain (Niang et al., 2009). Furthermore, STEVORs have been reported to bind to glycophorin C on the RBC surface to facilitate the formation of rosettes and merozoite invasion (Niang et al., 2014). STREVORs are expressed at various stages of the life cycle, some in merozoites and gametocytes similar to B-RIFIN. Antibodies to STEVORs weakly inhibit merozoite invasion suggesting a role in merozoite invasion (Khattab & Meri, 2011).

Overall, the exact function of those VSA required further study.

1.2.3 The antibody response to parasites

Human immunity to malaria

Naturally acquired immunity to malaria can develop in individuals after repeated expose to *P. falciparum* infection and confer protection to severe disease and death (Crompton et al., 2014). However, how this protection is formed and how to recapitulate this event through vaccination is still not clear. In order to address those questions, tools like animal models that employ rodent-specific parasite have been developed (Wykes & Good, 2009). Unlike other diseases in which the pathogenic elements are the same for both human and animal models, the fact that *P. falciparum* only infection humans limits the use of related animal models. One way to study human malaria is to look at the real infection in individuals. For example, volunteers will be exposed to the bites of laboratory-reared *P. falciparum*-infected mosquitos, a procedure that is strictly monitored (Roestenberg, de Vlas, Nieman, Sauerwein, & Hermsen, 2012). Alternatively, a direct intravenous administration of IEs in healthy individuals could be carried out to study early immunological response to blood stage infections which is also under strictly controlling of the parasitemia in the blood (Engwerda, Minigo, Amante, & McCarthy, 2012). Both approaches have been used during the past decades and provide many insights to the immunological response to parasites. Recently, approaches that challenge volunteers with either infectious sporozoites under chemoprophylaxis control or whole attenuated sporozoites were developed and were tested against controlled human malaria infection (CHMI) (Gómez-Pérez et al., 2015), a protocol that can be achieved by inoculation of sporozoites through a mosquito bite or by direct injection of sporozoites or *Plasmodium*-infected blood (Behet et al., 2014). All of these approaches mentioned above are utilized to address key questions in the context of malaria infection and I will focus on a few in this section: how is the antibody response to *P. falciparum* infection? How is the humoral response to blood-stage infection? What is the role of antibody response to VSA?

Antibody response

Antibody plays an essential role in host immunity to malaria was first described by Cohen *et al* in 1961 (Cohen, Mcgregor, & Carrington, 1961). They showed that transferring of purified IgG from malaria-immune adults to children who had acute malaria confers a profound reduction in parasitemia and resolution of fever (Cohen et al., 1961). *P. falciparum* has a complex genome with

more than 5,000 genes encoding for ∼ 5000 proteins (Cowman et al., 2016). Thus far, we have yet fully understood which are the target proteins among ∼ 5000 protein in *P. falciparum* of these protective antibodies, and the underlying mechanisms of protection. Another mystery is whether those antibodies have a uniform target or a cocktail of antigens. To address the second mystery, Newbold *et al* developed antibody agglutination assay to distinguish between antisera that contain variant specific antibody or cross-reactive antibody or both (Newbold, Pinches, Roberts, & Marsh, 1992). The results showed that the predominant agglutinating antibody response is variant specific (Newbold et al., 1992). Their finding and later evidence suggested that protective antibody can only be generated after years of exposure (Baird et al., 1991). Why could an individual only generate variant specific immunity? One plausible explanation is that the genetic diversity of variant parasite proteins, and parasite's ability to switch antigens on the surface of IEs only allow host immunity to see a specific antigen at a time, thereby only one specificity was generated. It indicates that an individual is required to experience a sufficient (large) number of parasite strains to be able to accumulate protective antibodies. In this regard, how can we design a vaccine that resembles this long history of multiantigens experience? To address this question, it will be crucial to understand what types of antibodies are generated and what is the corresponding targets during the infection.

Antibody to IE-VSA

Most antibodies that interfere with the development of parasites are generated during the blood stage, where VSAs are the potential targets for the immune response as they are exposed for almost half of the asexual cycle (Turner et al., 2011). VSA on IEs as I described in the previous section is involved in two important processes of malaria pathogenies: sequestration and inflammation (induction of proinflammatory cytokines). Naturally acquired antibody against VSA is able to block such events, and in particular, PfEMP1 is a primary antigenic target (Jensen, Adams, & Hviid, 2019).

Antibodies to PfEMP1 have been shown to be important mediators of protection against symptomatic malaria (del Pilar Quintana et al., 2018). Specifically, a study conducted in Tanzanian children found an association between high levels of PfEMP1 (containing an ICAM1-binding domain) specific antibodies and a reduced risk of severe malaria (Oleinikov et al., 2012). Consistent with this finding, a study carried out in children from Papua New Guinea described antibody re-

sponse to a specific ICAM1-binding domain in PfEMP1 was associated with reduced risk in high-density clinical malaria (Tessema et al., 2018). Furthermore, children who experienced severe malaria had significantly lower levels of antibodies to such domain, suggesting a protective role of this antibody (Travassos et al., 2018). Although PfEMP1 which contains ICMA1 binding domain appears to be the target, the exact gene/target of those protective antibodies is not easy to be assessed. Most of the studies are based on association analysis which could not specify a particular PfEMP1 variant. The only parasite gene products has been identified to have a causal relationship to severe disease is var2CSA, a PfEMP1 molecule that enables IEs to sequester in the placenta of pregnant women where the var2CSA ligand chondroitin sulfate A (CSA) is selectively expressed, causing damage to the placenta, fetus and mother (Barfod et al., 2007). The unique feature of var2CSA and its conservation in pregnant women makes it a potential vaccine for placental malaria. For this I will discuss in more detail in the malaria vaccine section.

Antibody against RIFIN

Although antibodies against RIFIN or STEVOR has been little studied as compared to PfEMP1 specific antibodies, collective evidence showed that naturally acquired RIFIN antibodies are a dominant component of the overall response against IE VSAs (Abdel-Latif et al., 2004; Abdel-Latif et al., 2002). Several studies have reported that high level of antibodies to recombinant RIFIN were detected in the majority of adult from high malaria transmission region, while the prevalence of RIFIN specific antibodies were low in children (Andersson et al., 2019; del Pilar Quintana et al., 2018; Turner et al., 2011). Moreover, it has been demonstrated by studies that the RIFIN antibody may has a protective effect on several malaria (Abdel-Latif, Dietz, Issifou, Kremsner, & Klinkert, 2003). For example, rapid parasite clearance was observed in children with elevated levels of RIFIN antibodies, and these antibodies could still be detected after 2 years (Abdel-Latif et al., 2003). Furthermore, asymptomatic children were found to have higher levels of RIFIN antibodies as compared to the children with severe disease (Abdel-Latif et al., 2003). In contrast, a recent investigation in the sera of Cameroon children showed an equivalent level of RIFIN-A specific antibodies in mildly infected group and the complicated infected group suggested that the association of RIFIN antibody and protection is more complicated. Given the large number of RIFIN variants, it is speculated that antibody repertoire is also diverse. Due to limited research tools, these questions will be addressed in future studies.

It is speculated that antibodies to STEVOR may have a role in clinical protection given the structural similarity of STEVORS and RIFINs. A study demonstrates that STEVOR specific antibody weekly inhibit merozoite adhesion and invasion in vitro. More studies are required to understand natural immunity to this protein (Niang et al., 2014; Niang et al., 2009).

1.2.4 Malaria vaccines

Introduction

Although we have yet developed a highly effective vaccine against *P. falciparum*, advances have been made during the last decades and are continuing to add new knowledge that needed for the establishment of next-generation vaccines (Campeotto et al., 2017; Fried & Duffy, 2015; Lyke et al., 2017; Modjarrad & Koff, 2016). In this section, I will briefly summaries currently available vaccine candidates and discuss how the use of information from neutralizing antibody molecular details could facilitate the development of an effective vaccine.

Vaccine candidates

In general, vaccines against malaria can be classified into two categories: pre-erythrocytic-stage vaccine and erythrocytic vaccine. Most efforts have been made in the vaccines against pre-erythrocytic-stage with a goal to induce sterile immunity. One of the most advanced vaccine candidate RTS, S/AS01 (RTS,S Clinical Trials Partnership, 2015). targets the circumsporozoite protein (CSP) on the surface of sporozoites has now passed through Phase III and move to pilot implementation trials. The vaccine is composed of part of CSP protein (including a region with NANP-repeat) that was fused to the hepatitis B antigen. This approach provides an example of using the CSP-based vaccine to fight against malaria, although the results showed only partial protection of modest duration, which required improvement. Following this idea, improved CSP-based vaccines that use infectious sporozoites under chemoprophylaxis control or whole attenuated sporozoites were developed and tested against CHMI (Seder et al., 2013; Tan et al., 2018; Tiller et al., 2008). The latter approach has been shown to have 53% protection against a heterologous challenge (Lyke et al., 2017). Previously, by investigating the antibody response at the molecular level, we showed that Tanzanian volunteers who were immunized with the Sanaria PfSPZ vaccine generate protective monoclonal antibodies that bind to CSP and recognizes unique

epitopes in both the N terminus and NANP-repeat region (Tan et al., 2018). The N terminus epitope was found to be absent in the RTS,S vaccine (RTS,S Clinical Trials Partnership, 2015). This study provides a proof of the concept that the molecular details of neutralizing antibodies against malaria could provide critical information for improving vaccine design.

While we are waiting for the good news from CSP-based vaccines, such strategy still faces challenges in terms of scalability, immunogenicity in Africans, and the breadth of protection. As a complementary approach, vaccines that target blood stage infection or more specifically, the invasive merozoite form would have potential value to the field (Chan et al., 2012). Recently, *P. falciparum* merozoite surface PfRH5-PfCyRPA-PfRipr (RCR) complex, a conserved elongated protein trimer that binds to erythrocyte basigin receptor, was characterized as a promising candidate (Alanine et al., 2019; Campeotto et al., 2017; Ragotte, Higgins, & Draper, 2020; Travassos et al., 2018). PfRH5-based vaccine, a leading target, is now underway of Phase I/II clinical trials and the RCR components PfCyRPA- and PfRipr-based vaccine are also under developing. Studies of the first clinical trial of PfRH5-based vaccine identified (Alanine et al., 2019) highly cross-reactive neutralizing human monoclonal antibodies that direct blocking basigin binding and revealed critical epitopes for the targeting. Moreover, a subset of non-neutralizing antibodies was isolated and found to be important for synergizing with a set of neutralizing antibodies by slowing the ability of parasite to invade erythrocytes (Alanine et al., 2019). These data provide another example of how atomic details of monoclonal antibody could provide a roadmap for structurally guided vaccine design which aims to elicit both types of antibodies (Julien & Wardemann, 2019).

However, beyond targeting the merozoites form of the parasite in the erythrocytic-stage, the vaccine against the IEs forms is rarely being brought up (Chan, Fowkes, & Beeson, 2014). This is partly due to the parasite's reliance on redundant host-pathogen interactions, which exploits multiple pathways for red blood cell invasion. In addition, the polymorphism of essential ligands on IE surface which often results in strain-specific immunity. However, one exception is the placental malaria vaccine candidate, which targeting a particular erythrocyte membrane PfEMP1, called var2CSA, that enables IEs to sequester in the placenta in pregnant women (Doritchamou et al., 2019). Evidence has shown that var2CSA adheres to chondroitin sulfate A (CSA), which is selectively expressed on placental proteoglycans, causing damage to the placenta, fetus and mother (Srivastava et al., 2010). After all, targeting blood stage IE is challenging due to the high degree of variability. More advances in the function and structure of IE VSA would allow us to open up such vaccine strategy.

1.2.5 Broadly reactive antibodies against malaria VSA

As I described in the previous sections, VSAs are important targets of naturally acquired immunity against malaria. Although antibody response to VSA is predominantly variant specific, it does not rule out the possibility of the existence of protective broadly reactive antibody. The identification of such antibody against VSA could be beneficial for generating new therapeutic tools and for guiding vaccine design. To investigate whether broadly cross-reactive antibody response to diverse variants are established in malaria-experienced individuals, our laboratory screened sera of a cohort of 577 adults living in the malaria-endemic region using improved mixed agglutination assay and isolated a number of human monoclonal antibodies that recognize a spectrum of IE isolates. By using the Liquid chromatography-mass spectrometry (LC-MS), our laboratory identified members of RIFIN as the target of these antibodies. Furthermore, by analyzing the antibody at the molecular level, our laboratory discovered that these antibodies acquired such broad reactivity through a novel mechanism of insertion of a large DNA segment between the V_H and D_H gene in Ig locus. The inserted fragment encodes for a mutant form of the extracellular domain of the LAIR1 inhibitory receptor, which is expressed on immune cells that specific for collagen. Mutations in the LAIR1 domain abolished the binding to host collagen while retaining the binding to RIFINs on IEs. This study demonstrates a novel mechanism of antibody diversification by DNA insertion in the VH exon and implies a new function of RIFIN in binding to host immune receptors. Abstract adapt from (Tan et al., 2016).

Follow-ups studies resolved the crystal structure of the LAIR1-containing antibody showing that the LAIR1 domain is presented at the tip of the VH region and exposed for IE recognition (Hsieh & Higgins, 2017). Owing to the high hydrophobic nature of RIFIN, the structure of RIFIN in complexed with LAIR1-containing antibody has yet to be identified. This will be addressed in my thesis work.

This study opens up many questions, for example, what is the prevalence of LAIR1-containing antibodies in African donors? Are there any other receptor-containing antibodies against malaria or other pathogens? What is the role of other RIFN variants? What is the conserved epitope on RIFINs targeted by LAIR1-containing antibody? I am trying my best to address some of these questions in my PhD study.

1.3 Rationale and aims of the project

1.3.1 Investigate the prevalence of LAIR1-containing antibodies

My PhD project has three main goals, all of which are following the discovery of LAIR1 insertion in the antibody against malaria, where we identified two individuals who produce broadly reactive antibodies with a LAIR1 insertion in the antibody VH region.

The discovery makes us wonder, how frequent is the LAIR1 insert that ends up in an antibody. Answering to this question may provide insights in how host immunity is shaped under malaria infection and the possibility of recapitulating such events via vaccination. To do so, we screened sera from two large cohorts from Tanzania and Mali by using the beads assay for fishing out LAIR1-specific IgG or IgM. Once a serum showed positive for LAIR1-specific beads, the corresponding B cell clone were analyzed for their genomic and cDNA organization to verify the LAIR1 insertion. This is the first goal of my PhD study.

1.3.2 Searching for receptor-based antibody

The goal of my second project is to investigate the possibility that *P. falciparum* targets host inhibitory receptors. Our laboratory has initially reported that somatic mutations in the LAIR1 domain are required for high affinity binding to certain RIFINs. Surprisingly, in our second study (the results of my first PhD project where we discovered LAIR1 insertion in the switch region of Ig and forming an elbow LAIR1 antibody), the LAIR1 antibodies generated by insertion in the switch region are not, or only minimally mutated, yet these cells are selected by natural infection. The data obtained so far suggest that certain parasites can indeed bind to unmutated LAIR1 (endogenous LAIR1 receptor), indicating that *P. falciparum* targets LAIR1, an inhibitory receptor that is broadly expressed by T cells, B cells and myeloid cells. Therefore, I hypothesize that targeting of inhibitory receptors may represent an evasion and suppression strategy used by the parasite to interfere and disable the immune response. Furthermore, a study from *Saito et al* described a new inhibitory receptor LILBR1 was able to be recognized by parasite isolated from malaria patients (Saito et al., 2017). I therefore screened sera from a Malian cohort for their possibility to obtain LILIRB1-containing antibodies during malaria infection. This project will provide insights in

the basic aspects of *P. falciparum* immune evasion and explores the antigen of naturally acquired antibody.

1.3.3 Engineering of bispecific antibodies

The last goal of my PhD project is to adapt the new modality of exon insertion into the immunoglobulin gene to the generation of novel formats of bsAbs for various therapeutic applications. Specifically, I tested different types of inserts, including Ig-like domains, VHH domains and scFvs. As it occurs for currently developed bsAbs, the new formats of bsAbs are required to meet the following criteria: they must be stable, easy to produce, persist longer in circulation and should not be immunogenic. In our settings, the IgG constant regions are preserved, which not only allow ADCC to be triggered, but also allow the antibody to be recycled by the FcRn (neonatal Fc repeptor). Moreover, the configuration of LAIR1-containing antibody is created by nature during malaria infection, thus it is likely that the antibody has the potential to be manufactured and be harmless to the host. Essentially, I aim to determine whether immunoglobulins edited to include additional domains in the antibody elbow region are able to exhibit bispecific binding to cognate antigens. This approach may serve as a proof of concept to demonstrate the potential of using this new bsAbs format for therapeutic and research purposes.

