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EXPLORING B CELL RESPONSES IN *PLASMODIUM FALCIPARUM* MALARIA

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Exploring B cell responses in *Plasmodium falciparum* malaria

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To my precious family

ABSTRACT

Plasmodium falciparum malaria remains one of the most devastating infectious diseases today. Small children in sub-Saharan Africa carry the heaviest burden of morbidity and mortality. Immunity to malaria is not well understood, although humoral immunity has proven an integral part of protection from disease. Antibodies are produced by B cells and have been directly linked to clinical immunity to malaria. There is a need to further characterize the development of B cell responses during and following a malaria infection, in order to understand the basis of clinical immunity. In **Study I** we developed a method for the identification of *P. falciparum*-specific B cells using Quantum dots in flow cytometry. We found almost a third of B cells from individuals living in a malaria-endemic area to be specific for *P. falciparum*. In **Study II** we followed a cohort of mothers and infants in Uganda with prospective blood sampling from birth up to nine months. Levels of the cytokine, B cell activating factor, were measured and in infants found to be highest in cord blood with a subsequent decrease, while the levels in mothers remained stable. Furthermore, B cell activating factor was inversely correlated with IgG^+ memory B cells and $CD27^-$ memory B cells at different time points in infants and mothers. **Study III** was a prospective study in Stockholm enrolling individuals with acute malaria with subsequent sampling over a year. We found that B cells responding to infection with *P. falciparum* expressed CD11c with a dynamic shift within B cell compartments. Differences between individuals with a primary malaria infection and those previously infected, revealed differential expansion with a higher frequency of atypical memory B cells in previously infected individuals. In **Study IV** we established a novel co-culture method for human B cells and *P. falciparum*-infected red blood cells to mimic *in vivo* conditions. Parasitemia increased more rapidly when parasites were cultured with B cells than when cultured alone, and B cells exhibited phenotypic changes after ten days in co-culture with *P. falciparum.* Within the scope of this thesis we provide new methodology for the study of B cell responses to malaria, and present longitudinal data on B cell remodeling after acute malaria, as well as B cell activating factor in an endemic area. These novel methods and findings contribute valuable knowledge and can be used to inform the design of future studies to increase our understanding of the immune system in malaria.

LIST OF SCIENTIFIC PAPERS

I. Allan Lugaajju, Sreenivasulu B. Reddy, **Caroline Rönnberg**, Mats Wahlgren, Fred Kironde and Kristina E. M. Persson

Novel flow cytometry technique for detection of Plasmodium falciparum specific B-cells in humans: increased levels of specific B-cells in ongoing infection

Malar J 2015, 14:370

II. **Caroline Rönnberg**, Allan Lugaajju, Anna Nyman, Ulf Hammar, Matteo Bottai, Christopher Sundling, Fred Kironde, Kristina E M Persson

A longitudinal study of plasma BAFF levels in mothers and infants in Uganda

Manuscript

III. Christopher Sundling*, **Caroline Rönnberg***, Victor Yman, Muhammad Asghar, Peter Jahnmatz, Tadepally Lakshmikanth, Yang Chen, Jaromir Mikes, Mattias N. Forsell, Klara Sondén, Adnane Achour, Petter Brodin, Kristina E.M. Persson, and Anna Färnert

B cell profiling in malaria reveals expansion and remodeling of CD11c+ B cell subsets

JCI Insight. 2019; 4(9):e126492

IV. Sreenivasulu B. Reddy, Noemi Nagy, **Caroline Rönnberg**, Francesca Chiodi, Allan Lugaajju, Frank Heuts, Laszlo Szekely, Mats Wahlgren, Kristina E. M. Persson

Direct contact between Plasmodium falciparum and human B-cells affects parasite growth and FcRL4 expression in novel long-term co-culture method

Manuscript, submitted

* Equal contribution

CONTENTS

LIST OF ABBREVIATIONS

ACT	Artemisinin-based combination therapy
AMA	Apical membrane antigen
APRIL	A proliferation-inducing ligand
ASC	Antibody secreting cell
aMBC	Atypical memory B cell
BAFF	B cell activating factor
BCMA	B cell maturation antigen
BCR	B cell receptor
CD	Cluster of differentiation
CHMI	Controlled human malaria infection
CpG	Deoxy-cytidylate-phosphate-deoxy-guanylate
CRP	C-reactive protein
CSP	Circumsporozoite protein
CSR	Class-switch recombination
CyTOF	Cytometry by time-of-flight
cMBC	Classical memory B cell
EBA	Erythrocyte-binding antigen
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immune absorbent spot
FBS	Fetal bovine serum
FDC	Follicular dendritic cell
FMO	Fluorescence minus one
GC	Germinal center
GiRBC	Ghost *Plasmodium falciparum* infected RBC
GLURP	Glutamate-rich protein
ICAM	Intracellular cell adhesion molecule
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
IMDM	Iscove Modified Dulbecco Media
IPT	Intermittent preventive treatment

iRBC	*Plasmodium falciparum* infected RBC
ITN	Insecticide-treated bed nets
KHC	Kasangati Health Center
LT	Lymphotoxin
mAb	Monoclonal antibody
MBC	Memory B cell
MHC	Major histocompatibility complex
MSP	Merozoite surface protein
MZ B cell	Marginal Zone B cell
NAI	Naturally aquired immunity
NK cell	Natural killer cell
OD	Optical density
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PfEMP1	*P. falciparum* erythrocyte membrane protein 1
PfRh	*P. falciparum* reticulocyte-binding homologs
Qdot	Quantum dot
RBC	Red blood cell
RDT	Rapid diagnostic test
RIFIN	Repetitive interspersed repeats
RPMI	Roswell Park Memorial Institute (cell culture medium)
STEVOR	Subtelomeric variant open reading frame
SURFIN	Surface-associated interspersed gene family
TACI	Transmembrane activator and CAML intercalator
TCR	T cell receptor
Tfh	Follicular helper T cell
TGF	Transforming growth factor
TLR	Toll-like receptor
TNF	Tumor necrosis factor
t-SNE	T-distributed stochastic neighbor embedding
VCAM	Vascular cell adhesion molecule
VFR	Visiting friends and relatives

1 INTRODUCTION

1.1 Background

Malaria is an ancient disease known to have tormented humanity for over 4000 years (1). In spite of tremendous historical efforts to eliminate the disease, malaria remains one of the biggest killers among infectious diseases today. According to the World Malaria Report, 2017 saw an estimated 219 million cases of malaria and 435 000 deaths (2). Sadly, this devastating morbidity and mortality affects primarily the most vulnerable of populations, namely small children and pregnant women in low-resource settings. Beyond the severe detrimental impact on the health of people subjected to disease goes the considerable hampering of economic development imposed by malaria in lesser developed countries. Malaria has a wide geographic distribution in tropical and sub-tropical regions although 92% of cases and 93% of deaths occur in the WHO African region. *Plasmodium falciparum* is the most prevalent species in the WHO African region accounting for 99.7% of cases (2). This thesis focuses on immunological aspects concerning B lymphocytes in the context of *Plasmodium falciparum* malaria.

1.2 The parasite and its life cycle

Malaria traverses most vertebrates of the animal kingdom from primates to birds and reptiles. This wide range of intermediate hosts, holds a multitude of species of the unicellular parasite *Plasmodium. Plasmodia* are apicomplexan protozoans dependent on the mosquito vector, and definitive host, *Anopheles* for their reproduction. Seven species of *Plasmodia* are known to cause human disease: *P. falciparum, P. vivax, P. ovale spp. (curtisi and wallikeri), P. malariae, P. knowlesi* and *P. cynomolgi (3).* The latter two fall into the simian *Plasmodium* species and are less often encountered in humans. *P. falciparum* causes the most severe forms of clinical disease and consequently most deaths. The life cycle of the parasite is complex and starts with an infected female *Anopheles* mosquito biting a human for a blood meal (Figure 1). The sporozoite stage parasites are thus deposited in the skin and subsequently taken up into the blood stream and lymphatic system to eventually end up in the liver. The sporozoites invade hepatocytes by means of the circumsporozoite protein (CSP) recognizing and engaging heparin sulfate proteoglycans on the hepatocyte surface, leading to invagination of the hepatocyte membrane with a resulting parasitophorous vacuole surrounding the sporozoite (4). Within the hepatocyte, sporozoites then transform and start replicating by schizogony eventually producing thousands of exoerythrocytic merozoites. At the completion of the liver stage, merosomes which are merozoites surrounded by hepatocyte plasma membrane, start budding off to be released into the blood stream via the sinusoids of the liver. Merosomes eventually break up liberating merozoites that continue

the cycle by invading red blood cells (RBCs) (4). The release of parasites into the blood stream marks the first stage at which clinical symptoms of the infection may occur (5). RBC invasion by merozoites is a complex but rapid process involving binding to the RBC, apical reorientation of the merozoite and junction formation. Merozoites house the apicomplexan organelles rhoptries, micronemes and dense granules containing multiple parasite proteins known as adhesins and invasins (6). RBC invasion inhibition studies have revealed a remarkable redundancy in the invasion pathways of *P. falciparum* which undoubtedly contribute to it virulence (7). A parasitophorous vacuole is formed by the invasion process and the cycle continues within RBCs. Merozoites develop into ring stages and trophozoites which mature into replicating schizonts, that subsequently rupture releasing about 20 new merozoites (7). Each of these continue the replication process by invading a new RBC. In the case of *P. falciparum* the RBC cycle takes about 48 hours typically producing bouts of fever as the parasite spills into the circulation (7).

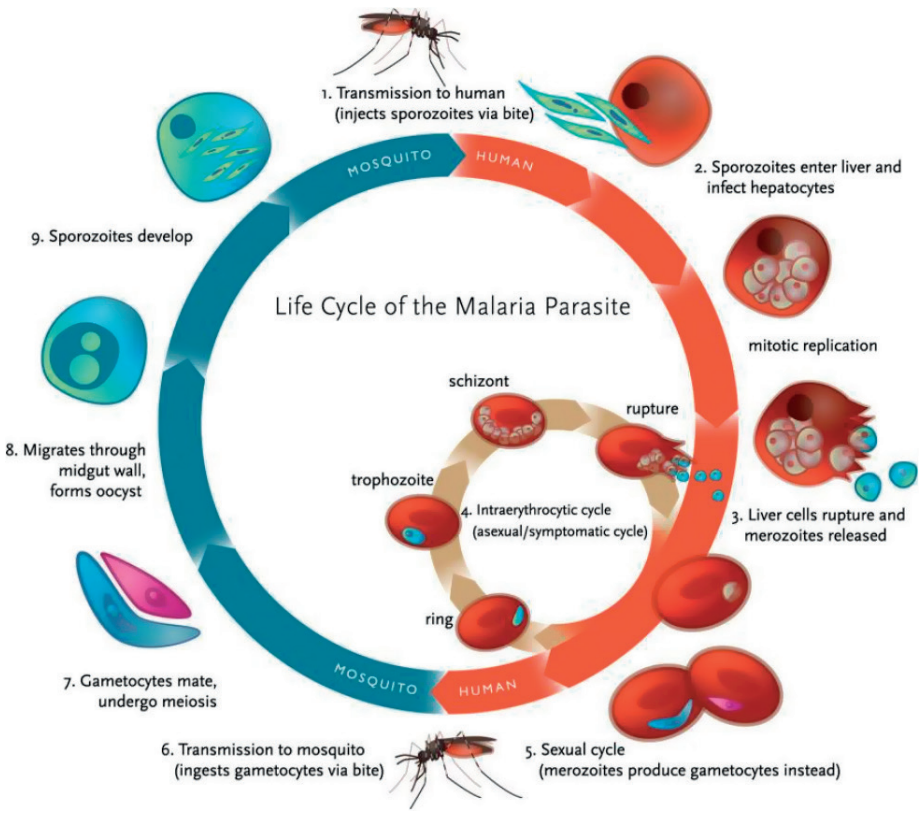

Figure 1. Plasmodium life cycle. *Reprinted from Antimalarial drug resistance: a review of the biology and strategies to delay emergence and spread, Vol 41, issue 4, April 2013, Pages 311-317, Eili Klein, Copyright (2019), with permission from Elsevier.*

A small proportion of the merozoites, however, have a different fate after entering RBCs. Rather than replicating, they mature into male or female gametocytes which are then taken up during the blood meal by another mosquito (8). In the gut of the mosquito, male and female gametocytes multiply by sporogony, eventually generating sporozoites which migrate to the salivary glands of the mosquito, ready to be injected into another human (9).

