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METHODS FOR STUDYING MEMORY B-CELL IMMUNITY AGAINST MALARIA

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Methods for studying memory B-cell immunity against malaria

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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In loving memory of my mother, Suzanne Andersson,

who taught me more than any scientific article ever could have.

POPULÄRVETENSKAPLIG SAMMANFATTNING (IN SWEDISH)

Malaria är en sjukdom som orsakas av parasiter från släktet *Plasmodium* och vållar över 420.000 dödsfall årligen. Malaria är ett globalt problem, men koncentrerat främst till afrikanska länder söder om Sahara där majoriteten av dödsfallen sker. Barn är speciellt i fara då de ännu inte hunnit utveckla skyddande immunitet mot parasiten. För att framställa nya effektiva vacciner behöver man förstå hur immunitet mot malaria utvecklas. Länge har man vetat att antikroppar mot parasiten är skyddande. Antikroppar produceras av s.k. B-celler som finns i bland annat blod, lymfkörtlar och mjälten. B-celler utgör en viktig del av immunförsvaret då de efter att ha träffat på något kroppsfrämmande, t.ex. en parasit, kan utvecklas till s.k. ”minnes B-celler” vars uppdrag är att snabbt producera antikroppar vid nästa infektion. Mycket tyder på att minnes B-celler är viktiga för immunförsvaret mot malaria, men studier har begränsats av det låga antal av dessa celler i blod. För att hitta och analysera dessa celler i blod, krävs därför känsliga metoder särskilt då det vid malaria är viktigt att kartlägga svaret mot flera olika parasitantigener.

Syftet med denna avhandling var att vidareutveckla metoden ”B-cells FluoroSpot” som möjliggör analys av minnes B-celler mot olika kroppsfrämmande proteiner s.k. ”antigen” och sedan använda metoden för att få djupare inblick i hur immunitet mot malaria utvecklas. I Studie **I**, utvecklade vi metoden ”reversed B-cell FluoroSpot” och visade med celler från möss att den kan användas för att detektera antigen-specifika B-celler mot fyra olika antigener samtidigt. I Studie **II** adapterade vi metoden för studie på minnes B-celler hos människa och för att samtidigt detektera minnes B-celler mot antigener från hepatit B (gulsot), tetanus toxoid (stelkramp) och cytomegalovirus (ett vanligt förekommande virus hos människor). Vi visade också att vi kunde använda metoden för att mäta minnes B-celler före och efter hepatit B vaccination. I Studie **III** adapterade vi metoden för att mäta minnes B-celler mot malariaparasitens antigener och använde metoden för att följa hur immunsvaret mot parasitantigen utvecklas över tid i personer som infekterats med malaria för första gången eller hos de som haft malaria vid flera tillfällen. I Studie **IV** använde vi metoden för att mäta minnes B-celler mot parasit-antigener i barn som bor i malaria-endemiska områden i östra Kenya, och identifierade att faktorer så som ålder, och antal kliniska malaria episoder påverkade det uppmätta immunsvaret, samt identifierade immunsvar som påverkade risken att få malaria vid ett senare tillfälle.

Sammanfattningsvis är den nya B-cell FluoroSpot som vi tagit fram en känslig metod för analys av lågfrekventa minnes B-celler mot malaria och andra infektioner/antigen. Studierna har också bidragit till kunskapen kring hur minnes B-celler utvecklas och bibehålls efter malaria. Metoden har användning för att kartlägga minnessvaret vid infektioner och efter vaccination. Denna kunskap kan komma att vara viktig vid framställandet av nya vacciner.

ABSTRACT

Plasmodium falciparum malaria remains one of the world's deadliest infectious diseases and the search for an effective vaccine is highly warranted. Memory B cells (MBCs) and the antibodies they produce, once activated, is believed to play an important role in the protective immunity against malaria, but the mechanism of acquiring and maintaining these cells is poorly understood. New and sensitive tools able of gathering detailed information regarding the development and maintenance of antigen-specific MBCs could increase the understanding of protective immunity but also be used for the evaluation of new vaccines. In Study **I**, we developed the reversed B-cell FluoroSpot assay, a new assay format based on an established technique for single-cell analysis. Using hybridomas and splenocytes from immunized mice together with a tag/anti-tag approach for detection, we showed proof-of-principle that the assay could be used for multiplex analysis of single B cells specific to four different antigens simultaneously, as well as detecting B cells displaying cross-reactivity against antigen variants. In Study **II**, we adapted the assay for studies on humans and measured MBC responses against hepatitis B virus, tetanus toxoid and cytomegalovirus. We also measured MBC frequencies before and after vaccination against hepatitis B and used new FluoroSpot reader functions to assess spot volume. We showed that the assay could be used to detect B cells against all of the antigens simultaneously and also changes in MBC frequencies and spot volume before and after vaccination. In Study **III**, we adapted the multiplex assay further for studies on *P. falciparum* antigen-specific MBCs and used it to study the kinetics of MBC responses in primary infected and previously exposed travelers diagnosed with malaria in Sweden. We showed that primary infected individuals could acquire and maintain *P. falciparum*-antigen specific MBCs as efficiently as previously exposed individuals during a one year follow up period, but that the maintenance and magnitude of antibody levels in plasma were higher in the previously exposed individuals. In Study **IV**, we used the assay developed in Study **III** to analyze *P. falciparum* antigen-specific MBCs in children living in areas with endemic transmission of malaria in Kenya. We identified that high levels of MBCs against certain *P. falciparum* antigens were associated with a reduced risk of a subsequent clinical malaria episode, and that proportions of MBCs specific to some, but not all, *P. falciparum* antigens, increase with age, but also some decrease with cumulative number of infections. We conclude that the multiplex FluoroSpot method developed in this thesis provide insights towards the acquisition and maintenance of *P. falciparum* malaria-induced MBCs. We believe that the reversed B-cell FluoroSpot assay is a sensitive and highly adaptable method to assess MBC responses against multiple antigens and will be a powerful tool for future studies on protective immunity to malaria, but also other fields of research.

LIST OF SCIENTIFIC PAPERS

I. **Peter Jahnmatz**, Theresa Bengtsson, Bartek Zuber, Anna Färnert, Niklas Ahlborg

An antigen-specific, four-color, B-cell FluoroSpot assay utilizing tagged antigens for detection

Journal of Immunological Methods, 2016, 433 (23–30)

II. **Peter Jahnmatz**, Christopher Sundling, Bartek Makower, Klara Sondén, Anna Färnert, Niklas Ahlborg

Multiplex analysis of antigen-specific memory B cells in humans using reversed B-cell FluoroSpot

Journal of Immunological methods, 2020, 478, (112715)

III. **Peter Jahnmatz**, Christopher Sundling, Victor Yman, Linnea Widman, Asghar Mohammad, Klara Sondén, Christine Stenström, Christian Smedman, Francis Ndungu, Niklas Ahlborg, Anna Färnert

Antigen-specific memory B-cell responses after acute Plasmodium falciparum malaria, assessed using a novel multiplexed FluoroSpot assay

Manuscript, submitted

IV. **Peter Jahnmatz**, Diana Nyabundi, Christopher Sundling, Linnea Widman, Jedidah Mwacharo, Jennifer Mysyoki, Niklas Ahlborg, Philip Bejon, Francis Ndungu*, Anna Färnert*

Memory B-cell responses to Plasmodium falciparum merozoite antigens in children living in an endemic area of Kenya

Manuscript

CONTENTS

LIST OF ABBREVIATIONS

AMA	Apical membrane antigen
BAFF	B-cell activating factor
BCR	B-cell receptor
BSA	Bovine serum albumin
CHMI	Controlled human malaria infection
CSP	Circumsporozoite protein
CMV	Cytomegalovirus
DMEM	Dulbecco's modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
ELISpot	Enzyme-linked immunospot assay
FBS	Fetal bovine serum
GPI	Glycosylphosphatidylinositol
HBsAg	Hepatitis B surface antigen
HEK	Human embryonic kidney cells
HR	Hazard ratio
HRP	Horseradish peroxidase
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
iRBC	Infected red blood cell
LLPC	Long-lived plasma cell
mAb	Monoclonal antibody
MBC	Memory B cell
MSP	Merozoite surface protein
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate buffered saline
PVDF	Polyvinylidene difluoride
RBC	Red blood cell
RPMI	Roswell Park Memorial Institute
RSV	Relative spot volume
SA	Streptavidin

SLPC	Short-lived plasma cell
Th cell	T helper cell
TMB	Tetramethylbenzidine
TT	Tetanus toxoid
VLP	Virus-like particle
WHO	World Health Organization

1 INTRODUCTION

1.1 THE BURDEN OF MALARIA

Malaria is a disease caused by parasites belonging to the protozoan genus *Plasmodium* that are spread by *Anopheles* mosquitoes (1). There are six major species of *Plasmodium* that infect humans and causes malaria: *P. falciparum, P. vivax, P. malariae, P. ovale curtisi, P. ovale wallikeri and P. knowlesi* (2, 3). According to estimates from the World Health Organization (WHO), approximately 228 million people were diagnosed with malaria and 405,000 people died of malaria in 2018 (4). Malaria is a global health problem but is concentrated in the Sub-Saharan region of Africa where most of the cases and 93% of deaths occur (Figure 1) (4). The parasite species *P. falciparum* is attributable to a majority of these deaths (5) and is the focus of this thesis.

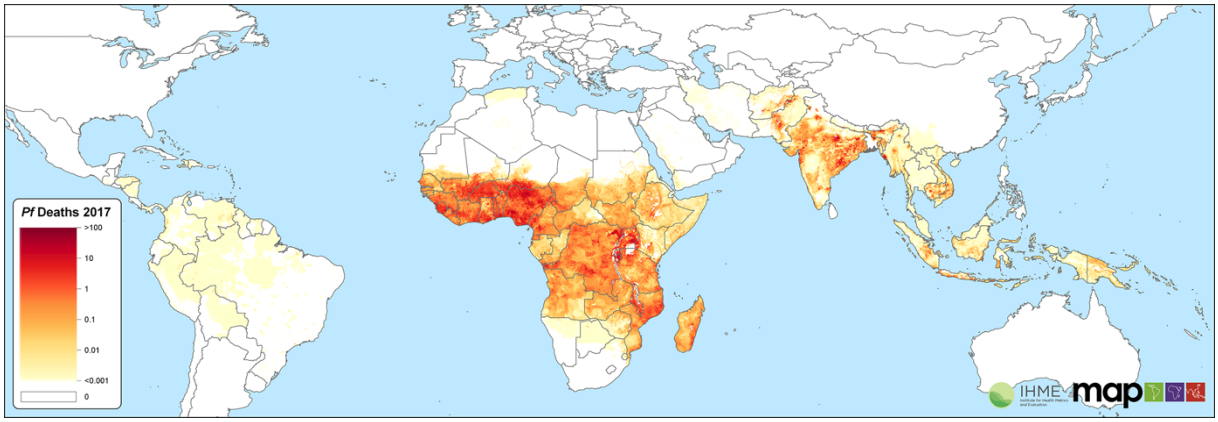

Figure 1. Predicted P. falciparum death count in 2017 in all age groups. Malaria Atlas Project. Available from https://malariaatlas.org, and reproduced with permission.

1.1.1 Malaria prevention

Great efforts have been made to stop the transmission of malaria. The widespread distribution of insecticide-treated bed nets, indoor residual spraying, mass drug administrations and effective monitoring of parasite transmission have led to a great decline of malaria cases in the last 15 years (4). However, parasite drug resistance, more recently also to artemisinin-based drugs, has been reported in south-east Asia and mosquito insecticide resistance has been widely observed (6, 7). An efficacious malaria vaccine is greatly needed, and much effort has been made to develop vaccines that are able to provide protection against malaria but also to stop the transmission of the disease. In 2019 the first licensed malaria vaccine, RTS,S/AS01 (called Mosquirix™) was launched in in Malawi, Ghana and Kenya as a part of a pilot vaccination program coordinated by the WHO (4).

1.1.2 The life cycle of *P. falciparum*

The life cycle of *P. falciparum* in humans begins when a parasite-carrying *Anopheles* mosquito takes a blood meal (Figure 2) (8). This leads to the injection of parasite sporozoites residing in the mosquito salivary gland into the human dermis. The sporozoites glide through the dermis and penetrate the blood vessels to enter the blood stream (9) where they then then migrate to the liver and infect hepatocytes (10). In the hepatocytes, the sporozoite uses the nutrients of the cell to differentiate into thousands of merozoites that are released into the blood stream upon cell rupture (11). As the merozoite encounters a red blood cell (RBC), it attaches using low-affinity receptors on the merozoite surface (12) which is most likely mediated by merozoite surface proteins (MSP) such as MSP-1 (13). The bound merozoite then undergoes apical re-orientation and express junction-forming proteins, such as apical membrane antigen 1 (AMA-1) and other proteins from the merozoite rhoptry that bind to receptors on the RBC membrane (14). A tight junction mediated by erythrocyte binding antigen (EBA) proteins and reticulocyte binding homolog (RH) proteins is then formed between the merozoite and RBC (15). The merozoite then penetrates the membrane of the RBC to complete invasion. After entering, the merozoite remodels the cell, feeds on its nutrients, and develops through several intermediate trophozoite stages, into a schizont containing between 8-32 new merozoites (16). As the infected RBC (iRBC) ruptures, the released merozoites infect other RBCs nearby. Merozoites in the iRBC can also go through a sexual stage and develop into either male or female gametocytes. These gametocytes can be transferred to another mosquito taking a blood meal (17). Male and female gametocytes in the mosquito gut develop into gametes that after fertilization become a zygote. This zygote later develops into an ookinete that penetrates the gut wall of the mosquito and continues to develop into an oocyst (18). In the oocyst, new sporozoites are formed. Upon rupture of the oocyst, the sporozoites are released and glide through the wall of the salivary gland where it waits for the mosquito to take a new blood meal (1).

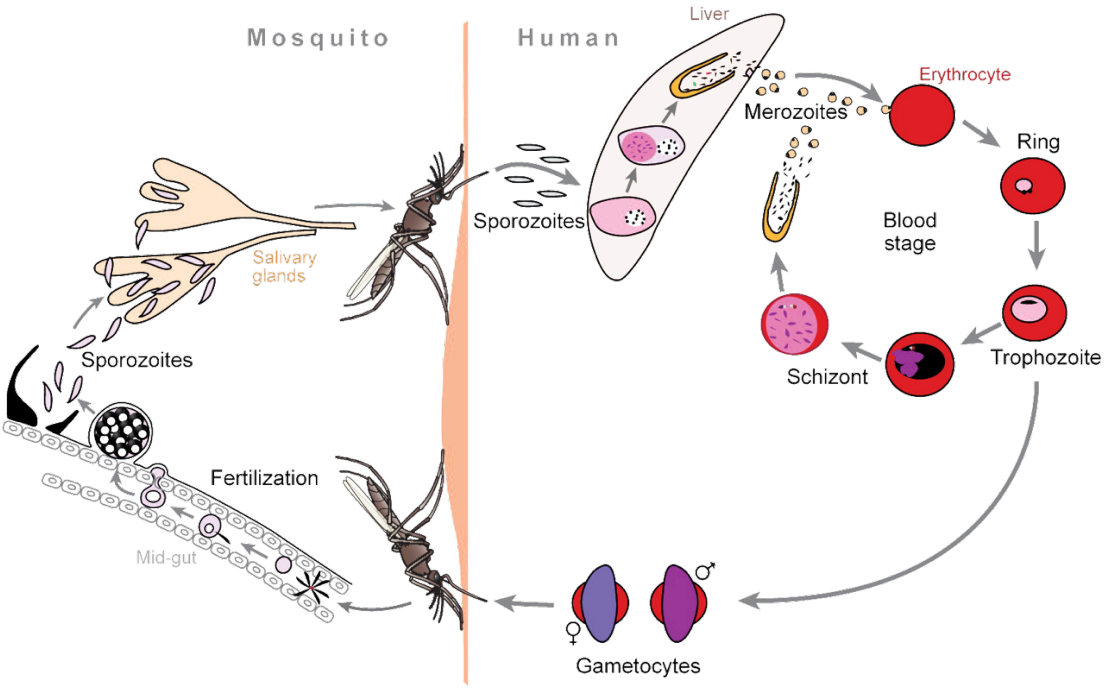

Figure 2. P. falciparum life cycle in humans and mosquito (Adopted with permission from Scherf et al., 2008 (8).