2

Results

2.1 Public antibodies to malaria antigens generated by two LAIR1 insertion modalities

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2.1.1 Author contributions

K.P. characterized genomic DNA, analysed the data and wrote the manuscript; J.T. isolated new LAIR1-containing antibodies, analysed the data and wrote the manuscript; L.P. produced mutant antibodies, analysed the data and wrote the manuscript; M.F. performed bioinformatics analysis; S.B. sequenced and expressed antibodies; Y.C. helped with genomic sequencing; C.S.-F. immortalized memory B cells; T.W. helped with MinION sequencing; D.J. performed cell sorting and analysis; M.A. performed protein analysis; A.A. performed P. falciparum culture; F.M.N., S.J., O.K.D., B.T., I.Z., C.D. and P.C.B. provided cohort samples; T.M.T. and P.D.C. provided cohort samples and analysed the relationship between LAIR1-containing antibodies and malaria risk; F.S. provided supervision; A.L. provided overall supervision, analysed the data and wrote the manuscript.

2.1.2 Results

Prevalence of LAIR1 antibodies in African donors

LAIR1-containing antibodies were initially isolated from two Kenyan donors who were selected from a large cohort of more than 500 individuals for their capacity to produce broadly reactive antibodies to *P. falciparum*-infected erythrocytes (Tan et al., 2016). To establish the prevalence of LAIR1-containing antibodies in malaria-exposed individuals, we screened plasma samples from two large cohorts in Tanzania (Mwakasungula et al., 2014) and Mali (Tran, Li, et al., 2013). To identify LAIR1-containing antibodies irrespective of their specificity for parasite isolates, we

developed a two-determinant immune assay using beads coated with anti-LAIR1 or control antibodies. Six out of 112 Tanzanian donors (5.4%) and 57 out of 656 Malian donors (8.7%) had detectable levels of LAIR1-containing IgG (Fig. 2.1 a). In addition, 2–4% of African donors had LAIR1-containing IgM, with no or variable levels of LAIR1-containing IgG (Fig. 2.1 b, c). By contrast, only 3 and 4 out of 1,043 European blood donors showed a low positive result in the assays for LAIR1-containing IgG and IgM, respectively. The presence of LAIR1-containing antibodies was confirmed by the isolation of 52 immortalized B cell clones from seven East and West African donors (data not shown), whereas we were not able to isolate LAIR1-containing monoclonal antibodies from four European donors that showed serum reactivity. The finding that 5–10% of individuals living in malaria-endemic regions produce LAIR1-containing antibodies is suggestive of a public antibody response.

To investigate the contribution of LAIR1-containing antibodies to the response to infected erythrocytes, we dissected this response at the polyclonal and monoclonal levels. Staining of infected erythrocytes with plasma from selected individuals with LAIR1-containing antibodies revealed that most infected erythrocytes were recognized by the LAIR1-containing antibodies, whereas only a minority of infected erythrocytes was recognized by conventional IgG (Fig. 2.1 d). Furthermore, when immortalized memory B cell clones from four donors were analyzed for reactivity to infected erythrocytes and for the presence of LAIR1, all of the infected erythrocyte-recognizing monoclonal antibodies from three donors and most of such antibodies from the fourth donor contained the LAIR1 insert (Fig. 2.1 e). These findings suggest that, in certain individuals, circulating antibody and memory B cell responses are dominated by LAIR1-containing antibodies, a finding that may be explained both by the breadth of these antibodies' reactivities and by clonal expansion of the B cells that produce these antibodies.

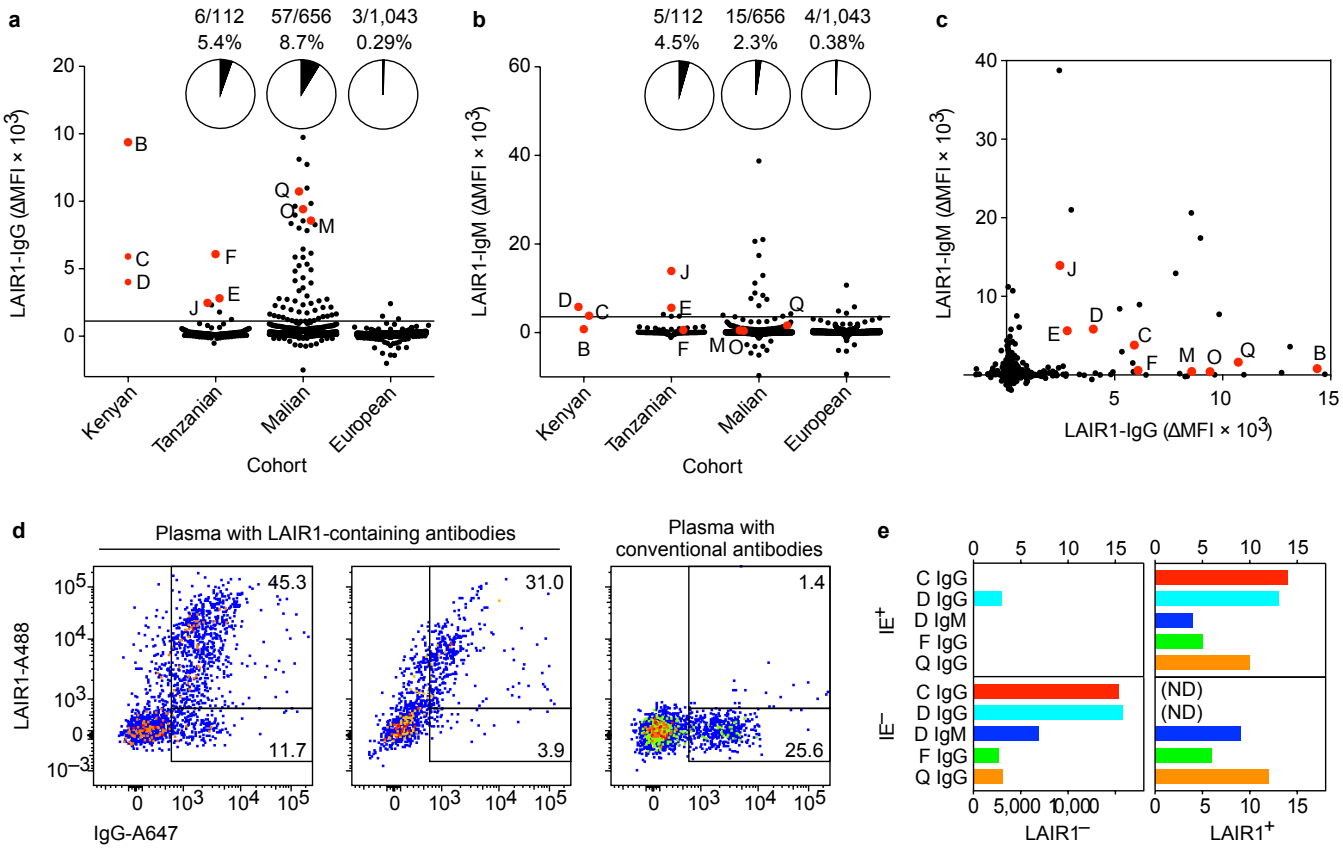


Figure 2.1. **Prevalence and dominance of LAIR1-containing antibodies in malaria-endemic regions.** a, b, Prevalence of LAIR1- containing IgG and IgM in African individuals living in malaria-endemic regions and in European blood donors. Donors from whom LAIR1-containing antibodies were isolated are highlighted in red. c, Comparison between LAIR1- containing IgG and IgM values. MFI, median fluorescence intensity. Data points with a Δ MFI value below − 2,000 are not shown. d, Staining of infected erythrocytes by LAIR1-containing IgG and conventional IgG from three representative donors. e, Dominance of LAIR1-containing B cell clones among memory B cells specific for infected erythrocytes. Monoclonal antibodies isolated from immortalized memory B cells were classified according to their ability to bind to infected erythrocytes and the presence of a LAIR1 insert. Bars show number of IgG or IgM monoclonal antibodies isolated from each donor.

Two insertion modalities of LAIR1

To investigate the nature of the LAIR1 insertion, we sequenced cDNA and gDNA from B cell clones isolated from different individuals. As reported for the first two Kenyan donors (Tan et al., 2016), the B cell clones isolated from four Malian and Tanzanian donors (E, F, O and Q) contained an insertion of the LAIR1 exon with flanking intronic sequences between the V and DJ segments, positioning the LAIR1 domain in the CDR3 loop (Fig. 2.2 a). The size of the insert and the partial splicing of the upstream intronic region differed between donors, but were identical in the sister clones isolated from each individual, indicating that in each donor the LAIR1-containing antibody response is monoclonal.

Strikingly, B cell clones from three additional donors showed a different insertion modality (Fig. 2.2 b–d). The cDNA of clones isolated from donors M (Malian) and J (Tanzanian) contained only the LAIR1 exon, which was precisely located between the J_H and CH1 domains. In both cases, gDNA analysis revealed that a DNA fragment comprising the LAIR1 exon flanked by intronic sequences was inserted into the switch μ region (Data not shown) and, by alternative splicing, gave rise to two mRNA variants with or without the LAIR1 insert. This was confirmed by the production of antibodies with or without LAIR1 in similar proportions by a single B cell clone (Fig. 2.2 e). Another example of LAIR1 insertion into the switch region was observed in donor B (Kenyan), from whom we isolated a B cell clone (MGB47) producing a truncated LAIR1-containing IgG3 heavy chain without an attached light chain (Fig. 2.2 f, g). In this clone, the gDNA carried multiple deletions that removed most of the VDJ and the entire CH1 region, leading to the production of a camel-like antibody (Bannas et al., 2017) (Fig. 2.2 d). Together, these findings highlight a new modality of exon insertion in the switch region that can add an extra domain to an antibody.

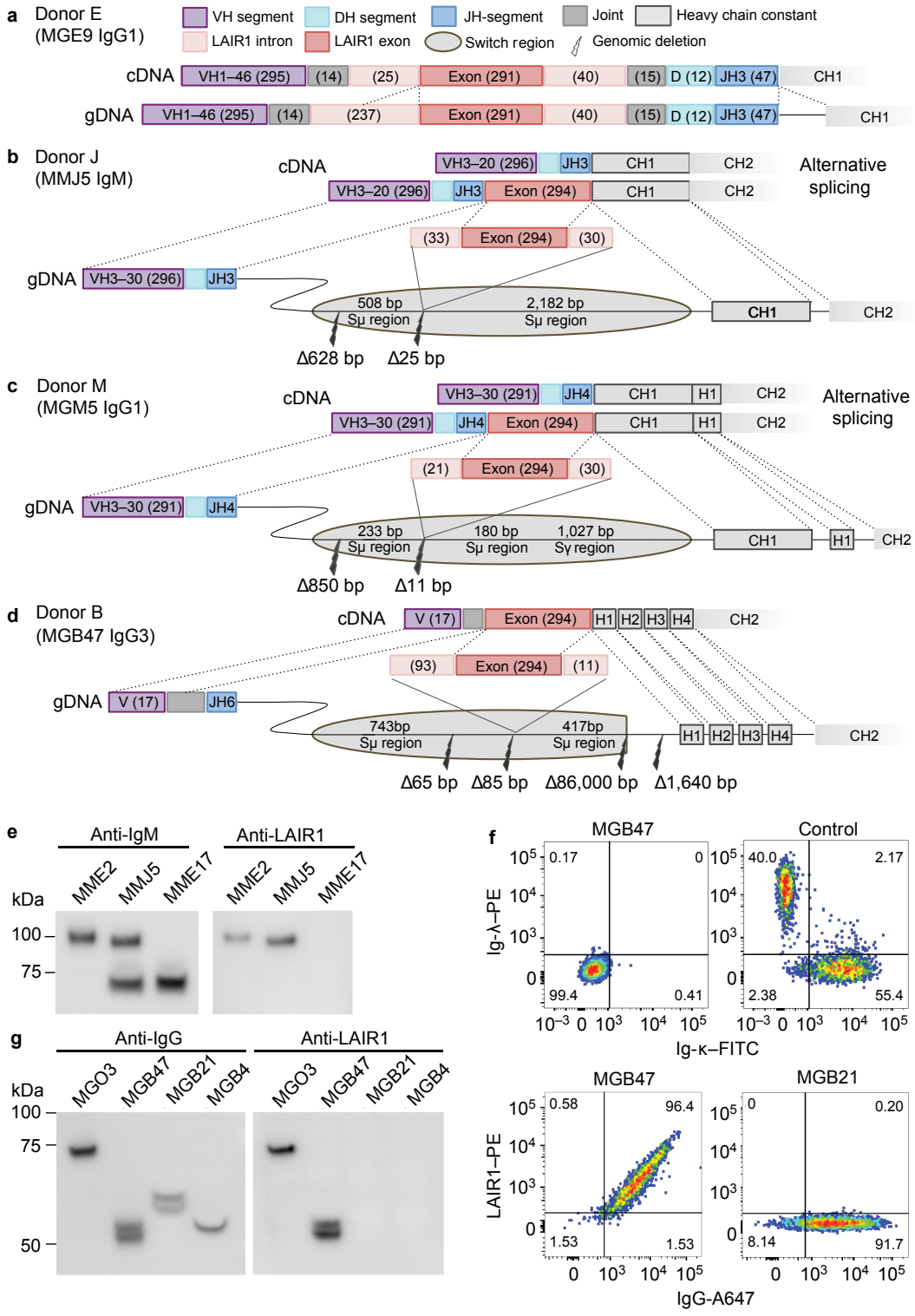


Figure 2.2. **LAIR1-containing antibodies produced by two insertion modalities.** a–d, cDNA and gDNA organization in representative B cell clones from different donors. Donors E and J are Tanzanian, donor M is Malian and donor B is Kenyan. e, Western blot analysis of culture supernatants of B cell clones with LAIR1 insertion in the VDJ (MME2, donor E) or in the switch region (MMJ5, donor J) or a conventional antibody (MME17) (n = 4). f, Surface staining of clone MGB47 showing co-expression of LAIR1 and IgG and lack of light chain (n = 3). A positive control and a LAIR1-negative clone (MGB21) are shown for comparison. g, Western blot analysis of culture supernatant of the camel-like clone MGB47 (n = 4). Also shown are supernatants from clones MGO3 (LAIR1 insertion in VDJ region), MGB21 (IgG3 control) and MGB4 (IgG1 control).

2.2 LILRB1-receptor-based antibodies target a unique set of *P. falciparum* RIFINs

2.2.1 Abstract

We recently discovered, in malaria-exposed individuals, a new type of antibodies with an insertion of LAIR1, an inhibitory receptor that is recognized by certain *Plasmodium falciparum* RIFINs expressed on infected erythrocytes (Pieper et al., 2017). These findings suggest a general mechanism for the generation and selection of receptor-based antibodies that can target the pathogen through the same binding element used for infection and pathogenesis. To address the general relevance of this phenomenon we searched for antibodies that incorporate LILRB1, an MHC class I-binding inhibitory receptor that was reported to bind to RIFINs (Saito et al., 2017). By screening sera from a Malian cohort, we identified six individuals with LILRB1-containing antibodies. Sequencing of isolated B cell clones showed insertions of large DNA fragments encoding LILRB1 domains 3 and 4 or domain 3 alone in the switch region leading to the display of the inserted domains in the VH-CH1 elbow. Using mass-spectrometry (MS), *in vitro* binding assays and information gained from the LAIR1-RIFIN structure (Kai Xu unpublished), we identified a novel set of RIFINs comprising PF3D7_1041200, PF3D7_1373400 and PF3D7_0937700 that bind to LILRB1 domain 3, which is not involved in binding to the host MHC-I. A cryo-EM structure of a LILRB1-containing antibody in complex with RIFIN-V2 domain at 3.4 Å revealed that the LILRB1 domain 3, a separate structural domain inserted between the VH-CH1 elbow, exclusively interacted with RIFIN, involving a RIFIN interface similar to that observed in the RIFIN-LAIR1 structure. Collectively, these findings support the general notion of receptor-based antibodies and identify LILRB1 domain 3 as the target of a unique set of RIFINs. A manuscript by Yiwei Chen, Kai Xu, Luca Piccoli, Mathilde Foglierini, Joshua Tan, Yaroslav Tsybovsky, Jay Gorman, Claudia Daubenberger, Peter Crompton, Peter Kwong and Antonio Lanzavecchia is ready for submission.

2.2.2 Introduction

P. falciparum genome-encoded repetitive interspersed families of polypeptides (RIFINs) are variable antigens expressed on the surface of infected erythrocytes (IEs). RIFINs are shown to mediate aggregation, rosetting and adhesion of IEs to endothelial cells, thus contributing to the pathogen-

esis of severe malaria (Goel et al., 2015). We previously reported that certain RIFINs, such as PF3D7_1400600 and PF3D7_1040300, bind to the collagen-specific inhibitory receptor LAIR1, suggesting an additional role of RIFINs in immune evasion (Tan et al., 2016). A subsequent study confirmed our observation and provided further evidence that RIFINs can bind to multiple inhibitory receptors expressed on myeloid and lymphoid cells, in particular to LILRB1 (Saito et al., 2017).

Based on the finding of LAIR1-containing antibodies, we hypothesized that B cell clones with insertions of other inhibitory receptors such as LILRB1 into antibody genes could be selected in the course of malaria infection.