1.3 Pathogenesis

In a malaria-naïve individual the release of parasites into the blood stream about 10 days after being bitten by the vector leads to strong immune activation with a cascade of proinflammatory cytokines such as tumor necrosis factor (TNF), interleukin-1 (IL-1), interferon-γ (IFN-γ), IL-6, IL-12 and chemokines such as IL-8, as well as anti-inflammatory cytokines like IL-10, IL-4, IL-5 and transforming growth factor-β (TGF-β) (10, 11). The sudden storm of circulating TNF will, as with many other inflammatory conditions, manifest as a fever with a selection of other possible symptoms seen in acute malaria (12). Cytokines are small proteins produced by cells of the immune system and function through a complex network of interaction and communication between cells (13). Pro-inflammatory cytokines are often produced by macrophages (13), although IFN-γ is known to originate from various T cells and natural killer (NK) cells (14). While TNF and IL-1 initiate pathways that ultimately keep coagulation in homeostasis, increased levels during malaria may be the cause of harmful parasite sequestration in the brain and placenta (11). A less studied cytokine known to upregulate TNF is lymphotoxin (LT). Like TNF, LT increases the expression of intracellular cell adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) in support of these cytokines enhancing cellular adhesion to endothelium (15). In contrast, IL-10, which is found at high circulating levels in malaria, has been proposed to suppress disease severity by inhibiting the inflammatory effects of TNF and thus contributing to malarial tolerance (16). Increased levels of IL-6 are seen in severe malaria and appear to be a bad prognostic factor (17, 18). IFN-γ enhances phagocytic activity and is known to have a protective role in animal-models of intracellular infections (19). A number of protective mechanisms, innate and adaptive, of IFN-γ have been proposed (14). In controlled human malaria infection (CHMI), high IFN-γ-responses have been associated with reduced parasite multiplication rates (20) and prospective field studies have found parasite-induced IFN-γ responses to be associated with reduced risk of fever and clinical malaria episodes (21-23). Interethnic differences in the susceptibility to malaria provide unique possibilities for studying immunological correlates of protection. The Fulani tribe in west Africa is known to suffer less from malaria than other sympatric tribes, and it has been shown that mononuclear cells from Fulani, stimulated with *P. falciparum* parasites elicit >10-fold higher levels of IFN-γ than cells from the Dogon tribe (24). Moreover, IFN-γ responses in both tribes have been inversely correlated with parasite density.

The *Plasmodium* parasite uses a number of mechanisms to "hide" from the immune system, known as immune evasion. Surface modifications like knobs on the host RBC, play a major role in this (25). To escape the host immune response, the parasite exports and presents a number of variant surface antigens on the RBC surface. The sequential expression of variant surface antigens known as antigenic variation is an effective strategy for immune evasion and is mediated by several multigene families in *P. falciparum*: *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) encoded by the var genes, repetitive interspersed family (RIFIN) proteins encoded by the rif genes, subtelomeric variant open reading frame (STEVOR) proteins encoded by the stevor genes and surface-associated interspersed gene family (SURFIN) proteins encoded by the surf genes (26). A well-characterized phenomenon is that of sequestration, which is the ability of the parasite to cause the infected RBC to adhere to endothelial tissue as well as to other RBCs known as rosetting (27). This cytoadherence is well-studied for PfEMP1 which is known to be particularly sticky (28-30). Sequestration in the brain can have severe consequences leading to cerebral malaria with altered consciousness and sometimes death, especially in small children in highly endemic settings (5). Anemia, resulting from the suppression of erythropoiesis and destruction of RBCs, further exacerbates the condition (7).

1.4 Clinical presentation and treatment

The clinical symptoms and signs of malaria are by no means specific, which makes the anamnesis and knowledge of epidemiology imperative in order not to miss this life-threatening diagnosis. Working as a clinician in a malaria-endemic area invariably creates an ever-present awareness of malaria when a patient presents with a fever. In non-endemic areas, however, when travel history is not recorded, malaria can be missed among other conditions causing fever, which could in the worst case of scenarios, lead to the death of a patient who could have been easily treated. Most people suffering from malaria will have a fever, while additional symptoms vary widely and include headache, chills, cough, diarrhea, nausea and vomiting, abdominal discomfort, dizziness and weakness (31). Upon examination, jaundice, hepatosplenomegaly, pallor, increased respiratory rate, hypotension and increased pulse rate can be noticed (31). Blood morphology may show anemia, thrombocytopenia and lymphopenia, while blood chemistry may reveal increased C-reactive protein (CRP), procalcitonin and lactate dehydrogenase (31). The progression from this initial acute phase to severe malaria with symptoms of altered consciousness or organ failure can be very fast. Severe malaria consists of a list of clinical conditions most often seen in children and in cases where treatment has been delayed. They include; impaired consciousness, prostration, convulsions, acidosis, hypoglycemia, severe anemia, renal failure, jaundice, pulmonary oedema, bleeding, shock and hyperparasitemia (32). Laboratory confirmation of the diagnosis must be

performed without delay. Microscopy remains the gold standard for malaria diagnostics (33). Slides of thick and thin blood smears stained with Giemsa or Fields stain, examined using a light microscope usually reveal ring form trophozoites in case of *P. falciparum* infection, although gametocytes can also sometimes be seen. Microscopy is robust and can be performed in a short amount of time but requires skilled personnel. In areas of inadequate laboratory services rapid diagnostic tests (RDTs) of a lateral flow type are commonly used (33). RDTs provide a fast and simple tool for diagnosing malaria by the detection of parasite antigens, but may present with issues of sensitivity and specificity (34). RDT-results should always be confirmed by light microscopy when possible. As a complement to microscopy and RDT, nucleic acid amplification methods such as polymerase chain reaction (PCR) can detect malaria on the species-level based on 18S ribosomal RNA (35). PCR is a valuable tool in cases of very low parasitemia due to its high sensitivity, and as a confirmation of species based on high specificity. PCR is, however, too time-consuming to be used as a primary diagnostic method since awaiting the result may delay treatment-initiation. Treatment for uncomplicated malaria with artemisinin-based combination therapies (ACT) is usually very effective and should be instituted as soon as possible. For severe malaria, initial intravenous or intramuscular administration of artesunate followed by ACT is recommended (32). There is, however, increasing concern over the rise of artemisinin-resistant strains of parasites, especially in Southeast Asia (36). Thus far, clinical resistance has not been identified in Africa, where a failure of artemisinin would be catastrophic (37).

1.5 Immunity to malaria

Much research has been directed towards understanding how immunity to clinical malaria develops. Sterilizing immunity prevents infection while clinical immunity only prevents disease. Achieving sterilizing immunity is desirable as it implies complete protection with no detectable pathogen within the host. It is well-known that clinical immunity to malaria is slow to develop and requires repeated exposure to be maintained (38). The development of naturally acquired immunity (NAI) to malaria is a function of age and exposure (39). That is; a child born in a malaria-endemic area acquires an increasing degree of clinical immunity with age, and based on the number of infections with the parasite. Clinical immunity thus also depends on transmission intensity and periodicity (40). Immunity develops gradually and slowly starting with a degree of "anti-disease" immunity mounted by children of 2-5 years of age who survived the first couple of bouts of malaria inflicted upon them (41). As the child is subjected to subsequent infections with the parasite, this immunity becomes stronger as evidenced by milder episodes of clinical malaria until, at the adolescent age, symptoms of disease are absent despite continuous inoculation with the parasite (Figure 2). One mechanism of these gradually milder episodes of malaria may be that exerted by IFN-γ (23).

The circumstance of malaria-naïve children and adults being suddenly exposed to sustained intense malaria transmission was extensively studied in migrants to Papua New Guinea (42). In a cross-sectional approach, acquired immunity as seen by parasitemia prevalence, did not appear to be the cumulative result of years of exposure but was rather attributed to intrinsic characteristics of the immune response changing with age (43). Interestingly, in that setting of primary exposure to the parasite, severe malaria was more often seen in adults than in children (42). From these observations it follows that children seem to be able to better handle the acute exposure, while adults can mount clinical immunity more rapidly and thus handle the chronic exposure. An altogether different approach to the matter of age versus exposure was undertaken in Kenya, where the timing of puberty rather than cumulative exposure to *P. falciparum* was linked to the onset of protective immunity (44), suggesting that the key to understanding the molecular basis of NAI to malaria lies in the maturing immune system.

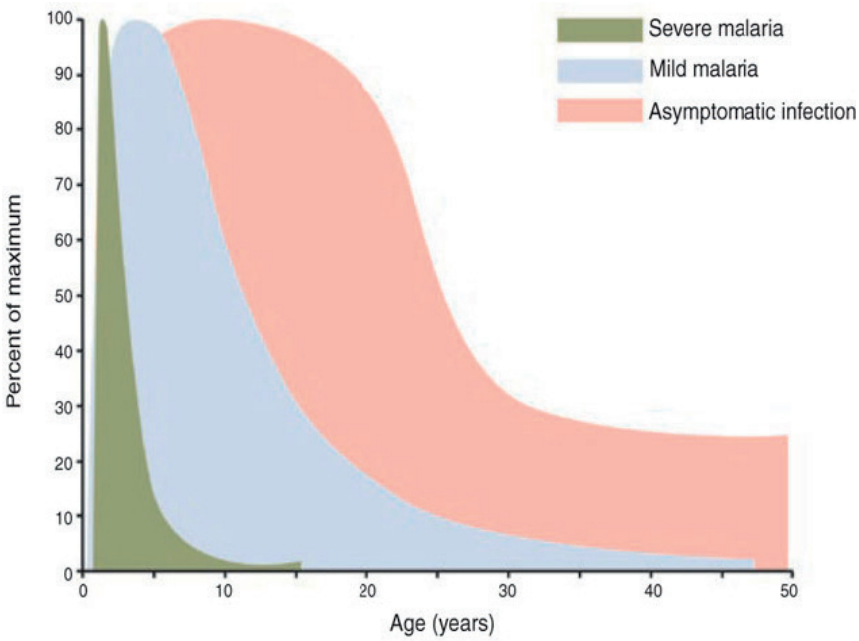

Figure 2. Naturally acquired immunity to malaria. *Reprinted by permission from Springer Nature: Nature Immunology; Immunity to malaria: more questions than answers; Jean Langhorne, Francis M Ndungu, Anne-Marit Sponaas, Kevin Marsh, Copyright (2019)*

Following the acquisition of anti-disease immunity is the slowly developing "anti-parasite" immunity, protecting older children against severe malaria and high parasitemia (41). Intriguingly, individuals who have acquired a high degree

of immunity as seen by solid protection against clinical malaria, may still harbor low-grade parasite concentrations in their blood at all times. This state, originally described by Koch in 1900, is referred to as "premunition" and is characterized not only by apparent good health, but also by protection against new infections (39, 45). Sterilizing immunity is thus unlikely fully achievable through naturally acquired infections (46). A model based on clinical and entomological surveillance data from Uganda, was used to show that both anti-parasite and anti-disease immunity develop gradually and in parallel (47). Older children are less prone to symptomatic malaria since they both tolerate higher parasite densities and are less likely to harbor high parasite densities.

The characteristics of NAI to malaria substantially change during pregnancy. In spite of accumulated immunity from growing up in an endemic area, pregnancy seems to offset protection from malaria leaving women once again susceptible to severe illness, particularly during first and second pregnancies (48-50). This loss of protection is likely due to general suppression of cell-mediated immunity during pregnancy and lack of immunity to pregnancy-specific parasite isolates that sequester in the placenta (51-53). Gradual clinical immunity is achieved with each subsequent pregnancy at least in part due to boosting of humoral immunity against PfVAR2CSA antigen (51, 52, 54-56).

Experience with malaria-immune individuals leaving an endemic area to live in a non-endemic country, shows that immunity wanes rather quickly in the absence of continuous reinfection. Migrants from, for example, sub-Saharan Africa often travel back to visit friends and relatives (VFR) without the precaution of malaria prophylaxis and end up contracting overt, and sometimes severe, malaria. There is however, a prevailing notion that VFR maintain a state of semi-immunity resulting in less severe malarial episodes upon returning to their country of residence (57-59). Lasting immunity is not always evident however (60), and it even seems that after a period of approximately 15 years in the absence of exposure to the parasite, previously clinically immune individuals have again become as susceptible to disease as malaria-naïve individuals (61). The trouble of establishing immune status post malaria exposure is that of a lack of robust biomarkers of immunity (62, 63).

1.6 B cells and antibodies

B cells, together with the antibodies they produce, constitute the humoral part of the adaptive immune system. As such, B cells are very effective in recognizing foreign antigens leading to the formation of high affinity antibodies (64). Immunity to an infectious agent depends on a memory response displaying three typical features: i) it is rapid and more robust than the primary antibody response, ii) it predominantly consists of high-affinity, isotype-switched antibodies and iii) it is long-lived

and self-sustaining (65). B cells are formed in the bone marrow, from which they exit as immature B cells and make their way to secondary lymphoid organs as the spleen and lymph nodes, via the blood stream (66). B cells express antibodies on their surface, in the form of a B cell receptor (BCR), already when leaving the bone marrow as immature B cells (67). They enter secondary lymphoid organs as transitional B cells and mature into naïve follicular, or marginal zone (MZ) B cells. B cell activation starts with the binding of an antigen to the BCR and can be T cell-dependent or independent (66). T cell-dependent activation generally leads to class-switching and a high-affinity antibody response. It starts with the binding of a protein antigen to the BCR and endocytosis of the antigen into the B cell (68). The processed peptides of the antigen are then presented on the B cell surface in complex with major histocompatibility complex (MHC) II (68). The presented complex is recognized by a T helper cell which binds through the T cell receptor (TCR) and provides co-stimulation by CD40-ligand binding to the CD40 receptor on the B cell (69). Cytokines such as IL-4 and IL-21 expressed by T cells serve as additional stimulatory factors. Following activation a differentiation process takes place generating either short-lived blasmablasts, or germinal center B cells, that can go on to further differentiate into long-lived plasma cells or memory B cells (MBCs) (70). T cell-independent activation typically occurs with polysaccharide antigens and involves the crosslinking of antigen-BCR complexes (type 2) or extensive activation through toll-like receptors (TLRs) (type 1) rather than binding to T helper cells (66). Activated B cells either differentiate into memory B cells or plasma cells at the T-B border, or continue into a secondary step within the germinal center (GC) (65). B cells undergoing T cell-independent activation proliferate outside lymphoid follicles and tend to differentiate into short-lived plasmablasts producing mostly IgM (71).