1.1.3 Pathogenesis of *P. falciparum* malaria

The clinical manifestations of a *P. falciparum* infection range from unspecific flu-like symptoms such as fever, headache, chills, nausea and muscle aches, to severe and potentially fatal presentations such as coma, severe anemia, respiratory distress, multi-organ failure or shock (19, 20). The onset of symptoms occurs during the blood-stage of the parasite life cycle (21, 22). The incubation time is usually between 1–4 weeks after infection, as demonstrated in an experimentally induced malaria challenge of human volunteers, in which the symptoms of malaria started 6–23 days after inoculation as the level of parasitemia increased (20). The clinical manifestation of disease can be divided into two categories: uncomplicated or severe malaria, with a set of criteria defined by WHO (4).

The severity of disease is dependent on preexisting host immunity, but also parasite factors such as the level of parasitemia i.e. the proportion of infected red blood cells (iRBCs) (23). High parasitemia in children can lead to severe anemia caused by factors such as hemolysis of RBCs, but also parasite-associated damage on the bone marrow which could ultimately lead to an ineffective production of new RBCs (24, 25). High parasitemia is also associated with liver dysfunctions, for instance jaundice or kidney dysfunctions, such as malaria acute renal failure (26). Another important pathogenic factor is cytoadhesion of iRBCs, also called sequestration or rosetting depending on the type of cells involved (27). Cytoadhesion occurs

when iRBCs express surface proteins (adhesins) such as *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) which bind to epithelial cells or uninfected RBCs in close proximity to the iRBC (28). By doing so, iRBCs avoid following the blood stream to areas of highly active immune functions, such as the spleen where they would likely be cleared by immune cells (29). Cytoadhesion occurs in most organs, and can lead to reduced oxygen delivery to the tissues resulting in lactic acidosis or respiratory distress (30). Cytoadhesion in the capillary vessels of the brain can lead to obstruction of the vessels and cause cerebral malaria (31). Cerebral malaria can lead to coma, cortical blindness and convulsions and has the highest fatality rate (19, 32).

According to treatment guidelines set by the WHO, uncomplicated malaria should be treated with artemisinin-based combination therapy to clear the parasite. In severe malaria, treatment involves intravenous injection of artesunate followed by a full course of artemisinin-based combination therapy (33).

1.2 NATURALLY ACQUIRED IMMUNITY TO *P. FALCIPARUM* MALARIA

Even though a primary *P. falciparum* infection can give rise to a strong immune response, development of clinical immunity takes time and is complex (34). In high transmission areas, children under 5 years of age are at particular risk of severe malaria due to the lack of immunity (35). With increasing age and exposure to the parasite, clinical immunity, i.e. protection from disease, is gradually acquired leading to a higher incidence of mild or asymptomatic malaria in older children and adults (34, 36). Even though studies have shown that sterilizing immunity can be achieved by inoculation of sporozoites in experimental human models (37, 38), the general consensus is that sterile protection, i.e. complete clearance of the infecting parasite and protection against new infections, is never truly achieved by naturally acquired immunity (39).

In order to maintain clinical immunity towards malaria, continuous exposure to the parasite seems to be required (40, 41). This has been demonstrated in studies showing that antibody levels against malaria antigens follow the transmission seasons with high levels during the rainy seasons, with high exposure and low levels during the dry season with low exposure (21). Similar to this study, another longitudinal study, following a cohort of Kenyan children, showed that anti-merozoite antibodies declined rapidly when transmission intensity decreased (40).

Acquired immunity against clinical malaria declines in the absence of re-infection as demonstrated by previous studies of African immigrants moving from endemic to malaria-free areas (42). Also, in a retrospective medical chart review of over 900 patients treated for malaria in Sweden, it was shown that the risk of developing severe malaria for African adults, returning to endemic areas, increased with time spent in Sweden (43). Furthermore, loss of clinical immunity has also been observed during malaria elimination programs in remote islands with extensive vector control and mass drug administration (44).

The process of acquiring immunity towards malaria is also delayed by the fact that many of the *P. falciparum* antigens display extensive genetic diversity with polymorphisms and allelic variation (45). It is therefore widely considered that immunity to malaria is "strain-specific" (46, 47), meaning that immunity can differ against parasites with different genotypes. In accordance with this, increasing age together with exposure to a multitude of *P. falciparum* parasite variants/clones has been shown to correlate with protective immunity (48, 49). It is therefore believed that repeated bouts of malaria gradually lead to clinical immunity as the immune system recognizes more variants of malaria antigens, and progressively develops an efficient repertoire of protective antibodies (50-52).

In summary, the process of acquiring clinical immunity to malaria is multi-factorial and largely dependent on an experienced immune system with broad recognition of parasite antigens and antigen variants.

1.2.1 The humoral immune response against malaria

There is substantial evidence that B cells and the antibodies they produce upon stimulation are highly important for the development and maintenance of immunological protection against clinical malaria (34, 51). The protective role of anti-malaria antibodies has been known since at least 1961, when Cohen *et al*., showed that purified immunoglobulin (Ig) G antibodies from malaria-immune adults transferred to malaria-infected children reduced parasitemia and symptoms of disease (53).

Antibody responses are mounted to almost every stage of the *P. falciparum* life cycle (21). At the time of a second sporozoite infection, the humoral immune response, with anti-sporozoite antibodies in co-operation with CD8+ T cells, γδT-cells and natural killer cells, combat the invading sporozoite to prevent infection of hepatocytes or clearance of infected cells (54, 55). It has also been suggested that antibodies binding to sporozoite antigens, can alter the

morphology of the sporozoite by inducing precipitation of sporozoite surface proteins and thereby affecting the migration and entry into hepatocytes (56-58).

During the blood stage of the *P. falciparum* life cycle, antibodies directed against surface proteins on the merozoite can block merozoite invasion and parasite growth (59, 60). Furthermore, the binding of antibodies to merozoites can initiate opsonization and monocyte-mediated phagocytosis of the merozoite (61, 62). Antibodies having bound to antigens on the merozoites can induce complement-mediated lysis of the merozoite by the formation of the membrane attack complex (63). Finally, it has also been shown that antibodies play an important role in binding to adhesion molecules on the iRBC thereby preventing cytoadherence (64, 65).

1.2.2 B-cell differentiation

B cells develop in the bone marrow and leave into the peripheral blood as immature B cells expressing membrane bound IgM and IgD as the B-cell receptor (BCR) (66). The immature B cells migrate via the blood stream to secondary lymphoid organs, such as the spleen and lymph nodes where they transition into a mature naïve follicular B cell (67, 68), which are the largest subset of B cells and reside in B-cell follicles of the secondary lymphoid organs (69). The differentiation of naïve B cells into memory B cells (MBCs) or long-lived plasma cells (LLPCs) starts when an antigen is bound by the BCR on the naïve follicular B cell (Figure 3). The cells will then become activated and migrate to the border of the B-cell follicle and T-cell zone, where they receive co-stimulatory signals from antigen-activated T helper (Th)-cells (70). This co-activation leads to extensive proliferation of the B cells and Ig-class switching of the BCR, supported by follicular Th cells. As the cells proliferate, they will take one of three paths (71, 72).

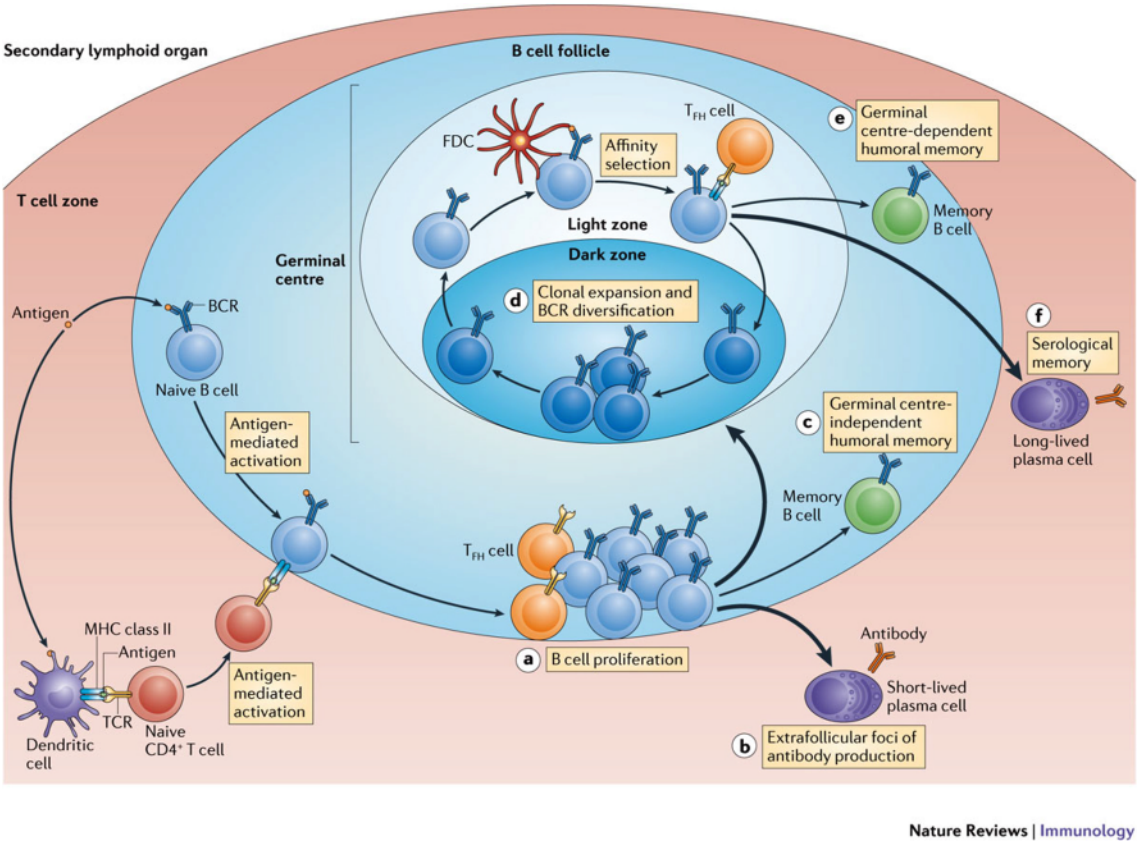

Figure 3. T-cell dependent memory B-cell generation. Reprinted by permission from Macmillan Publishers Ltd: Memory B cells, Tomohiro Kurosaki, Kohei Kometani, Wataru Ise, Nature Reviews Immunology Vol 15, Feb 2015, pages 149-159, copyright (2017).

One path is to exit from the B-cell follicle and differentiate into short-lived plasma cells (SLPCs) producing high levels of low-affinity antibodies against the antigen (73). The second path is further proliferation and formation of a germinal center (74). The germinal center consists of the dark zone and the light zone. In the dark zone, B cells go through somatic hypermutation; a process in which mutations occur in the antigen-binding region of the genes coding for the BCR. These mutations can either increase or decrease affinity for the antigen or even introduce stop codons, removing BCR expression altogether (75). The B cells with a retained BCR migrate to the light zone where the affinity of the BCR is tested on follicular dendritic cells presenting the cognate antigen (76). The B cells are also tested for their ability to process and present antigen-specific peptides to follicular Th cells (77). The mechanisms for B-cell peptide presentation and other related processes are largely unknown but eventually lead to four different fates of the B cell: 1) apoptosis, 2) return of the B cell to the dark zone for further somatic hypermutation, 3) migration of the B cell out of the follicle to become a MBC or 4) differentiation into a LLPC (78). MBCs are quiescent cells that upon antigen recognition can differentiate into antibody-producing plasma cells that secrete high

levels of antibodies with enhanced affinity compared to naïve B cells. In a healthy state, MBCs can be found in circulation while LLPCs reside in specialized niches in the bone marrow where they produce high-affinity antigen-specific antibodies released into the blood stream (73). Antigen-specific MBCs and LLPCs can exist for a very long time. This has for instance been demonstrated after smallpox vaccination, where functional antigen-specific MBCs were found over 50 years after vaccination (79).

1.3 DEVELOPMENT OF B-CELL MEMORY AGAINST *P. FALCIPARUM*

P. falciparum is almost as old as the human species (80). Since the parasite has co-evolved together with humans, it has been a constant arms race between the parasite and the immune system (81). In order to develop immunological memory against an antigen, the immune system requires an effective acquisition of antigen-specific MBCs and LLPCs able to produce protective amounts of high-affinity antigen-specific antibodies. In general, LLPCs and MBCs seem to be generated in a complex manner dependent on both age and parasite exposure. Interestingly, several studies have shown that the acquisition and maintenance of *P. falciparum* antigen-specific MBCs are more stable in areas of low transmission (82-84), while antigen-specific MBCs and LLPCs have been shown to be ineffectively acquired in individuals with recurrent *P. falciparum* infections, especially children (85, 86). For instance, studies on children living in endemic areas have shown that the half-life of antibodies against malaria antigens is much shorter than the half-life of antibodies after vaccination against tetanus (87, 88) suggesting that a *P. falciparum* induce the generation of SLPCs rather than LLPCs or MBCs in children living in endemic areas (89). However, studies comparing antibody half-lives of vaccine antigens and parasite-induced antibodies are challenging in endemic areas due to new infections, leading to continuous activation and generation of SLPCs producing short-lived antibody responses, whilst vaccines are boosted less frequently.

1.3.1 Parasite-mediated modulation of B-cell memory

The slow acquisition of MBCs and LLPCs in frequently exposed individuals is believed to be linked with a dysregulation of B cells following excess parasite exposure (81). Several mechanisms for how *P. falciparum* affect the development of immunological memory have been described (89, 90). For instance, the PfEMP1 domain cysteine-rich interdomain region 1α (CIDR1α), can cross-link the BCR on B cells, which leads to a T-cell independent polyclonal activation of B cells (91). This activation can in turn lead to differentiation of

naïve B cells into SLPCs rather than MBCs and LLPCs (91). Furthermore, during a *P. falciparum* infection, activated monocytes have been shown to produce high levels of the ligand B-cell activating factor (BAFF) (90). BAFF and its receptors are important for maintaining B-cell homeostasis, and enhanced levels of BAFF are associated with the induction of regulatory B cells (92). In turn, regulatory B cells along with other cells, have been shown to produce the immunoregulatory cytokine interleukin 10 (IL-10) shown to suppress B–T-cell interactions and the activation of B cells (93, 94).