2.2.3 Results

Discovery of LILRB1-containing antibodies

To Identify LILRB1-containing antibodies, we developed a two-determinant immunoassay by incubating beads coated with anti-LILRB1 or control antibody with serum or plasma followed by anti-human IgG antibody. Using this method, we screened 672 sera from a Malian cohort and identified 6 donors which had detectable levels of LILRB1-containing IgG (Fig. 2.3 a). From 3 of the positive donors, we immortalized memory B cells and isolated 11 B cell clones producing LILRB1-containing antibodies. When tested on different parasite isolates, the LILRB1-containing antibodies stained IEs (Fig. 2.3 b).

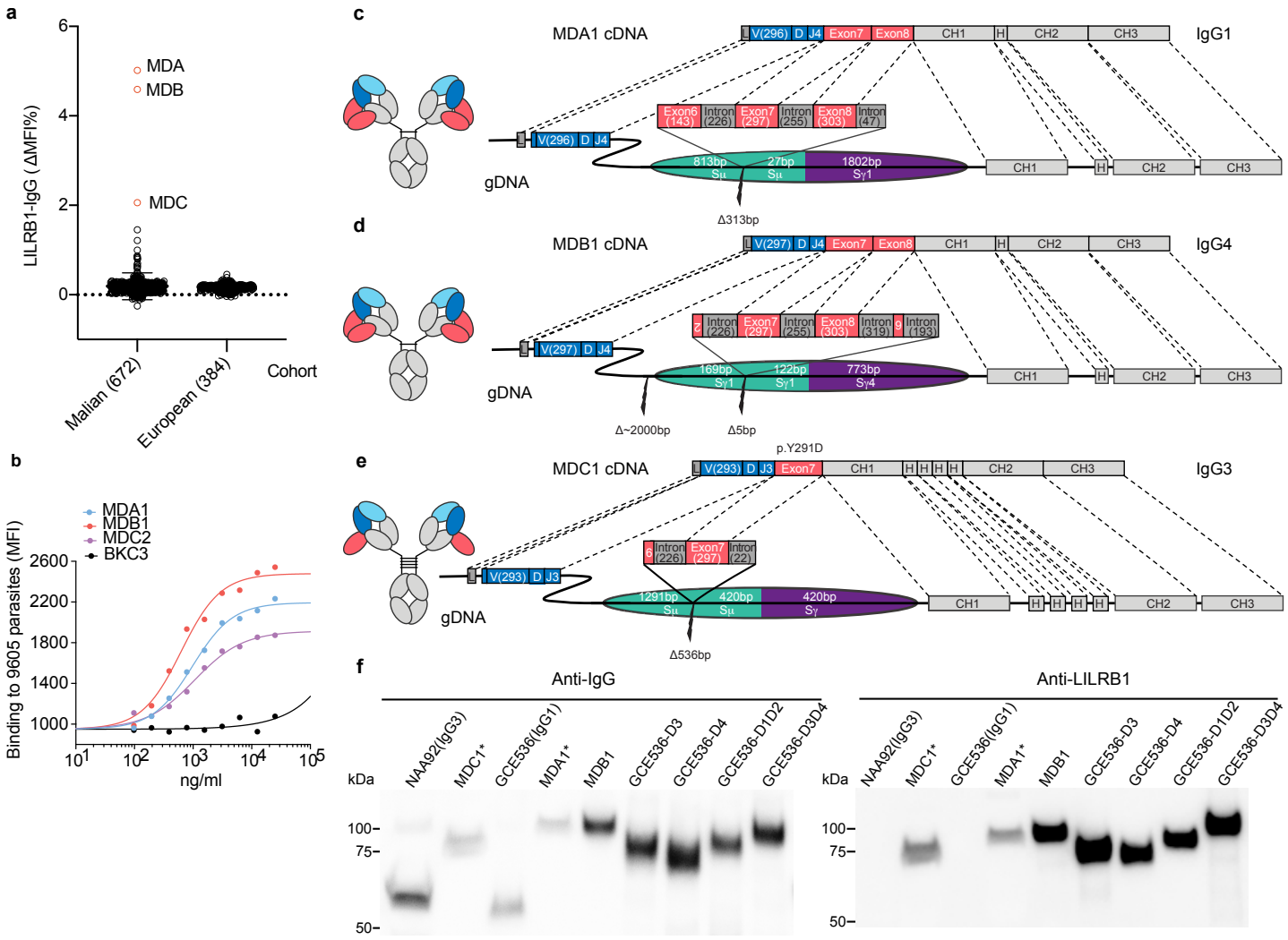


Figure 2.3. **Isolation and identification of LILRB1-containing antibodies in malaria endemic regions.** a, Prevalence of LILRB1-contaning IgG in plasma of Malian and European donors. Donors from whom LILRB1-containing antibodies were isolated are shown in red. b, Binding of LILRB1-containing antibodies to infected erythrocytes (representative of 3 independent experiments). c-e, cDNA and genomic DNA organization in representative B cell clones from 3 donors. LILRB1 inserted DNA segments are colored in red (exon) or dark gray (intron). Adjacent switch regions are shown in green and purple. f, Western blot analysis of culture supernatants of B cell clones MDC1* and MDA1* or recombinant antibody MDB1. Recombinant IgG1 antibodies with the same backbone (GCE536) and in elbow insertions of different LILRB1 domains were also analyzed. Antibodies form B cell clones are marked with an asterisk.

Analysis of cDNA reveals insertions of D3-D4 or D3 between VH and CH1

To investigate the nature of LILRB1 insertion, we sequenced cDNA from B cell clones isolated from positive donors. Sequencing analysis showed the B cell clones in each donor belonged to a single clonal family as demonstrated by the same insert and VDJ rearrangement (Fig. 2.4, Table 2.1). The LILRB1 gene comprises 16 exons. Exon 5-8 encode the extracellular domains (D1-D4) and exon 9 encodes a linker before the transmembrane domain. In all cases, templated LILRB1 insertions were found between the VH and CH1 and comprised, in MDA and MDB clones, exons 7 and 8 (encoding LILRB1 domains D3 and D4) and, in MDC clones, exon 7 alone (Fig. 2.3 c-e). These findings are consistent with the expression of the extra LILRB1 domains in the VH-CH1 elbow. Interestingly, while the VH regions were somatically mutated, the LILRB1 inserts were not, with exceptions of exon 7 in MDC1 that carried a single mutation leading to a p.Y291D substitution and in few MDA clones that carried two mutations leading a p.E306G substitution of exon 7 (Fig. 2.3 e, Fig. 2.5 a). Surprisingly, in MDA8, an insertion of an intronic fragment between exons 7 and 8, as well as a deletion at the beginning of exon 8 were detected in the cDNA (Fig. 2.4 a). Given that the clone belongs to a single clonal family, and the observation of cryptic splicing sites, this cDNA variant is likely a resultant of alternative splicing (Fig. 2.4 a).

a

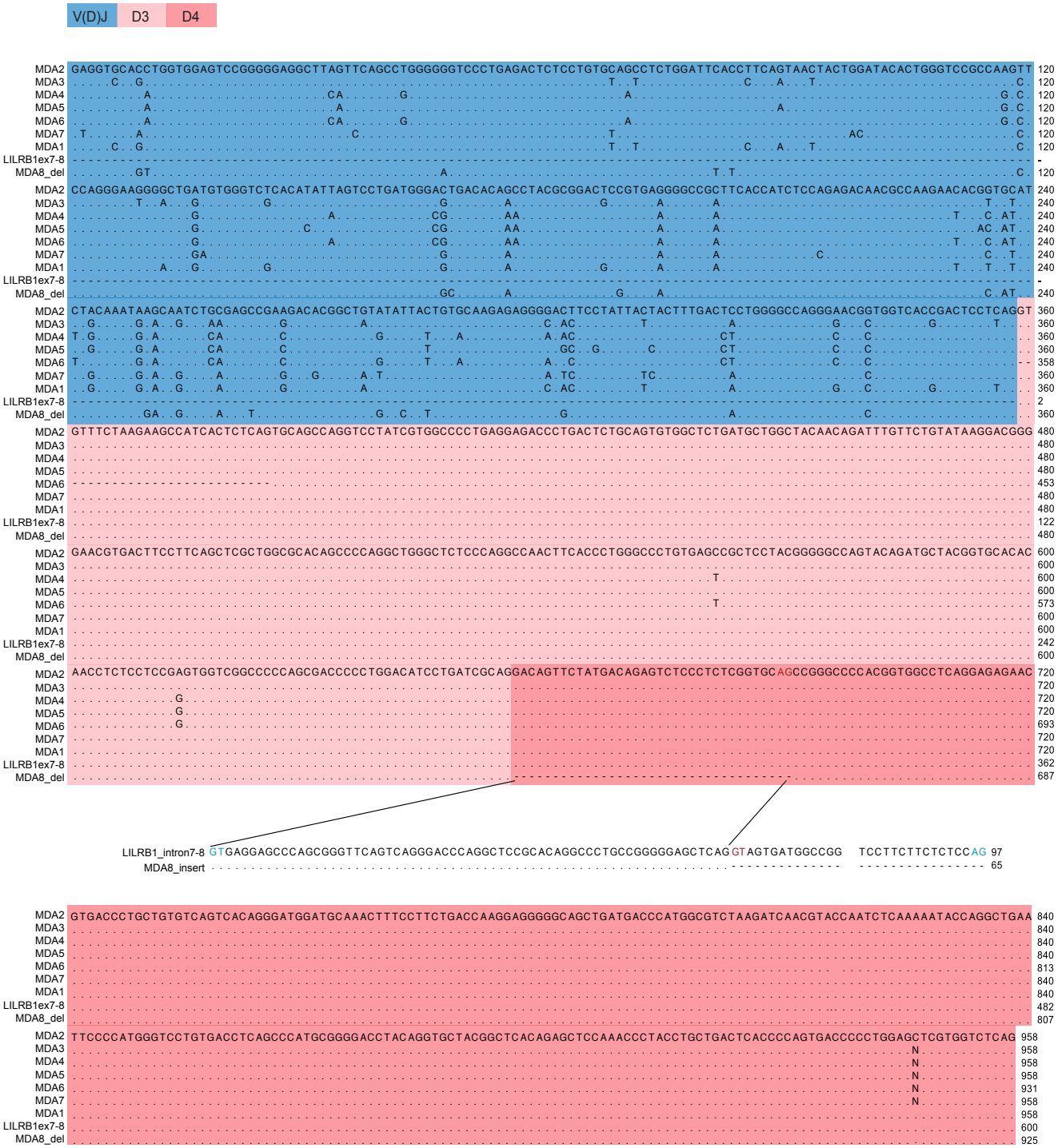

V(D)J
D3
D4
MDA2
MDA3
MDA4
MDA5
MDA6
MDA7
MDA1
LILRB1ex7-8
MDA8_del
LILRB1_intron7-8 GTGAGGAGCCCAGCGGGTTCAGTCAGGGACCCAGGCTCCGCACAGGCCCTGCCGGGGGAGCTCAGGTAGTGATGGCCGG TCCTTCTTCTCTCCAG 97
MDA8_insert 65

Figure 2.4. **Alignment of cDNA sequences of LILRB1-containing antibodies from each donor.** a, MDA (donor A), one of the clones MDA8 employs a different splicing site in the intron between LILRB1 exon 7 encoding the D3 domain and the exon 8 encoding the D4 domain, results in insertion of a piece of intronic sequence (between exon7 and 8) before the exon. Cryptic splicing sites (GT-AG) are marked in blue, alternative splicing sites are highlighted in red; b, MDB (donor B); c, MDC (donor C).

Donor	mAb	Isotype		Heavy chain VDJ genes (% identity to GL)					Light chain VDJ genes (% identity to GL)				Inserted LILRB1 domain	LILRB1 mutations (% identity to GL)
A	MGA1	IgG1	λ	VH3-74	(92.4)	D2-21	JH4	(87.5)	VL1-51	(95.4)	JL2	(86.1)	D3-D4	(100)
B	MGB1	IgG4	λ	VH2-5	(90.7)	D5-24	JH4	(87.5)	VL3-25	(89.6)	JL1	(86.8)	D3-D4	(100)
C	MGC1	IgG3	κ	VH7-4	(94.8)	D4-23	JH3	(94)	VK4-1	(91.9)	JK2	(91.9)	D3	(99)

Isotype and *V(D)J* gene usage of heavy chain and light chain of mAbs containing LILRB1 domain/domains in the switch region. GL, germline.

Table 2.1. V gene and insert usage of representative LILRB1-containing antibodies

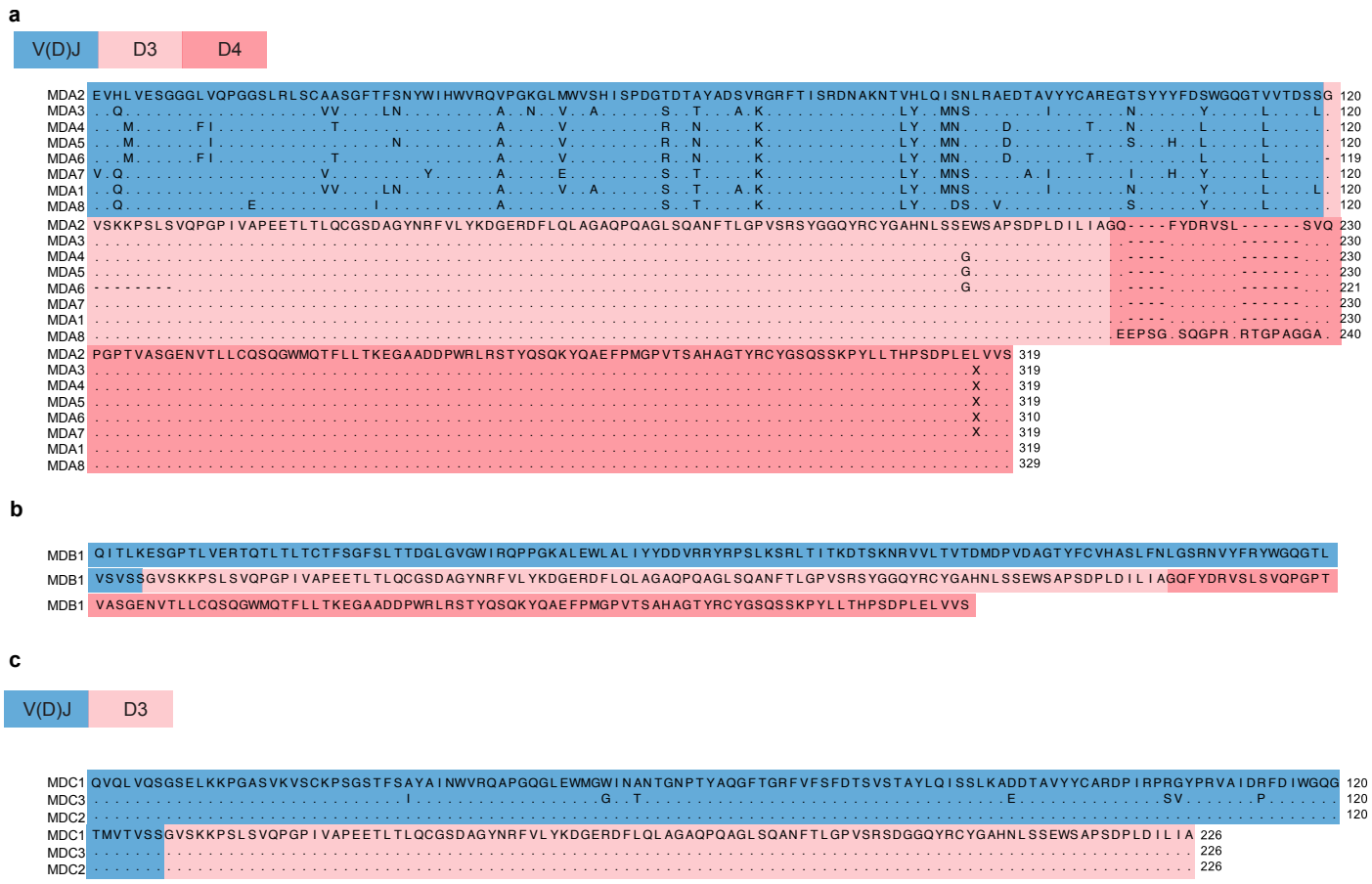


Figure 2.5. **Alignment of amino acid sequences of LILRB1-containing antibodies from each donor.** a, MDA (donor A). b, MDB (donor B). c, MDC (donor C).

Large genomic LILRB1 inserts are found in the switch region

To understand the mechanism that generates LILRB1 containing antibodies we sequenced the gDNA from representative B cell clones (Fig. 2.3 c-e and Fig. 2.6). In donor MDA, the antibody was an IgG1 and the insert found in the switch μ region comprised a 1271 bp LILRB1 genomic fragment starting from exon 6 and ending after exon 8. In donor MDB, the antibody was an IgG4 and the insert was found in the switch $\gamma 1$ region and comprised a 1723 bp genomic fragment spanning exons 7, 8 and 9 with flanking intronic sequences plus a short and inverted sequence at the 5' end originates from exon 2. Finally, in donor MDC, the antibody was an IgG3 and the insert was in the switch μ region and comprised a shorter 645bp fragment spanning exon 7 with flanking intronic sequences plus a small portion of exon 6. Importantly, the conservation of the splice sites flanking exons 7 and 8 allowed in all cases the precise splicing observed in the cDNA, while exon 2 and 9 in MDB1 were not spliced in. The junctional analysis didn't reveal any homology sequences (>3bp) between inserted DNA and donor DNA, suggesting that a conventional non-homologous end join repair pathway (c-NHEJ) was employed (Data not shown).