The Germinal Center

Class-switch recombination (CSR) is the process of immunoglobulin isotype switching occurring in T cell-dependent activation. Once thought to be a major event in the germinal center (GC), it has recently been shown to occur mainly prior to differentiation into GC B cells (72). GCs are compartments within the secondary lymphoid organs where the key processes of B cell clonal expansion, somatic hypermutation and affinity maturation take place (73). In the dark zone of the GC of a lymphoid follicle, an initial process of B cell proliferation and diversification of the B cell receptor known as somatic hypermutation takes place, resulting in mature B cells with increased affinity for a specific antigen (Figure 3). Further differentiation and selection involving follicular dendritic cells (FDCs) and T follicular helper (Tfh) cells, occur in the light zone of the GC (65). GC B cells are then either recycled to the dark zone, or exit the GC as MBCs or plasma cells. Long-lived plasma cells home primarily to the bone marrow in which they reside in specialized niches, continuously releasing antibodies into the blood stream (70).

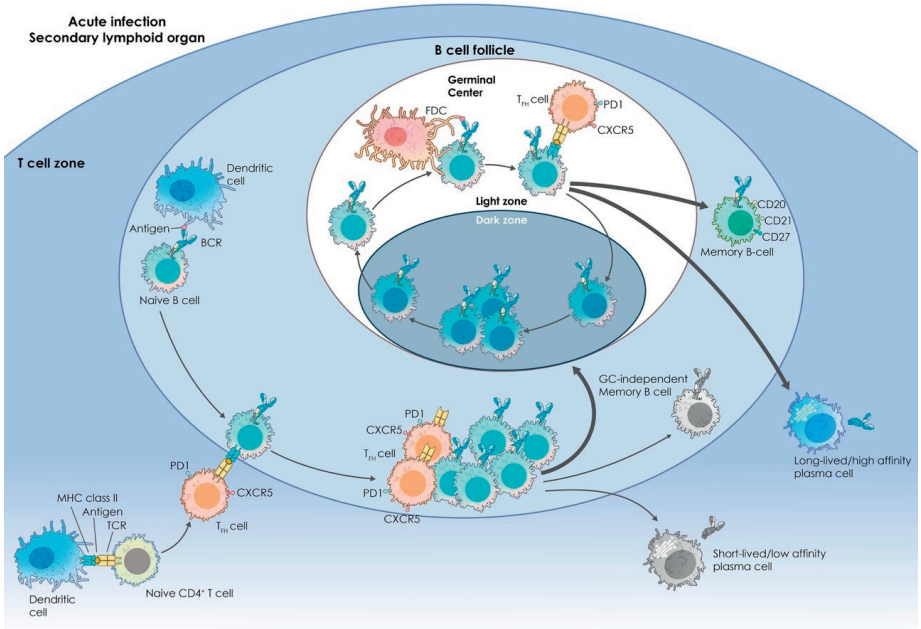

Figure 3. Generation of T cell-dependent B cell memory. *Reprinted from Atypical memory B cells in human chronic infectious diseases: An interim report, Vol 321, November 2017, Pages 18-25, Silvia Portugal, Nyamekye Obeng-Adjei, Susan Moir, Peter D. Crompton, and Susan K. Pierce, Copyright (2019), with permission from Elsevier*

MBCs become tissue-resident or circulate in the periphery, poised to recognize the antigen by which they were primed (65). In response to antigen encounter they will more readily proliferate and differentiate into short-lived plasmablasts temporarily increasing antibody levels dramatically (65).

B cell Activating Factor

Circulating B cells depend on the TNF family ligand, B cell activating factor (BAFF), during the establishment of immune tolerance (74). BAFF is instrumental in the development and maintenance of B cells and provides survival signals for immature and mature B cells (74). It is suggested that cross-linking the BCR and BAFF-receptor (BAFF-R) results in cross-talk and activation of classical- and alternate NF-κB pathways ultimately providing survival signals for the cell (75). BAFF has also been shown to support survival of plasma cells (76), and to promote class switch recombination (77). BAFF, the related a proliferation-inducing ligand (APRIL) and three receptors; BAFF-R, transmembrane activator and calcium-modulating cyclophilin ligand intercalator (TACI), and B cell maturation antigen (BCMA), are members of the "BAFF system molecules" (78). BAFF binds all three receptors

whereas APRIL only binds TACI and BCMA (78). BAFF-R is expressed on most mature B cells except plasma cells, whereas TACI and BCMA are expressed on both MBCs and plasma cells (79, 80). BAFF-R and TACI expression is regulated by physiological and microbial signaling (81-84). BCR engagement in conjunction with Toll-like receptor activation or CD40 ligation induce TACI expression on B cells, render B cells more sensitive to BAFF and APRIL, and stimulate plasma cell differentiation and antibody secretion (81-85). Some insight into BAFF biology has been gained in the malaria field. For instance, monocytes in co-culture with B cells stimulated with *P. falciparum* schizont extract and the malarial pigment hemozoin, secrete BAFF (86). In line with this finding, a study in children in Kenya found increased BAFF plasma-levels during acute malaria, reflecting disease severity (87). Concomitantly, BAFF-R expression was decreased on peripheral B cells while TACI and BCMA expression increased (87). Similarly, in CHMI a significant increase in plasma BAFF was detected on the day of thick smear positivity (88).

Antibodies to *P. falciparum* antigens

Antibodies acquired through repeated infections with the malaria parasite are known to protect against clinical disease. This has been shown in studies with passive gamma-globulin transfer from Gambian adults to children with acute malaria as evidenced by a decrease in parasitemia and subsiding fever (89). Antibodies to *P. falciparum* function by several mechanisms such as invasion inhibition of the parasite into the RBC (90), blocking egression from schizonts (91), complement activation leading to merozoite lysis (92) and opsonization for phagocytosis (93). The knowledge of humoral immunity playing a key role in clinical immunity to malaria has sparked substantial interest in further research characterizing the B cell- and antibody response during acute and chronic malaria. *P. falciparum* expresses a wide variety of antigens, exhibits antigenic variation and to some degree strain specific immunity, all of which contribute to the slow development of immunity and make the targeting of protective antibodies challenging. A rather large body of literature concerning antibodies to *P. falciparum* has accumulated, demonstrating that protective immunity depends on both breadth and magnitude of the antibody repertoire (94, 95). The antibody response to malarial antigens is known to be short-lived, especially in children (96, 97). Antibody responses have also been shown to differ between previously exposed and malaria-naïve individuals (98), although anti-PfEMP1 antibody responses in previously malaria-naïve adults experiencing a single *P. falciparum* infection have been detected 20 weeks after infection (99). The kinetics of other antibodies like the ones against apical membrane antigen 1 (AMA1) and merozoite surface protein 2 (MSP2), have also been shown to differ in magnitude, breadth and longevity depending on previous malaria exposure (100). Blood-stage antigens on the merozoite and those expressed on the RBC surface have been identified as immune targets (26, 101). Some of the antigens evaluated as potential vaccine candidates are; AMA1, MSP2 and MSP3. These have been

ranked among the top ten *P. falciparum* antigens potentially inducing protective antibodies (94, 101). Others including antibodies to erythrocyte-binding antigens (EBAs) (102), *P. falciparum* reticulocyte-binding homologs (PfRh) (103) and GLURP (104) have also been associated with protection.

Determining whether investigated antibodies are actual correlates of protection or simply mirror exposure is however challenging, given the chronic nature of the infection in endemic areas. Furthermore, it seems that breadth of the antibody response is a better correlate of protection than responses to individual antigens (94), which likely reflects a cumulative exposure to malaria parasites suggesting that a combination of antibodies is necessary for protection. The affinity of antibodies has also been shown to be of importance; when time to clinical malaria during follow-up was investigated, high affinity antibodies against merozoite antigens were associated with protection (105). A prospective study in Mali showed that *P. falciparum*-specific MBCs and antibodies were acquired gradually in a stepwise fashion (106), and an age-stratified cross-sectional study in Gambia demonstrated that the breadth of *P. falciparum*-specific MBC responses increased with age while the magnitude did not (107). Moreover, antibody levels did not correlate with the prevalence or median number of MBCs, a finding also reported from Kenya where *P. falciparum*-specific antibody responses declined to undetectable levels while their cognate MBCs were long-lived (108). The maintenance of long-lasting MBCs in the absence of reexposure has indeed been demonstrated after 16 years in a non-endemic setting (109). Thus, it appears that memory to *P. falciparum* is maintained while protection from clinical malaria is not.

Atypical Memory B Cells

Protective antibodies produced in response to an infection require functional B cells. Several studies involving individuals living in malaria-endemic areas have led to the conclusion that malaria disrupts B cell homeostasis creating a dysfunctional immune response (110-114) (Figure 4). A subpopulation of atypical MBCs (aMBCs) devoid of the classical MBC (cMBC) marker CD27 was initially identified in children and adults exposed to intense malaria transmission in Mali (112). These atypical cells were originally described in patients with HIV and were, based on a decreased ability to proliferate and differentiate into antibody secreting cells (ASCs), termed "exhausted" MBCs (115). Exhausted MBCs were found to express the immunoregulatory receptor FcRL4 previously described to be expressed by tissue-like MBCs found primarily in tonsils (116). Interestingly, in the context of HIV, exhausted MBCs were found in normal levels in individuals treated with antiretrovirals (115). Subsequent studies showed that compared to *P. falciparum*-naïve controls, aMBCs were increased in Peruvian adults exposed to low *P. falciparum* transmission, and further increased in Malian adults exposed to intense *P. falciparum* transmission (114). When comparing Kenyan children from

an area of ongoing malaria transmission with children from an area of historical transmission, the proportion of aMBCs was significantly higher in children from the area of ongoing transmission (110) prompting the conclusion that chronic exposure to *P. falciparum* is associated with phenotypic evidence of B cell exhaustion. The function of aMBCs is under investigation with some insight gained in recent years. Circulating Tfh cells have been shown to exhibit impaired B cell help, and to be preferentially activated during acute malaria in Malian children (111). This mechanism was suggested to contribute to suboptimal antibody responses. A study where parasite-specific monoclonal antibodies were characterized in semi-immune Gabonese individuals, however, suggested that cMBCs and aMBCs originate from different precursors and that aMBCs actively secrete antibodies in vivo and thus contribute to the humoral response (117). There is, nevertheless, no consensus regarding the function, whether beneficial or detrimental, of aMBCs in malaria.