Malaria has also been associated with the differentiation of B cells into a subset referred to as atypical MBCs (95-99). Although highly studied, the immunological role of this B-cell subset in malaria remains unclear. Some studies have shown that atypical MBCs have impaired effector functions *in vitro*, such as reduced BCR signaling, cytokine expression, activation and IgG production (100). In contrast, others have suggested that both classical and atypical MBCs can produce broadly neutralizing antibodies during a *P. falciparum* infection and that an increased proportion of atypical MBCs is associated with protection from malaria (101, 102). Recently, Aye *et al*., showed a greater expansion of atypical MBCs in children persistently exposed to *P. falciparum* compared to previously exposed children, but also that these atypical MBCs were specific against *P. falciparum* antigens MSP-1 and AMA-1 (103), suggesting that these cells could have an important function in the response against malaria, or, in contrast, were diverted away from more important functions, such as becoming conventional MBCs and LLPCs.

1.4 MALARIA VACCINES

Although *P. falciparum* infections can alter the humoral immune response in many ways, protective immunity to the parasite can still be achieved. The protective functions of antibodies in malaria have led to the belief that a vaccine against malaria is feasible. Therefore, efforts to develop a potent vaccine against malaria have been highly prioritized. Many types of vaccines have been evaluated and several are currently in clinical trials (104, 105). The malaria vaccines are usually divided into three types: pre-erythrocytic vaccines, sexual stage vaccines and asexual stage vaccines.

1.4.1 Pre-erythrocytic vaccines

Vaccines aiming to elicit an immune response against the sporozoite are normally called pre-erythrocytic vaccines. More than fifty years ago it was shown that irradiated sporozoites from the parasite *P. berghei* injected in mice, provided some degree of protective immunity when mice were challenged with viable sporozoites from the same parasite strain (106). More recent studies have also shown that with controlled human malaria infections (CHMI) inoculation of *P. falciparum* sporozoites followed by chloroquine treatment, can result in long-term protection against new infections (37, 38).

The first vaccine that was launched in areas of high malaria transmission was RTS,S/AS01. This vaccine aims to elicit an immune response against the sporozoite antigen *P. falciparum* circumsporozoite protein (CSP) thereby preventing infection of liver cells. The CSP antigen is delivered using a virus-like particle (VLP) platform based on hepatitis B surface antigen (HBsAg) that displays repeats of the CSP antigen (107). Randomized clinical trials in African children have shown that administration of this vaccine gives rise to a protection efficacy between 25-50% (108). Within the RTS,S Phase 3 trial showed that in the 6000 children aged 5-7 months having received the vaccine, the number of clinical or severe malaria episodes were reduced by half during the first year (108, 109). However, a more recent study measuring the efficacy of the vaccine in children after seven years, has shown that the protection wanes over time to be only 4-7% in moderate transmission areas (110). The protection was even lower in children with higher-than-average exposure to malaria (110). Attempts have also been made to increase the efficacy of the RTS,S vaccine. For instance, the reduction of HBsAg expression in the VLP have been shown to increase magnitude of antibodies and also efficacy of the RTS,S vaccine in preclinical studies (111). In addition, pre-clinical studies have also been made where RTS,S is administered concomitant with other pre-erythrocytic antigens such as the thrombospondin-related adhesion protein (TRAP) (112).

1.4.2 Asexual stage vaccines

Vaccines based on antigens expressed on the surface of the merozoite or iRBC are usually called asexual stage vaccines. Much focus has been on MSP-1 (59, 113), MSP-2 (114, 115), MSP-3 (116, 117), AMA-1 (118, 119), EBA-175 (120) as well as RH5 (121). An antibody response directed against these antigens has been linked with protection and has shown to be associated with blocking the merozoite invasion of the RBC, thereby reducing the severity of disease (122). Some of the vaccines candidates contain combinations of these antigens (123). Delivery of the recombinant antigen can be performed using a prime-boost strategy with a

viral vector, such as the simian adenovirus 63 vector that induce the expression of small amounts of the antigens in the host (124). These types of vaccines, e.g. ChAd63/MVA MSP1 or ChAd63.AMA1/MVA.AMA1, have been highly successful and immunogenic when tested in CHMI and are currently in clinical trials (4, 104, 105).

1.4.3 Sexual stage vaccines

Vaccines targeting the sexual stages of the parasite life cycle i.e. gametocytes, are also called "transmission blocking vaccines". The transmission blocking vaccines aim to elicit an immune response able of either blocking the mosquito uptake of the gametocyte or blocking the parasite development in the mosquito (125). Three of the most promising candidate antigens are PfHAP2, expressed on the surface of the gametocyte (126), Pfs230, expressed before zygote formation, and Pfs25, expressed after zygote formation in the mosquito (127, 128). To date, two vaccines targeting Pf25 are currently in pre-clinical trials (129, 130).

1.5 *P. FALCIPARUM* ANTIGENS

The genome of *P. falciparum* encodes for over 5300 proteins (131). The identification of protein antigens to which immune responses are linked with protection, or markers of exposure, is highly important for vaccine development or epidemiological studies. Several *P. falciparum* antigens such as MSP-1, MSP-2, MSP-3, AMA-1 and CSP have been extensively studied in order to understand their function and the effect of antibody responses against them (122, 132-137). However, the high degree of polymorphism and allelic variation displayed by these antigens are a major challenge for vaccine development (138, 139). If vaccines are to be developed targeting these antigens, knowledge regarding antigen structure and diversity is important.

1.5.1 MSP-1

The specific function of the merozoite surface protein 1 (MSP-1) is still unknown, but it is believed to have a role in the cytoadhesion to RBCs (15) although studies have yet to confirm this. Studies have shown that antibodies directed against MSP-1 can block the entry of the merozoite into the erythrocyte (140). MSP-1 is produced as a ~190 kDa precursor protein that is attached to the merozoite surface via C-terminal GPI anchor proteins (141). MSP-1

undergoes proteolytic cleavage into several fragments on the surface just before rupture of the schizont (142). One of the C-terminal fragments, MSP-1_{42}, is then further cleaved into the fragments MSP-1_{33} and MSP-1_{19} (143). Only MSP-1_{19} remains on the merozoite surface during the invasion of erythrocytes. According to the amino acid structure analysis made by Tanabe *et al.* in 1987, MSP-1 can be divided into 17 blocks containing both conserved and non-conserved parts (144). Based on differences in the non-conserved regions, the allelic variants of MSP-1 block 2 can be divided into three major groups: KI, R033 and MAD20 (145).

1.5.2 MSP-2

MSP-2 is expressed as a ~30 kDa glycoprotein and like MSP-1, attached via C-terminal anchor proteins to the surface of the merozoite. MSP-2 consists of non-repetitive conserved N- and C-terminal regions flanking a highly polymorphic repetitive domain as well as semi-conserved dimorphic parts that define the two major allelic families 3D7 and FC27 (146). MSP-2 is often referred to as an intrinsically unstructured protein that under physiological conditions has the conserved N- and C-terminal region close to the merozoite surface, while the variable dimorphic and polymorphic parts of the protein protect the conserved part from antibody binding (147). A challenge for vaccine development is that MSP-2 has been reported to undertake an amyloid like form when expressed recombinantly (147) and have to be coupled to a lipid membrane in order to assume its native form (148). Furthermore, due to its extensive polymorphism, MSP-2 has frequently been used for genotyping in order to assess the types and number of parasite clones in blood during an infection (149-151).

1.5.3 MSP-3

Unlike MSP-1 and MSP-2, MSP-3 is considered to be a soluble antigen, and believed to be attached to the merozoite membrane via protein-protein interactions (152). MSP-3 is expressed as a 62 kDa protein but is cleaved at its N-terminal site to its mature 42-44 kDa size (153). MSP-3 has an N-terminal region containing three blocks of four tandem-repeated heptad motifs (AXXAXXX) and a conserved C-terminal (153). Based on sequence variations in the N-terminal heptad motifs, MSP-3 is divided into two major allelic families 3D7 and K1 (154). *In vitro* studies have shown that antibodies directed against MSP-3 might be associated with inducing antibody-dependent cellular inhibition of the merozoite (155).

1.5.4 AMA-1

AMA-1 is believed to play an important role in the invasion process of the RBC (156). Apart from being expressed on the merozoite, studies have also proposed that AMA-1 is expressed on the sporozoite surface (157). AMA-1 is expressed as an 83 kDa precursor protein that undergoes proteolytic cleavage and is converted into the 42 kDa protein, which is believed to mediate merozoite invasion of the RBC (158). The amino acid sequence of AMA-1 is divided into three domains, and differs from other malaria antigens, as repetitive parts are absent (159). The genetic variation of AMA-1 is instead due to point mutations and deletions in domain 1 that define the two major allelic groups 3D7 and K1 (160).

1.5.5 CSP

CSP is the most abundant protein expressed on the surface of the sporozoites and has several functions in the development of the sporozoite but also mediates adhesion and invasion of hepatocytes (161). CSP can be divided into three domains: the conserved N-terminal domain containing region I, followed by a central repeat domain that contains the NANP repeat region which is the major site for antibody- and T-cell recognition after RTS,S vaccination (162). The C-terminal domain contains the thrombospondin-like type I repeat but also the GPI anchor proteins that mediates linkage to sporozoite membrane (163).

1.6 IMMUNOASSAYS TO MEASURE HUMORAL IMMUNE RESPONSES

Several immunoassays have been used to study antibody reactivity to the malaria antigens MSP-1, 2, 3, AMA-1 and CSP (84, 86, 164). For studies on antibody responses and reactivity in plasma samples, immunoassays such as Enzyme-linked immunosorbent assay (ELISA) or bead based multiplex assays (e.g. Luminex) are most widely used. Studies on cellular responses can be also be assessed using assays like flow cytometry, B-cell Enzyme-linked Immunospot (ELISpot) assay, and more recently B-cell FluoroSpot. Immunoassays analyzing antibody- or cellular responses both have strengths and weaknesses.

1.6.1 ELISA

ELISA is a plate-based immunoassay that allows fast and sensitive detection of an analyte of interest in a solution (165). Serological analysis can be performed by coating an antigen in protein-binding polystyrene plates, followed by the addition of antibody-containing samples such as plasma. The antigen-specific antibodies in the plasma bind to the coated antigen and can later be detected using a secondary detection antibody labeled with an enzyme, commonly horseradish peroxidase (HRP) or alkaline phosphatase. In a final step of the assay, a colorimetric substrate is added to the wells. The enzyme cleaves the substrate and generates a substrate product. The level of substrate product can then be measured by an ELISA reader and is proportional to the amount of bound enzyme-labeled detection reagent in the well.

1.6.2 B-cell ELISpot

The B-cell ELISpot assay is performed in 96-well PVDF membrane plates and can be used to gain information on single antibody-producing B cells in e.g. PBMC samples (166). ELISpot can for example be used to study the frequency of IgG-producing cells as well as the antigen specificity and antibody subclass. In contrast to ELISA, ELISpot plate wells contain a membrane on which antigens are immobilized (Figure 4). Also, instead of adding an antibody-containing sample, cells are directly added to the wells when assessing antigen specificity. The added B cells produce antibodies that bind to the antigen nearby the position of the cell. The antigen-bound antibodies and the position of the B cell can then be visualized using enzyme-labeled detection antibodies and a precipitating substrate, creating a spot on the membrane. In the context of malaria, the B-cell ELISpot has been used in malaria research in order to determine frequency of MBCs reactive with different malaria antigens (82, 167-171).

1.6.3 Reversed B-cell ELISpot

In 2009, Dosenovic *et al*., described the reversed B-cell ELISpot assay from the analysis of antibody-producing B cells (172). Instead of using antigen-coated wells, the assay utilizes anti-IgG antibody-coated wells (Figure 4). The coated antibody captures the antibodies secreted from the added B cells, followed by the addition of soluble biotinylated antigens to the wells. Antigen-specific B cells can then be detected using enzyme-labeled streptavidin (SA) followed by a precipitating substrate. The benefits of this approach were described as an improvement of spot quality, but also a large reduction in the amount of antigen needed (172).

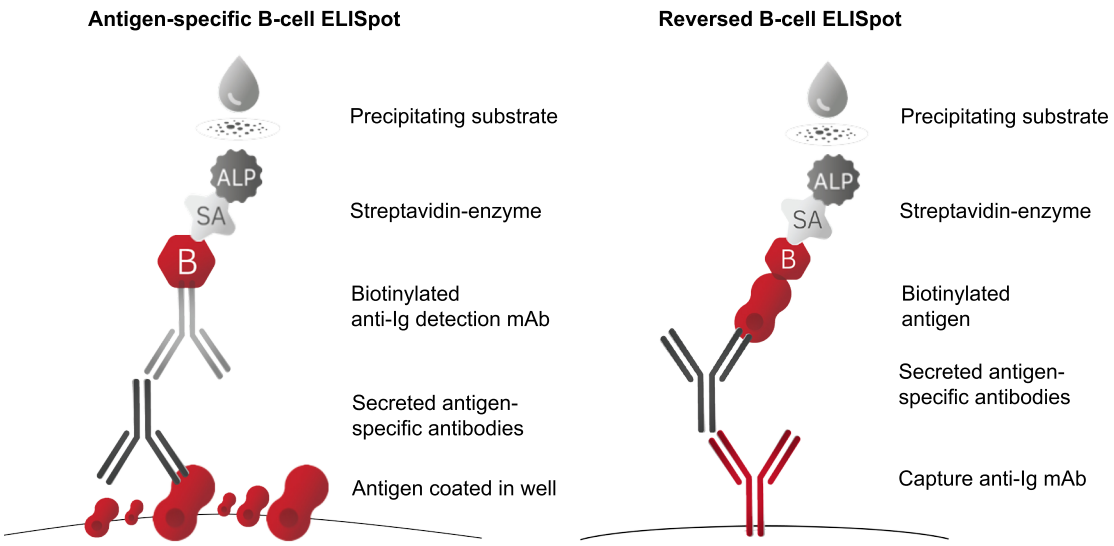

Figure 4. Variants of the B-cell ELISpot assay for the detection of antigen-specific B cells.

1.6.4 FluoroSpot

The methodology of the ELISpot is limited to the analysis of only one parameter (e.g. an antigen) at a time. The FluoroSpot assay, on the other hand, allows for multiplex analysis of several analytes at the single-cell level since it utilizes multiple different fluorescent detection systems rather than a precipitating substrate (173).

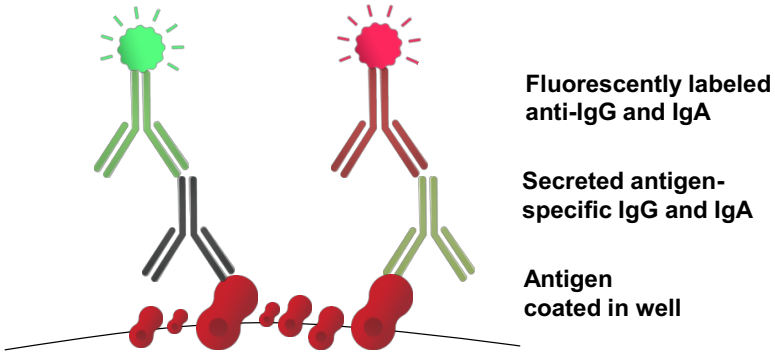

Figure 5. B-cell FluoroSpot assay for determination of antigen specificity and isotype of antibodies produced by B cells.