Figure 2.6. **Genomic sequences showing the junction of switch regions containing LILRB1 domains.** Annotation of representative gDNA from each donor, the reference sequences used are indicated at the bottom. a, MDA (donor A). b, MDB (donor B), The gene coordinates of the inserted exon 2 are indicated in red at the bottom. c, MDC (donor C).

Analysis of LILRB1 antibodies at the protein level

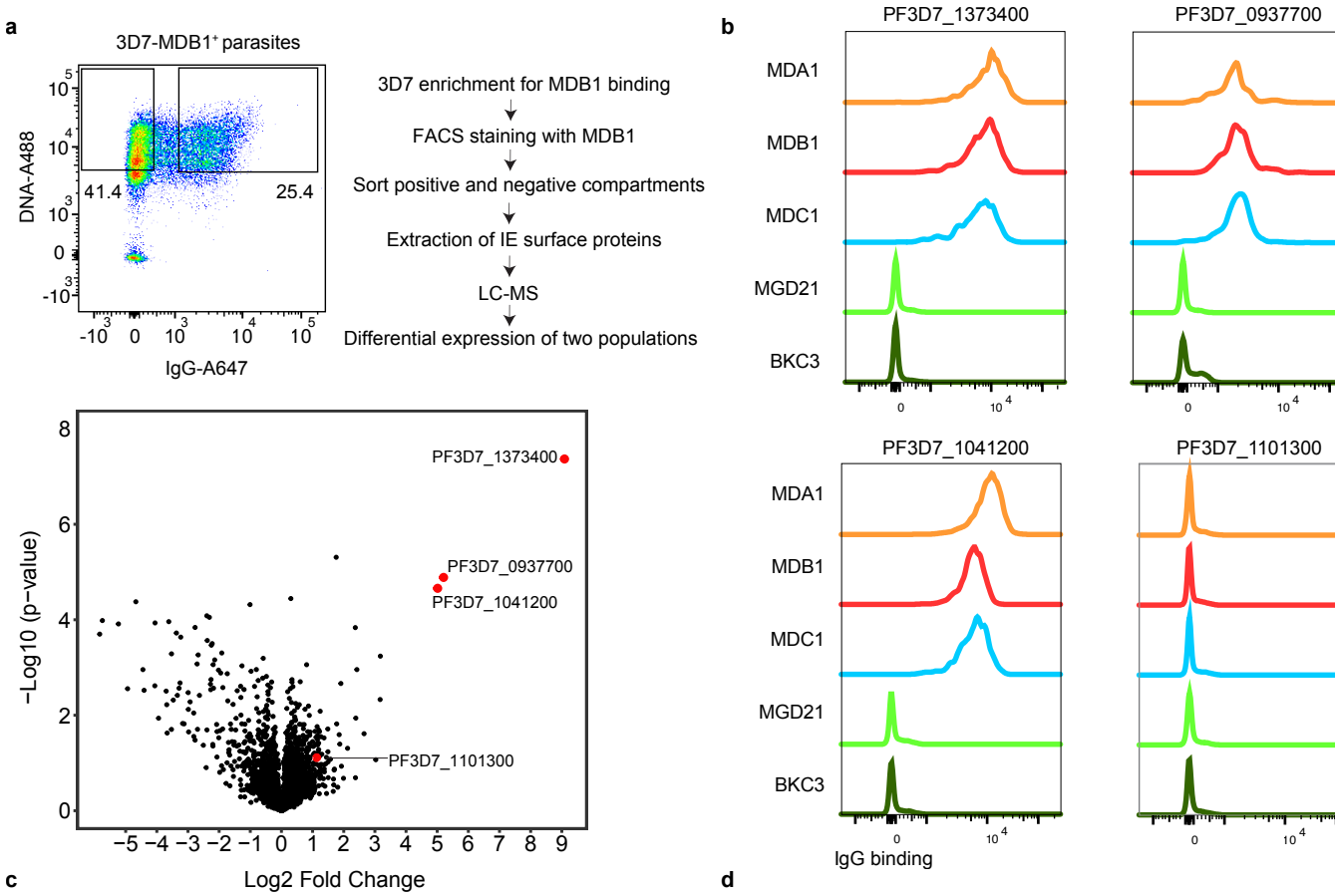
Consistent with the cDNA data, western blot analysis of the antibodies secreted by immortalized B cell clones demonstrated the presence of a heavy chain of higher molecular weight that was blotted by anti-LILRB1 polyclonal antibodies (Fig. 2.3 f). Strikingly, this was the only form of heavy chain present in the B cell culture supernatant of the MDA1 and MDC1 B cell clones, suggesting a very high efficiency of LILRB1 exons splicing in these cells. The MDB1 antibody could not be isolated from the culture supernatant due to poor growth of the B cells and was therefore produced only in a recombinant form, as were other recombinant antibodies with LILRB1 D3 and/or D4 insertions.

Identification of certain RIFINs as targets of LILRB1-containing antibodies

To identify the targets of LILRB1-containing antibodies, we performed multiple rounds of enrichment of 3D7-IEs with MDB1. The reason behind this was that the laboratory line of parasites usually expresses low level of a limited size of RIFIN repertoire, the enrichment of MDB1-binding parasite stained better with MDB1 and other LILRB1-containing antibodies. Subsequently, we isolated MDB1-positive and negative IEs by cell sorting (Fig. 2.7 a). In this way, we could determine, in an unbiased fashion, the molecules enriched in the MDB1-positive fraction. Analysis of the two IE compartments by LC-MS/MS revealed expression of 30 RIFINs, of which some were most highly expressed in the positive IEs (Fig. 2.7 a and Fig. 2.8). Although RIFIN variants shared high homology to each other, unique peptides were identified during the LC-MS/MS and sufficient for determining the identity of specific RIFIN molecule (Fig. 2.8 a).

To validate whether enriched RIFINs are the *bone fide* targets for LILRB1-containing antibodies, we expressed candidate RIFIN-V2s on the surface of HEK cells using the P-display method (Tan et al., 2016). The reason for using the V2 region of RIFINs (as defined by alignment of full RIFIN protein sequences in Fig. 2.8) is based on the finding that the V2 region of LAIR-specific RIFINs is the minimal epitope for LAIR1 binding ((Tan et al., 2016) and Kai Xu personal communication). Staining of HEK cells transfected with RIFIN-V2 domains confirmed specific binding of LILRB1-containing antibodies to the 3 RIFINs that were most highly differentially expressed: PF3D7_1041200, PF3D7_1373400 and PF3D7_0937700 (Fig. 2.7 b). As a control, a low score RIFIN in the LC-MS/MS assay, PF3D7_1101300, did not binds to any of the tested antibodies. Furthermore, the LAIR1-contaning antibody MGD21 did not stain the LILRB1 RIFINs, indicating

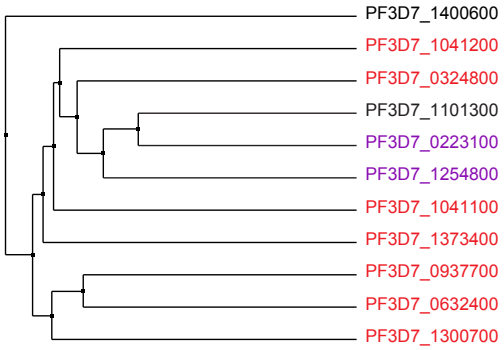
that LAIR1 and LILRB1 target different sets of RIFINs.



c

	Shared hotspots	LILRB1⁺ rifins	LILRB1⁻ rifins	Total
LC-MS rifins	14	3		3
Ranking of 221 3D7 rifins sharing hotspots	14	1		1
	11	1		1
	10	1	1	2
	9		2	2
	8	1	1	2
	7		2	2
	5		1	1
	4		1	1
	3		1	1
	2		2	2
	1		1	1
	Total	7	12	19

d



e

Low-resolution signature	A108	V113	I114	F125	N172	Y173	A176	I219	F235	P282	I283	C285	I328	V332
PF3D7_1041200														
PF3D7_1373400														
PF3D7_0937700														
PF3D7_0632400														
PF3D7_0324800														
PF3D7_1041000														
PF3D7_1300700														
High-resolution signature				F125		Y173		I219		P282		C285	I328	V322

Figure 2.7. **LILRB1-containing antibodies bind to distinct RIFINs.** a, Volcano plot showing the differential expression of 3D7 *P. falciparum* proteins in MDB1$^+$ and MDB1$^-$ IEs, as determined by LC-MS/MS analysis (from n = 4 independent experiments). Statistical significance was evaluated by Welch tests. MDB1$^+$ and MDB1$^-$ IEs were defined by FACS gating showing in the up panel and processed using the workflow on the upper right .b, Staining of HEK cells transfected with high score RIFINs (PF3D7_1041200, PF3D7_1373400 and PF3D7_0937700), with low score RIFIN (PF3D7_1101300) with LILRB1-containing antibodies (MDA1, MDB1, MDC1), with a LAIR1-containing antibody (MGD21) or with an irrelevant antibody (BKC3, anti-influenza). c, RIFINs were ranked according to the number of shared hotspots and for each rank a few were transfected into HEK cells and stained with LILRB1-containing antibodies. d, Phylogenetic tree showing distance between LILRB1$^+$ RIFINs (red), LILRB1$^+$ RIFIN reported prior (purple), LAIR1$^+$ RIFIN (black) or other RIFINs (black). Trees showing percentage identity (PID) are calculated on the basis of a measure of similarity between each pair of sequences in the alignment. e, Low resolution (red) and high resolution (blue) signatures identify seven 3D7 LILRB1-binding RIFINs.

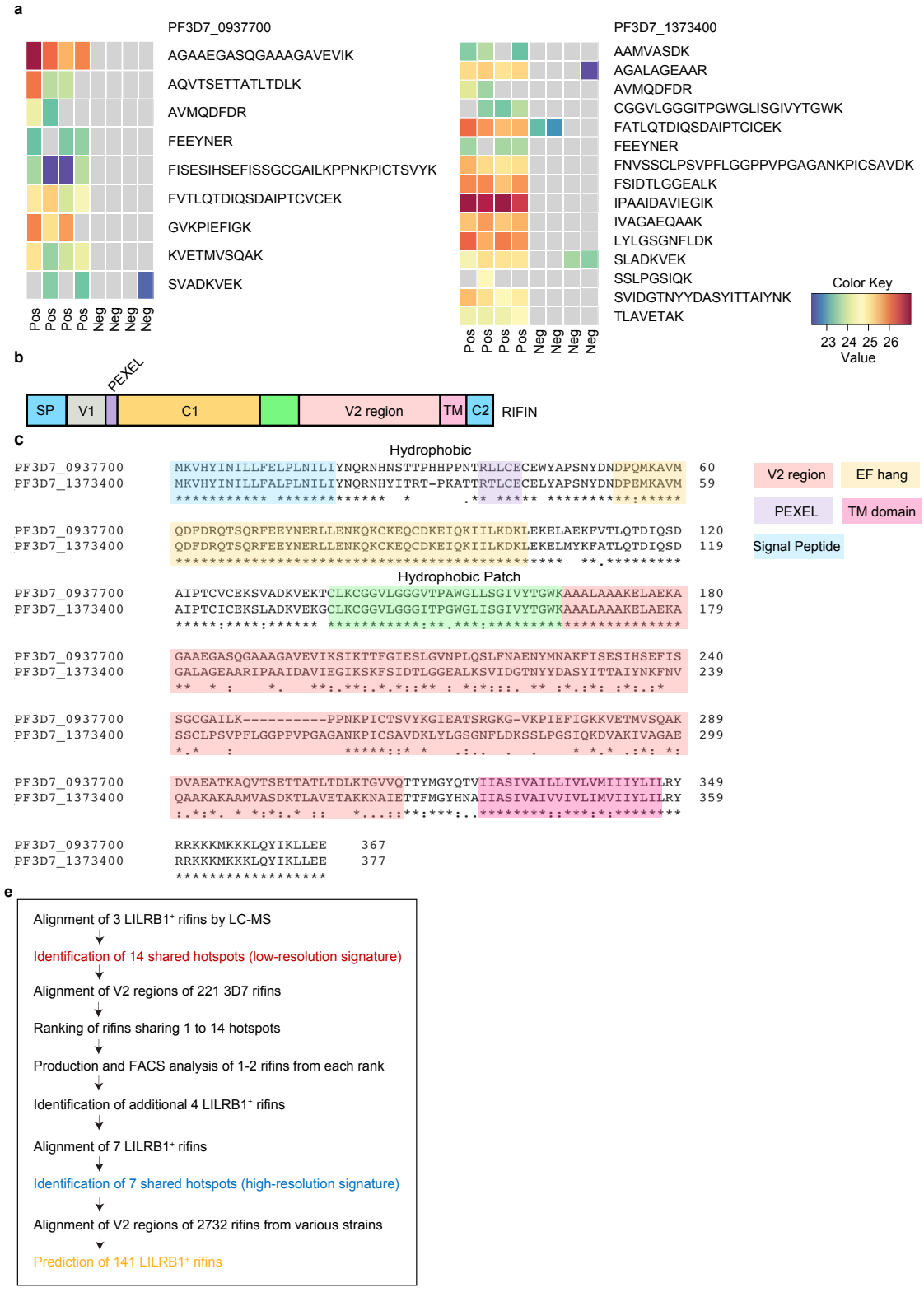


Figure 2.8. **LC-MS/MS peptide calling and define of RIFIN-V2.** a, Heatmap showing the enrichment of peptides in 3D7-MDB1$^+$ IEs from the LC-MS analysis; b, Representation of RIFIN protein. SP, signal peptide; V, variable region; PEXEL, PEXEL motif; C, constant region; TM, transmembrane region. The images are not to scale. c, Alignment of representative full length RIFINs and V2 region is highlighted in light pink. e, Workflow to identify additional RIFINs recognized by LILRB1-containing antibodies. Prediction of 141 LILRB1$^+$ RIFINs from various strains can be found in supplementary materials upon request.

Identification of a large cluster of RIFINs as targets of LILRB1

Given the limited expression of RIFINs in cultured parasites, possibly due to lack of selective pressure, we were interested to determine the spectrum of RIFINs that bind to LILRB1. For this purpose, we used a homology-based approach to identify additional LILRB1-binding RIFINs (or referred to as LILRB$^+$ RIFIN). For the first step, we performed homology analysis on the 3 positive RIFINs and the alignment revealed 14 shared hotspots in the V2 region that defined a low-resolution LILRB1 binding signature (Fig. 2.7 e). This signature was used to screen in silico the remaining 221 3D7 RIFINs that were ranked according to the number of shared hotspots. For each rank, 1 or 2 RIFINs were expressed and tested using the surface display analysis (Fig. 2.7 c). This approach allowed us identified four additional 3D7 RIFINs that were recognized by the LILRB1-containing antibodies. Using the new set of 7 LILRB1 positive RIFINs we defined a higher resolution signature that was applied to predict 141 LILRB1-specific RIFINs of a variety of parasite strains out of 2732 present in the database (Supplementary item is provided if requested). To further understand how LILRB1$^+$ RIFINs related to each other in an evolutionary point of view, we applied average distance to assess the closest relative in the context of few selected RIFINs, including an irrelevant RIFIN PF3D7_1101300, LAIR1$^+$ RIFIN PF3D7_1400600 and two LILRB1-specific RIFINs previously reported (PF3D7_1254800 and PF3D7_0223100). As shown in Fig. 2.7 d, the average distance tree was generated using Percentage Identity (PID) method, an algorithm which creates tress by comparing the similarity of the actual sequences of homologs (Fig. 2.9 b). The tree revealed that the V2 region of LILRB1$^+$ RIFINs are generally close to each other and split into 3 clusters, LAIR1$^+$ RIFINs are relative far from the rest of the RIFINs (Fig. 2.7 d, Fig. 2.9 a). To our surprise, the two previously reported LILRB1$^+$ RIFIN were clustered together with the negative control (Fig. 2.7 d). These results were also confirmed in an extended phylogenetic tree analysis of the entire 3D7 RIFIN-V2 pool, which revealed 3 clusters of LILRB1$^+$ RIFIN away from the LAIR1$^+$ RIFIN cluster as well as the two reported LILRB1$^+$ RIFINs (Fig. 2.9 a). Together, these results highlight that LILRB1-containing antibodies target a specific set of RIFINs. These RIFINs are closely related to each other in an evolution perspective, suggesting the existence of similar RIFINs in other parasite stains.