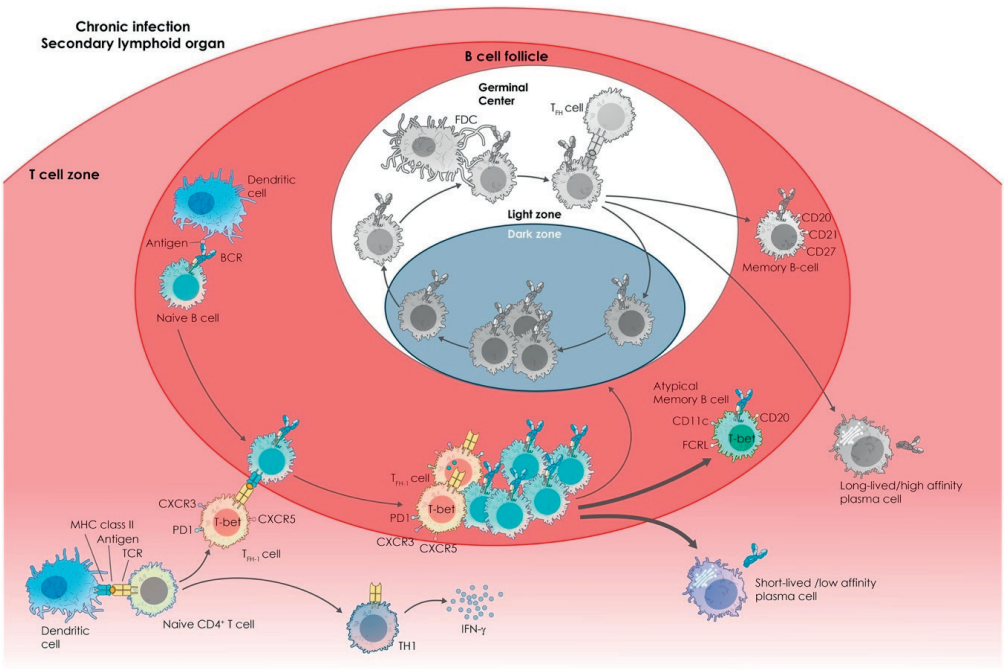

Figure 4. Generation of atypical memory B cells during chronic infection. *Reprinted from Atypical memory B cells in human chronic infectious diseases: An interim report, Vol 321, November 2017, Pages 18-25, Silvia Portugal, Nyamekye Obeng-Adjei, Susan Moir, Peter D. Crompton, and Susan K. Pierce, Copyright (2019), with permission from Elsevier*

1.6 Malaria vaccines

A key intervention that would markedly ameliorate the burden of malaria and contribute to its elimination, is that of an efficacious and safe vaccine. To date, however, vaccine successes have been limited in the malaria field. One strategy showing a high degree of protection in controlled human malaria infection (CHMI) is that of whole sporozoite immunization (118), a rather complicated regimen that would need optimization for large-scale application in the field. Vaccine approaches in malaria can be divided into i) pre-erythrocytic vaccines targeting the parasite before entering the liver-stage ii) blood-stage vaccines directed towards parasite antigens on merozoites and on the RBC and iii) transmission blocking vaccines aiming to interrupt gametocyte transfer from an infected individual. A pre-erythrocytic vaccine appears most attractive as it aims to neutralize the parasite already at the sporozoite stage preventing the infection altogether. The most advanced vaccine to date, RTS,S, is based on this approach using the major sporozoite surface antigen, circumsporozoite protein (CSP) (119). In a large phase 3 trial across several African countries, efficacy at 12-month follow-up was assessed at 50% in children aged 5-17 months, and 30% in newborns of 6-12 weeks (120). Subsequent follow-up over 3-4 years showed a decline in efficacy to 28% and 18% in the respective groups, with a booster dose at 18 months pushing efficacy to 36% and 26% respectively (120). Based on these trials, the WHO has launched the Malaria Vaccine Implementation Programme as a pilot implementation in Malawi, Ghana and Kenya to assess the feasibility of administrating the four required doses, safety profile and prevention of malaria-deaths in children (121). Numerous other malaria vaccine trials are ongoing, notably MSP3 reaching phase 2b among the blood-stage candidates (122). Aside from the obvious question of safety, a vaccine must hold a certain degree of efficacy for its' implementation into vaccine programs to be judicious and cost-effective. While every single life saved is worth the vaccine for the individual, experience with vaccine trials and correlates of protection studies indicate that on a public health level, a satisfactory malaria vaccine may need to consist of a combination of pre-erythrocytic and blood-stage antigens.

2 AIM

The overall aim of this thesis was to investigate how B cells respond to *P. falciparum* infection, in order to contribute to the overall knowledge on naturally acquired immunity to malaria, which may serve as a basis for future vaccine development.

The specific aims were:

I. To develop a method to identify B cells specific for *P. falciparum.*

II. To prospectively investigate the plasma levels of B cell Activating Factor (BAFF) in infants and mothers in a malaria-endemic area, and correlate these to B cell proportions.

III. To explore the B cell profiles and dynamics in individuals with a primary *P. falciparum* infection compared to individulas with previous exposure, over the course of one year.

IV. To develop a method for co-culturing *P. falciparum*-infected red blood cells and B cells, in order to study the immunological effects of infection in vitro.

3 MATERIALS AND METHODS

3.1 Study populations

3.1.1 Kasangati, Uganda (Study I and II)

Study **I** and **II** were conducted using blood samples collected from individuals coming to Kasangati Health Center (KHC), Uganda. Kasangati is a peri-urban town located 20 km north-east of Kampala and sees meso-endemic malaria transmission after the two rainy seasons (Feb-Mar and Sep-Oct). KHC antenatal clinic is a public, free-of charge referral unit of the Wakiso district, receiving about 60 patients and 7 deliveries per day. For study **I**, samples were collected from adults in Kasangati (n=57) primarily during the high-transmission season. Samples from healthy Swedish blood donors were used as negative controls (n=25). For study **II**, samples from mother-infant pairs (n=131) were collected prospectively over a 17-month period. Inclusion criteria were normal deliveries and healthy newborns. Sampling was performed at birth, ten weeks, six months and nine months for infants, and at delivery and nine months after delivery for mothers. Clinical examination was performed at each visit and data entered into a study questionnaire. Pregnant women visiting the antenatal clinic were given long-lasting insecticide-treated bed nets (ITN) and offered intermittent preventive treatment (IPT).

3.1.2 Stockholm, Sweden (Study III and IV)

Study **III** was performed by enrolling adults (n=51) diagnosed with acute *P. falciparum* malaria at Karolinska University Hospital in Stockholm, Sweden after travelling to malaria-endemic areas. Study participants were stratified according to previous exposure to malaria parasites to enable comparison between immune responses in malaria-naïve individuals (n=17) and individuals originating from malaria-endemic countries (n=34). Prospective blood sampling was done at the time of acute infection and further at ten days, one month, three months, six months and twelve months post infection. Clinical data were extracted from medical records, and a questionnaire relating to health status, previous traveling, and malaria exposure was filled in by all participants. Healthy volunteers (n=14) with no prior travels to tropical areas were sampled for negative controls.

Study **IV** was entirely performed using blood from anonymous healthy blood donors in Stockholm, Sweden, as well as cell lines.

3.2 Ethical considerations

Written informed consent was obtained from all study participants (the mothers in study II) prior to study enrollment. Study I and II were approved by the Research and Ethics Committee (SOMREC) of Makerere University School of Medicine, Uganda National Council of Science and Technology, and by the Regional Ethical Committee in Stockholm, Sweden. Study III was approved by the Regional Ethical Committee in Stockholm, Sweden. Study IV did not require ethical approval.

3.3 Study I

3.3.1 *P. falciparum*-infected RBC (iRBC)

P. falciparum parasites of the FCR3S1.2 line were cultured in human blood group 0+ RBCs as previously described (123). Briefly, cultures were maintained at pH 7.4 in sealable flasks at 3% haematocrit in RPMI malaria-medium in an atmosphere of 1% O_2, 4% CO_2 and 95% N_2 at 37°C. Cultures were kept synchronized using 5% sorbitol.

3.3.2 Ghost *P. falciparum*-infected RBC (GiRBC)

Trophozoite-stage parasites were treated with Streptolysin O (SLO) (Sigma) to obtain GiRBC according to the protocol in (123). After release of the RBC cytosol, the resulting intact parasitophorous vacuole was homogenized using sonication (Q500, Fisher Scientific), and the protein concentration measured using Nanodrop (ND2000, Thermoscientific).

3.3.3 Quantum dot (Qdot) conjugation of iRBC and GiRBC

iRBC and GiRBC were conjugated to both amino Qdots and carboxyl Qdots (both Invitrogen) for method-optimization purposes. In short; Qdots were cross-linked and purified, and then allowed to react with iRBC or GiRBC. The resulting conjugate was filtered, washed and diluted 10 times before storage at 4°C.

3.3.4 Immunophenotyping of *P. falciparum*-specific B cells

Cryopreserved peripheral blood mononuclear cells (PBMCs) were thawed, washed and initially incubated with Fc block (CD16/CD32mAB, Biolegend) to remove non-specific binding. GiRBC-Qdot conjugate was then added and further incubated on ice for 30 min. After washing, the cells were stained with CD19 PE fluorochrome-conjugated mAb (BD Horizon), washed and resuspended in flow buffer. Analysis was carried out on a LSRII flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, USA). Data was analyzed using FLOWJO software (Tree Star Inc., San Carlos, CA, USA).

3.3.5 Parasite detection

All samples from Uganda were subject to rapid diagnostic testing (RDT) using Combo Rapid Diagnostic Test (pLDH/HRP2, Premier Medical Corporation Limited, India). Giemsa-stained thick and thin blood films were examined in case of RDT positivity.

3.4 Study II

3.4.1 BAFF ELISA

Plasma samples were tested for the concentration of BAFF (Quantikine ELISA kit, R&D Systems) according to the manufacturer's instructions. Optical density was determined at 450 nm using a SPECTRA max340PC384 microplate reader. BAFF concentrations (pg/mL), were calculated using SoftMax pro software.

3.4.2 Schizont extract ELISA

Total anti-*P. falciparum* IgG and IgM in plasma were measured as previously described (124). In short; diluted plasma samples were added to microtiter plate wells coated with schizont extract and incubated for 1 hour in room temperature. After washing, peroxidase-conjugated goat anti-human IgG or IgM (Sigma) was added followed by incubation and washing. Bound secondary antibody was quantified using TMB substrate (Promega), and optical density (OD) was read at 450 nm.

3.4.3 B cell immunophenotyping

PBMC were phenotyped on a LSRII flow cytometer (Becton-Dickinson Immunocytometry Systems, San Jose, USA) as detailed in (124). The following flourochrome monoclonal antibodies were used: CD19 PE-CF594, CD20 V450, CD27 PE-Cy7, IgG FITC (all from BD Horizon) and FcRL4 APC (Biolegend). B cells specific for *P. falciparum* were identified using Qdots (Invitrogen) conjugated to extract of trophozoite-stage parasites as described in study I. Proportions of B cells specific for *P. falciparum* were defined in the following cell compartments: IgG-positive MBC ($CD19^{+}CD20^{+}CD27^{+}IgG^{+}$), IgG-negative MBC ($CD19^{+}CD20^{+}CD27^{+}IgG^{-}$), naïve B cells ($CD19^{+}CD20^{+}CD27^{-}IgG^{-}$), plasma cells/blasts ($CD19^{+}CD20^{-}CD27^{+}IgG^{-}$), and $CD27^{-}$ MBC, including aMBC ($CD19^{+}CD20^{+}CD27^{-}IgG^{+}$). Data was processed using FLOWJO software (Tree Star Inc, San Carlos and Ca, USA).

3.4.4 Parasite detection

All samples were subject to rapid diagnostic testing (RDT) using Combo Rapid Diagnostic Test (pLDH/HRP2, Premier Medical Corporation Limited, India). Giemsa-stained thick and thin blood films were examined in case of RDT positivity.

3.5 Study III

3.5.1 Parasite detection

Parasites were detected and quantified by light microscopy of Fields-stained thick and thin blood smears, and species was confirmed by PCR (125).

3.5.2 Leukocyte phenotyping

White blood cell differential counts were performed on the Sysmex XS-1000i platform, Sysmex Corporation in the department of clinical chemistry at Karolinska University Hospital.

3.5.3 Mass cytometry

Mass cytometry, also referred to as cytometry by time of flight, CyTOF™, relies on antibodies labelled with heavy-metal isotopes detecting the resulting signals using a time-of-flight detector (Figure 5). Each marker is coupled to a lanthanide with a unique atomic mass. The stained cells are injected into the instrument after which they are vaporized into a cloud of ions by argon gas. Particles over 100 Da are then separated and characterized based on mass/charge ratio in a time-of-flight channel. The resulting profile of spectra is interpreted and grouped into populations of cells expressing the same set of markers. PBMCs were thawed, stained, and subsequently analyzed by CyTOF (Fluidigm Inc.) using a predefined 29-marker panel targeting B cell–associated cell surface molecules. Samples were normalized to equal cell numbers. T-distributed stochastic neighbor embedding (tSNE) was used for dimensionality reduction of the data followed by Phenograph for clustering data into subpopulations. The resulting data files were gated for live $CD45^{+}CD3^{-}CD19^{+}$ B cells using FlowJo X version 10.4.2 and analyzed using the Cytofkit version 1.12.0 (126).

3.5.4 Flow cytometry

Frozen PBMCs were thawed and stained with an antibody mix targeting primarily B cell surface antigens including CD19 PECy7, CD20 APC-H7, CD10 APC-R700, CD21 BV421, CD27 BV650, IgG PE, IgD FITC, CD38 PerCpCy5.5, CXCR3 PECy5, FcRL5 PE, CD11c BV786, CD85j Biotin with SA-BUV395 as well as the intracellular markers T-bet and Ki67. Cells were acquired on a BD LSRFortessa flow cytometer and gating was done using FlowJo X version 10.4.2.

3.5.5 B cell culture

B cell culture was performed according to (127) with some modifications. PBMCs were thawed and stained for IgG-switched CD11c+ and CD11c- B cells to enable sorting. Cells were sorted on BD influx at 2 cells per well into a 384-well plate containing enriched medium and irradiated CD40L feeder cells. Cells were incubated for 10-16 days in 37°C and 5% CO_2.