Multiplex analysis is facilitated by the use of different fluorophores with distinct excitation- and emission spectrums. By using a FluoroSpot reader equipped with wavelength specific filters, each fluorophore can be analyzed separately (174). As this light exposure causes excitation of the selected fluorophore, the fluorophore-emitted light then passes through a second filter after which it is detected by a camera revealing the location of the cell as a

fluorescent spot. The FluoroSpot assay was first designed to study cytokine-secreting cells, but has since then been adapted for studies on B cells (175). The B-cell FluoroSpot has for instance been used to simultaneously study the antigen specificity as well as Ig subclass of antibodies from single B cells (175-177) (Figure 5). In malaria research, the FluoroSpot assay has been used to study cytokine secretion during CHMI following vaccination with live-attenuated sporozoites (178). However, to our knowledge, no studies had previously been reported using the B-cell FluoroSpot assay in malaria research.

1.7 MEASURING HUMORAL IMMUNE RESPONSES TO *P. FALCIPARUM* ANTIGENS

For long, ELISA has been the standard method when studying humoral responses against malaria. The pros of ELISA are that it is fast, easy and highly sensitive. However, in recent years, this method has been partly replaced by other serological assays offering multiplex analysis such as Luminex (179, 180) or protein microarrays (181-183). For instance, one of the first proteome arrays contained 2320 *P. falciparum* peptides (184). However, protein microarrays are highly expensive and any analyses of immune responses in plasma will be affected by the often reported short-lived antibody responses to *P. falciparum* antigens (88, 185). Hence, studies on immunological memory based solely on anti-malaria antibodies in circulation carries the risk of drawing inaccurate conclusions regarding previous exposure and memory. Also, predicting exposure and protection by the analysis of circulating anti-malaria antibodies can be challenging due to the transient nature of antibody levels in individuals living in endemic areas (87).

In order to overcome this constraint, a few studies have combined the analysis of plasma antibody responses with the assessment of MBCs in circulation (83, 84, 171). The B-cell ELISpot assay has been used for studies on *P. falciparum* MBCs (167, 168, 170, 186) and proven to be an important complement to studies on circulating antibodies. This was for instance suggested by a five-year follow-up study of two cohorts of Kenyan children, where antigen-specific MBCs were detected in the absence of antibody levels (83). Similarly, in another study on travelers diagnosed with malaria in Sweden, ELISA and B-cell ELISpot were used to study *P. falciparum* antigen-specific antibodies and MBC responses several years after an acute infection (82). The results showed that even if the *P. falciparum* antigen-specific antibody levels had waned, MBCs could be found up to 16 years after infection (82).

In addition to ELISpot, flow cytometry has also been used to assess MBC responses to *P. falciparum* antigens (187, 188). Flow cytometry has the advantage over ELISpot that it enables the possibility to phenotype cells based on surface markers, but also potentially the opportunity to isolate cells for further transcriptomic or antibody sequence analysis. Nevertheless, the ELISpot has been described to be more robust and less laborious compared to flow cytometry (189), and to a higher extent, allow high throughput analysis of MBC responses for screening studies involving multiple individuals.

Several techniques have been described for expression of *P. falciparum* antigens used for immunoassays. Some of the most commonly used expression systems have been *E. coli* (184, 190), a wheat germ cell-free system (191), or mammalian cell lines such as human embryonic kidney cells (HEK) (192). Even though techniques have successfully been used to express a variety of *P. falciparum* antigens, the major challenge when expressing recombinant proteins for use in immunoassays, as well as for vaccines, is to secure the structure and functionality of the expressed protein. However, securing structure can prove challenging due to the complexity of many extracellular *P. falciparum* antigens in regard to highly repetitive amino acid sequences, as well as unclear structural domains (131). This has favored the use of mammalian expression systems that, unlike bacterial systems, can add disulfide bonds, does not require protein refolding after expression, and can add post-translational modifications of the expressed protein (192, 193).

In order to study individual MBCs and their role in *P. falciparum*, robust and sensitive tools are needed in order to get a broad and detailed understanding of the fine specificity of individual MBCs towards a multitude of *P. falciparum* antigens and variations of these. The reversed approach for the B-cell ELISpot assay allows for new possibilities with the B-cell FluoroSpot assay. By combining the reversed approach together with fluorescent detection systems, it would be possible to detect B cells specific for different antigens simultaneously. The information gained by studying individual cells in terms of specificity and cross-reactivity during the acquisition of immunity to *P. falciparum*, could potentially provide important knowledge for vaccine development.

2 AIM

The overall aim of this project was to develop a new multiplex FluoroSpot assay for the analysis of antigen-specific B cells at a single cell level. The aim was further to use this new methodology to increase the understanding of factors influencing the acquisition and maintenance of *P. falciparum* MBCs, as well as their role in protection against malaria.

Specific aims:

The specific aims of the papers presented in this thesis were:

I. To develop the FluoroSpot methodology for multiplex-based enumeration of antigen-specific B cells utilizing a tag/anti-tag approach for detection and then investigate the potential of this assay.

II. To adapt the FluoroSpot technology for multiplex analysis of human MBCs specific against multiple different antigens, and then evaluate the functionality of the assay by assessing MBC responses to common virus- and vaccine antigens.

III. To further develop the multiplex B-cell FluoroSpot assay to detect and analyze antigen-specific MBCs against multiple different *P. falciparum* antigens in terms of frequency and specificity. Then to use the assay to study the kinetics of *P. falciparum* antigen-specific MBCs in travelers treated for malaria in Sweden, and with different history of exposure.

IV. To assess MBC responses against *P. falciparum* antigens in Kenyan children living in malaria endemic areas in order to understand factors influencing the acquisition of *P. falciparum* antigen-specific MBCs, but also investigate the association between MBCs and the risk of subsequent clinical malaria.

3 MATERIALS AND METHODS

3.1 STUDY POPULATIONS

3.1.1 Swedish blood donors (Study II)

This study was conducted on 23 anonymized Buffy coats received from the Blood bank at the Karolinska University Hospital, Stockholm, Sweden. The study also included six individuals scheduled for a hepatitis B vaccination (Engerix®-B, GlaxoSmithKlein, Rixenart, Belgium). These individuals were all students at the Karolinska Institutet who were enrolled to the study and were asked to donate venous blood samples before and 18-21 days after planned vaccination. Individuals were also asked to fill in a form regarding vaccination history and current health status.

3.1.2 Cohort of travelers diagnosed with *P. falciparum* malaria in Sweden (Study III)

Study **III** was conducted on 20 *P. falciparum* infected travelers followed in a longitudinal cohort in Sweden. These individuals were asked to participate in this study at the time of diagnosis of a *P. falciparum* malaria infection at the Karolinska University Hospital in Stockholm. Venous blood samples were collected before treatment, and then at follow-up visits after ten days, one, three, six and finally twelve months after treatment. Ten selected individuals were born in Sweden and were treated for a primary infection whereas the remaining ten individuals originated from Sub-Saharan Africa and reported previous malaria episodes and residency in areas with endemic malaria transmission (Figure 6). The median time since last infection for these individuals were nine (range 2-32) years. The selected individuals were all infected during travels to African countries. This study also included five individuals with no previous travel to malaria endemic areas and thus no exposure to *P. falciparum* malaria as controls.

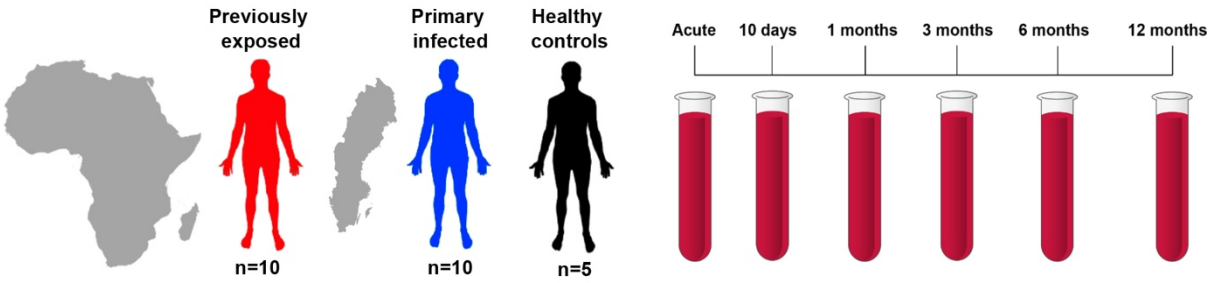

Figure 6. The cohort of travelers in Study III and their origin as well as history of exposure. Peripheral blood mononuclear cells (PBMC) and plasma were collected at time of diagnosis (acute), and then at the 10 days, 1-, 3-, 6- and 12-month follow-up visits.

3.1.3 Kenya (Study IV)

Study **IV** was performed in Kenya on 116 samples collected from children living in two longitudinal cohorts within the regions Junju and Ngerenya. The regions are located within 20 kilometers from each other and separated by an Indian Ocean creek on the coast of Kenya (Figure 7).

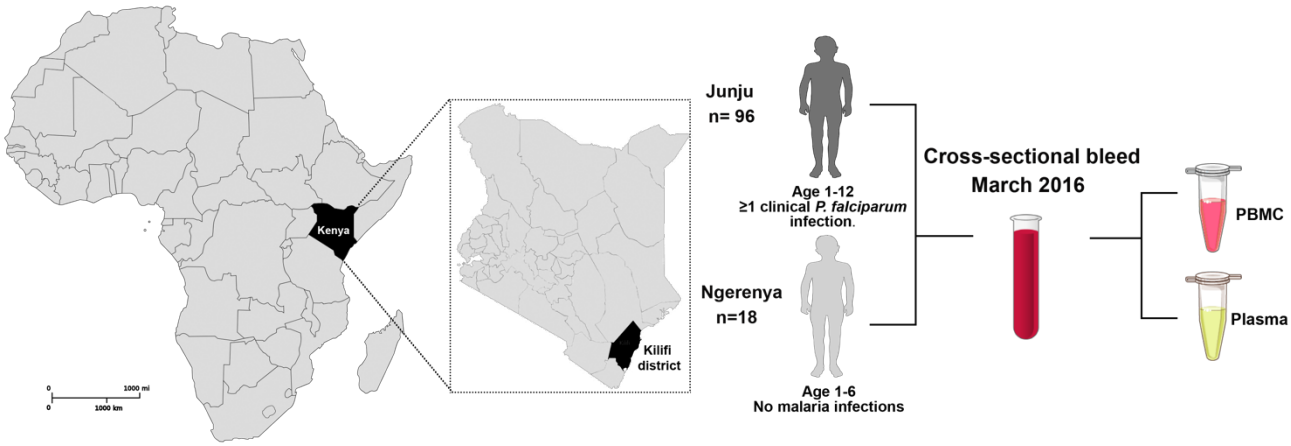

Figure 7. Geographical location and study design of Study IV.

Since 1998, children in the Junju region have been longitudinally monitored with weekly home visits for malaria surveillance and treatment upon infection. Children living in the Ngerenya region were actively monitored from 1998 until 2005 when transmission of malaria declined to zero. In contrast, the region of Junju experiences stable malaria transmission with a parasite prevalence of approximately 30% during the rainy season. Each year in March-April before the start of the rainy season, a baseline blood sample is taken from each child. For our study, baseline samples collected in 2016 were used from 96 children from the Junju

region and 20 children from Ngerenya. From these samples, PBMCs and plasma samples were used (Figure 7). The inclusion criteria for Junju children were age (1-12 years old) and at least one confirmed clinical malaria episode before baseline 2016. The inclusion criteria for Ngerenya children were age (1-6 years old) and no documented malaria episodes since birth. The median cumulative number of clinical infections in Junju children were 8 (range 1-28). Study **IV** involved all accumulated clinical data collected during the active monitoring and annual blood sampling.

3.2 ETHICAL CONSIDERATIONS

The animal Study **I** was performed in accordance with the guidelines of the Swedish Ethical Committee for Animal Protection. For the immunization of mice, ethical approval was given by Stockholms Norra Djurförsöksetiska nämnd. Study **II** and **III** were approved by the Regional Ethical Review Board in Stockholm, Sweden. Informed consent was given from participants in Study **III** when responses after vaccination was analyzed. Study **IV** was ethically approved by the Kenya Medical Research Institute National Ethics Committee and the Regional Ethical Review Board in Stockholm, Sweden.

3.3 DEVELOPMENT OF ANTI-TAG DETECTION SYSTEMS

3.3.1 Monoclonal antibody development (Study I)

The monoclonal antibody directed against a synthetic peptide CPDYRPYDWASPDYRD (designated WASP) was developed and used in Study **I**, and also used in Study **III** and **IV**. The synthetic peptide tag was first conjugated to keyhole limpet hemocyanin (KLH) using the ImjectTM Maleimide-Activated mcKLH kit (Thermo Fisher Scientific Waltham, MS, USA) according to the manufacturer. Development of anti-WASP monoclonal antibody (mAb) was performed by immunizing a female BALB/c mouse housed at Karolinska Institutet, Stockholm, Sweden on three occasions with two weeks interval using purified 100 µg/mL WASP-KLH and 60 µg/mL AbISCO-100 adjuvant (Novavax, Uppsala, Sweden) in 200 µL PBS. Three days before splenectomy, the mouse was boosted with 100 µg/mL WASP-KLH in PBS only. Hybridomas were then developed by fusing splenocytes with the myeloma cell line Sp2/0 (194) and supernatants recovered after cultivation were

for screening in ELISA against WASP-conjugated bovine serum albumin (BSA) by ImjectTM Maleimide-Activated BSA system (Thermo Fisher Scientific). Hybridomas producing antibodies with strongest reactivity against the peptide were subcloned in order to secure monoclonality. Hybridomas were then cultivated before harvest of supernatant followed by affinity purification of monoclonal antibody using Protein G sepharose columns (GE Healthcare, Uppsala, Sweden). The tag-specific mAbs anti-BAM and anti-GAL, also used in Study **I** (Table 1) had previously been developed by Mabtech, Nacka Strand, Sweden in same way as mAb anti-WASP. In Study **II**, fluorescently labeled SA was used to detect biotinylated TT. In Study **III** and **IV**, StrepTactinXT (IBA Lifescience, Goettingen, Germany) was also used to detect the peptide tag TWIN-Strep-tag (IBA Lifescience).

3.4 ANTIGEN EXPRESSION

3.4.1 Development of recombinant peptide tagged antigens

The addition of peptide tags to antigens enables the subsequent detection of the antigen in immunoassays by using tag-specific detection systems.

The recombinant antigens tagged with a peptide tag, were expressed using transient transfection of HEK293/T17 cells (used for Study **I**), or the Expi293F expression system (Thermo Fisher Scientific) according to a previously described protocol (195). Briefly, the genes coding for the protein sequence of antigen, together with tag sequence (Table 1) placed recombinantly either C- or N-terminally of the protein sequence, were synthesized and cloned into a pcDNA3.1/Zeo(-) plasmid (Life technologies Carlsbad, CA, USA). In addition, the mouse IgG kappa leader sequence (METDTLLLWVLLLWVPGSTGD) was also inserted to facilitate protein secretion. Synthesizing and cloning of the protein sequences in to a pcDNA3.1/Zeo(-) plasmid were made by GeneScript (Piscataway, NJ, USA). HEK293/T17 and Expi293F transfected with plasmids were cultivated for six days before supernatant was harvested, centrifuged and then treated with 0.1% sodium azide and stored in 4 C^{o} until use.