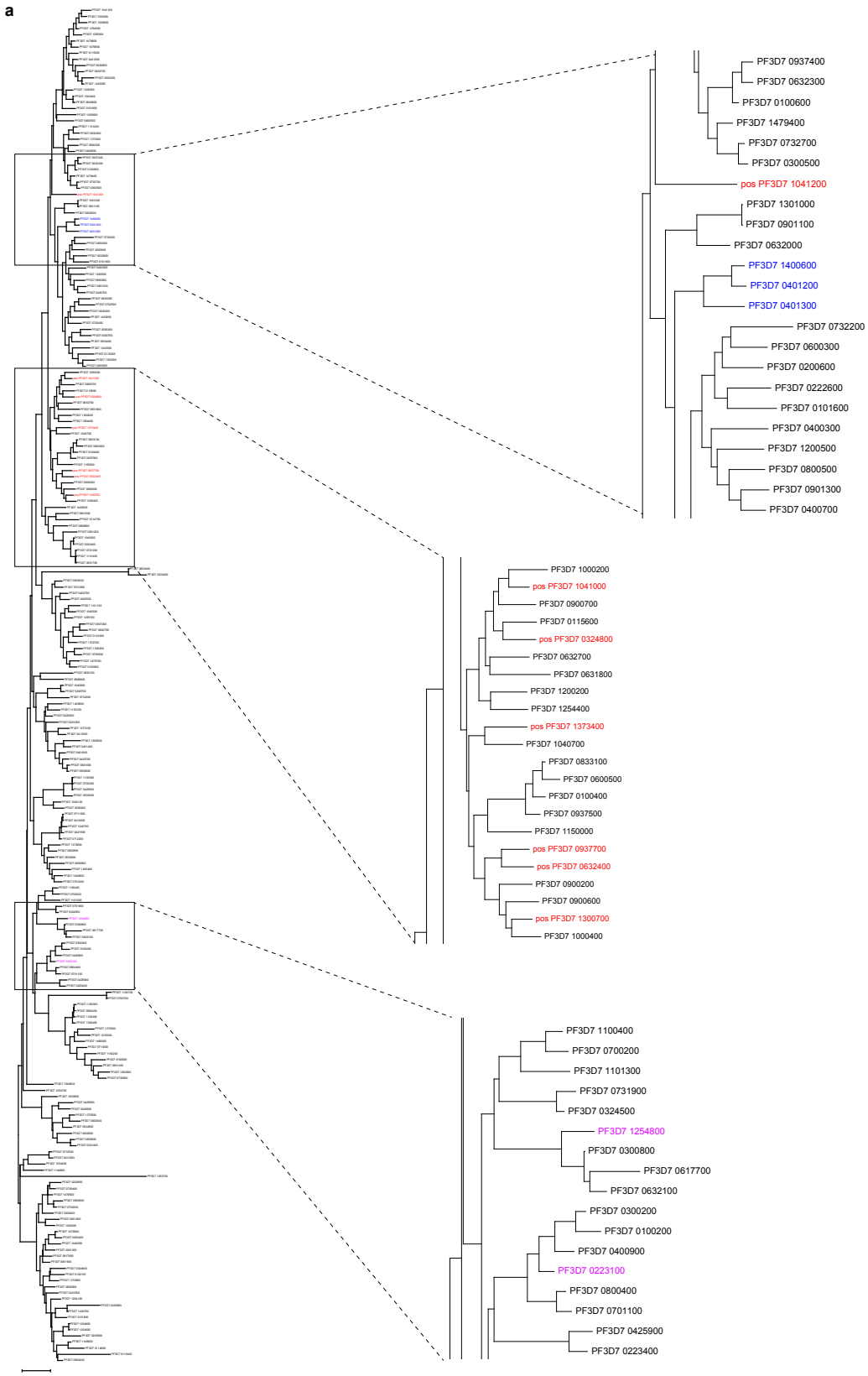


Figure 2.9. **3D7 RIFIN-V2 homology analysis.**

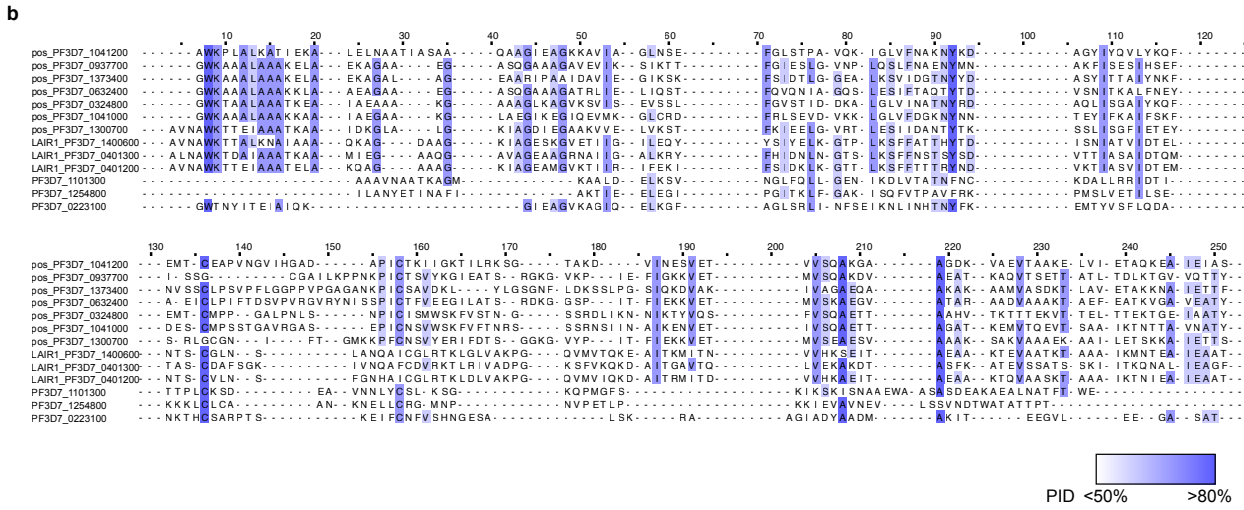


Figure 2.9. **3D7 RIFIN-V2 homology analysis.** a, Phylogenetic tree of 221 3D7 RIFIN-V2. LILRB1$^+$ RIFINs are highlighted in red. LAIR1$^+$ RIFINs are highlighted in blue. Previously reported LILRB1$^+$ RIFINs are highlighted in purple. b, Homology analysis of LILRB1$^+$ RIFINs and other RIFINs. Amino acid conservation is shown in gradient blue (darker indicates higher conservation); PID, percentage identity.

The D3 domain is uniquely responsible for the binding to RIFINs

A previous study described two 3D7 RIFINs (PF3D7_1254800 and PF3D7_0223100) that bound by an Fc fusion protein containing the full length extracellular LILRB1 spanning D1 through D4 domains (Saito et al., 2017). To confirm these findings and to dissect the LILRB1 domains involved in binding to different RIFINs, we tested a panel of RIFIN transfectants with natural and recombinant antibodies and fusion proteins containing different LILRB1 domains (Fig. 2.10, Fig. 2.11). Antibodies containing LILRB1 D3-D4 or D3 elbow inserts bound to the 7 identified RIFINs and deletion of the inserted domains completely abolished binding, indicating a critical role for LILRB1 D3 domain. Antibodies containing LILRB1 D1-D2 or D4 elbow inserts did not bind to any RIFIN. Importantly, an Fc fusion protein containing all the 4 LILRB1 domains bound to all 7 RIFINs, demonstrating that the D3-mediated binding (in LILRB1-containing antibody) is not dependent on the molecular context. Surprisingly, however, neither the D3-containing antibodies, nor the full length LILRB1 fusion protein bound to PF3D7_1254800 and PF3D7_0223100 that were reported by Saito *et al* to be LILRB1 binders (Saito et al., 2017). This finding was fur-

ther confirmed by staining of LILRB1 Fc fusion proteins to the 2 reported RIFINs, which showed negative binding profile to any part of LILRB1 domain (Fig. 2.11 c). In contrast, 2 representatives of LILRB1^{+} RIFIN identified in our study showed positive binding to all D3-containing fusion constructs. Binding of D3-containing antibodies was also demonstrated using full length RIFINs displayed on IE (Fig. 2.10 b). Collectively, these data identify a number of RIFINs that bind to LILRB1 D3 and D3 as an exclusive site of binding for RIFINs.

a

RIFIN	MDB1	MDB1-ΔD3D4	GCE536-D3D4	GCE536-D1D2	GCE536-D3	GCE536-D4	GCE536	LILRB1-Fc	MGDUCA (LAIR1)	MGD21 (LAIR1)	BKC3
PF3D7_1373400	+	-	+	-	+	-	-	+	-	-	-
PF3D7_0937700	+	-	+	-	+	-	-	+	-	-	-
PF3D7_1041200	+	-	+	-	+	-	-	+	-	-	-
PF3D7_0632400	+	-	+	-	+	-	-	+	-	-	-
PF3D7_0324800	+	-	+	-	+	-	-	+	-	-	-
PF3D7_1041000	+	-	+	-	+	-	-	+	-	-	-
PF3D7_1300700	+	-	+	-	+	-	-	+	-	-	-
PF3D7_1400600	-	-	-	-	-	-	-	-	-	+	-
PF3D7_1101300	-	-	-	-	-	-	-	-	-	-	-
PF3D7_1254800	-	-	-	-	-	-	-	-	-	-	-
PF3D7_0223100	-	-	-	-	-	-	-	-	-	-	-

b

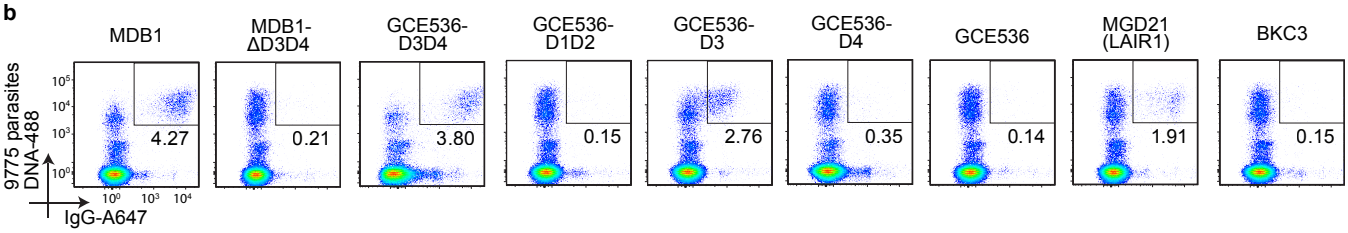


Figure 2.10. **The LILRB1 extracellular domain 3 (D3) is responsible for binding to a set of RIFINs.** a, Identification of LILRB1 minimum binding domain by staining selected RIFIN transfectants with natural and recombinant LILRB1-containing antibodies or control antibodies (MDB1, MDB1 lacking D3D4 domains, LILRB1-elbow-fusion antibodies, LAIR1-containing antibody (MGD21, or germline reverted MGDUCA)). b, Example of staining of parasite isolate with natural and recombinant LILRB1-containing antibodies or control antibodies.

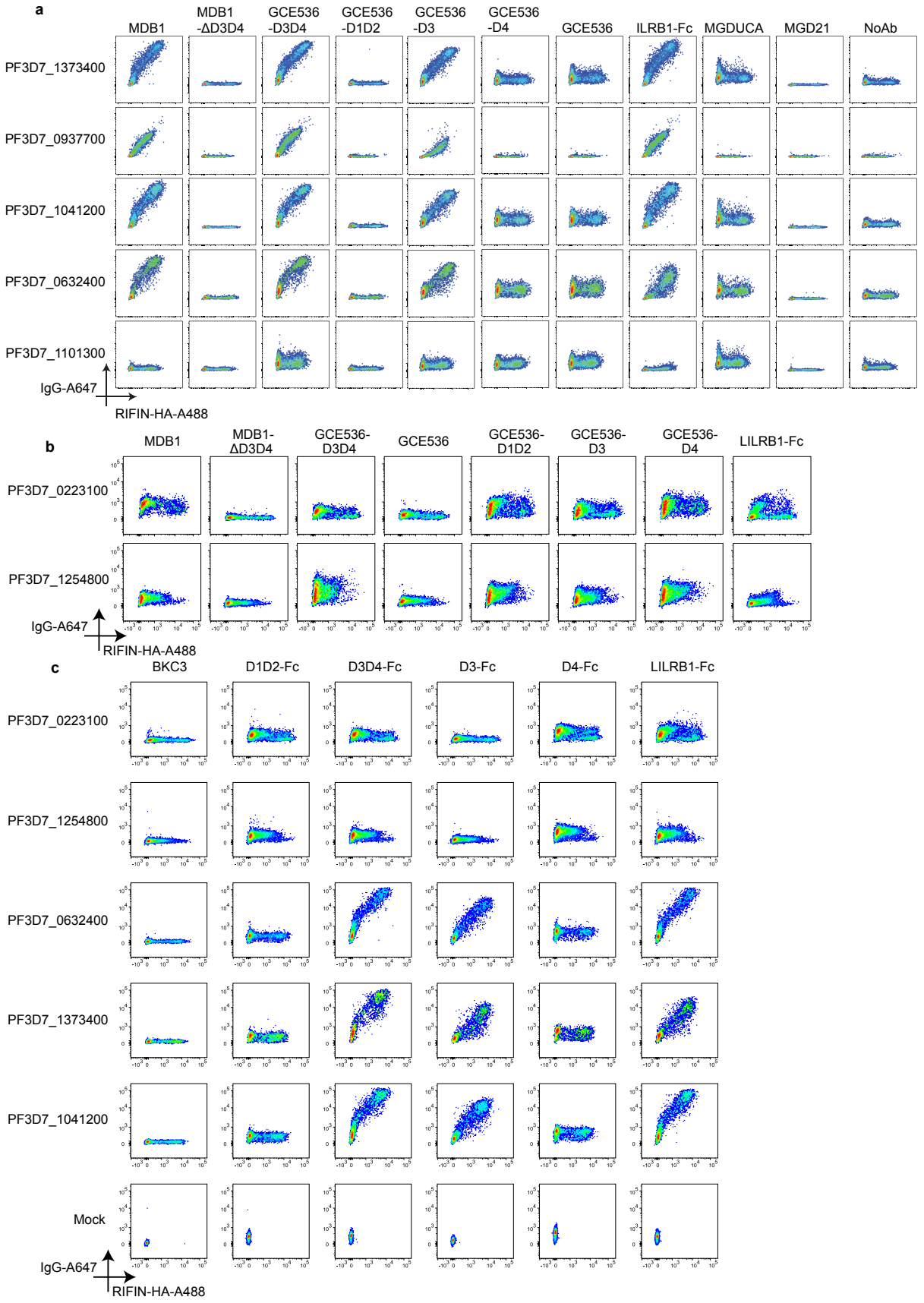


Figure 2.11. **Representative FACS analysis of RIFINs binding to various constructs.** a-b, FACS analysis of representative RIFINs bind to natural or recombinant antibodies or control antibodies. c, FACS analysis of representative RIFINs bind to Fc fusion protein containing the LILRB1 domain/domains.

Structure of LILRB1-containing antibody in complex with RIFIN PF3D7_1373400

Thus far, we demonstrated that certain RIFINs target the D3 domain of LILRB1 (Fig. 2.12 a). To elucidate the mechanism by which LILRB1-containing antibodies recognize RIFIN, we determined a cryo-EM structure of MDB1 Fab in complex with V2 domain of RIFIN PF3D7_1373400 at 3.5 Å resolution (Data not shown). The structure revealed an unconventional triangular shape Fab architecture (Fig. 2.12 b-d) due to the elbow insertion of LILRB1 D3D4 domain. The VH-VL, D3D4 and CH-CL domains compose the three apexes of the triangle. These three individual domains superimpose well with their apo structures, suggesting the rigidity within each domain. However, the angle of light chain elbow region swings outward almost 90 degree, in order to accommodate the LILRB1 insertion in the heavy chain elbow region. In addition, MDB1 Fab formed a homodimer through the interaction between the two light chains, as well as between VH and D3D4 insertion on the other copy of MDB1 Fab (Fig. 2.12 b). The structure further indicated RIFIN-V2 interacted only with the D3 domain of LILRB1. Due to the inter-domain flexibility of MDB1-RIFIN complex revealed by 3D variability analysis, RIFIN electron microscope (EM) map density is largely unclear except the region around MDB1-RIFIN interface. We therefore combined this cryo-EM structure and the crystallographic structure of LILRB1-RIFIN to generate a full model (Fig. 2.12 c, d). Together, the elbow of VH-CH1 opens up completely to accommodate the LILRB1 domains while preserving VH/VL and CH1/CL interactions.