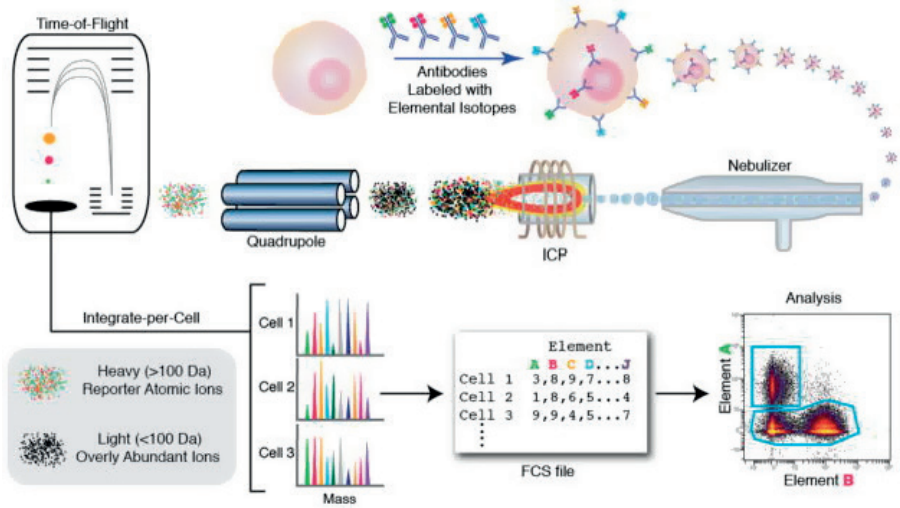

Figure 5. Schematic overview of mass cytometry. *Mass cytometry allows single-cell atomic mass spectrometry of heavy elemental (> 100 Da) reporters. An affinity product (e.g., antibody) tagged with a specific element binds to the cellular epitope. The cell is introduced into the ICP by droplet nebulization. Each cell is atomized, ionized, overly abundant ions removed, and the elemental composition of remaining heavy elements (reporters) is determined. Signals corresponding to each elemental tag are then correlated with the presence of the respective marker and analyzed using conventional cytometry platforms. Reprinted from A deep profiler's guide to cytometry, Vol 33/7, July 2012, Pages 323-332, Sean C. Bendall, Garry P. Nolan, Mario Roederer, Pratip K. Chattopadhyay, Copyright (2019), with permission from Elsevier*

3.5.6 Screening ELISA

B cell culture supernatants were screened for total IgG as well as *P. falciparum*-specific IgG by ELISA. In short; 384-well plates were coated with anti-human IgG/IgM polyclonal antibody or schizont extract (3D7 clone) followed by blocking with 1% bovine serum albumin (BSA, MilliporeSigma). Supernatant was then added to the wells and detected with goat anti-human IgG conjugated to HRP diluted in PBS with 0.1% BSA. The signal was developed with TMB and OD was read at 450 nm.

3.5.7 Schizont extract ELISA

P. falciparum-specific IgG in plasma was measured as previously described (94). Briefly; diluted plasma samples were added to flat-bottom plates coated with schizont extract of the 3D7 clone and incubated. Bound malaria-specific IgG was detected using HRP-conjugated rabbit anti-human IgG (Dako). OD was read at 492 nm.

3.5.8 B cell half-life calculation

Linear mixed-effects models were used to determine the rate of contraction within the $CD21^{-}CD27^{+}$ activated memory, the $CD21^{-}CD27^{-}$ atypical, and the $CD11c^{+}$ compartments after infection.

3.6 Study IV

3.6.1 Isolation and activation of $CD19^{+}$ B cells

B cells were positively selected from PBMCs using $CD19^{+}$ Dynabeads (Invitrogen, Massachusetts, USA), and then incubated with irradiated CD40L-expressing fibroblasts for activation.

3.6.2 Co-culture of *P. falciparum* and B cells

Activated B cells and 0+ RBCs infected with either FCR3S1.2 or 3D7 parasites were cultured together in 12- or 24-well plates (TPP, Techno Plastic Products AG, Switzerland) in co-culture medium (90% IMDM, 5% Human AB+ serum from healthy Stockholm blood donors, 5% FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) at 37°C in 5% CO_2. Concomitantly, 0.4 µm pore transwell plates were used (Corning Incorporated COSTAR) with B cells in receiver wells and iRBCs in inserts. Recombinant CD40L and IL4 was added at the start of the culture and further at every second media change. From day seven, IL2 was added at every second media change. Co-cultures were maintained for ten days with daily parasite quantification using light microscopy and acridine orange staining. When parasitemia exceeded 1.0-1.5% in at least one well, 5-10 µL of fresh RBCs were added to all wells. As a negative control, deoxy-cytidylate-phosphate-deoxy-guanylate (CpG) (Hycult Biotech, Pennsylvania, USA) was added to selected wells instead of parasites. Vybrant carboxyfluorescein diacetate succinimidyl ester (CFDA-SE) Cell Tracer Kit was used to label RBCs.

3.6.3 Flow cytometry

At the end of the co-culture, red blood cell lysis buffer was added after which cells were spun down, washed and transferred to V-bottom plates (Nunc, New York, USA) for staining. Samples were aliquoted into unstained controls, single-colour controls, fluorescence minus one (FMO) controls and test samples. Cells were initially incubated with Fc block (CD16/CD32 mAb, BD Biosciences, New Jersey, USA) and subsequently with CD19 PE-CF594 (BD Biosciences) and FCRL4 APC (Biolegend, California, USA).

3.6.4 Confocal microscopy

Confocal microscopy was used for visualization of the co-culture as described (128).

3.7 Statistical methods

Statistical analyses were carried out using Graphpad prism (version 6), STATA 13, R (version 3.4.1) and SAS JMP (version 14.0.0). P-values less than 0.05 were considered statistically significant. In study II, a linear random-intercept model was used to estimate mean BAFF concentration across different time-points and individuals, as well as the change in mean proportions of B cell subsets. Pearson and Spearman correlation analyses were used to correlate BAFF levels to antibody levels and mean cell proportions. Unpaired *t* test with Welch's correction was used to compare mean levels of BAFF in children and mothers. In study III, all statistical tests were two-tailed. Comparisons between controls and infected individuals at selected time points were done using ANOVA followed by Tukey's HSD test, while variables within the same individual were evaluated using Wilcoxon's matched pairs test, and between infected individuals with the Mann-Whitney *U* test. Overall differences in group dynamics were outlined using a linear mixed-effects model with restricted maximum likelihood. Specific comparisons between previously exposed or primary infected individuals were done with a least squares mean differences Student's *t* test.

4 RESULTS

4.1 Study I

We developed a novel flow-cytometry technique for the detection of *P. falciparum*-specific B cells. For this purpose, we used Qdots which are highly fluorescent, nanometer-size, single crystals of semiconductor material with narrow emission-spectrum and good photostability. Qdots can be conjugated to a variety of bio-molecules and analyzed by flow cytometry. We evaluated two kinds of Qdots and found that carboxyl Qdots are better for identifying *P. falciparum* specific B cells compared to amino Qdots .

To circumvent the issue of unspecific binding of the Qdot-iRBC conjugate to haemoglobin, GiRBC were prepared which increased the mean percentage of *P. falciparum*+ B cells in Ugandan blood donors from 1.1% to 4.6% (Figure 6). This increase can be ascribed to the increased availability of binding sites when unspecific binding to haemoglobin was reduced.

The potential impact of cryopreservation of PBMCs on this technique was evaluated by running the same sample of PBMCs both directly after ficoll preparation, and after one week of standard cryopreservation. No difference in the yield of *P. falciparum*+ B cells was observed between the conditions.

Comparative analysis of PBMC samples from Ugandan (n=57), and Swedish blood donors (n=25) showed that a mean of 22% of CD19+ cells were *P. falciparum*+ in Ugandan samples, as opposed to 1.7% in Swedish samples.

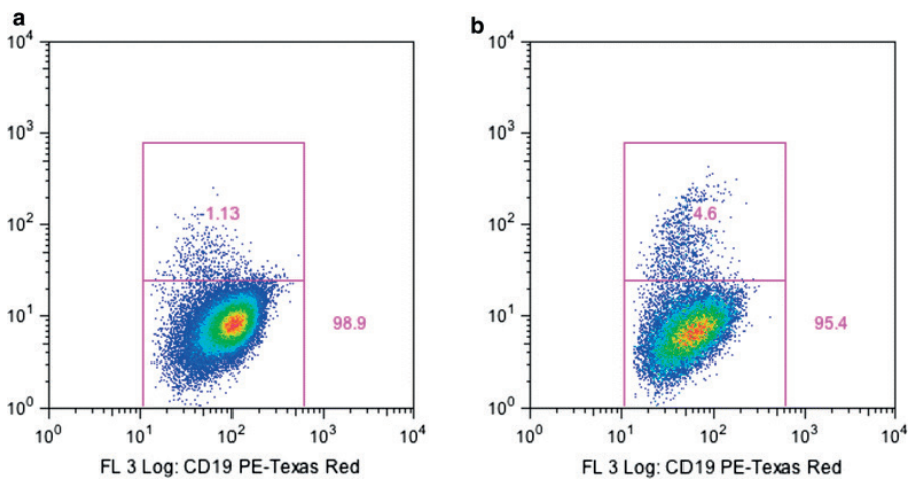

Figure 6. Percentage of P. falciparum+ B cells *using a) carboxyl Qdot-iRBC and b) carboxyl Qdot-GiRBC in a representative Ugandan donor.*

Repeatability of the method was ensured by running ten of the Swedish samples in four separate immunophenotyping experiments with the same conditions, resulting in very little variability of the mean and standard deviation. Furthermore, two separate experiments including seven samples from individuals with positive RDT, five samples from individuals with other inflammatory conditions and negative for malaria, and one Swedish sample as negative control were performed (Figure 7). The mean percentage of *P. falciparum*+ B cells in individuals with ongoing infection was 26.7% in the first, and 26.8% in the following experiment. The corresponding fraction in samples from individuals with inflammatory conditions were 3.6% and 3.5%.

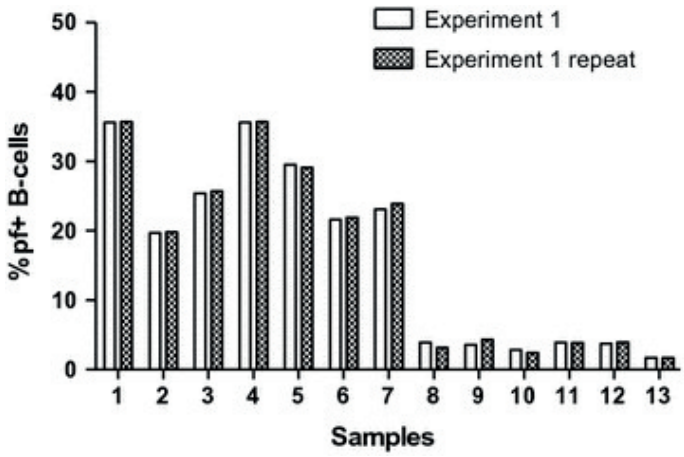

Figure 7. Percentage of P. falciparum+ B cells. *Samples 1–7: ongoing malaria infection, 8–12: inflammatory conditions other than malaria and 13: negative control from non-endemic area for comparison. 1–12 were collected during low transmission season.*

When samples from Ugandan donors were stratified into parasite positive (n=13) and parasite negative (n=44) the mean proportions of *P. falciparum*+ B cells were 27.9% and 20.6% respectively ($p<0.0001$).

In conclusion; carboxyl Qdot conjugation to GiRBC schizont extract, is a good method for the separation of *P. falciparum*-specific B cells using flow cytometry. Parasitemia at the time of sampling increases the yield of *P. falciparum*-specific B cells, demonstrating the suitability of this method for the detection of B cells responding to infection.

4.2 Study II

We performed a longitudinal study in which blood samples from 109 mother-infant pairs in Uganda were collected for the analysis of BAFF concentration in plasma. Infants were sampled at birth, ten weeks, six months and nine months. Mothers were sampled at delivery and nine months post-partum. The highest mean level of BAFF was found in cord blood; 2075 pg/mL, and subsequently decreased to 1265 pg/mL at ten weeks, then levelled off from that point onward (Figure 8).

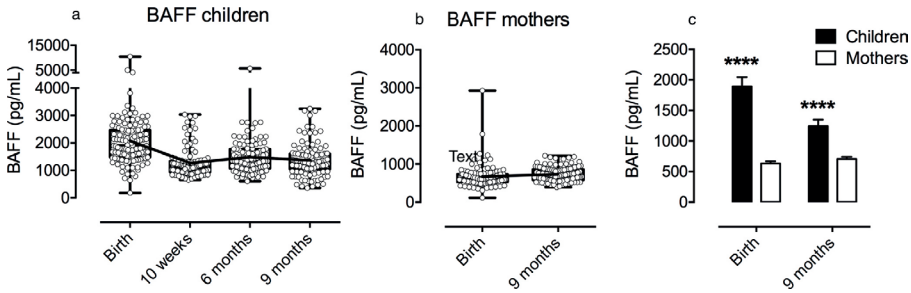

Figure 8. Distribution of individual and mean levels of measured BAFF concentrations for children and mothers at each time point. *a) BAFF levels in children over the course of the study. b) BAFF levels in mothers at birth and nine months post-partum. Mean values indicated by interconnecting line. c) Comparison of mean BAFF levels in children and mothers at birth and at nine months using unpaired t test with Welch's correction. **** $p<0.0001$.*

We found that mean BAFF-levels in children were significantly higher than in mothers at the time of birth, with a difference of 1405 pg/mL ($p<0.0001$). The difference between mean BAFF-levels was smaller at nine months; 631 pg/mL, but remained statistically significant ($p<0.0001$).