In addition, in Study **I** and **II**, purified antigens were biotinylated using long-chain biotinyl-N-hydroxysuccinimide ester sulfonic acid (Thermo Fisher Scientific) according to the manufacturer's instructions.

Table 1. Peptide tags used for recombinant expression of antigens

Tag	Amino acid sequence	Detected by
BAM	DAEFRHDSGY	**mAb anti-BAM**
GAL	YPGQAPPGAYPGQAPPGA	**mAb anti-GAL**
WASP	CPDYRPYDWASPDYRD	**mAb anti-WASP**
TWIN-Strep®	WSHPQFEKGGGSGGGSGGSAWSHPQFEK	**Strep-Tactin®**

3.4.2 Recombinant antigens used in different studies

The tagged antigens used for the different studies and the Uniprot accession number, expression system and tag for respective antigens are described in Table 2. In Study **I**, we recombinantly expressed the cytokines bovine, woodchuck and dog interferon gamma (IFN-γ), as well as sooty mangabey and rhesus macaque IL-2. In addition, we used purified biotinylated human IFN-γ (Peprotech, Rocky Hill, NJ, USA), cat IFN-γ (RnD Systems, Minneapolis, MN, USA), bovine IFN-γ (Thermo Fisher Scientific) as well as human IL-2 (Peprotech). For Study **II**, we expressed the HBsAg which is the major protein for the hepatitis B VLP formation. We also expressed the abundant tegument protein pp65 of cytomegalovirus (CMV.pp65). Furthermore, biotinylated purified tetanus toxoid (TT) (Statens Serum institut, Copenhagen, Denmark) was also used. In Study **III** and **IV**, we expressed the *P. falciparum* merozoite surface proteins MSP-1 (the 19kDa fragment), MSP-2 (isolate 3D7), MSP-2 (isolate FC27), MSP-3 (isolate 3D7) and AMA-1 (isolate 3D7) as well as the sporozoite antigen CSP. To enable secretion, all *P. falciparum* antigens were expressed without the amino acid sequence for GPI anchor proteins. Also, amino acids thyrosines and serines of potential N-linked glycosylation sequons (NXT/S) were replaced by alanines in order to avoid glycosylation of *P. falciparum* antigens when expressed in human cells. For all studies, expressed antigens were codon optimized for expression in human cells.

Table 2. Tagged antigens used in studies

	Antigens	Uniprot acc.nr	Expression system	Peptide tag FluoroSpot	Peptide tag ELISA
Study I	Bovine IFN-γ	P07353	HEK293/T17	BAM	-
	Woodchuck IFN-γ	O35735	HEK293/T17	GAL	-
	Dog IFN-γ	P42161	HEK293/T17	WASP	-
	Sooty Mangabey IL-2	P46649	HEK293/T17	BAM	-
	Rhesus macaque IL-2	P68291	HEK293/T17	BAM	-
	Human IFN-γ	-	purified*	Biotin	-
	Cat IFN-γ	-	purified*	Biotin	-
	Human IL-2	-	purified*	Biotin	-
Study II	HBsAg	Q773S4	Expi293F	BAM	-
	CMV.pp65	P06725	Expi293F	GAL	-
	Tetanus toxoid	-	purified*	Biotin	-
Tag control	Bovine IFN-γ	P07353	Expi293F	BAM	-
Tag control	Woodchuck IFN-γ	O35735	Expi293F	GAL	-
Tag control	Cat IFN-γ	-	purified*	Biotin	-
Study III	MSP-1_{19}	Q8I0U8	Expi293F	BAM	TWIN-Strep®
	MSP-2 (3D7)	P50498	Expi293F	GAL	TWIN-Strep®
	MSP-2 (FC27)	P19599	Expi293F	GAL	TWIN-Strep®
	MSP-3	Q8IJ55	Expi293F	WASP	TWIN-Strep®
	AMA-1	Q7KQK5	Expi293F	TWIN-Strep®	TWIN-Strep®
Tag control	Bovine IFN-γ	P07353	Expi293F	BAM	-
Tag control	Woodchuck IFN-γ	O35735	Expi293F	GAL	-
Tag control	Dog IFN-γ	P42161	Expi293F	WASP	-
Tag control	Horse IFN-γ	P42160	Expi293F	TWIN-Strep®	TWIN-Strep®
Study IV	MSP-1_{19}	Q8I0U8	Expi293F	BAM	TWIN-Strep®
	MSP-2 (3D7)	P50498	Expi293F	GAL	TWIN-Strep®
	MSP-2 (FC27)	P19599	Expi293F	TWIN-Strep®	TWIN-Strep®
	MSP-3	Q8IJ55	Expi293F	WASP	TWIN-Strep®
	AMA-1	Q7KQK5	Expi293F	TWIN-Strep®	TWIN-Strep®
	CSP	P19597	Expi293F	WASP	TWIN-Strep®
Tag control	Bovine IFN-γ	P07353	Expi293F	BAM	-
Tag control	Woodchuck IFN-γ	O35735	Expi293F	GAL	-
Tag control	Dog IFN-γ	P42161	Expi293F	WASP	-
Tag control	Horse IFN-γ	P42160	Expi293F	TWIN-Strep®	TWIN-Strep®

** Purified antigens were obtained commercially.*

3.5 CELL HANDLING

3.5.1 Cultivation of cells

In Study **I**, hybridomas recovered from liquid nitrogen were thawed, washed and cultivated in DMEM supplemented with 10% fetal bovine serum (FBS) and 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Life technologies). Cells were then cultivated at 500,000 cells/mL before use in the FluoroSpot assay. Splenocytes from immunized mice were isolated by passing spleen through a cell strainer (BD/Falcon, Becton Drive Franklin Lakes, NJ, USA). Isolated splenocytes were then washed in DMEM supplemented with 100 U/mL

penicillin and 100 µg/mL streptomycin (all from Life Technologies) before use or storage. In Study **II**, **III** and **IV**, Buffy coats or blood samples collected in EDTA tubes were processed into PBMCs and plasma using Ficoll-Paque Plus density gradient centrifugation according to manufacturer's instructions (GE Healthcare, Uppsala, Sweden) before storage.

3.5.2 Storage of cells

In Study **I**, hybridomas and splenocytes were reconstituted in 20% FBS (Life technologies) 10% dimethyl sulfoxide (Sigma-Aldrich, Saint Louis, MO, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin (both from Life technologies) and frozen in cryogenic vials at −80 °C and then stored in liquid nitrogen until use.

3.5.3 Determination of viability and concentration of cells.

Before use in the FluoroSpot, cells were counted and analyzed for viability. In Study **I**, concentration and viability of hybridomas and splenocytes were measured using a Guava ViaCount® assay (Guava Technologies, Hayward, CA, USA). In Study **II** and **III**, a Muse® Cell Analyzer (Merck, Darmstadt, Germany) was used to analyze PBMCs, whereas for Study **IV**, concentration and viability were assessed using a Countess® Automated Cell Counter (Merck Millipore, Burlington MA, USA).

3.5.4 Stimulation of cells

In Study **II, III** and **IV**, frozen PBMCs were recovered from liquid nitrogen, thawed and then washed twice in RPMI, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Life technologies). After the cells had been rested for 1 hour, cells were stimulated by adding 1 µg/mL R848 and 10ng/mL recombinant IL-2 (both from Mabtech) in 20% FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (all from Life technologies) before cultivation for 5 days in 37 °C and 5% CO_2.

3.6 ANTIBODY ASSAYS

3.6.1 Indirect ELISA (Study I, II and III)

In Study **I**, two anti-human IL-2 mAbs; MT2A91 and MT8G10 (both from Mabtech) were tested against human IL-2, sooty mangabey IL-2 as well as rhesus macaque IL-2 according to a previously described protocol (195). Briefly, mAbs were separately coated to microtiter plate wells and allowed to bind recombinant human IL-2–biotin, sooty mangabey IL-2–BAM, or rhesus macaque IL-2–BAM, and then detected with SA–HRP or biotinylated mAb anti-BAM followed by SA–HRP and Tetramethylbenzidine (TMB) substrate (all from Mabtech).

In Study **II**, sandwich ELISA was used to investigate whether the recombinantly expressed HBsAg had assumed a VLP formation. In short; mAb anti-BAM (Mabtech) was coated to microtiter plate wells and was allowed to bind BAM-tagged HBsAg in serial dilutions and then detected subsequently by biotinylated mAb anti-BAM followed by SA–HRP and TMB substrate (all from Mabtech).

In Study **III**, IgG reactivity of semi-immune plasma to *E.coli*-derived *P. falciparum* antigens MSP-1_{19}, MSP-2 (isolate 3D7), MSP-2 (isolate FC27), MSP-3 (isolate 3D7) and AMA-1 (isolate 3D7) (196) was measured. Antigens were coated to microtiter plate wells followed by the addition of serially diluted plasma. *P. falciparum* antigen-specific IgG were subsequently detected by anti-human IgG-HRP and TMB substrate (both from Mabtech).

3.6.2 Peptide tag-based ELISA (Study III and IV)

In Study **III**, a new ELISA format named Peptide tag-based ELISA was developed. In this assay, microtiter plates were coated with StrepTactinXT® (IBA Lifesciences) and incubated overnight. After blocking and washing of plates, TWIN-Strep® tagged antigens diluted 1:5 were added and the plates were incubated for 1 hr in RT. Following another washing step, diluted plasma samples were added to wells and incubated for 1 hr. After a last wash of plate, antigen-specific IgG were subsequently detected by anti-human IgG-HRP and TMB substrate (both from Mabtech). In Study **III** the reactivity of plasma samples against TWIN-Strep® tagged *P. falciparum* antigens MSP-1_{19}, MSP-2 (isolate 3D7), MSP-2 (isolate FC27), MSP-3 (isolate 3D7) and AMA-1 (isolate 3D7) were measured.

3.6.3 Reversed B-cell FluoroSpot

In Study **I**, reversed B-cell FluoroSpot assay was used to detect antibody-producing hybridomas against human IFN-γ, dog IFN-γ, woodchuck IFN-γ and cow IFN-γ. Hybridomas was also used in the assay to detect B-cell cross-reactivity against human IL-2 and sooty mangabey IL-2 or rhesus macaque IL-2. Splenocytes from a hyperimmunized mouse were also used to detect B cells displaying cross-reactivity against dog IFN-γ and cat IFN-γ. In Study **II**, the assay was used to enumerate the frequency of MBCs in PBMC samples to recombinant expressed antigens HBsAg, CMV.pp65 or purified TT. In Study **III** and **IV**, PBMCs were used to enumerate the frequency of MBCs specific to recombinant expressed *P. falciparum* MSP-1_{19}, MSP-2 (isolate 3D7), MSP-2 (isolate FC27), MSP-3 and AMA-1. In addition, in Study **IV**, the assay was also used to detect MBCs against *P. falciparum* CSP.

In order to capture IgG secreted by antibody-secreting cells, low fluorescent PVDF plates (Merck Millipore, Burlington, MS, USA) were coated with a polyclonal goat anti-mouse IgG antibody (Mabtech) for Study **I**, whereas for the remaining studies, a mouse-anti-human IgG mAb (Mabtech) was used. The concentration of cells, antigens and detection systems were then defined for each of the studies.

3.6.4 Analysis of FluoroSpot plates

The analysis of FluoroSpot plates requires readers equipped with wavelength specific filters for excitation and emission of light in order to analyze each fluorophore separately. For Study **I** and **II**, plates were analyzed using an ELISpot/FluoroSpot reader system (iSpot Spectrum, AID, Strassberg, Germany), with software version 7.0 (build 14,790). In Study **II, III** and **IV**, plates were analyzed using Mabtech IRIS™ with Apex™ software version 1. Both readers were equipped with filters equivalent for DAPI, FITC, Cy3 and Cy5 to detect fluorophores absorbing and emitting light at 350/470, 490/520, 550/ 570 and 640/660 nm, respectively.

3.6.5 Assessment of relative spot volume (RSV)

The relative spot volume (RSV) is a new type of data provided by the Apex™ software in the Mabtech IRIS™ FluoroSpot reader (Mabtech). The value provides additional information on the size and intensity of single spots in the well and corresponds to the amount of analyte secreted by the cell. In Study **II** the mean RSV value of spots from HBsAg-specific MBCs was assessed before and after vaccination with Engerix®-B (GlaxoSmithKlein). In Study **III**, we assessed the kinetics of median RSV of spots from MBCs specific for *P. falciparum* MSP-1_{19}, MSP-2, MSP-3 and AMA-1 up to one year after treatment of acute malaria in travelers. In Study **IV** the mean RSV values of spots from MBCs specific for *P. falciparum* MSP-1_{19}, MSP-2 (3D7), MSP-2 (FC27), MSP-3, AMA-1 and CSP were assessed in children living in malaria-endemic areas.

3.7 STATISTICAL ANALYSIS

Statistical analysis was carried out using STATA MP (version16.0), R (version 3.6.1) and GraphPad Prism (version 8.3) (GraphPad Software, La Jolla, CA). In Study **III**, Mann-Whitney U-test was used to compare reproducibility between tests, duplicates and singleplex *vs* multiplex analysis and differences in MBC and antibody responses between specific timepoints. Spearman correlation was used to analyse association between proportion of MBC, RSV and antibody levels throughout the study period, as well as variability between replicate wells. A mixed-effects linear regression model was used to compare differences between groups regarding MBC and antibody responses. In Study **IV**, Kruskal-Wallis test with Dunn's correction for multiple comparisons was used to compare MBC and antibody responses between two age groups. Spearman correlation was used to determine association between MBC and antibody responses, whereas Partial Spearman correlation was used to determine the association of MBC and antibody responses with age, number of clinical malaria episodes since birth and parasite density at baseline in separate multivariate analysis. A Cox-regression model was used to investigate the risk of subsequent clinical malaria after baseline (date of sample collection in March 2016) until 365 days later, and similarly for time since last clinical infection until baseline. Proportional hazards were tested using Schoenfeld's residuals. A hazard ratio (HR) with 95% confidence interval (CI) not passing 1 as well as a p-value below 0.05, were considered significant.

4 RESULTS

4.1 STUDY I

In Study **I**, we developed the reversed B-cell FluoroSpot assay and demonstrated the use of this assay for various applications. We used capture antibodies to trap antibodies from hybridomas producing mAbs with known specificities as well as splenocytes from a hyperimmunized mouse, followed by detection with biotinylated or peptide-tagged recombinant antigens as well as fluorescently labeled SA and anti-tag mAbs, respectively (Figure 8).