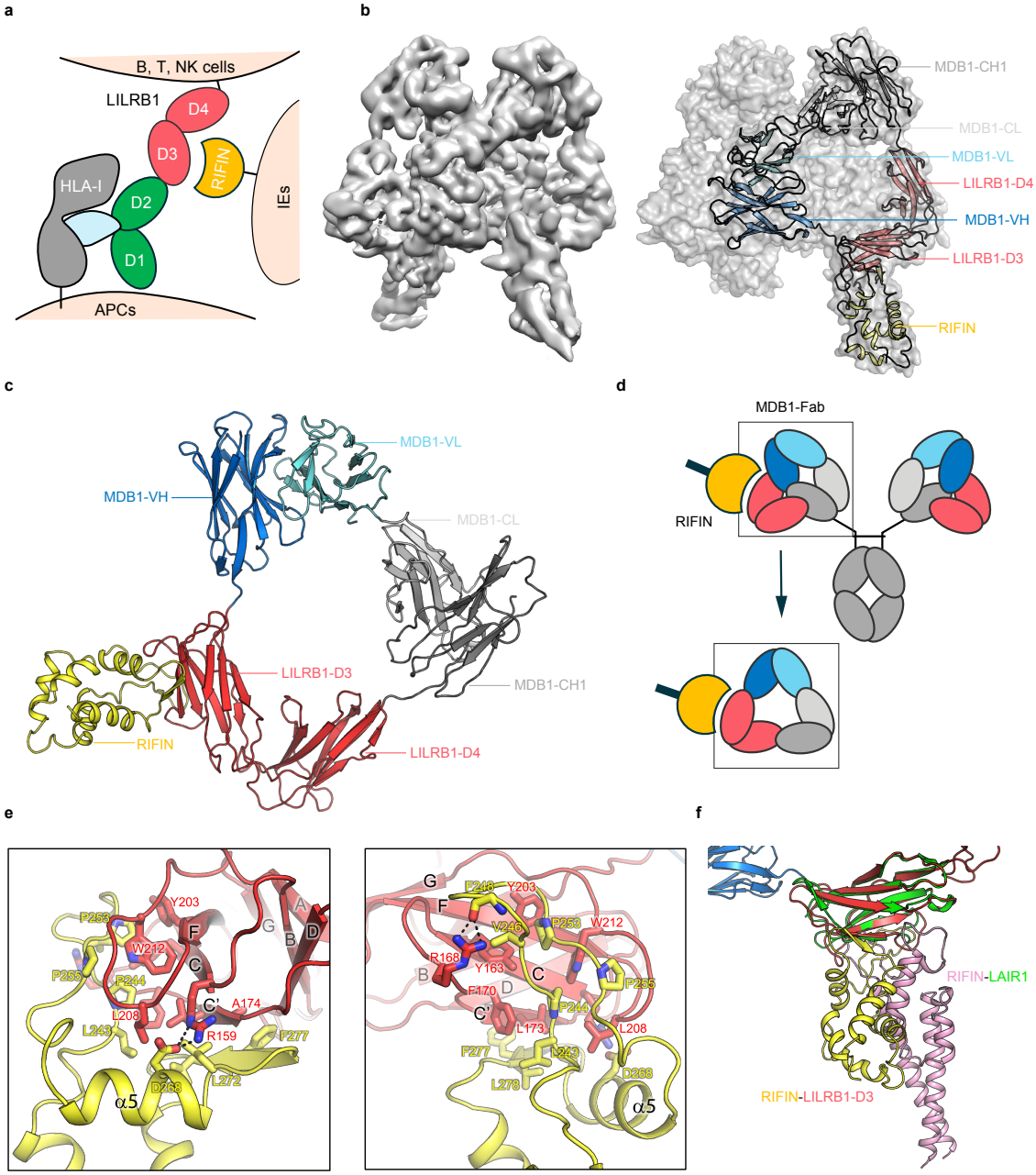


Figure 2.12. **Structure of MDB1 Fab and overall architecture of MDB1-RIFIN.** a, Schematic of endogenous LILRB1 receptor binding to peptide-HLA-I on antigen presenting cells, LILRB1 extracellular domains (D1D2 is indicated in green, D3D4 is indicated in red). D1D2 is responsible for HLA-I binding, while D3D4 acts as a scaffold. b, RIFIN-MDB1 Fab Forms 2:2 Dimer Complex, 3D Variability Analysis to track movement in cryo-EM structure (left) and RIFIN-MDB1 Fab Structural Model (right). c, Overall architecture of MDB1 Fab in complex with RIFIN PF3D7_1373400. d, schematic showing conformational accommodation of MDB1 Fab upon binding to RIFIN. e, The interface of MDB1-RIFIN complex. RIFIN is colored in yellow and LILRB1 D3 is colored in red. f, Comparison of RIFIN binding between LILRB1 and LAIR1. LILRB1 D3 and LILRB1^{+} RIFIN is colored in red and yellow; LAIR1 and LAIR1^{+} RIFIN is colored in green and pink.

The interface of MDB1-RIFIN PF3D7_1373400 complex

The interface of MDB1-RIFIN is formed by the RIFIN-V2 apex region and one side of LILRB1 D3 Ig domain β-sandwich (Fig. 2.12 e). RIFIN-V2 apex region involves α5 helix and its two adjacent loops. The contacting side of LILRB1 D3 domain involves the strands C, C' and F, together with the neighboring loop regions. The interface composes of mostly hydrophobic interaction, which involves the side chain contact between RIFIN residues L243, P244, V246, F248, P253, P255, L272, F277, L278 and MGZ4 residues Y163, F170, L173, A174, Y203, L208, W212. However, hydrophilic interaction also plays important roles, including salt-bridge formed between D268RIFIN and R159MDB1; hydrogen bond formed between F248RIFIN main chain carbonyl and R168MDB1 side chain guanidium group; and β-sheet formed between α5-α6 region of RIFIN and C' strand of LILRB1 D3 domain (Fig. 2.12 e). It is worth noting that there are four glycine and six proline residues locating in RIFIN α4-α5 loop region, including P244, P247, G250, G251, P252, P253, P255, G256, G258 and P262 (Fig. 2.12 e). Together they increase the flexibility of α4-α5 loop to enable better fit into the hydrophobic groove on LILRB1 D3 domain.

To compare the similarity between LILRB1$^+$ RIFIN and LAIR1$^+$ RIFIN, the secondary structure components were first analyzed. Overall, they shared similar components including helix hundle and disulfide bond stabilization module. We then superimposed two complexes. As shown in Fig. 2.12 f, both V2 apex regions (α5 and two adjacent loops) are used to target LAIR1 or LILRB1 D3 domain. In addition, they both interact with strands C, C' and F of LILRB1/LAIR1 Ig domain. However, subtle differences are observed such as the binding angle and interface residues determining the specificity.

2.3 Engineering bispecific antibodies by insertion of binding domains in the elbow

2.3.1 Rationale

Given the discovery of the unique conformation of naturally acquired malaria antibodies based on the insertion of one or two Ig-like domains in the VH-CH1 elbow, we hypothesized that this elbow and possibly other elbows of the antibody molecule might be used to insert additional antigen binding domains to make bispecific antibodies (bsAbs).

2.3.2 Introduction of VHH or scFv into the different elbows to make bsAbs

To test this hypothesis, I engineered a series of antibodies with VHH or scFv insertions in various elbows of the antibody. In particular I tested the possibility to make insertions not only in the VH-CH1 elbow, as found in natural LAIR-1 and LILRB1 containing antibodies, but also in the VL-CL elbow and in the CH2-CH3 elbow.

As a proof of concept, I used an antibody specific for the influenza H1 hemagglutinin, FI174 (Pappas et al., 2014), as the backbone for in elbow insertions of VHH or scFv specific for tetanus toxoid, namely T3-VHH (Rossotti et al., 2015) and TT39.7-scFv (Lanzavecchia's lab). The engineered constructs were expressed efficiently in HEK cells at levels comparable with those of conventional antibodies (data not shown). The bsAbs were tested for their capacity to bind to H1 and tetanus toxoid using a quantitative ELISA. As shown in Fig. 2.13 a, the binding of the bsAbs to H1 is retained as in the original FI174 antibody, while binding to tetanus toxoid is gained at levels comparable to that of the original T3-VHH or TT39.7-scFv. Strikingly, insertions in the CH2-CH3 elbow resulting in display in the Fc portion of the antibody give rise to bsAb of comparable binding property as antibodies with insertions in the VH-CH1 elbow (Fig. 2.13 a). Although insertions in the VL-CL elbow showed slightly decreased binding affinity to cognate antigens as compared to the ones in the VH-CH1 elbow format, the overall data suggested that the elbows are editable in an antibody.

I obtained comparable results in a second series of constructs where scFv specific for respiratory syncytial virus (RSV) (Corti et al., 2013) was inserted into several positions of the FI174 antibody (Fig. 2.13 b). It is not clear why the light elbow format showed less good performance than VH-CH1 elbow format; one plausible explanation is that the space for accommodation of extra domains in the light while preserving pairing with heavy chain is less flexible. Nevertheless, in the second example, the dual specificity to H1 and RSV confirmed that the suitability of using heavy chain elbow format as a new modality for bsAb engineering. Collectively, these results indicate that an extra domain or even two domains can be accommodated in various elbows of an antibody to confer an additional specificity while preserving the original specificity. This approach extends current strategies to engineering bsAbs.

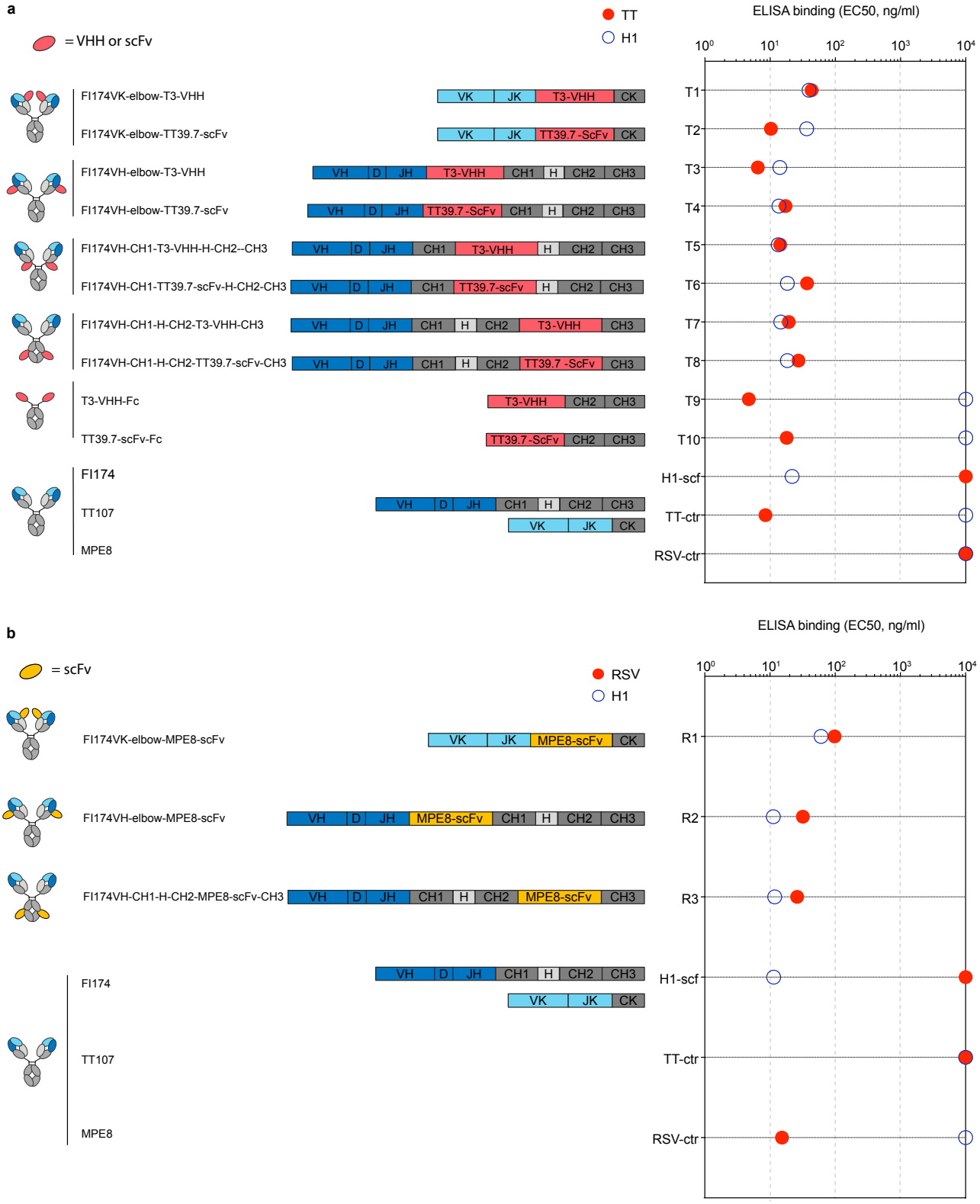


Figure 2.13. **Validation of novel bispecific antibody (bsAb) constructs based on in elbow insertions.** a, VHH or scFV specific for tetanus toxoid (TT) were inserted in the backbone of an anti-influenza hemagglutining (H1) antibody. The positions of the inserts are indicated in the protein and in the constructs. Shown is the binding to H1 and TT as determined by quantitative ELISA. b, scFv specific for RSV was inserted in the backbone of an anti influenza hemagglutining (H1) antibody. Shown is the binding to H1 and RSV F protein as determined by quantitative ELISA. FI174, TT107 and MPE8 are the control antibodies specific for H1, TT and RSV, respectively. EC50, half-maximum effective concentration.

2.3.3 Use of the new elbow format to engineer bsAb against PfCSP

To investigate whether the elbow format could be applied to improve neutralizing antibodies for various applications, I engineered *P.falciparum* circumsporozoite protein (PfCSP) specific antibodies isolated in a previous study (Tan et al., 2018) using the new elbow format or the classical DVD-Ig format (Jakob et al., 2014). The previous study from our laboratory showed that the most effective antibodies against pfCSP bound not only to the NANP repeat region but also to a N-terminal NPDP peptide (Tan et al., 2018) (Fig. 2.14 a). Therefore, we were interested to use a bispecific format to determine if we were able: i) to combine two distinct single-specific antibodies into one bispecific molecule and ii) to improve binding by increasing the antibody avidity through the insertion of an additional binding site.

To investigate whether we could see synergy between antibodies of different specificities we first used two antibodies, MGU1 specific for NANP and MGV3 specific for NPDP (Fig. 2.14 b). To investigate whether we could increase the potency of an antibody by doubling the binding sites we used MGU1 or MGU10 (that binds to both NANP and NPDP) to produce bsAbs with identical binding sites (Fig. 2.14 b). We refer to these formats as MGU1/MGV3 elbow and 2X MGU1 elbow or 2X MGU10 elbow, respectively. In all cases, DVD-Ig formats were also generated as positive controls (Fig. 2.14 b).

As the first step to analyze these engineered antibodies, we determined the antibody expression levels and confirmed that elbow-containing antibodies are well expressed in HEK cells at levels comparable to those of the original antibodies (data not shown). To assess the antibody affinity, all engineering antibodies along with their original counterparts were tested against full PfCSP, junctional NPDP peptide, or NANP peptide respectively by ELISA (Fig. 2.14 c). Interestingly, all the elbow-containing antibodies showed increased binding affinity to full PfCSP as compared with their original counterparts. Specifically, the results demonstrate that the MGU1/MGV3 elbow antibody binds with increased affinity to both NANP and NPDP, consistent with its dual specificity (Fig. 2.14 c). Furthermore, the 2X MGU1 and 2X MGU10 elbow antibodies showed increased binding to all antigens tested as compared to the original antibodies. Collectively these findings illustrate the possibility of using the new in elbow format to engineer bsAbs with improved specificity and avidity (Fig. 2.14 c).