In order to investigate potential associations between BAFF concentration and *P. falciparum*-specific antibody titers, Pearson and Spearman correlation analysis was performed. The correlation analysis for BAFF and antibodies to schizont extract generated only a few weak albeit significant associations for IgM, and no associations for IgG.

We further correlated mean BAFF levels with total and *P. falciparum*+ B cell phenotyping data previously depicted in (124). In children at nine months, BAFF concentration was negatively correlated with IgG^+ MBC and $CD27^-$ MBC (Pearson correlation coefficient -0.48; $p<0.0001$ and -0.51; $p<0.0001$, respectively), (Spearman correlation coefficient -0.46; $p<0.001$ and -0.48; $p<0.0001$, respectively) including *P. falciparum*+ proportions for both these compartments

(Pearson correlation coefficient -0.51; $p<0.001$ and -0.46; $p<0.001$, respectively) (Spearman correlation coefficient -0.45; $p<0.001$ and -0.42; $p<0.0001$, respectively). In mothers at delivery, BAFF concentration was negatively correlated with IgG^+ MBC and $CD27^-$ MBC (Pearson correlation coefficient -0.39; $p<0.0001$ and -0.51; $p<0.0001$, respectively), as well as with *P. falciparum*+ $CD27^-$ MBC (Pearson correlation coefficient -0.43; $p<0.0001$).

In conclusion; the concentration of BAFF is considerably higher in the cord blood of infants than in mothers blood at the time of birth. Levels of BAFF in infants decrease over time but remain significantly higher than in mothers at the age of nine months. There is an inverse correlation between BAFF and IgG^+ MBC and $CD27^-$ MBC in infants at nine months, as well as in mothers at the time of delivery.

4.3 Study III

In study III we investigated the B cell response of 51 individuals sampled prospectively at the time of acute *P. falciparum* malaria and then at ten days, one month, three months, six months and twelve months after infection. All study participants were included at the time of diagnosis in Sweden and were stratified into "primary infected" (n=17) and "previously exposed" (n=34). Consistent with a B cell memory response, we observed a more extensive *P. falciparum* schizont-specific IgG response in previously exposed individuals compared with those infected for the first time (5.5-fold difference, p=0.0005).

In an initial exploratory approach we used mass cytometry to characterize the phenotype of circulating B cells in two primary infected and two previously exposed individuals (Figure 9). Data analysis resulted in the identification of ten B cell clusters out of which naïve B cells defined as $CD21^+CD27^-IgD^+$, made up the largest cluster with 31% of B cells. Memory B cells, defined as $CD21^+CD24^+CD27^+$, constituted three clusters further defined by BCR isotype. One cluster constituting 18.7% of B cells expressed IgM and IgD, but lacked expression of CD21 and CD27 which may represent transitional B cells. Three clusters were characterized by high expression of CD11c, two of which lacked CD21 and CD27, likely representing atypical B cells. The remaining two clusters consisted of cells with low expression of CD20, high expression of CD38 and variable expression of CD27 and were defined as plasmablasts. We compared the dynamics of these subpopulations of B cells between study individuals and three healthy controls and found that naïve and memory B cell frequencies were similar between study individuals and controls at the acute time point, while plasmablasts and $CD11c^+$ cells had expanded in study individuals. As expected, the plasmablast expansion was transient and had returned to baseline frequency ten days after the acute infection. In contrast, $CD11c^+$ B cells remained in circulation for more than three months in previously exposed individuals.

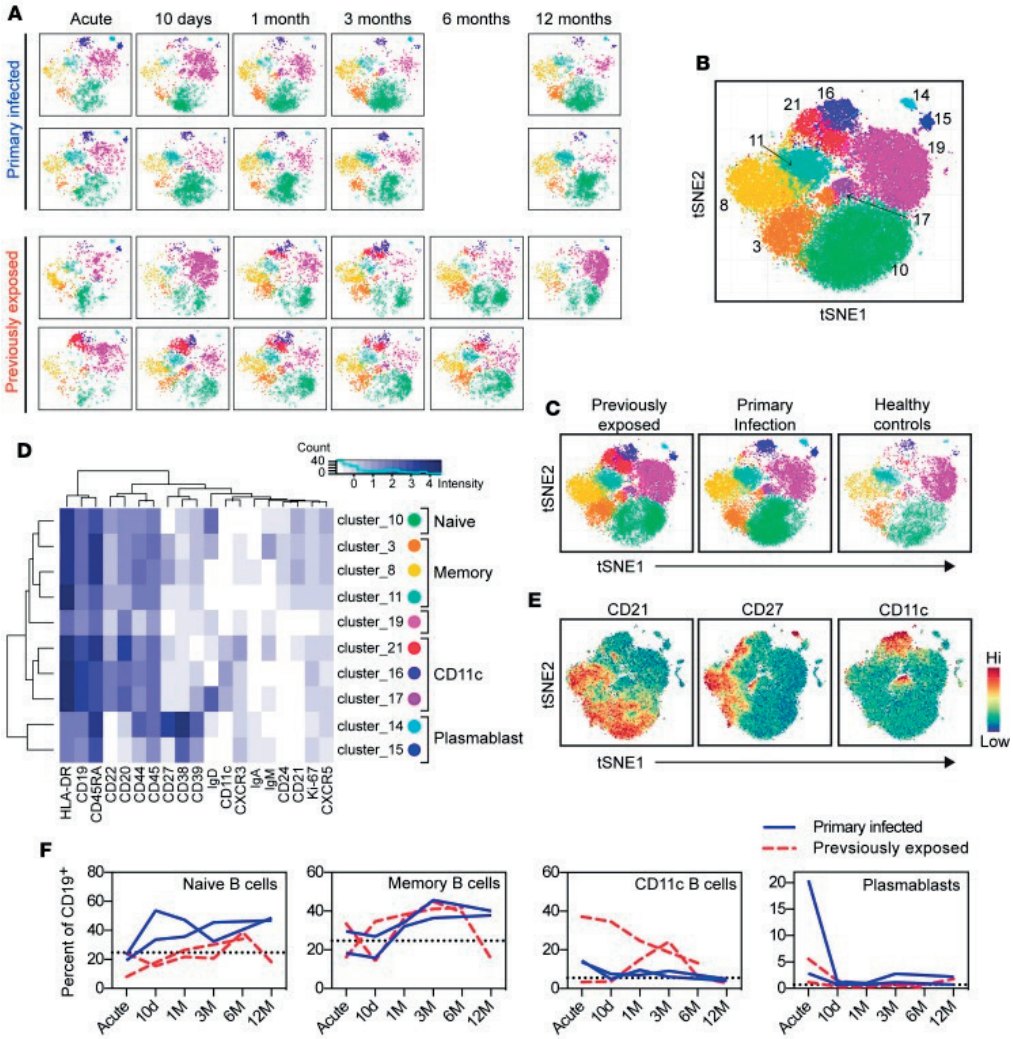

Figure 9. B cell phenotypic analysis by mass cytometry. *(**A**) tSNE analysis followed by clustering of mass cytometry data for individual primary infected (n = 2) and previously exposed (n = 2) donors. (**B**) Merged tSNE plot for all donors and time points (n = 24 samples), with each cluster indicated by number and color. (**C**) Merged tSNE plots for each group (previously exposed: n = 11 samples; primary infection: n = 10 samples; healthy controls: n = 3 samples). (**D**) Heatmap showing marker expression within each cluster. Circles are colored as cluster plots. (**E**) Expression of CD21, CD27, and CD11c for analyzed cells. (**F**) Contribution of each B cell subset to total CD19⁺ B cells at different time points after infection. Blue solid lines indicate individuals with a primary infection; red dashed lines indicate individuals previously exposed to malaria. The dotted black line indicates the mean frequency of each population in healthy controls.*

The mass cytometry findings were suggestive of differential B cell regulation between the groups and were further investigated in a larger cohort using a 13-colour flow cytometry panel. We included 63 individuals (primary infected: n=16; previously exposed: n=33; healthy controls: n=14) and evaluated longitudinal B cell subset dynamics. Plasmablast frequencies were expanded in both malaria-infected groups at the acute time point compared to healthy controls (primary infected: 11.1-fold increase, $p<0.0001$ and previously exposed: 8.3-fold increase, $p< 0.0001$), and contracted rapidly after treatment. The frequency of immature B cells ($CD19^+CD20^+CD10^+$), was similar to that of healthy controls at any time point for both malaria-infected groups. Mature B cells ($CD19^+CD20^+CD10^-$) were separated into the following populations: Activated MBC ($CD21^-CD27^+$), Resting MBC ($CD21^+CD27^+$), Naive B cells and $CD27^{lo}$ MBC ($CD21^+CD27^-$), and Atypical B cells ($CD21^-CD27^-$). Activated MBCs were elevated for both individuals with primary infection and previously exposed individuals compared with healthy controls at the acute time point (2.2-fold increase, p=0.0042 and 2.3-fold increase, p=0.0004, respectively), and remained elevated for three months in individuals with primary infection, while it took twelve months for the previously exposed group to reach similar levels as in healthy controls. Resting MBCs increased over time following infection without any significant difference in the infected groups. Naive B cell and $CD27^{lo}$ MBC frequencies were highly variable within infected groups and healthy controls. Atypical B cells rapidly expanded, both in individuals with a primary infection and in those with previous exposure at the acute time point compared with healthy controls (2.8-fold increase, p=0.0002 and 3.9-fold increase, p<0.0001, respectively). Atypical B cells reached peak values on day ten, where the previously exposed group showed a significantly higher frequency compared with individuals with primary infection (1.6-fold higher, p= 0.02). Primary infected individuals, reached background levels within six months after infection, whereas previously exposed individuals maintained a higher frequency at twelve months compared with controls.

The frequency of $CD11c^+$ B cells was highly correlated with the frequency of $CD21^{lo}$ B cells ($r^2 = 0.82$, $p<0.0001$), indicating that CD11c was expressed by $CD21^{lo}$ activated memory and atypical B cells. To further understand the composition and dynamic of the $CD11c^+$ compartment, we measured the frequency of $CD11c^+$ mature B cells expressing a naïve/$CD27^{lo}$ MBC ($CD21^+CD27^-$), resting memory ($CD21^+CD27^+$), activated memory ($CD21^-CD27^+$), and atypical ($CD21^-CD27^-$) phenotype at each time point. The distribution of B cell subsets within the $CD11c^+$ compartment was highly dynamic, with a rapid expansion of activated and atypical B cells coinciding with a reduction in the frequency of resting memory B cells. Normalization of cell numbers to gated live lymphocytes indicated that the remodeling within the $CD11c^+$ B cell compartment was primarily due to expansion of cells, rather than a redistribution between compartments.

To investigate the effect of de novo versus memory responses, $CD11c^+$ B cells were separated based on expression of IgD. The overall frequency of $CD11c^+IgD^+$ B cells was significantly higher for individuals with a primary infection compared with those with previous malaria exposure (1.4-fold increase, p=0.0049). Cell numbers and dynamics of IgD^+ B cells in each subset were similar between primary infected and previously exposed individuals, whereas the number of IgD^- B cells was significantly higher in all $CD11c^+$ B cell subsets in previously exposed individuals, with a dynamic response in atypical and activated memory B cells.

Further phenotypic assessment showed that $CD11c^+$ B cells expressed higher levels of CD19, CD20, CD85j, CXCR3, and FcRL5 (ratio > 1) and lower levels of CD21 (ratio < 1) compared with $CD11c^-$ B cells. FcRL5 and CXCR3 were highly expressed by $CD11c^+$ B cells. We assessed this expression over time and found FcRL5 in approximately 70% of $CD11c^+$ B cells at the time of acute infection with a slow decrease to 50% at twelve months after infection. FcRL5 was expressed in almost all $CD21^-CD27^+$ activated MBCs and $CD21^-CD27^-$ atypical B cells. CXCR3 was slower to be expressed and peaked at approximately 60% of $CD11c^+$ B cells at ten days to one month after acute infection. CXCR3 displayed similar kinetics in all $CD11c^+$ B cells, although the frequency of $CXCR3^+$ cells was highest among $CD21^-CD27^+$ activated MBC.

T-bet is a transcription factor suggested as a key driver in the formation of atypical B cells (129). We found that, at the time of acute infection, T-bet was expressed in 33% of $CD11c^+$ B cells, while only 1.1% of $CD11c^-$ B cells expressed T-bet ($p<0.0001$, n=26). T-bet was primarily expressed in cells with high levels of CD11c and low levels of CD21.