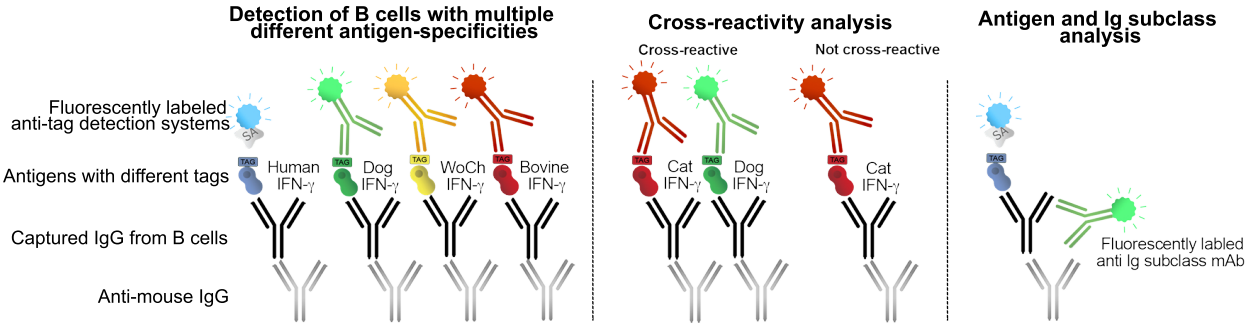

Figure 8. Overview of the different FluoroSpot assay formats described in Study I.

We showed proof of principle that the assay could be used for multiplex analysis of single antibody-producing cells specific against different antigens. This was possible by combining hybridomas producing mAbs to four different species of IFN-γ as well their respective IFN-γ antigen either tagged with a peptide tag or biotin (Figure 8). By using anti-tag specific mAbs or SA labeled with fluorophores with dissimilar emission/excitation spectrums, and a reader equipped with wavelength specific filters, the position of the four different hybridomas was detected on the membrane as a spot. The assay was validated by demonstrating functionality also with other combinations of hybridomas and antigens.

We also showed that the assay could be used to study cross-reactivity of single antibody-producing cells. For this, we used two separate hybridomas (MT8G10 and MT2A91) producing mAbs against human IL-2 but with dissimilar cross-reactivity with IL-2 from non-human primates sooty mangabey and rhesus macaque. While hybridoma MT8G10 cross-reacted with both species of non-human primates, MT2A91 only recognized rhesus macaque IL-2. Detection of cross-reactive hybridoma mAbs could be demonstrated by combing the two hybridomas together with human IL-2 and either of the non-human primate IL-2. The combination of human and sooty mangabey IL-2 resulted in single positive spots (likely from

hybridoma MT2A91) and co-positioned/double positive spots (likely from hybridoma MT8G10), while the combination of human and rhesus macaque IL-2 resulted in only double positive spots. The possibility of cross-reactivity analysis using the assay was further demonstrated by using splenocytes from a mouse immunized with cat IFN-γ and tested against a combination of cat and dog IFN-γ homology 87%) (Figure 8). The analysis resulted in 15% double positive spots for both antigens whereas the remaining 85% spots were positive for cat IFN-γ. When the test was repeated, the analysis resulted in 14% double positive spots.

Lastly, we showed that the assay could be used to simultaneous analyze the specificity of antibodies secreted by antibody secreting cell as well as isotype/subclass of the antibodies (Figure 8). For this, we used two separate hybridomas producing mAbs with known dissimilar antigen-specificities and IgG subclass. Next, corresponding antigens tagged with peptide tag as well as fluorescently labeled anti-subclass and anti-tag mAbs were used. Co-positioned/double-positive spots were obtained for the respective antigens and their corresponding IgG subclass.

4.2 STUDY II

In Study **II**, we adapted and optimized the assay for detection of human MBCs. For this, we expressed recombinant tagged antigens based on amino acid sequences for HBsAg and CMV.pp65. We also included a third antigen, TT, which was used biotinylated (Figure 9).

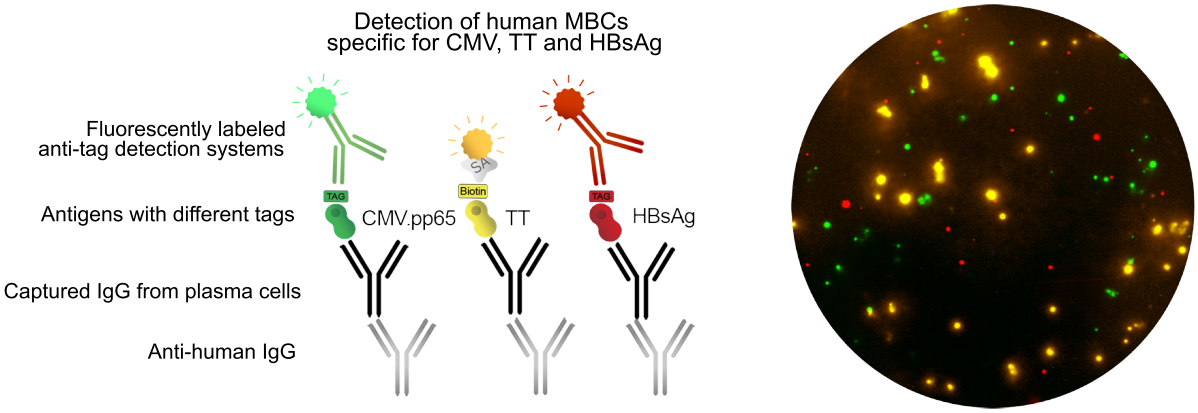

Figure 9. Schematic presentation of FluoroSpot layout for Study II. The image to the right represents a well from an individual with spots detected for all antigens tested (197).

The expression of tagged HBsAg and CMV.pp65 was verified using Western Blot and ELISA. While the concentration of TT was already known, the concentration of HBsAg was measured to 18900 IU/mL by electrochemiluminescence method whereas the concentration of CMV.pp65 was estimated to 19 µg/mL in a semiquantitative Western Blot. The optimal concentration of each antigen for use in the FluoroSpot assay was established by titrating each antigen in serial dilutions and set to 1890 IU/mL of HBsAg, 5 µg/mL for CMV.pp65 and 1 µg/mL for TT.

The functionality of the assay was demonstrated by screening PBMC samples from 23 healthy anonymized blood donors. Median proportion of MBC/total IgG for TT was 0.14% with a range of 0–2.25%, for CMV.pp65 0.03% with a range of 0–0.91%, and for HBsAg 0.07% with a range of 0–0.38% (Figure 10).

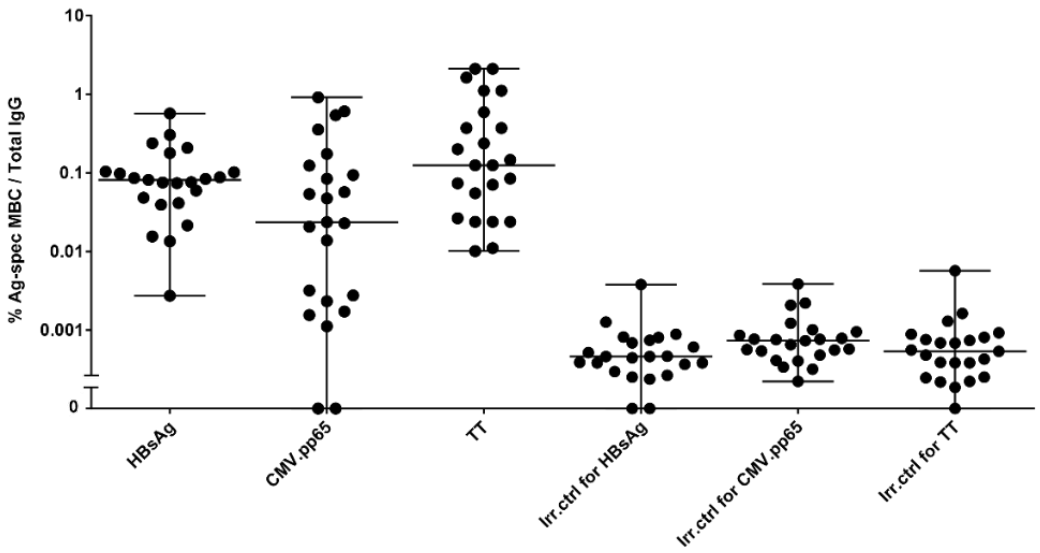

Figure 10. Proportion of antigen-specific B cells against HBsAg, CMV.pp65, TT as well as irrelevant control antigens in 23 donors with unknown vaccination history (197).

Since history of exposure or vaccination were unknown in the 23 anonymized blood donors, a small study was conducted where the assay was also used to assess frequencies of MBCs before and after planned vaccination. For this, PBMC samples from six individuals were collected before and 18–21 days after vaccination with HBsAg (Engerix®-B, GlaxoSmithKlein) and analyzed in the FluoroSpot assay. Before vaccination, one previously vaccinated individual had 0.24% HBsAg-specific MBC/total IgG while the remaining five individuals with no prior hepatitis B vaccination had proportions similar to irrelevant controls

ranging from 0–0.03% MBC/total IgG. No individuals were considered seropositive before vaccination when anti-HBsAg antibodies were measured in plasma determined by electrochemiluminescence method. After vaccination, the proportion of MBCs increased 3-fold in the individual with detectable levels of MBCs before vaccination, while no change was measured for the remaining individuals. Three individuals seroconverted after vaccination.

In addition, we also measured the RSV of spots before and after vaccination and observed tendencies of an increase of the mean RSV of spots in three individuals despite that frequency of spots did not increase.

4.3 STUDY III

In Study **III**, we adapted the reversed B-cell FluoroSpot assay for the detection of human MBCs specific for *P. falciparum* antigens (Figure 11). We then studied the kinetics of *P. falciparum* antigen-specific MBC and antibody responses in Swedish travelers treated for an acute *P. falciparum* malaria episode at Karolinska University Hospital and then followed prospectively over the course of one year. In total, 20 individuals were included, where ten individuals were primary infected, and ten individuals had lived in malaria endemic areas under an extended period of time and reported previously exposure to *P. falciparum* malaria. We also included five healthy malaria unexposed individuals as negative controls and five individuals previously exposed to *P. falciparum* that had displayed strong antibody responses towards the *P. falciparum* antigens in previous studies to be used for optimization of the assay.

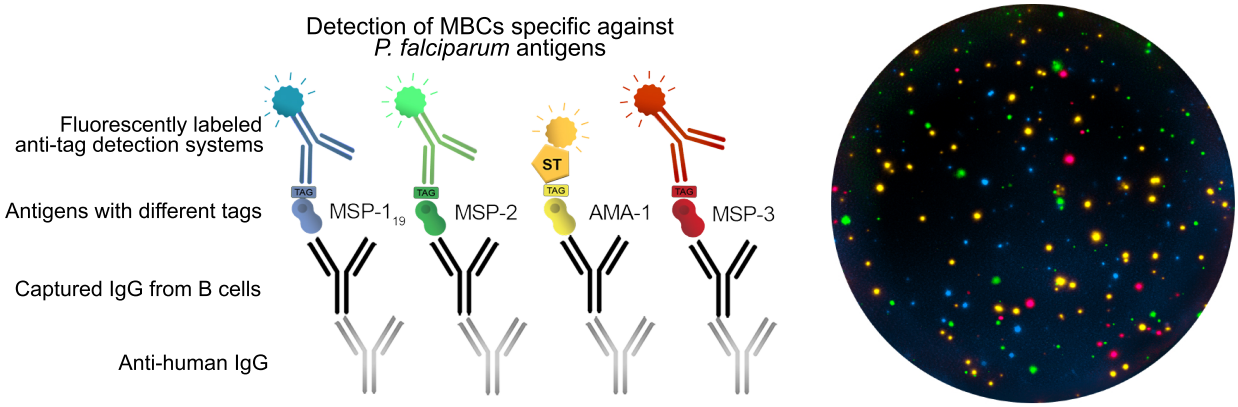

Figure 11. Schematic presentation of FluoroSpot layup for Study III. The image to the right represents a well from an individual with spots detected for all antigens tested.

P. falciparum antigens MSP-1$_{19}$, MSP-2 (allelic variant 3D7 as well as FC27), MSP-3 and AMA-1 were recombinantly expressed with peptide tags, and antigen expression was confirmed by Western Blot and ELISA. The reactivity of *P. falciparum* semi-immune plasma with the expressed tagged antigens was fully comparable with the reactivity to corresponding *E.coli*-derived antigens used for previous studies, verifying the quality and antigenicity of the expressed peptide-tagged antigens. Optimization of the FluoroSpot assay included establishing optimal number of cells as well as optimal dilution of supernatant antigens needed for analysis. The optimization was performed using PBMCs from the five *P. falciparum*-exposed individuals having displayed strong reactivity to antigens in previous studies. We established that 250,000 cells per well were optimal for analysis and that the optimal dilution of antigen supernatant was 1:50 for all antigens with the exception of AMA-1 where the optimal dilution was 1:500.

A linear mixed effects model adjusted for time was used to compare antigen-specific responses between primary infected and previously exposed individuals throughout the study period. Proportions of MBCs or median RSV of spots were not different between the groups to any *P. falciparum* antigen throughout the study period. In both groups, proportion of MBCs peaked at the 10 day follow-up and showed tendencies of increasing with time for MSP-1$_{19}$ and AMA-1 whilst more stable for MSP-2 and MSP-3 (Figure 12).

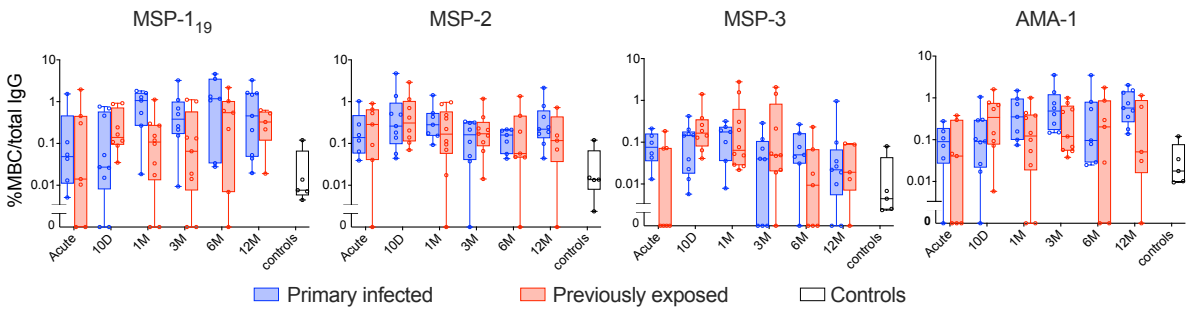

Figure 12. Proportion of MBCs to P. falciparum antigens over time in primary infected and previously exposed individuals.

For the primary infected group, proportions of antigen-specific MBC were significant increase between 10 day and 1 month follow-up for MSP-1$_{19}$ (Mann-Whitney U-test p=0.03), and from the acute time point until the 12 month follow-up for AMA-1 (Mann-Whitney U-test p=0.003). In the previously exposed group proportions of MBCs against MSP-3 (Mann-

Whitney U-test p=0.02) and AMA-1 (Mann-Whitney U-test p=0.04) significantly increased from the acute time point until the 10 day follow-up.

Furthermore, for the primary infected group, there was a significant increased in median RSV against AMA-1 between the 10 day until the 1 month follow-up (Mann-Whitney U-test p= 0.03) as well as between the acute time point until the 12 month follow-up (Mann-Whitney U-test p=0.005).

We also developed a new type of ELISA format to analyze antibody reactivity to peptide tagged antigens. In this assay, plates were coated with StrepTactinXT® (IBA Lifesciences) followed by the addition of recombinant *P. falciparum* antigens tagged with TWIN-Strep®-tag (IBA Lifesciences) that subsequently will be captured by StrepTactinXT®. By then adding an antibody containing sample, such as plasma, followed by enzyme-conjugated anti-antibodies, reactivity of the antibody containing sample could be measured.

