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MEMORY B-CELL FUNCTION AND ANTIBODY PROFILES IN MALARIA: THEIR ROLE IN PROTECTION AGAINST DISEASE IN INFANCY

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Memory B-cell function and Antibody profiles in malaria: Their role in protection against disease in infancy
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

Malaria infection is of huge public health importance in developing countries, including Uganda. Antibody immunity assumes a paramount part in the disease control however there's a great need to study components of antibody sustenance and how natural immunity is elicited. Here in this thesis we studied development of *P. falciparum* specific B-cells and antibodies.

First and foremost we developed a novel flow cytometry method using nano particles (quantum dots) and parasitized red blood cell ghosts that can directly detect *P. falciparum* specific B-cells in humans. This technique is advantageous for it does not require growth and activation of cells. For the first time using flow cytometry we were able to quantify how much of the B-cell response was directed against malaria. We detected a statistically significant difference in *P. falciparum* specific B-cells between immune samples (22.3%) and non-immune samples (1.7%). The highest percentage of malaria parasite specific B-cells (27.9%) were observed in individuals with an ongoing malaria infection.

Using the novel flow cytometry technique we studied the development of *P. falciparum* specific B-cell sub-populations during infancy and in adults. The babies showed increases in *P. falciparum* specific IgG memory B-cells (MBCs), atypical MBCs, and plasma cells/blasts over time, but the proportion of these cells were still lower than in the mothers who displayed stable levels (5, 18, and 3 %, respectively). *P. falciparum* specific non-IgG+ MBCs and naïve B-cells binding to *P. falciparum* antigens were higher in the babies compared to the mothers (12 and 50%). In ELISA there was an increase in IgG and IgM antibodies over time in babies, and stable levels in mothers.

Finally, in another collaborative study, we investigated the antibody anamnestic or memory reaction evoked by a solitary episode of *P. falciparum* infection. We found that in a larger part (70%) of people, an infection of *P. falciparum* evokes not less than 20% expansion in level of anti-parasite IgG. This boost in anti-*P. falciparum* IgG is neither influenced by parasite density on the day of malaria diagnosis, nor HIV status.

In conclusion this thesis advances current knowledge on the development of anti *P. falciparum* antibodies and *P. falciparum* specific B-cell sub-populations during infancy and in adults. This information is vital for understanding malaria immunity and future malaria vaccine studies.

LIST OF SCIENTIFIC PAPERS

I. **Lugaajju A**, Reddy SB, Rönnberg C, Wahlgren M, Kironde F, Persson KEM: Novel flow cytometry technique for detection of *Plasmodium falciparum* specific B-cells in humans: Increased levels of specific B-cells in ongoing malaria. Malar J. 2015; 14: 370

II. **Lugaajju A**, Reddy SB, Wahlgren M, Kironde F, Persson KEM: Development of *Plasmodium falciparum* specific naïve, atypical, memory and plasma B-cells during infancy and in adults in an endemic area. Malar J. 2017; 16: 37

III. Kaddumukasa M, Lwanira C, **Lugaajju A**, Katabira E, Persson KEM, Wahlgren M, Kironde F: Parasite specific Antibody increase induced by an Episode of Acute *P. falciparum* uncomplicated Malaria. PLoS One.2015; 10(4)

CONTENTS

LIST OF ABBREVIATIONS

WHO	World Health Organization
CD	Cluster of differentiation
DC	Dendritic cell
EBA175	Erythrocyte binding antigen-175
ELISA	Enzyme linked immunosorbent assay
ELISPOT	Enzyme linked immunospot
IgG	Immunoglobulin G
IgM	Immunoglobulin M
Pf+	*Plasmodium falciparum* specific

1 INTRODUCTION

My thesis is mostly about the detection of *Plasmodium falciparum* specific B-cells in humans and their development during infancy and adults in an endemic area. But before I go into the details of my work, I will briefly introduce malaria in general, the *Plasmodium* parasites, as well as the general human immune system and malaria immunology.

1.1 GLOBAL BURDEN OF MALARIA

It has been evaluated that 1.13 and 1.44 billion individuals worldwide are at risk of unstable and stable *P. falciparum* malaria respectively (Gething et al., 2011). 70% of these clinical occasions ascribed to *P. falciparum* occur in Africa (WHO, 2010). In sub-Saharan Africa, malaria is the dominant tropical parasitic disease and one of the three main killer transmittable illnesses (Lopez and Mathers, 2006). Around 90% of the deaths due to malaria occur in sub-Saharan Africa, mostly among children below five years of age (Black et al., 2010). The worldwide *P. falciparum* endemicity is shown in Fig 1.