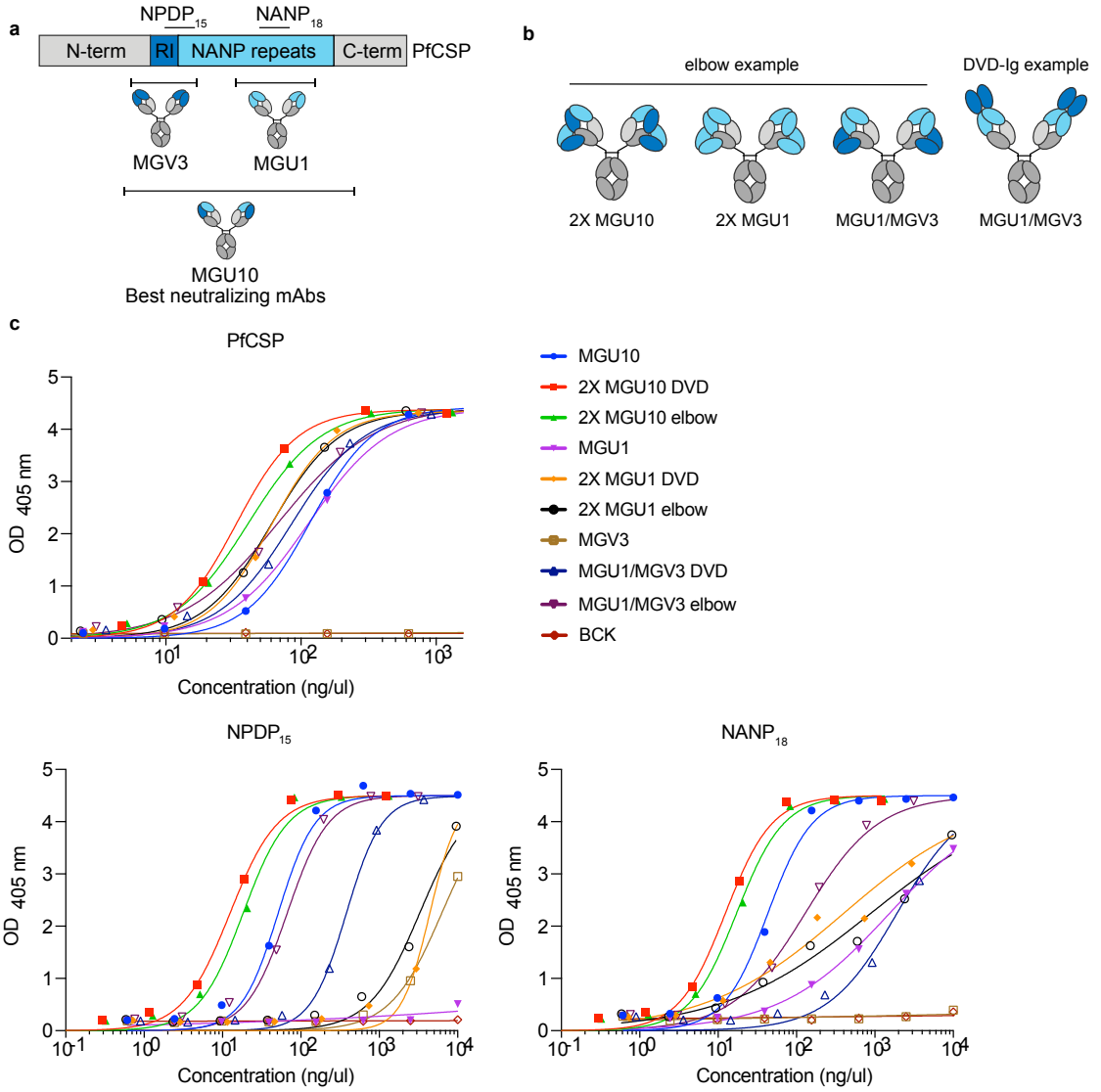


Figure 2.14. **Dual specificity and increased avidity achieved by in elbow insertions.** a, Schematic of PfCSP protein and the specificity of neutralizing antibodies against NANP and NPDP. b, Schematic of design of elbow antibodies and an example of bsAb DVD-Ig format. c, Antibody binding specificities and affinities were tested against PfCSP, $NANP_{18}$, $NPDP_{15}$ by ELISA.

3

Discussion

Our findings reveal a new example of templated insertions giving rise to receptor-based antibodies that are selected by the stimulation with malaria parasites (Fig. 3.1). The sequencing of cDNA and gDNA revealed in all cases the simple principle of insertion of one or more exons that could be efficiently spliced in leading to the display of antigen-binding domains in the VH-CH1 elbow of an antibody. Our study also provides genetic and structural evidence for the interaction of RIFINs with host inhibitory receptors which is relevant to understand malaria pathophysiology. These findings opened up questions on the versatile functions of RIFIN variants as well as the potential of host generation of receptor-based antibodies against various diseases. Furthermore, we have shown a proof-of-concept, that elbow-containing antibodies can confer dual-specificity or increase the affinity of PfCSP neutralizing antibodies. This new model extended current strategy for antibody engineering. This section aims to discuss some unanswered questions based on the finding of the study.

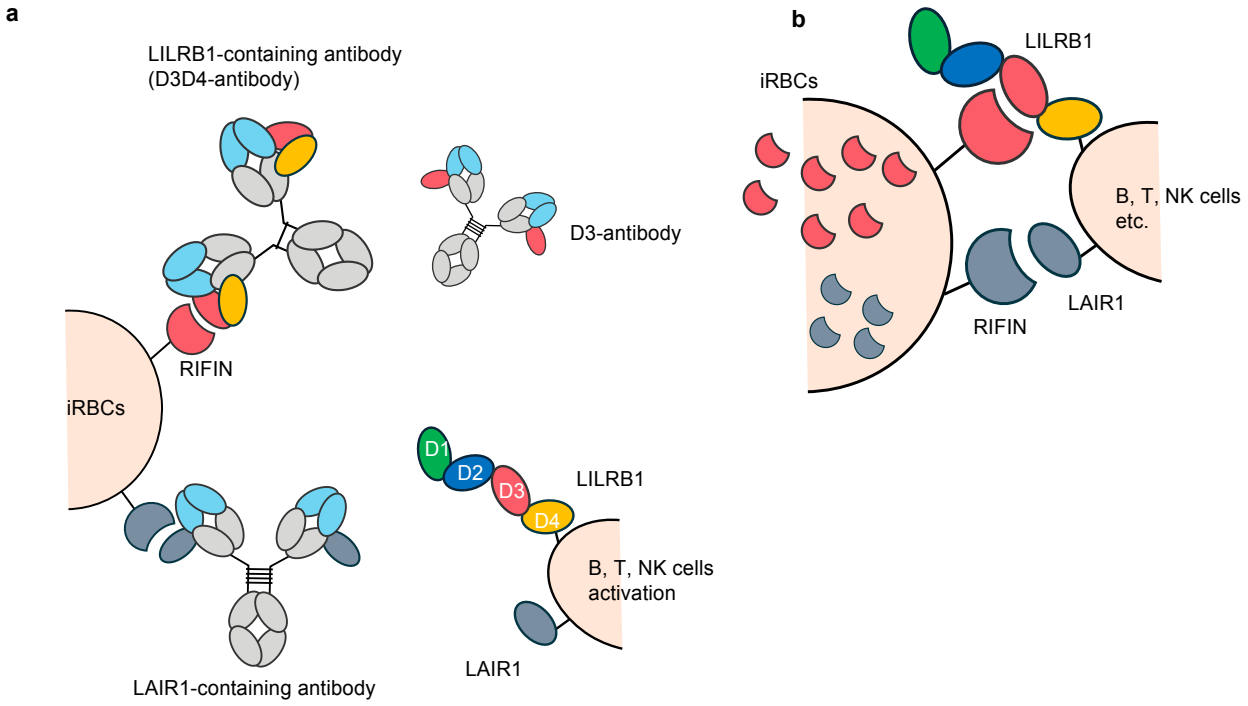


Figure 3.1. **Scheme of receptor-based antibodies hijacking binding of RIFIN to host inhibitory receptors.** a, Immune cells such as B, T, Natural killer cells express inhibitory receptors LILRB1 or LAIR1 on the cell surface. IEs express surface variant antigen RIFIN that can target host inhibitory receptor for immune invasion. Host immune system developed natural receptor-based antibody to combat this evasion mechanism. b, LILBR1-containing antibody do not crossreact with RIFINs that are specific for LAIR1.

3.1 Templated insertion in the switch region generates receptor-based antibodies

Open questions given the insertions described regards: where the origin of inserted DNA is and what is the mechanism of DNA insertion. In a previous study we found that in B cells producing LAIR1-containing antibodies both LAIR1 alleles were present on chromosome 19, suggesting a "copy and paste" rather than a "cut and paste" mechanism (Tan et al., 2016). In the following section, I will first discuss the similarity of LAIR1 and LILRB1 insertions in the switch region and then I will consider a plausible mechanism that generates such receptor-based inserts.

The leukocyte receptor cluster on chromosome 19 encompasses at least 29 genes encoding leukocyte-expressed receptors of the immunoglobulin superfamily, including LAIR1 and LILRB1

that have inhibitory function mediated by a tyrosine-based inhibitory motifs (ITIMs) present in the cytoplasmic tail (Hirayasu & Arase, 2015; Meyaard, 1999; Yoder et al., 2001).

Both receptors are broadly expressed on human leukocytes including B cells, T cells, natural killer (NK) cells and myeloid cells (Arora et al., 2018). LAIR1 is a small glycoprotein of 32 kDa with a single extracellular Ig-like domain that binds with high affinity to collagen and to proteins with collagen-like folds, such as the C1q (Son, Santiago-Schwarz, Al-Abed, & Diamond, 2012). The inserted LAIR1 domain would cause the antibody to become autoreactive. It was therefore interesting to find that in all clones analyzed the LAIR1 insert was mutated in such a way that it could no more bind collagen. This was true even when the insert was in the switch region which unlike the V region is not targeted by AID and somatically mutated. The loss of self-reactivity through somatic mutations has been previously described in antibody V genes and dubbed "B cell redemption" (Sabouri et al., 2014). Thus, we speculate that only those inserts that fortuitously mutate away from self-reactivity can be tolerated in a developing B cells clone, while those that do not mutate are deleted.

Endogenous LILRB1 inhibitory receptor binds human class I molecules (HLA-I) through its N terminal D1-D2 domains while D3 and D4 simply serve as a scaffold for the receptor and do not have physiological function (Qihui et al., 2019). The insertions of LILRB1 in the switch region follow a similar theme as observed for LAIR1, with two major differences. The first is that the insert is much larger and comprised several domains, including D3 or D3-D4 at protein level. The second is that the insert does not carry significant mutations, a finding that is explained by the fact that the two inserted domains are not self-reactive. On this ground we speculate that just every developing B cell with an insertion of a spliceable LILRB1 D3 domain can be selected and respond to the malaria parasite.

With regard to effector function and protective mechanisms, LAIR1 and LILRB1 containing antibodies may interfere with parasite evasion mechanisms based on triggering of host inhibitory receptors (Arora et al., 2018; Saito et al., 2017). We should consider however that those mechanisms may be very redundant since different RIFIN may engage different receptors. Another possibility is that these antibodies opsonize IE and thus promote their removal from the circulation by phagocytic cells (Tan et al., 2016).

We can only speculate on the reasons why LAIR1 and LILRB1 are frequent donors for templated insertions. Both genes are both located at the subtelomeric region of chromosome 19 (Hsieh & Higgins, 2017; X. Kang et al., 2016). The subtelomeric DNA has been shown as preferential sites of

segmentally duplicated DNA and correlate with genomic instability (Riethman, 2003). This class of repeat DNA is characterized by very high sequence similarity and is estimated to comprise 5% of the human genome (Riethman, 2003). Although the underlie mechanism of DNA duplication is unknown, it was suggested that such duplication activity are still active due to its physical location and the long arm of chromosome 19 is one of the hotspots for duplication (Riethman, 2003). Furthermore, the subtelomeric region is known to be unstable, where replication stall or DSBs often occur (Miller, Reynolds, Mejia, Stark, & Murnane, 2011; Yatsenko et al., 2012). It is likely that during a rare duplication event or a failure of replication, a piece of double stranded LAIR1 or LILRB1 DNA were generated which contributes to the source for elbow-containing antibodies. Lastly, both genes are actively expressed in B cells during the development (X. Kang et al., 2016; Meyaard, 1999), which provide an open DNA structure for the fragmentation to occur. These properties are likely to contribute to the propensity of LAIR1 and LILRB1 to donate DNA templates for insertion into antibody genes.

Concerning the timing of this insertion event, we found inserts primarily end up in the Smu region, indicating that such insertion occurs during class switching. S region is most active for DSBs during the CSR under the influence of specific cytokines. In this regard, it is likely that this process occurs during the primary immune response when antigen-specific B cells are activated by T_{FH} cells and undergo class switching. Junctional analysis of the S region insertions showed no homology between donor sequence (S region) and template DNAs (LAIR1 or LILRB1), suggesting a simple break and rejoining event via the c-NHEJ pathway (Deriano & Roth, 2013; Richardson, Ray, Bray, & Corn, 2016). It is interesting that in clone MDB1 who makes an IgG4 antibody the LILRB1 insert was found in the Sg1 region, suggesting that this B cell has underwent at least two rounds of class switching with insertion occurring during the secondary switching event (Jabara et al., 1993).

The fact that S region is fragile has been demonstrated by numerous studies (Chaudhuri & Alt, 2004; Luby, Schrader, Stavnezer, & Selsing, 2001). We and other have shown that insertion could happen in the S region (Baar & Shulman, 1995; Pieper et al., 2017). Moreover, S region insertion have been seen more often when associate with genomic instability, for example, it has been observed that in malignant B cells that Smu region have several DNA insertions from various chromosomes (Gostissa, Alt, & Chiarle, 2011; Lenz et al., 2007). Although evidences have shown that DNA fragments (less than 500bp) can be inserted into the V(D)J or S region of the antibody heavy chain locus, to the best of our knowledge the insertion of LAIR1 or LILRB1 is the first

example of insertion that gives rise to functional antibodies.

3.2 RIFIN targets inhibitory receptor

Given the fact that RIFINs are highly polymorphic, the function of RIFIN maybe as well. To the best of our knowledge, we are the first to propose that RIFIN function to target host inhibitory receptors to facilitate immune evasion. This observation resonates with the finding that tumor cells upregulate ligands of inhibitory receptors such as PD-L1 to target host inhibitory receptor PD-1 on effector cells, thereby alleviating the host immune response and evade immune surveillance (Blank, Gajewski, & Mackensen, 2004).

We have shown that LILRB1-specific RIFINs cannot recognize LAIR1, and it is true also for LAIR1-specific RIFINs, which cannot be recognized by LILRB1-containing antibody. It appears that a subset of RIFINs is only responsible for one particular receptor, whereas the other subset of RIFINs is responsible for a different receptor. Since RIFIN has more than 200 copies per parasite genome, it is likely that the remaining RIFIN variants target other host receptors despite LAIR1 and LILRB1. Prior studies, including us, have the attempt to screen a series of inhibitory receptors for their ability to bind IEs; however, only LILRB1 was identified (Saito et al., 2017), (data not shown). Candidate inhibitory receptors such as LILRB2, CTLA4 (Cytotoxic T-lymphocyte-associated antigen 4), BTLA (B and T lymphocyte attenuator), KIR (killer cell inhibitory receptors) family (Valiante et al., 1997) were fused with an Fc domain respectively failed to bind to the testing parasites (Saito et al., 2017). What could be the reason for these results? Given that D1-D2 is critical for the function of LILRB1, it is surprising that inserted domains in the LILRB1-containing antibody turn out to be the D3-D4 domain (scaffold) rather than the D1-D2 domain. Most host receptors have more than one extracellular domain, and the functional domain/domains are expected to be the target for a certain ligand. However, RIFIN exploits a binding mode to a membrane-proximal region of the LILRB1 receptor (D3-D4), suggesting that the non-functional domains of potential inhibitory receptors may interact with RIFIN. It is possible that the use of Fc-fusion protein limited the exhibition of the domain of interests as compared to its native structure and leading to the such results. To overcome this limitation, an elbow format could be used to perform better pathogen binding domain presentation.

3.3 From the structure of elbow antibody to bispecific antibody engineering

Our structural data revealed an extraordinary triangle architecture where the VH-CH1 elbow opens up completely to accommodate the LILRB1 D3-D4 domains while preserving VH/VL interaction. The structural basis explains why engineered elbow antibodies with Ig-like domain insertion are comparable to the conventional antibodies in terms of their expression and function.

The interface of MDB1-RIFIN PF3D7_1373400 complex revealed a series of residues on the RIFIN-V2 involving the contact to the LILRB1 D3 domain. Interestingly, these residues are not conserved among identified LILRB^{1+} RIFINs, suggesting MDB1 recognizes other RIFINs not via specific residues. Together with the information of LAIR1^{+} RIFIN binding pattern, the conformation of V2 apex region is likely to be essential for the interaction with the receptor, rather than a conserved epitope. Given that RIFIN-V2 are even more polymorphic in various strains of parasite, it is conceivable that to possess a set of RIFINs shared a similar conformation is easier than sharing specific residues in the context of evolutionary adaptation. Reciprocally, this finding explained why LAIR1 and LILRB1-containing antibodies have a broadly reactivate propensity (Tan et al., 2016).

These data provide a route for using RIFIN as an immunogen that ought to elicit an antibody response targeting a conserved confirmation of RIFIN-V2. Moreover, LAIR1^{+} RIFIN and LILRB1^{+} RIFIN shared a similar binding mode but possessed specificity towards distinct receptor. The balance between the specificity and crossreactivity of anti-RIFIN antibodies is essential to be determined in further detailed analysis.

4

Outlook

This section aims to discuss the future path that can be taken to identify novel receptor-based antibodies against malaria or other diseases. Furthermore, I will also discuss the possibility of using RIFIN as a target for passive and activate vaccination against malaria.