Using this assay, plasma antibody levels to *P. falciparum* antigens could be measured in groups throughout the study period. In both groups, antibody levels to all antigens peaked at the 10 day follow-up followed by declined over the study period. However, the magnitude as well as maintenance of response were significantly higher for MSP-2 (FC27), MSP-3 and AMA-1 in the previously exposed group compared to the primary infected group. While median RSV and proportion of MBC were often correlated, limited relationship was observed for antibody levels and proportion of MBC or RSV.

In addition, during the optimization of the FluoroSpot assay for detection of MBCs specific against *P. falciparum* antigens, we also investigated the possibility of detecting MBCs displaying cross-reactivity to variants of a polymorphic *P. falciparum* antigen. For this, we extracted DNA from parasites isolated from patients. We then amplified and sequenced the gene coding for the polymorphic region of MSP-2. The sequence was then used to recombinantly express patient specific MSP-2 with a peptide tag. Combinations of tagged MSP-2 variants were then tested with PBMCs from patients in the FluoroSpot assay for homologous and heterologous responses. Preliminary results showed that cross-reactivity displayed by single B cells to different MSP-2 antigens was detected in variants within the two respective allelic families FC27 and 3D7, but not between allelic families. Although interesting, these results were preliminary and were therefore not included in any of the papers (Figure 13).

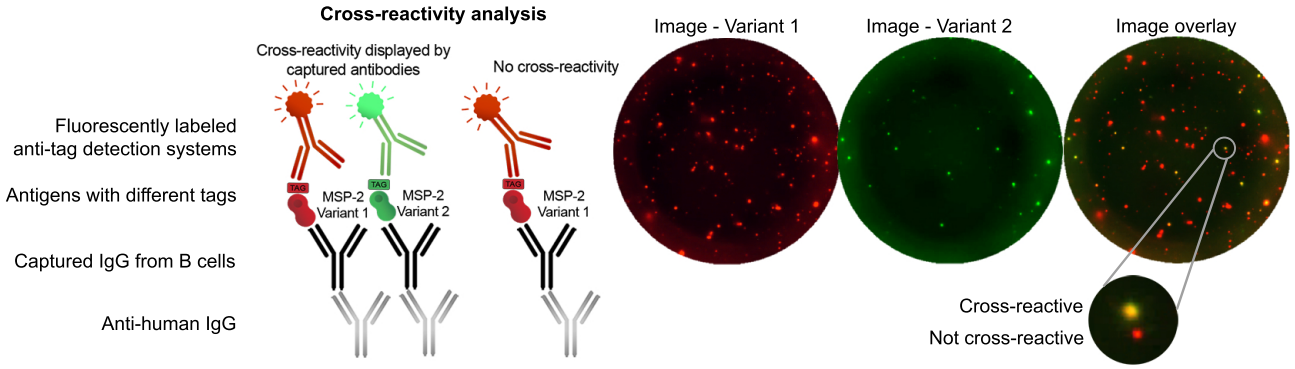

Figure 13. Detection of B cells displaying cross-reactivity against variants of the polymorphic P. falciparum antigen MSP-2 using the reversed B-cell FluoroSpot assay.

4.4 STUDY IV

In this study, we investigated how factors such as age, cumulative exposure or asymptomatic parasitemia influence MBC and antibody responses to *P. falciparum* antigens in children living in an endemic area of Kenya. Furthermore, we also investigated the role of *P. falciparum* antigen-specific MBCs and antibodies in protection against subsequent clincial infections. For this, we used samples from children in two longitudinally monitored cohorts, Junju and Ngerenya, located in close proximity but experiencing different transmission of malaria during rainy seasons. While parasite prevalence during rainy season is appoximately 30% in Junju, Ngerenya has remained at 0% since 2005 (198). We used blood samples collected in 2016 from 96 children from Junju (age 1-12) with at least one documented malaria episode since birth, and 20 children from Ngerenya (age 1-6) with no documented malaria episodes, to be used as negative controls. MBC and antibody levels were measured for *P.falciparum* antigens expressed in Study **III**, as well as the sporozoite antigen CSP using reversed B-cell FluoroSpot assay and ELISA respectively (Figure 14).

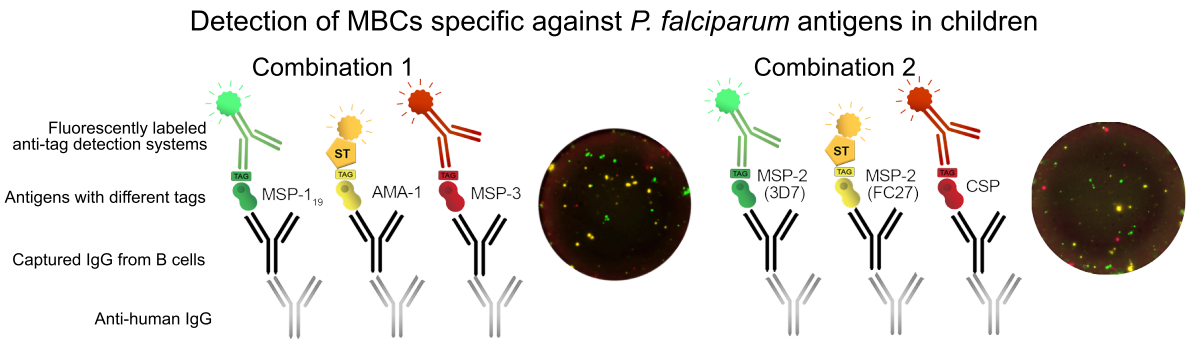

Figure 14. Schematic presentation of FluoroSpot layup for Study IV. The image to the right represents a well from child with spots detected for all antigens tested in each combination.

When the effect of age was investigated, a positive correlation was found for proportions of MBCs to MSP-2 (3D7) and MSP-3 (Spearman correlation coefficient r_s 0.258, p=0.015, and 0.212, p=0.048, respectively) and age, while the breadth of MBC response (i.e. the number of antigens an individual was considered positive for) was not associated with age. No association was observed between age and mean RSV for any antigen. Similarly, a weak positive correlation was also measured for antibody levels and age with regard to MSP-2 (3D7) (r_s 0.24, p=0.025) and MSP-3 (r_s 0.23, p=0.031), as well as age and breadth of antibody response (r_s 0.244 p=0.023).

High cumulative number of clinical malaria episodes since birth were associated with a low proportion of MBCs to MSP-3 (r_s -0.252, p=0.018), as well as for antibody levels for MSP-1_{19} and MSP-3 (r_s -0.292, p=0.006 and -0.211, p=0.05, respectively). When the effect of asymptomatic parasitemia was investigated, a postive correlation was found for proportions of MBCs and parasite density at baseline for AMA-1, as well as breadth of MBC response (r_s 0.561, p=<0.001 and 0.244, p=0.022, respectively) and average RSV for AMA-1, and MSP-2 (FC27) (r_s 0.340 p=<0.001 and 0.228 p=0.033, respectively).

Children considered positive for MBC responses against MSP-3 had a reduced risk of a subsequent malaria episode (HR 0.47, 95%CI 0.24-0.91, p=0.024). When children were sub-divided into the age groups 1-6 and 7-12 years old, only older children positive for MBC responses against MSP-3 had a reduced risk of clinical malaria (HR 0.29, 95%CI 0.11-0.82 p=0.019). A MBC breadth of ≥3 antigens appeared to be associated with a reduced risk of clinical malaria (Figure 15), although this was only borderline significant for children aged 7-12 years when children were subdivided into age groups (HR 0.32 95% CI 0.1-1.03 p=0.058). As for antibody levels, children considered postive for MSP-2 (3D7) and MSP-3 (HR 0.36 95% CI 0.15-0.86 p=0.021, and HR 0.36 95%CI 0.14-0.92, p=0.033, respectively) were associated with a reduced risk of clincial malaria, and predicted a higher level of protection compared to MBCs. The protective properties of antibodies were measured for children aged 7-12 years where children antibody positive for MSP-2 (3D7) (HR 0.33 95%CI 0.11-0.99, p=0.048) and AMA-1 (HR 0.27 95%CI 0.09-0.79, p=0.024) had a reduced risk of clinical malaria. Furthemore, an antibody breadth of ≥3 was associated with a reduced risk of clinical malaria in a model including all children as well as in the subgrouped analysis with children aged 7-12 years with (HR 0.2, 95% CI 0.06-0.67, p=0.009 and HR 0.15, 95% CI 0.03-0.72, p=0.018, respectively).

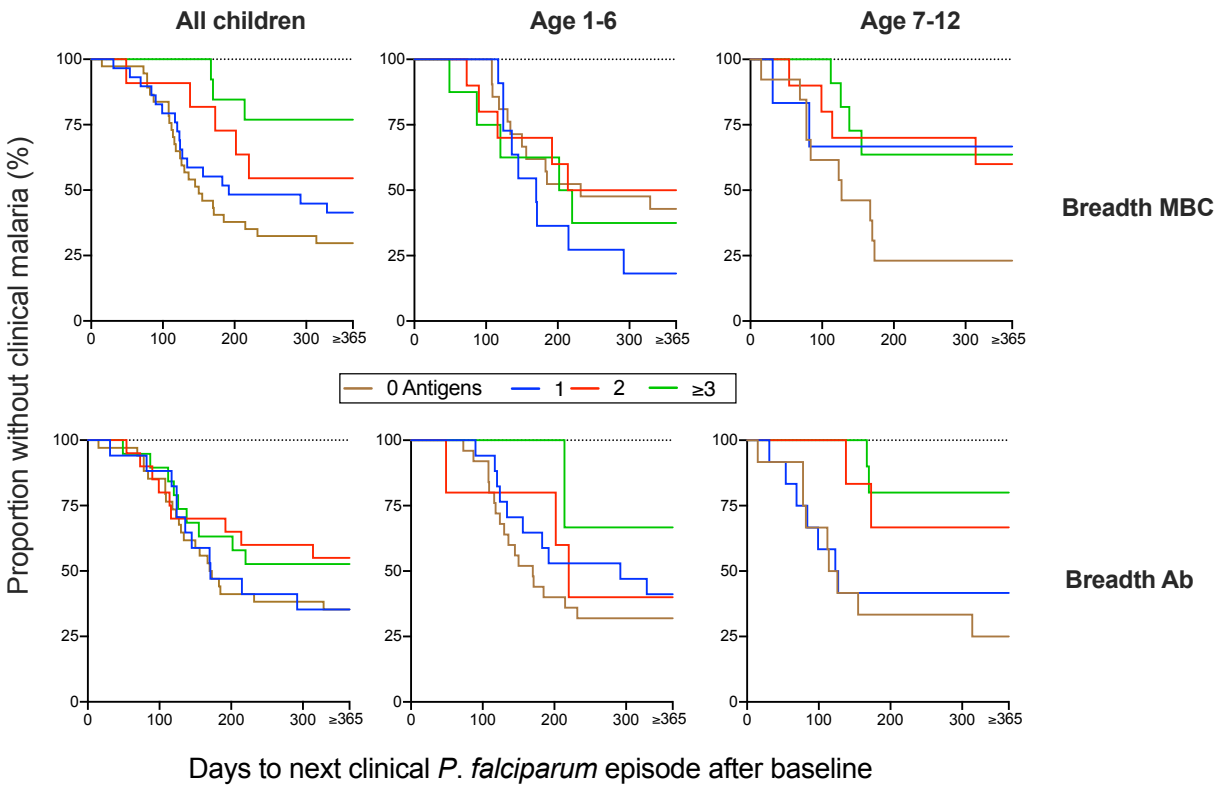

Figure 15. Time to next clinical P. falciparum infection based on MBC and antibody breadth in different age groups. Kaplan-Meier curves with subgroups of individuals with different breadth of response. Breadth was defined by the number of antigens an individual had responses above threshold for positivity.

5 DISCUSSION

MBCs and the antibodies they produce once activated are believed to be important for immunity, but the acquisition and protective role of these cells are poorly understood. The development of sensitive and adaptable technologies able to detect and analyse these cells, is therefore of great importance if the protective role of MBCs in malaria is to be unravelled.

Collectively, the four studies presented in this thesis describe the development of novel methodology aiming at facilitating new insights towards the acquisition and maintenance of MBCs to *P. falciparum* antigens. In particular, the studies describe the development of the reversed B-cell FluoroSpot, and the broad field of application for this assay for multiplex analysis of MBC responses after exposure to pathogen or vaccine. Furthermore, the included studies describe how the reversed B-cell FluoroSpot assay can be used for clinical as well as epidemiological studies. By applying this assay in malaria research, the studies contribute to the further understanding of how MBCs are acquired and maintained after acute infection of *P. falciparum*. The studies also provide new insights to the protective role of MBCs specific to *P. falciparum* antigens and identifies factors influencing the frequency and magnitude of MBCs specific to *P. falciparum* antigens in children living in malaria endemic areas.

When studying MBC responses against malaria, given the large number of antigens presented to the immune system during the parasite life cycle (131), there is often a need to study responses to a multitude of antigens in order to understand how immunity to malaria develops. However, the ELISpot assay, which is commonly used for the analysis of *P. falciparum* antigen-specific MBC responses, do not allow the analysis on MBCs responses to a multitude of antigens.

Within the scope of this thesis, in Study **I**, we therefore developed a reversed B-cell FluoroSpot assay utilizing a tag/anti-tag approach for detection. Compared to the conventional B-cell FluoroSpot where antigens are absorbed to the membrane of a well, the reversed B-cell FluoroSpot instead uses immobilized anti-IgG antibodies able to capture IgG antibodies produced by stimulated B cells. The captured IgG is then exposed to antigens tagged with unique peptide tags or biotin followed by secondary anti-tag reagents labeled with different fluorophores. We demonstrated that the assay could be used for multiplex analysis of up to four different antigen specificities of B cells, detection of B cells displaying antigen cross-reactivity, as well as simultaneous detection of B-cell Ig subclass and antigen-specificity. The tag/anti-tag approach increases the adaptability of the assay since they can be

applied on variety of antigens. Therefore, the reversed FluoroSpot assay utilizing tag/anti-tag for detection could be a useful tool when assessing MBC responses after vaccination with multivalent or multicomponent vaccines.

Compared to ELISpot assay which relies on an enzymatic reaction for development of spots, FluoroSpot utilizes fluorescently labeled detection antibodies thereby facilitates multiplex analysis. Other techniques such as, flow cytometry can be used to assess both antigen-specificity and subpopulations of cells (177, 199). However, while flow cytometry has the advantage over FluoroSpot in the sense that cells can be phenotyped and isolated (199), analysis of high number of samples in flow cytometry is often laborious. Also compared to flow cytometry, the FluoroSpot assay makes it possible to analyse antibody secretion over a cumulative time rather at one specific time point (200), potentially increasing the sensitivity of the assay. Furthermore, when studying antigen specificity of B cells in flow cytometry, a common approach is to use tetramers of the antigen coupled to a SA carrier protein in order to increase sensitivity of the assay (201). However, a common problem with this approach has been unspecific binding of B cells to SA (202). The problem with unspecific binding has been partly solved by for instance using decoy tetramers (199), but unspecific binding still remains an issue that potentially could lead to an increased uncertainty of results. In comparison, no carrier protein is necessary to increase sensitivity in the described tag/anti-tag approach, and the use of secondary detection gives rise to an amplification of the signal in FluoroSpot (203).