As CD11c was expressed in B cells expanding during clinical malaria, we wanted to evaluate if the responding B cell clones were specific for *P. falciparum*. We sorted switched $CD11c^+$ and $CD11c^-$ B cells from three previously exposed donors (n = 4606 wells) at ten days after acute infection and two healthy controls (n = 1000 wells). Following two weeks of incubation, we assessed IgG production by ELISA. To further determine if the produced antibodies were malaria specific, IgG from high producing wells (OD > 0.2–0.3 in IgG ELISA) were screened for binding to schizont extract. Out of 509 $CD11c^+$ wells, 41 wells were schizont-specific, corresponding to 8.1% of responding wells. In contrast, out of 566 $CD11c^-$ wells, 15 were schizont-specific, corresponding to 2.7% of responding wells. Accordingly, schizont-specific B cell clones were significantly enriched in $CD11c^+$ B cells compared with $CD11c^-$ B cells (3-fold, $p<0.0001$). To investigate the possibility of nonspecific binding to schizont extract, we screened 500 $CD11c^+$ wells from two healthy controls. 174 out of 500 wells, produced high levels of IgG whereas none showed binding to schizont extract. This finding clearly indicates that binding to schizont extract was not due to polyreactivity, but rather indicative of parasite-specific B cell clones.

Taken together, our data indicate that mass cytometry is a powerful exploratory tool to characterize human PBMCs in an unbiased approach. $CD11c^+$ B cells highly correlate with $CD21^{lo}$ B cells which implies that this population encompasses activated MBCs as well as atypical B cells. Following acute *P. falciparum* malaria, atypical B cells expand rapidly with a 1.6-fold higher frequency in previously exposed individuals compared with primary infected individuals. The magnitude of B cell populations, rather than rate of contraction, is the mechanism behind sustained levels in previously malaria-exposed individuals. The number of switched $CD11c^+$ B cells is significantly higher in previously exposed than in primary infected individuals. FcRL5 is expressed in 70% of $CD11c^+$ B cells at the time of acute infection. T-bet is expressed in 33% of $CD11c^+$ B cells, while only 1.1% of $CD11c^-$ B cells express T-bet. Finally, schizont-specific B cell clones are significantly enriched in $CD11c^+$ B cells compared with $CD11c^-$ B cells.

4.4 Study IV

We aimed to establish a method of co-culturing *P. falciparum* with human B cells in order to study the effects exerted on both parasites and B cells over a period of time sufficient for B cells to differentiate into ASC. Numerous trial experiments were performed with subsequently adjusted protocols in order to create an environment supporting both *P. falciparum*-infected RBCs and B cells for ten days. Factors requiring gradual optimization included B cell activation, parasitemia starting concentration, type of medium, serum proportions and duration of the experiment. When culture conditions were finally optimized, the starting concentration of parasites was 0.01%, and the co-culture medium included recombinant CD40 ligand, IL-4 and IL-2 with continuous addition of new RBCs to avoid overgrowth of parasites. Under these conditions, more than 90% of B cells were viable on day ten.

The co-cultures were monitored daily by microscopy (Figure 10). B cells and iRBC could often be seen in close contact. The concentration of parasites consistently increased more rapidly and reached a higher peak in wells with B cells, compared with wells with parasites only (Figure 11). These results clearly show that *P. falciparum* parasites can multiply under the given co-culture conditions for ten days and moreover, exhibit enhanced growth in the presence of B cells. Similar results were achieved with FCR3S1.2 (a rosetting parasite line), 3D7 and FCR3S1.6 (both non-rosetting).

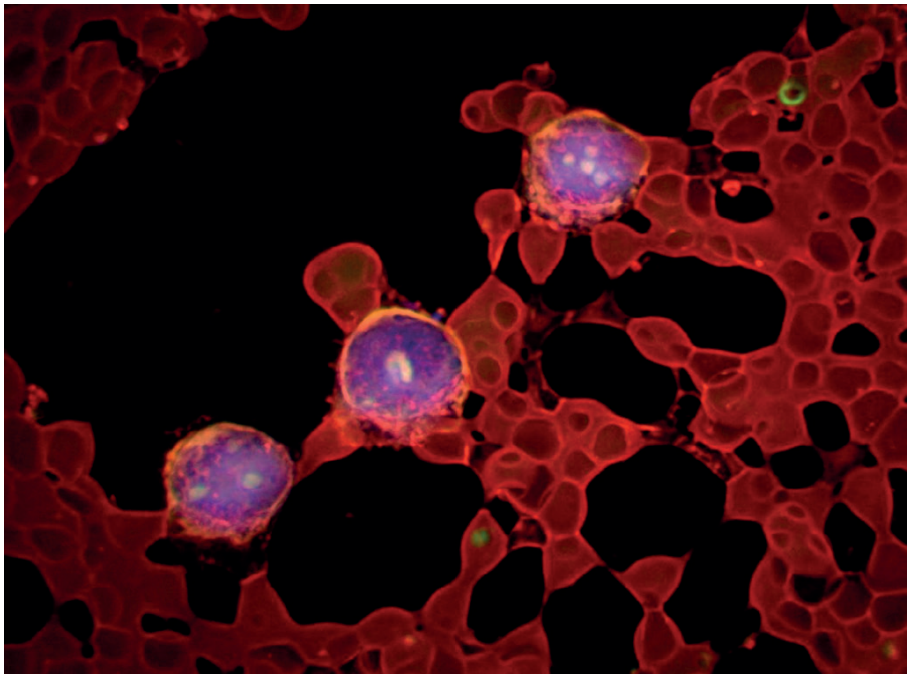

Figure 10. *Confocal microscopy on day ten of P. falciparum-infected RBC and B cell co-culture.*

To investigate the role of direct contact between iRBC and B cells, co-cultures were set up using transwell plates. While B cells and parasites were still kept in the same conditions, the membrane pore size of 0.4 μm only allowed small soluble molecules to pass, precluding contact between iRBC and B cells. Over the ten-day course of the experiment, parasitemia in transwells increased at a slower rate than in regular wells with B cells, but peaked significantly higher than parasites grown on their own (Figure 11).

B cells were monitored for viability and counted during, and at the end of the culture. After ten days, B cells in wells with parasites, had proliferated with a 7-fold increase in the number of cells compared with day zero. Control wells with B cells only showed a 1.2-fold increase, while in wells where CpG was added B cells increased 5-fold.

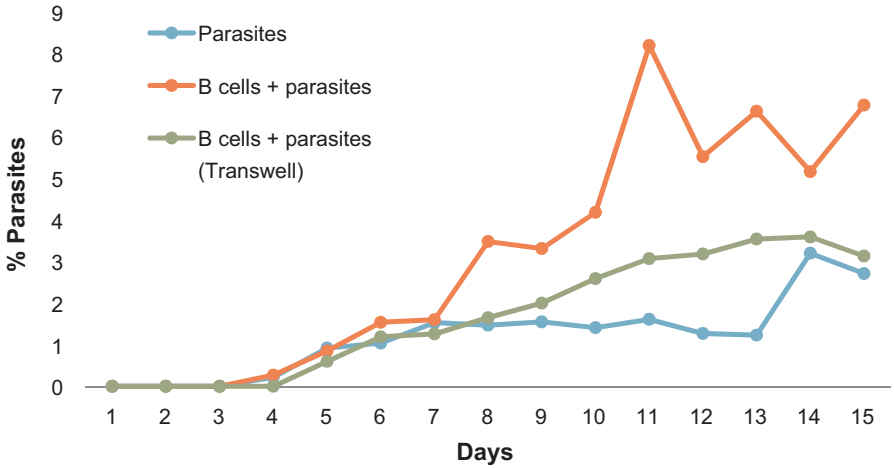

Figure 11. *Outline of parasite concentrations during P. falciparum-infected RBC and B cell co-culture.*

At the conclusion of the ten-day co-culture, B cells were collected and stained for CD19 and FcRL4 for analysis by flow cytometry. B cells grown together with parasites showed a marked increase in the proportion of cells expressing FcRL4 compared with B cells pre-activation. B cells in transwells and those cultured with CpG did not result in FcRL4-expression.

In conclusion; human B cells and *P. falciparum*-infected RBCs can be co-cultured for ten days retaining high viability of both components. Parasitemia increases more rapidly and peaks higher when *P. falciparum*-infected RBCs are cultured with B cells than when cultured alone. Direct contact between B cells and *P. falciparum*-infected RBCs is advantageous for parasite replication. B cells co-cultured with *P. falciparum*-infected RBCs demonstrate a marked increase in the expression of FcRL4 compared with the same cells pre-activation.

5 DISCUSSION

This thesis encompasses four studies that contribute new methodology and shed light upon the B cell and BAFF dynamics in the context of *P. falciparum* malaria. Herein, a novel technique for labelling *P. falciparum*-specific B cells has been developed, and a new method for co-culturing human B cells and *P. falciparum*-infected RBCs has been established. Furthermore, unique longitudinal data on B cell dynamics following acute malaria, and BAFF levels correlated to B cell compartments in individuals living in a malaria-endemic area are outlined. Taken together, the findings reported in this thesis contribute to the knowledge on humoral immunity in malaria, as well as providing new tools in malaria research.

In order to study mechanisms of immunity, it is desirable to identify memory B cells with specificity for a given pathogen. For *P. falciparum* this is an arduous and tricky matter since the parasite encodes over 5300 genes (130) and consequently only a fraction of the proteins have been described. *Plasmodium* parasites express this wide variety of antigens across the different stages of their complex life-cycle which has recently been extensively mapped (131). Memory B cells specific for *P. falciparum* blood-stage antigens can be analyzed using enzyme-linked immune absorbent spot (ELISpot) (132). We sought to develop a method with high accuracy that can be applied directly to B cells from human blood without prior activation. The Qdot method allows phenotypic analysis of antigen-specific cells. Qdots conjugated to schizont extract proved to be a useful method to quantify *P. falciparum*-specific B cells. Their suitability for this purpose was demonstrated by the increased frequency of *P. falciparum*-specific B cells in individuals with patent parasitemia. The rationale for decreasing unspecific binding by ridding the RBCs of haemoglobin-containing cytosol showed an increased yield of *P. falciparum*-specific B cells. Unspecific binding is a notorious complication in immunological assays both in clinical and research laboratories. RBCs which are a necessity for culturing *Plasmodium* may express a variety of antigens that can be targeted by antibodies with a broad affinity. Inflammatory conditions such as systemic lupus erythematosus (SLE) give rise to immunogenic factors that can result in unspecific binding. We tested the specificity of the Qdots by including five individuals from the same endemic area with different inflammatory conditions, who were negative for malaria at the time of sampling. The frequencies of *P. falciparum*-specific B cells were indeed significantly lower in the malaria-negative individuals with inflammatory conditions when compared to individuals with ongoing malaria. However, compared to healthy individuals from the same area the difference was less obvious. This new method for identifying B cells specific for malaria has the potential to become an important laboratory method to investigate the humoral response, and has been successfully applied in a subsequent study in Uganda (124). However, it could benefit from further optimization to reduce potential unspecific binding.

A cellular model to mimic human in vivo conditions allows continuous insight during all phases of the replication and differentiation processes. With a variety of laboratory methods available, the study of what happens on a cellular, as well as on a soluble level becomes feasible. We set out to create an environment that would support the proliferation of B cells, as well as replication of *P. falciparum* parasites for up to two weeks. After numerous trials and optimizations of the method we managed to set up a co-culture of B cells and *P. falciparum*-infected RBCs for ten days. Parasitemia was monitored daily and interestingly, increased at a more rapid rate and reached a higher parasitemia in wells where parasites were co-cultured with B cells than in control wells with parasites only. These results were obtained in numerous consecutive co-cultures. Furthermore, parasitemia in transwells also peaked at a higher level than in control wells, however, the increase was slower than in regular wells. From these results we can conclude that direct contact between B cells and iRBCs has a stimulatory effect on the replication of parasites, and further, that soluble factors are, to a lesser degree or perhaps at a later stage, also involved in this process.