4.1 Receptor-based antibodies beyond malaria

After the initial discovery of LAIR1-containing antibodies, the LILRB1-containing antibodies that we discovered in this study provide a second example of antibodies where the antigen binding moiety is generated by templated insertion of a pathogen receptor into Ig genes, in this particular case in the switch region. Thus, our findings illustrate with a second example, a new category of receptor-based antibodies. In a historical perspective, it is interesting to note that such antibodies were initially conceived by Paul Ehrlich in his “side chain theory” of antibody generation (Witebsky, 1954). On this ground, we hypothesize that other receptor-based antibodies will be found in the future. The basis of this hypothesis lies in the frequent occurrence of templated insertions in the switch region. In a previous study, we estimated that 3.5% of these inserts contain splicable exons that could lead to the insertion of a variety of domains in the Ig elbow (Pieper et al., 2017). Although these insertions are the product of sporadic clonal events, the large number of B cells present in the human immune system combined with the power of clonal selection could result in the expansion of these clones. Interestingly, in all cases studied so far, the antibody response that was readily detected in the serum was generated by a single B cell clone (Pieper et al., 2017; Tan et al., 2016). On this ground, we expect that further examples of receptor-based antibodies will be found, not only in response to the malaria parasites where other inhibitory or adhesive receptors

may provide a pathogen binding site, but also in the case of anti-viral responses in all cases where the virus targets a host protein using high affinity ligands. For instance, *in vivo*, ICAM1 function to provide adhesion between endothelial cells and leukocytes after injury or stress by binding to leukocyte function-associated antigen or macrophage-1 antigen (Philippe G Frank, 2008). However, ICAM1 is also used as a receptor by the major group of human rhinoviruses and is a catalyst for the subsequent viral uncoating during cell entry (Shukla et al., 2017). Of note, ICAM1 also composed of Ig like domains (Yang et al., 2004). We could screen for ICAM1-containing antibodies in individuals and assess their ability to neutralize rhinoviruses. Another receptor that could be interesting is basigin, which also belongs to the Ig superfamily (Wright et al., 2014). Basigin is the receptor for many ligands in the host, such as cyclophilins, S100A9, and platelet glycoprotein VI. Concurrently, basigin serves as the receptor for the parasite invasion reticulocyte-binding protein 5 (Rh5) and is required for invasion by all *P. falciparum* strains (Wright et al., 2014). The discovery and characterization of the basigin-containing antibody will be one of the future directions.

There are several factors that may increase the discovery of alternative naturally acquired receptor-based antibodies based on current evidence. First of all, the inserted DNA fragments should be actively transcribed, or the encoding receptor protein is expressed during the B cell development. Secondly, the encoding protein structure should have lg-like folds that be easily accommodated in the antibody Ig domains. Moreover, the inserted DNA should encode a protein that has the ability to bind foreign antigens such as virus spike protein or parasites VSAs, etc.

Nevertheless, even if it is challenging to identify naturally acquired receptor-based antibodies, using the elbow format to generate competing antibodies would be beneficial for both therapeutical and research purposes.

4.2 The role of RIFINs in the parasite life cycle and as vaccine candidates

The identification of RIFIN as the target of inhibitory receptor-based antibodies reveals a plausible role of RIFIN to evade immune surveillance and suggests the potential of using RIFIN as a suitable malaria vaccine candidate. Nanoparticle-based vaccine strategy incorporating RIFIN could complement ongoing efforts to develop pre-erythrocytic malaria vaccines (Bannas et al.,

2017). A major problem lies in the high degree of diversity due to the presence of multiple genes ($>$ 150) and to their extraordinary polymorphism. We expect that phylogenetic and structural studies such as those reported in this study will help clarify the role of these molecules in the parasite life cycle. We have shown that LAIR1 containing antibodies can opsonize IE expressing specific RIFINS suggesting a role of adaptive immunity in protection from blood stage parasites (Tan et al., 2016). It is interesting to speculate that the high degree of polymorphism in the RIFIN multigene family may reflect an attempt to escape from the antibody response. In this context, the receptor-based antibodies offer an extra degree of protection are not compatible with pathogen escape. Further studies will be required to address questions related to the possibility of RIFIN targeting other host receptors.

Other Plasmodium species infecting human, rodent or simians also express RIFIN analogs known as PIR (Plasmodium interspersed repeat) multigene family, which includes rif (200), stevor (35) in *P. falciparum* and cir (200), bir (180), yir (800), kir (68), cyir (256), vir (350) in *P. chabaudi*, *P. berghei*, *P. yoelli*, *P. knowlesi*, *P. cynomolgi* and *P. vivax* respectively (Yam, 2016). The function of PIR family remains to be determined despite their discovery more than a decade ago. It is possible that LILRB1 or LAIR1-containing antibody crossreactives with a subset of PIR in species besides *P. falciparum*. To test this hypothesis, we plan to stain plasmodium species such as *P. knowlesi*, *P. berghei* with LAIR1 or LILRB1-containing antibody then perform LC/MS to identify antigens. Moreover, without suitable animal models for *P. falciparum*, it is not feasible to validate RIFIN functions in vivo. To determine the importance of RIFIN or other PIRs, using a rodent infected malaria parasite, such as *P. chabaudi* as a robust animal model for studying the interaction of host cells and immune responses with malaria would be an integrative strategy (Mackinnon, Walker, & Rowe, 2002). Furthermore, human inhibitory receptors ortholog often found in other animal species. For example, LAIR1 is also expressed in mouse, and LILRB1 has a murine ortholog immunoglobulin-like receptors-A (PIR-A) (van der Touw, Chen, Pan, & Chen, 2017). It has been shown that murine monocytes and macrophages acquire memory specific to MHC-I antigens and identify PIR-A as the MHC-I receptors necessary for the memory response (Dai et al., 2020). Interaction between malaria PIR surface antigen with inhibitory receptor PIR-A may shield the light on the new role of RIFIN in the context of malaria infection. These approaches could complement ongoing effects to validate and explore RIFIN's function in vivo.

RIFIN may also have other unknown functional roles apart from immune evasion, as RIFIN has been shown to be expressed at various stages of the parasite life cycle, including merozoites and

sporozoites stage (Mwakalinga et al., 2012; Wang et al., 2010). It would be interesting to study whether LAIR1 or LILRB1-containing antibody has a role in different life stages. To address this question, we can use a mouse model as described above.

4.3 Explore the mechanism of templated insertions

Templated insertions of spliceable exons encoding a pathogen receptor in the switch region generate naturally occurring antibodies where pathogen binding domains are inserted in the VH-CH1 elbow. Further studies are required to understand the underlying mechanism. The finding of templated insertions in European blood donors suggests that this mechanism is not triggered by malaria infection (Pieper et al., 2017). Rather malaria selects those rare clones that carry an appropriate insert. While AID-mediated DSBs are likely to provide the site for insertion, a major question remains on the origin of the inserted DNA. The presence of introns is incompatible with its origin from mature mRNA as suggested (Onozawa et al., 2014). A hypothesis that we are contemplating is that DNA fragments originated from the resolution of stalled replication forks may be used by NHEJ to repair AID-induces DSB. A systematic analysis of insertions in gDNA and cDNA will be required to address this mechanism.

Based on our observation, for an insertion to occur, several mechanistic factors are required. First, DSBs must be present at Ig loci. Second, the broken Ig loci must be in proximity to the template DNA or be moved into proximity to be joint. Finally, the cellular DNA repair pathways must be available to join the two broken ends to complete the insertion. During CSR, DSBs are induced, which likely served as acceptors for template DNA to be inserted. However, to meet the second requirement, it is unlikely that fragmented DNA just swims near chromosome 14 (Ig loci) to be in close proximity to the breakpoint. We speculate that chromosome 19 where the DNA fragments originated is in spatial proximity with chromosome 14 during the germinal center B cell activation. To test this hypothesis, an approach for the disclosure of spatial interactions of specific genomic loci called 4C (chromosome conformation capture-on-chip) could be applied in class switching B cells and may provide a hint to this part of the puzzle (van de Werken et al., 2012).

4.4 Conclusion

In conclusion, on a personal ground, I believe that the discovery of receptor-based antibodies is only the tip of the iceberg, and it could be that many exciting findings lie ahead.

5

Material and Methods

5.1 Serum plasma samples and cells

The majority of the Malian sera were obtained from a cohort in Kalifabougou, Mali (Tran, Ongoiba, et al., 2013), 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). Sera from healthy individuals (various ages and genders) are collected based on standard regulation. The donor gave written informed consent for the use of these blood samples, fooling approval by the Cantonal Ethical Committee of Canton Ticino, Switzerland.

5.2 *P. falciparum* parasite cultures

The P. falciparum laboratory line 3D7 clone was cryopreserved at the ring stage and was thawed and cultured to >= 3% parasitemia in blood group O erythrocytes using the following culture medium (RPMI 1640 medium with 10% heat-inactivated serum from healthy donors and supplemented with 25 μ g/mL gentamicin, 0.2% glucose, 2 mM L-glutamine, 37.5 mM HEPES and 0.05 mg/mL hypoxanthine). Parasite cultures were routinely tested for Mycoplasma contamination. To select for MDB1-positive infected erythrocytes (IEs), IE cultures that have reached to late trophozoite stage were collected into a small volume (1.5ml) and were incubated with MDB1

(100ug/ml) for 30min at room temperature, washed and coated with Protein G magnetic beads (Life Technologies) for 30min at room temperature. Protein G binding parasites were collected via magnetic sorting and enriched fractions were returned to in vitro culture. Post 24 hours, the magnetic beads were removed from the parasite culture and discarded (Tan et al., 2016). The enrichments were established for several rounds until a clear positive population of MDB-positive parasites was observed using FACS analysis. Binding curves are fitting to a sigmoidal curve model using Graphpad Prism 8.

5.3 B cell immortalization and isolation of monoclonal antibodies

IgG memory B cells were isolated from donor's frozen PBMCs (peripheral blood mononuclear cells) by first staining with anti-CD19-PECy7 antibodies (BD, 341113) and mouse anti-PE microbeads (Miltenyi Biotec, 130-048-081) and subsequently with a magnetic cell sorting. Enriched CD19-B cells are further stained with goat Alexa Fluor 647-conjugated anti-human IgG (Jackson ImmunoResearch, 109-606-170) for FACS sorting. Sorted B cells were immortalized with Epstein-Barr virus (EBV) and plated in single cell cultures in the presence of CpG-DNA (2.5 μ g ml -1) and irradiated PBMC-feeder cells. After two weeks, the supernatants of cell culture were tested for the presence of LILRB1-containing antibody using beads based immunoassay as previously described (Pieper et al., 2017). For the conformation of LILBR1-containing antibody culture, supernatants were tested for the capability of binding to IEs from parasites. Briefly, fresh or cryopreserved late trophozoite parasites were stained with 10X SYBR Green I for 30 min at room temperature on a rotation rack. The parasites were washed and incubated with the B-cell supernatants for 1 hour at 4° C. The antibody binding was detected by FACS with 2.5 μ g ml -1 Alexa Fluor 647-conjugated goat anti-human IgG staining.

5.4 Analysis of monoclonal antibodies

cDNA was synthesized from selected B cells, and their heavy chain and light chain sequences were captured using a specific primer mix as previously described (Tiller et al., 2008). The usage of variable region genes and somatic mutations accounts were analyzed in IMGT. Genomic DNA

was extracted from selected B cell clones using conventional molecular cloning method and was amplified using the REPLI-g Single Cell Kit (QIAGEN) before performing PCR. The insertions of LILRB1 were determined using LILRB1-specific primers and VH specific primers based on cDNA data or constant region-specific primers. After PCR amplification with LongAmp Taq Polymerase (New England Biolabs), the $\sim$ 6000bp amplicons were cloned into a TOPO XL vector (TOPO XL PCR cloning kit, ThermoFisher) and sequenced by plasmid-NGS-sequencing (Microsynth, Switzerland).

5.5 Western blot analysis

The western blot was performed using standards protocols as described previously. Briefly, B cell supernatants or recombinant antibody constructs were diluted in H2O, 4x sample loading buffer (Pieper et al., 2017) (Life Technologies) and 10x sample reducing agent (Life Technologies) were loaded onto precast gels with a 4-12% acrylamide gradient (Invitrogen). The proteins were transferred to PVDF membranes followed by blocking with 2% milk in TBS buffer. The membrane was incubated with primary and secondary antibodies diluted in 2% milk TBS buffer for 1 hr at room temperature. After washing with the TBS-tween buffer, the membranes were develop using a chemiluminescent substrate (Thermo Scientific). The primary antibody for detection of IgG or LILRB1 was goat anti-human IgG (southerntech) used at 2ug/ml or goat anti-human LILRB1 used at 5ug/ml (southerntech). The secondary antibody for both western analyses was Rabbit anti-goat HRP used at 0.2ug/ml.

5.6 Recombinant antibody expression and purification

The heavy chain and light chain of antibodies (all the LILBR1-containing antibodies, BKC3, MGD21, and MGDUCA, etc.) were cloned into human IgG1, Igκ or Igλ expression vectors (Tiller et al., 2008) and were transfected into the Expi293F cells (ThermoFisher Scientific) using polyethyleneimine (PEI) or Lipofectamine 3000 Reagent (ThermoFisher Scientific). Routine tests for Mycoplasma contamination were performed. The antibodies were affinity purified by protein A chromatography or protein G chromatography using AKTA (GE Healthcare). Variants of MDB and GCE536 were constructed by inserting or deleting LILRB1 domains based on Ensemble genomic database. The LILRB1-Fc fusion protein was purchased from R& D Systems or made in the lab. The antibody

constructs were tested for staining of 9775 IEs on FACS.

5.7 Identification of target antigens

Lab-line 3D7 IEs cultures that have reached to late trophozoite stage at >5% parasitemia were enriched using MACS magnetic beads to avoid early stage parasites. Enriched IEs were stained with 10X SYBR Green I for 30 min at room temperature on a rotation rack. Followed by four times of washing, parasites were stained with 100ug/ml LILBR1-containing antibody MDB1 at room temperature for 20min and with 2.5 μ g ml -1 Alexa Fluor 647-conjugated goat anti-human IgG as secondary antibody at the same condition. Subsequently, FACS sort MDB1 binding positive and negative compartments, respectively. Positive and negative binding IEs were harvested at 10,000g for 5min, then the pellets were treated with hypotonic lysis buffer (5 mM KH2PO4, pH 7.4) for the RBC membrane extraction based on the previous protocol (Methods in Malaria Research). The collected RBC membranes were subject to sonication with Bioruptor® Plus Sonication System (Cat. No. B01020001) using the following program: 15cycle, 30s on, 30s off, high. After sonication, the solubilized proteins were treated with a series of chemicals (10mM DTT, 50mM IAA, LysC, trypsin, etc.) based on standard mass spectrometry sample preparation procedure. After trypsin digestion, peptides were analyzed on a Q-Exactive instrument at the IRB. Raw files were analyzed using the MaxQuant software (Cox & Mann, 2008), and MS/MS spectra were searched against 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). Data were visualized by Interactive Graphics Glimma (Su et al., 2017).

5.8 Antigen validation assay

To validate whether certain RIFIN is the antigen for LILRB1-containing antibodies, gene encoding full length or V2 region of RIFIN candidates were produced by gene synthesis (Genescript) and cloned into pDisplay vector (Invitrogen). The vector contains a hemagglutinin (HA) tag, which will be fused to the C-terminal of RIFIN antigens as previously described (Tan et al., 2016). RIFIN-containing pDisplay vectors were transiently transfected into HEK293F cells respectively

(ThermoFisher Scientific) using PEI. Cell lines were routinely tested for mycoplasma contamination. Briefly, one day before transfection, HEK293F cells were seeded at 0.7 x 10^6 cells ml −1 in 10ml Expi293™ Expression Medium (ThermoFisher Scientific). On the day of transfection, 6ug construct DNA was diluted in OPTI-PRO SEM Medium (Invitrogen) and mixed with 60ug PEI for 20min at room temperature. The DNA-PEI complexes were added to the cells, which were cultured in a CO_2 shaker incubator at 37degree, X r.p.m. Seventy-two-hour post-transfection, cells expressing RIFINs were collected and stained with LILRB1-containing antibodies or control antibodies and tested by flow cytometry. Briefly, 5 μ g ml −1 of LILRB1-containing antibodies were added to the RIFIN-transfected cells for 30min at 4 degree and followed by washing steps before staining with a secondary of 2.5 μ g ml −1 of Alexa Fluor 647-conjugated goat anti-human IgG antibody (Jackson ImmunoResearch, catalog no. 109-606-170). The cells were washed again, then stained with 5 μ g ml −1 of rabbit anti-HA tag for the same condition followed by washing before adding Alexa Fluor 488-conjugated goat anti-rabbit IgG (Life Technologies, catalog no. A11034) for the detection of RIFIN. Dead cells were excluded by gating (show gating in extended data).

5.9 Sequence homology analysis

The occurrence of different amino acid identities at the RIFIN V2 region was analyzed by downloading all RIFIN protein sequences present in National Center for Biotechnology Information (NCBI) Entrez Protein database (Wheeler et al., 2007) as of December 18, 2019 or PlasmoDB database (2008). After alignment of the RIFIN protein sequences using Clustal Ω (Sievers & Higgins, 2014), the V2 region was defined as the region from amino acid 1924 to 2137. We extracted the V2 region of each sequence and aligned them again using Clustal Ω . Hotspots were identified as the shared amino acids between the different RIFIN proteins binding to LILRB1 (PF3D7_1041200, PF3D7_0937700, and PF3D7_1373400). The remaining RIFIN protein sequences were analyzed and ranked by their number of shared hotspots for each position (108-A, 113-V, 114-I, 125-F, 172-N, 173-Y, 176-A, 219-I, 235-F, 282-P, 283-I, 285-C, 328-I, and 332-V).

The average distance tree assumes a constant rate of evolution across lineages. The average distance trees here were generated using alignments of homologous sequences to infer closest relatives in context of the gene of interest, and the tree was calculated in MEGA (S. Kumar, Stecher, Li, Knyaz, & Tamura, 2018).

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