Similar to the use of tetramers in flow cytometry, there are also risks involved when tagging antigens with tags for use in the FluoroSpot assay developed here. As an example, the addition of peptide tags to antigens could potentially interfere with folding of protein. However, studies have shown that addition of small size peptide tags, similar to the tags used for our studies, have minimal impact on protein folding (204). In Study **III**, we verified the quality of the expressed *P. falciparum* antigens, in regard to antigenicity, by comparing the reactivity of *P. falciparum*-reactive plasma to purified *E.coli*-derived variants of antigens used previously for other studies. This demonstrates that the structure and quality of the antigens are sustained when expressing antigens recombinantly in a human expression system, which has also been reported by other studies expressing tagged *P. falciparum* antigens using similar expression systems (192).

Recombinant expression of antigens with peptide tags also has other benefits including that the number of tags attached to an antigen is known and can be defined and controlled, which is challenging when performing e.g. chemical biotinylation of antigens that potentially could

lead to steric hindrance when performed on small antigens. Addition of peptide tags also enables for semi-quantification of the level of non-purified proteins as seen for CMV.pp65 in Study **II**, further demonstrating the benefits of expressing peptide tagged antigens for use in immunoassays such as FluoroSpot.

During a healthy state, MBCs in circulation are quiescent, but can rapidly proliferate and differentiate into antibody-producing plasma cells after stimulation. For the purpose of analysing in ELISpot/FluoroSpot assays, resting MBCs require pre-stimulation in order to differentiate into antibody-producing cells. This stimulation can be performed *in vitro* by cultivation MBCs in the presence of polyclonal mitogens such as toll-like receptor (TLR) agonists (170, 205). For this purpose, in Study **II**, human PBMCs were cultivated in the presence of the TLR7/8 agonist R848 and recombinant IL-2. This combination has previously been described to be a more potent activator of MBCs compared to other established protocols (206). However, *in vitro*-stimulation of MBCs has some caveats. For instance, the frequency of MBCs or rate of stimulation into plasma blasts could potentially differ between samples/individuals, suggesting an inaccurate reflection of the *in vivo* state. In order to partly adjust for this, we displayed results as proportion of antigen-specific spots per total IgG spots, instead of spots per number of cells in well which is a common approach to present ELISpot/FluoroSpot data. Nevertheless, by cultivation of PBMC in the presence of R848 and recombinant IL-2 before use in the reverse FluoroSpot assay, we showed in Study **II** that human MBCs producing antibodies for antigens on hepatitis B (HBsAg), cytomegalovirus (CMV.pp65) and tetanus (TT) could be detected simultaneously within a single well.

The relative spot volume (RSV) assessed by a newly developed FluoroSpot reader (Mabtech IRIS™, Mabtech), was used for the first time in Study **II** to study B-cell responses. The RSV value provides additional information on the volume of single spots and is affected by the relative amount of analyte secreted (207). Analysis of individual spots in FluoroSpot have previously been made by extracting FluoroSpot data into a flow cytometry software in order to analyse spots for studying spot size, intensity and circularity (200). Although interesting, this strategy is laborious and involves settings defined by the user, which potentially could lead to user-to-user variability. In contrast, here the RSV value is defined using a mathematical diffusion model performed by the software algorithms to define three dimensional spot shape, and RSV is then calculated as the area under curve (207). When the RSV value was evaluated in spots from HBsAg-specific MBCs before and after vaccination, indications of an increase in average RSV was found in two individuals. Similarly, in Study **III**, we observed tendencies of an increase in RSV over time in spots from MBCs specific to

MSP-1_{19} and AMA-1. Given that the spot size and intensity is affected by both amount of antibody secreted and possibly also the affinity of the antibody (200), this could propose an affinity maturation of MBCs but larger studies have to be performed in order to confirm this.

In Study **III**, we observed tendencies of a heterogeneity in MBC responses to the selected antigens. This heterogeneity of the response demonstrates dissimilarities in the ability of MBCs to respond to the selected antigens in the FluoroSpot assay. These differences in MBC responses between antigens have also been described by other studies on MBC responses in travelers (82). Furthermore, we also observed indications that the kinetics of the MBC responses to the included antigens differed over time within individuals after an acute malaria episode. A possible reason for this difference in kinetics could be that some merozoite antigens, such as AMA-1, are also expressed on the sporozoite (157), or at the liver-stage development, such as for MSP-1 (208), suggesting an earlier activation of MBCs to these antigens. Collectively, this demonstrates the need of including multiple antigens in the analysis, but also the benefits of studying responses at several time points.

The possibility for multiplex analysis of B-cell responses to multiple antigens is highly relevant in many scenarios of malaria research. For instance, several *P. falciparum* antigens have been associated as targets candidates for vaccine and some vaccine candidates contain multiple antigens (104). Also, natural acquired immunity to malaria is believed to be dependent on the development of a progressively increasing panel of LLPCs and MBCs able of producing antibodies against the many antigens expressed on the surface of the parasite (51). This highlights the usefulness of the FluoroSpot assay for multiplex analysis of MBCs against multiple different antigens. Another benefit of multiplex analysis is the decreased need of cells for the analysis which is an important parameter in e.g. studies involving children.

Genetic diversity and polymorphism displayed by the antigens on the parasite is a major problem for vaccine development (138, 139, 209). Therefore, the possibility of detecting B cells displaying broad cross-reactivity after, e.g. natural exposure or vaccination, could in a simple manner be addressed using B-cell FluoroSpot.

By combining fluorescently labeled subclass-specific antibodies with the tag/anti-tag approach, we demonstrated in Study **I**, that the assay could be used for simultaneous detection of B-cell antigen-specificity as well as Ig subclass determination of the secreted antibody. In malaria, studies have identified dissimilarities in *P. falciparum* IgG subclasses effector functions (210-212) and half-lives (88, 132). For instance, responses dominated by

subclasses IgG1 and IgG3 have been shown to be more protective against *P. falciparum* compared to IgG2 and IgG4 (210). The B-cell FluoroSpot assay described in Study **I**, could thus be used for a simple assessment of antigen-specific B cells to *P. falciparum* as well as their subclass in order to measure the effectiveness of the response after e.g. vaccination.

When we assessed MBCs specific to HBsAg before and after vaccination in Study **II**, HBsAg-specific MBCs could be detected before vaccination in a seronegative individual vaccinated against hepatitis B 12 years earlier. This is in line with other studies demonstrating detectable HBsAg-specific MBCs in the absence of cognate antibodies (213) and suggests that antigen-specific MBCs in circulation are a more accurate marker of immunological memory compared to circulating antibodies. Also, when screening 23 blood donors for MBC reactivity against TT, CMV.pp65 and HBsAg, we found that eight of the 23 individuals (35%) had no detectable MBCs to CMV.pp65. Given that CMV is a persistent virus, this suggests that these individuals had not been exposed to CMV, supporting observations in other studies estimating the CMV prevalence in Sweden to approximately 83% (214).

Several examples have been shown where vaccination can lead to the acquisition of long-lived MBCs (215-217). For instance, studies have shown that functional MBCs can be found over 50 years after smallpox vaccination (79). In contrast, the ability to acquire and maintain long-lived MBCs to *P. falciparum* antigens have been found to be more complex and could thus benefit from better understanding. Furthermore, studies on the maintenance of MBCs in malaria endemic areas are also challenging because of the risk of re-infection. In Study **III**, we therefore set up a longitudinal cohort of Swedish travelers treated for an acute *P. falciparum* malaria episode and followed prospectively over the course of one year in a malaria-free setting. We showed that primary infected individuals could mount and maintain MBCs as efficiently as previously exposed individuals, demonstrating that previous exposure is not a requirement for eliciting high levels of MBCs in circulation. We also showed a higher magnitude and extended maintenance of antibody levels in the previously exposed, supporting the findings of previous studies using a larger number of samples from the same cohort (132). The higher magnitude in antibody levels suggest that the previously exposed individuals had pre-existing LLPCs and MBCs. Upon activation, pre-existing MBCs could then rapidly proliferate and differentiate into plasma blast to increase antibody levels in circulation, while the antibody levels in primary infected came primarily from newly developed SLPCs and LLPCs. Interestingly, at the end of the one–year study period, the majority of primary infected individuals had detectable levels of MBCs against MSP-1_{19} and AMA-1 while their cognate antibody levels were similar to negative controls. These results

support previous studies demonstrating effective generation of MBCs to *P. falciparum* antigens in areas of minimal/low transmission (82-84) but also further demonstrates that MBCs may be a more accurate marker of past exposure.

It is widely recognised that repeated infections lead to the development of a subset of B cells termed atypical MBCs (97, 102, 218). Atypical MBCs have been linked with the exhausted phenotype of MBCs seen in chronic HIV infected individuals (98), and are believed to be one of the reasons for the slow development of immunity to malaria in areas with high transmission (95, 100). Other studies have addressed the level of atypical MBCs in the cohort of travelers used in Study **III**, and found that previously exposed individuals have higher levels of atypical MBCs compared to primary infected (99). Future studies should therefore investigate whether the differences in level of atypical MBCs influenced the MBC response in patients included in Study **III**.

While some studies have described the development of antigen-specific MBCs to *P. falciparum* in adults, much less is known about the acquisition and of antigen-specific MBCs to *P. falciparum* in children living in endemic areas. The reason for this is partly due to the low frequencies of MBCs in circulation but also the limited volume of blood one is allowed to take from children. In Study **IV**, we therefore studied the MBC and plasma antibody response a in longitudinal cohort living in an endemic area of eastern Kenyan. Since these children have been actively monitored for fever and malaria episodes since birth, we could correlate MBC and antibody responses to factors such as age, number of clinical episodes since birth but also estimate the risk of subsequent clinical malaria based on time to next clinical infection. By using combinations of antigens, multiplex analysis of MBC responses to 6 *P. falciparum* antigens was possible. We observed indications that MBCs and antibodies to *P. falciparum* antigens MSP-2 (3D7), MSP-3 and AMA-1 were associated with a reduced risk of infection in older, but not younger children. Similarly, we also showed that MBC and antibody levels to MSP-2 (3D7) and MSP-3 were correlated with increasing age, even when adjusting for number of clinical episodes. Together, as detected in peripheral blood, this suggest that the development of MBCs and LLPCs is inefficiently acquired in younger children, potentially leading to lower protection. It is possible to speculate that a reason for this could be that younger children compared to older children, humoral responses are focused on the induction of SLPCs rather than MBCs and LLPCs, supporting the findings of previous studies (87, 88, 219). We also observed indications that increased breadth of MBCs and antibody responses were linked with reduced risk of clinical malaria in older children. Breadth of response is often characterized as the possession of a broad antibody repertoire

able to recognize a variety of antigens (122), and often linked with protection from clinical disease (122, 196, 220, 221). While breadth of MBC- and antibody responses have been linked with increasing age (171, 222), our data showed that only breadth of antibodies were more clearly associated with age.

Since children included in Study **IV** had been monitored for clinical malaria episodes since birth, we were able to investigate how the immune responses were affected by previous cumulative clinical exposure. Surprisingly, we showed indications that high numbers of clinical infections had a negative impact on the levels of antigen-specific MBCs and antibodies in circulation. This observation is in conflict with the hypothesis that repeated infection expands the pool of MBCs and LLPC described by other studies (86) and instead implies that children subjected to multiple infections have impaired development of immunological memory. Recent studies involving children from the same cohort, have identified increased levels of the immunoregulatory cytokine IL-10 in children with high number of clinical infections (223). In malaria, IL-10 has been described as a double-edged sword since it both reduces immunopathology, but also block the antigen-presentation of T- and B cells (94). In the light of our results, it is possible that multiple clinical infections seen in children led to an increase in immunoregulatory effects that in turn led to reduced interaction between T- and B cells resulting in lower levels of MBCs and LLPCs.

In summary, the studies included in this thesis have presented the methodological development of the reversed B-cell FluoroSpot assay and demonstrated its use for both clinical and epidemiological studies on *P. falciparum* malaria. We have provided insights to the acquisition and maintenance of MBCs after acute malaria and the effect of pre-existing immunity. We have also highlighted factors affecting the level of MBCs and antibodies in children living in malaria-endemic areas and the role of MBCs and antibodies for the protection against clinical malaria. We believe that this highly adaptable multiplex B-cell FluoroSpot assay can be a powerful tool when assessing MBC responses to a multitude of antigens and will contribute to further improving the understanding of the acquisition of MBCs to *P. falciparum,* but also in other fields of research.

6 CONCLUSIONS AND FUTURE PERSPECTIVES

- The reversed B-cell FluoroSpot assay can be used for several purposes including: detection of antibody-producing cells specific against multiple different antigens, cross-reactivity, and simultaneous detection of different B cells with separate antigen specificities and Ig subclasses.

- The tag/anti-tag approach for detection increases the adaptability of the assay and facilitates analysis of B-cell responses to a variety of antigens and pathogens.

- The reversed B-cell FluoroSpot can detect HBsAg-specific MBCs over a decade after vaccination without detectable plasma antibodies. The RSV value, describing the volume of spots, increase after vaccination against HBsAg.

- The reversed B-cell FluoroSpot can detect MBCs specific against four different *P. falciparum* antigens simultaneously.

- Primary infected individuals can mount and maintain MBC responses against merozoite antigens as efficiently over one year as previously exposed individuals after acute malaria, while antibody responses to the same antigens are stronger in previously exposed individuals.

- The RSV value shows tendencies of increasing over time after an acute infection of *P.falciparum* malaria.

- MBC responses appear to be less pronounced in children with high cumulative number of clinical malaria episodes since birth.

- MBCs and antibody responses to merozoite antigens MSP-2 (3D7), MSP-3 and AMA-1 are associated with a somewhat reduced risk of subsequent malaria in older children living in a malaria endemic area, whereas MBCs and antibodies to CSP show tendencies of increasing risk.

- Breadth of MBC and antibody responses tend to be associated with a reducing risk of subsequent malaria in older children, but not younger children.

- The reversed B-cell FluoroSpot is a sensitive and adaptable tool that can be used to analyze MBC responses in exploratory, clinical and epidemiological studies.

In recent years, technological advancements have made it possible to dissect the immune responses after *P. falciparum* exposure. Knowledge regarding the function of specific cells as well as their protective roles have been invested down to the molecular level. Yet, there are still vital parts of the immune response that the scientific community not yet fully understand. One of the most crucial parts that we need to understand is the mechanisms leading to the acquisition of long-lasting protection against the parasite and disease in children. We also need to understand why some children develop mild or asymptomatic malaria while others develop severe malaria. The development of a vaccine able of inducing long-lasting protection in children during the most vulnerable years, would undoubtfully be a game-changer for the global burden of malaria. In this thesis we have described a robust, sensitive and highly adaptable assay, able to acquire detailed information about the B-cell response using a low number of cells. The insights gained from these studies contributes to a better understanding of the acquisition and maintenance of immunological memory against malaria. Given the possibility of cross-reactivity analysis using the reversed B-cell FluoroSpot assay, future studies should investigate MBC cross-reactivity to polymorphic antigens or variations and the role these cells have for the protection against disease.

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