The identification of the phenotypic marker FcRL4 on exhausted B cells in patients with HIV (115) sparked malaria researchers to hypothesize that the same mechanism may be behind the slow and incomplete development of NAI to malaria. By including the FcRL4 marker in the flow cytometry analysis following the co-culture, we could show that B cells co-cultured with *P. falciparum*-infected RBCs for ten days, demonstrated a marked increase in the expression of FcRL4 compared with the same cells before culture. Several research groups have characterized atypical B cells in relation to malaria exposure (56, 88, 110, 117), and some have shown increased levels of FcRL4 on B cells from individuals in malaria-endemic areas (112, 117). FcRL4 is an immunoregulatory member of the Fc receptor homologue family and was described in human tonsillar tissue on a subpopulation of MBCs referred to as “tissue-like” (116). The monoclonal anti-FcRL4 antibodies generated in these studies were initially used in the malaria-research field but have subsequently proved to be cross-reactive (133, 134). In extensive genome-wide expression profiling studies using B cells from Malian adults with life-long malaria exposure, it was shown that aMBCs express FcRL5 rather than FcRL4 (133), and further that the expression of FcRL5 was associated with markedly reduced BCR signaling. Moreover, by ELISpot, it was suggested that aMBCs from Malian donors did not show evidence of antibody production. Concomitantly, a similar approach was undertaken by another research group applying microarray-based transcriptome analysis of aMBCs from Ugandan children (134). The investigators demonstrated that FcRL3 and FcRL5, but not FcRL4 transcripts were present at higher levels in aMBCs than cMBCs. In addition, subjects living in areas of high malaria transmission had a significantly higher proportion of $FCRL5^{+}$ aMBCs than subjects living in areas with lower transmission. The authors further concluded that FcRL5-expression defined strongly inhibited subsets of both classical and

aMBCs, with only 1.1% of FcRL5^{+} cMBCs and 0.2% of FcRL5^{+} aMBCs capable of antibody production after stimulation, as opposed to 6.3% of FcRL5^{-} cMBCs. These comprehensive investigations contributed significantly to our understanding of aMBCs. At the time of the development of our co-culture, however, the notion that FcRL4 might be a key factor in the inhibition of a functional B cell response in malaria was still prevailing. Again, the issue of cross-reactivity must be considered. Whether or not the FcRL4 clone used in our co-culture experiments cross-reacted with FcRL5 is unknown. The possibility of a cross-reaction with the less studied FcRL3 can also be considered. Altogether, our data is in support of Fc receptor homologues characterizing distinct B cell subsets responding to *P. falciparum*.

BAFF is a crucial survival factor for B cells (74) and its overexpression or dysfunctional receptors can have severe consequences on B cell responses in conditions such as systemic lupus erythematosus (SLE) (135) or common variable immunodeficiency (CVI) (136, 137). The expression of, and release of BAFF is regulated and enhanced by IFNγ and IL-10 (138). Increased levels of plasma BAFF can be seen not only in chronic viral infections such as HIV and hepatitis C, but also in protozoan infections as *Trypanosoma cruzi* and *Leishmania* (78). In our longitudinal study of plasma BAFF levels in a malaria endemic area in Uganda, we found BAFF levels in infants to exceed those of mothers by >3-fold at the time of birth. While levels of BAFF decreased over time in children, levels in the mothers were unchanged, although still remaining significantly lower (1.6-fold) compared to levels in children at nine months. Our findings are in line with previous data on cord blood, both from non-endemic and a malaria endemic area (139-142). A study in Kenya found cord blood BAFF levels in healthy newborns to be higher than those in healthy US newborns (142). Only one previous study outlined longitudinal data in infants, in which a decrease of BAFF levels over time, similar to our findings, was seen (141). The exceedingly high levels of BAFF in cord blood compared to levels in mothers, may reflect an immature immune system. TACI, the receptor for BAFF on plasma cells and MBCs has been found to be reduced on neonatal B cells (143), a finding which has been connected with impaired BCR-signaling in newborn mice (144, 145). In accordance with these previous findings, the high levels of BAFF in our cohort of newborns may reflect a mechanism to saturate BAFF-binding B cells in order to fortify signaling aimed at inducing immunoglobulin production. In the context of acute malaria, Kenyan children were found to have significantly higher levels of BAFF than healthy controls, with levels progressively increasing with disease severity (87). In that study, BAFF-R was decreased in children with malaria compared to healthy controls, and like BAFF, normalized within four weeks following acute infection. In addition, elevated levels of IL-10 and IFNγ were seen with a positive correlation between BAFF and IFNγ. Analogous to our results, no correlation between BAFF and parasitemia was seen (87). Increased BAFF levels were also found on the day of thick smear positivity in CHMI studies (88). Peak BAFF levels were preceded by elevated IFNγ which

peaked two days before BAFF. Interestingly, peak IFNγ-levels were positively correlated to peak parasitemia levels while peak BAFF levels only showed a weak, non-significant correlation (88). Thus, our results corroborate previous findings which indicate that the cytokine cascade of the immune response to acute disease rather than the parasite itself, causes increased levels of BAFF.

In the cohort of mother-infant pairs we further correlated BAFF levels to subsets of B cells characterized by flow cytometry. In addition to immunophenotyping, the Qdots method previously described was applied in this cohort to determine the proportion of *P. falciparum*-positive B cells (124). We found IgG^+ MBCs and $CD27^-$ MBCs to be inversely correlated to levels of BAFF in both mothers and infants, however at different time points. In mothers the correlation was apparent at delivery while in infants the correlation was found at the age of nine months. Furthermore, in infants the correlations applied to *P. falciparum*+ proportions of IgG^+ MBCs and $CD27^-$ MBCs alike, while in mothers exclusively to $CD27^-$ MBCs. A longitudinal study of BAFF levels in Swedish infants found significantly higher levels of BAFF in dairy farmers children than in non-farmers children and moreover, a tendency towards a positive association between BAFF and $CD27^+$ B cells at 3-5 days after birth in farmers children (141). In our study, BAFF levels dropped significantly from the cord blood sampling to the subsequent sampling at ten weeks, after which they leveled off. Simultaneously, the proportion of naïve B cells decreased as IgG^+ MBCs and $CD27^-$ MBCs increased. These dynamics may reflect a maturation of the immune system driven by the exposure to pathogens. Pregnancy entails a particular immune state of which we only understand a fraction. While we found BAFF levels unchanged between delivery and nine months later, there was a negative correlation with IgG^+ MBCs and $CD27^-$ MBCs at delivery. One could speculate that the use of IPT preventing clinical malaria kept the BAFF levels low while aMBCs within the $CD27^-$ MBC compartment expanded as previously found in malaria endemic areas (56, 146). What also needs to be taken into consideration is the dynamic character of a cytokine being either in soluble or bound form while what we measure by ELISA is merely a snapshot of the current levels in plasma.

To step away from the biased design of B cell marker panels, we took an exploratory approach with a wide selection of markers using CyTOF. The CyTOF methodology offers several advantages over flow cytometry based on the labelling of antibodies with rare earth metals rather than fluorochromes. Metals in the lanthanide group of elements are especially suitable for the labelling of surface and intracellular markers since they are absent in organic material such as human cells, circumventing the issue of background signaling (147). Furthermore, as the method does not utilize fluorescence for detection, the complication of spectral overlap is overcome allowing for a large number of markers being analyzed simultaneously. CyTOF also poses some disadvantages compared to flow cytometry such

as a slower throughput, and immense data output requiring specialized software programs and appropriate expertise for the analysis. We found CyTOF to be a powerful exploratory tool to characterize human PBMCs in an unbiased approach allowing further targeted analysis with a point of entry different from that most probably generated by direct flow cytometry.

In our setting, free from malaria, we created a unique longitudinal cohort of individuals presenting with acute malaria, who were followed for a year in the absence of reexposure to the parasite. These individuals were travelers returning from malaria-endemic areas mostly in Africa, who either contracted malaria for the first time or, who were known to be previously exposed to malaria while growing up in an endemic country. B cell dynamics following acute *P. falciparum* malaria was investigated in these individuals allowing for a characterization of the humoral response based on a primary or secondary infection. By CyTOF we found a strong activation of B cells expressing high levels of CD11c and low levels of CD21. The expansion was stronger in previously exposed individuals compared with individuals with a primary infection. By sorting and culturing $CD11c^+$ and $CD11c^-$ cells from previously exposed individuals we evaluated the specificity of the response to *P. falciparum* by schizont extract ELISA. We found a 2- to 4-fold enrichment of *P. falciparum*-specific cells among $CD11c^+$ cells which precludes the possibility of simple polyclonal activation.

The expansion of the $CD11c^+$ subset peaked ten days after acute infection with a significantly stronger expansion in previously exposed individuals while the subsequent rate of contraction did not differ between the groups, suggesting that once generated, cells were similarly regulated. Characterization of the $CD11c^+$ subset based on CD21 and CD27 revealed a majority of resting MBCs and aMBCs within this compartment, each constituting between 30-40% of $CD11c^+$ cells. Further characterization indicated that both primary infected individuals and previously exposed ones, displayed a similar expansion of unswitched $CD11c^+$ cells, while an expansion of switched cells was only observed in previously exposed individuals indicating a memory response derived from preexisting *P. falciparum*-specific B cells. We found FcRL5 to be expressed on 70% of $CD11c^+$ cells, while T-bet was expressed in 33% of $CD11c^+$ B cells. T-bet expression in B cells has previously been associated with aMBCs in malaria-exposed children in Mali, concomitantly expressing FcRL5 and CD11c (112, 133). Moreover, it was shown that in $CD21^-CD27^-$ aMBCs, T-bet expression inversely correlated with phosphorylation of BCR signaling molecules, suggesting reduced BCR signaling and effector function (129, 133). In a study of malaria-exposed women in Papua New Guinea, extensive phenotypic characterization of B cells suggested that aMBCs are maintained in an anergic state rather than proceeding to apoptosis, helping to reduce the immune activation, and establishing a tolerogenic-like profile (148).

We conclude that CD11c is a marker of cells responding to malaria rather than solely a marker of aMBCs. The CD11c compartment encompasses resting MBCs, activated MBCs as well as atypical B cells, but nevertheless strongly correlates with $CD21^{lo}$ B cells. Following acute *P. falciparum* malaria, atypical B cells expand rapidly with a 1.6-fold higher frequency in previously exposed individuals compared with primary infected ones. The magnitude of B cell populations, rather than rate of contraction, is the mechanism behind sustained levels in previously malaria-exposed individuals. Importantly, we show that schizont-specific B cell clones are significantly enriched in $CD11c^{+}$ B cells compared with $CD11c^{-}$ B cells.

Taken together, we have provided novel laboratory methods available for further investigations into the immune response to malaria. The application of these methods has defined antigenic specificity among given subsets of B cells, and has revealed intriguing effects of the interaction between B cells and *P. falciparum*. Unique longitudinal data both from a malaria endemic area and from a malaria-free area has provided insight into the B cell dynamics following acute malaria as well as the relationship between BAFF and subsets of B cells during infancy. Our findings will hopefully be of value along the way to understanding the immunology of malaria.

6 CONCLUSION AND FUTURE PERSPECTIVES

- Carboxyl Qdot GiRBC conjugated to schizont extract is a good method for the separation of *P. falciparum*-specific B cells using flow cytometry
- Parasitemia at the time of sampling increases the yield of *P. falciparum*-specific B cells when using the Qdot method
- The concentration of BAFF is considerably higher in the cord blood of infants than in mothers at the time of birth, and remains significantly higher at the age of nine months
- BAFF is negatively correlated with IgG^+ MBCs and $CD27^-$ MBCs in infants at nine months, as well as in mothers at the time of delivery
- Human B cells and *P. falciparum*-infected RBCs can be co-cultured for ten days retaining high viabilty of both components
- Parasitemia increases more rapidly and peaks higher when *P. falciparum*-infected RBCs are cultured with B cells than when cultured alone
- B cells co-cultured with *P. falciparum*-infected RBCs demonstrate a marked increase in the expression of Fc receptor homologues
- $CD11c^+$ B cells highly correlate with $CD21^{lo}$ B cells implying that this population encompasses activated MBCs as well as atypical MBCs
- Following acute *P. falciparum* malaria, atypical MBCs expand rapidly with higher frequency in previously exposed individuals compared with primary infected individuals
- The magnitude of B cell populations, rather than rate of contraction, is the mechanism behind sustained levels in previously malaria-exposed individuals
- The number of switched $CD11c^+$ B cells is significantly higher in previously exposed individuals than in primary infected individuals
- At the time of acute infection, a high proportion of $CD11c^+$ B cells express FcRL5 and T-bet
- Schizont-specific B cell clones are significantly enriched in $CD11c^+$ B cells compared with $CD11c^-$ B cells

The immune response to a complex organism must be correspondingly complex. Indeed many researchers before us have studied *P. falciparum* in an effort to elucidate how immunity arises and yet, much remains obscure. The ambiguous fascination with this unicellular organism has, however, brought us considerable knowledge since the day of its discovery by Leveran in 1880. In the face of artemisinin resistance and an apparent stall in the efforts to decrease malaria morbidity and mortality, it becomes painfully obvious and beyond all doubt, that a safe and efficacious vaccine is as urgently needed as ever. Working on all levels from clinicians to entomologists, from basic researchers to epidemiologists, from politicians to engineers, from governments to philanthropists, the burden of malaria is acknowledged. The perpetrator is nonetheless omnipresent. And its existence spanning all vertebrates makes its elimination unlikely. Interventions on all levels must be maintained in an effort to firstly, prevent infection and secondly, to provide treatment. Continued efforts by the scientific community will bring about a deeper understanding which will provide us with new approaches to hamper malaria. The methods and findings presented in this thesis contribute to a better understanding of acquired immunity to malaria. By outlining the differences in the B cell response between individuals infected for the first time, and individuals previously exposed, we have gained some insight into the mechanisms underlying immunity. Furthermore, our novel methods provide tools for the study of B cells and parasites in the quest for defining protection from disease. Identifying correlates of protection is a crucial step in vaccine design. Ultimately, a vaccine with enhanced efficacy in combination with sophisticated entomological measures could lead to a favorable setting in which malaria is no longer a constant threat.

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