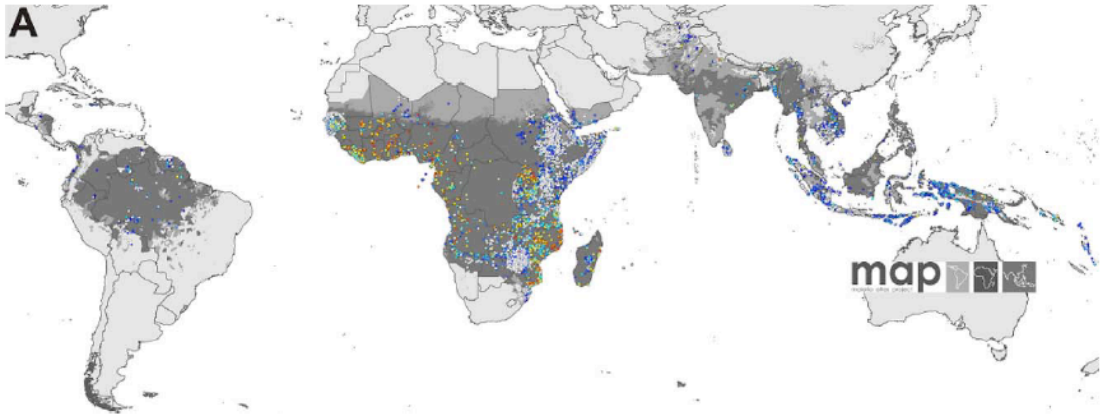

Figure 1. The spatial dissemination of Plasmodium falciparum malaria endemicity in 2010.

Areas were defined as stable; dark grey areas where PfAPI (P. falciparum annual parasite incidence) ≥0.1 per 1,000 pa. Unstable; medium grey areas where PfAPI< 0.1 per 1,000 pa. No risk; light grey where PfAPI = 0 per 1,000 pa). Adapted from Gething et al (Gething et al., 2011).

Malaria is among the leading causes of the burden of disease, measured in disability-adjusted life-years (Lopez and Mathers, 2006). Survivors of malaria may suffer a combination of anaemia and immune suppression that leaves them vulnerable to other fatal illnesses (Snow et al., 1999). Malaria is endemic in 95% of Uganda with 5% epidemic prone areas in the highlands of southwest and eastern Uganda. Malaria is the main cause of morbidity and mortality in Uganda and it accounts for 25-40% of all outpatient visits at health facilities, 20% of clinic confirmations, and 9-14% of inpatient deaths. Child deaths due to malaria in Uganda account for approximately 70,000– 110,000 per year (Health, 2005). Youngsters under five and pregnant ladies bear the best burden of the disease and, within these groups,

the poorest are most vulnerable. Destitution appears responsible for part of the severe malaria transmission due to limited access to insecticide treated bed nets (ITNs), indoor residual spraying (IRS) and reduced access to control programs benefits (Sachs and Malaney, 2002).

1.2 MALARIA ELIMINATION

Measures to control and dispose of malaria on an expansive scale go back to the late nineteenth century, with the revelation of the plasmodium parasite and its transmission by Anopheline mosquitoes. The exceptional accomplishment in malaria eliminating nations was driven by the Global Malaria Eradication Program, propelled by WHO in all parts of the world separated from Africa in 1955 (Feachem et al., 2010). The program depended on vector control, primarily through indoor residual spraying, and precise identification and treatment of cases. But in light of authoritative, money related, and specialized issues, it was deserted in 1969 (WHO, 1969). Substantial financial increments for malaria and the widespread use of long-lasting insecticide-treated bed nets, artemisinin-based combination therapies and rapid diagnostic tests have brought about advance towards disposal in a few nations since the early part of the 21st century (Snow et al., 2010). Uganda has gained ground in executing key malaria control measures. However, malaria co-infections, frequency of malaria illness, and mortality from rigorous malaria all stay high (Yeka et al., 2012). Real difficulties to malaria control in Uganda include high malaria transmission intensity, a feeble public health service, limited comprehension of malaria disease transmission and the effect of control mediations, parasite resistance to drugs and mosquito resistance to insecticides.

In spite of these difficulties, progress toward the control of malaria in Uganda is notable.

1.3 PLASMODIUM SPECIES

Malaria is caused by protozoan parasites called Plasmodia which belong to the parasitic phylum Apicomplexa. More than 200 types of Plasmodium have been recognized that are parasitic to reptiles, winged creatures, and warm-blooded animals. Four *Plasmodium* species commonly cause human malaria: *P. falciparum, P. vivax, P. ovale, and P. malariae.* A fifth one, *P. knowlesi* causes human disease in numerous nations of Southeast Asia (Cox-Singh et al., 2008, Daneshvar et al., 2009, Kantele and Jokiranta, 2011, Sabbatani et al., 2010).

Investigations of the mitochondrial DNA shows that *P. knowlesi* originated from a genealogical parasite that existed prior to human settlement in Southeast Asia, and expanded 30,000–40,000 years back. *P.knowlesi* malaria is ancient in Southeast Asia and is principally a zoonosis with wild macaques as the reservoir hosts. Continuous biological changes due to deforestation together with related increase in human population could empower *P. knowlesi* to change to people as the favored host (Lee et al., 2011).

1.4 MALARIA PARASITE LIFE CYCLE

P. falciparum and to a much lesser degree *P. vivax* are the primary drivers of sickness and demise from malaria. Mosquitoes infuse parasites (sporozoites) into the subcutaneous tissue, and to a less extent into the circulatory system (Figure 2); from that point sporozoites go to the liver where sporozoites are believed to pass through a number of hepatocytes (Lee et al., 2011, Mota and Rodriguez, 2004, Mota et al., 2001). The co-receptor on sporozoites that enables intrusion includes the thrombospondin areas on the circumsporozoite protein and on thrombospondin-related anonymous protein (TRAP).

Every sporozoite forms into a schizont containing 10,000–30,000 merozoites (or more in the case of *P. falciparum*) (Amino et al., 2006, Gueirard et al., 2010, Jones and Good, 2006, Kebaier et al., 2009).

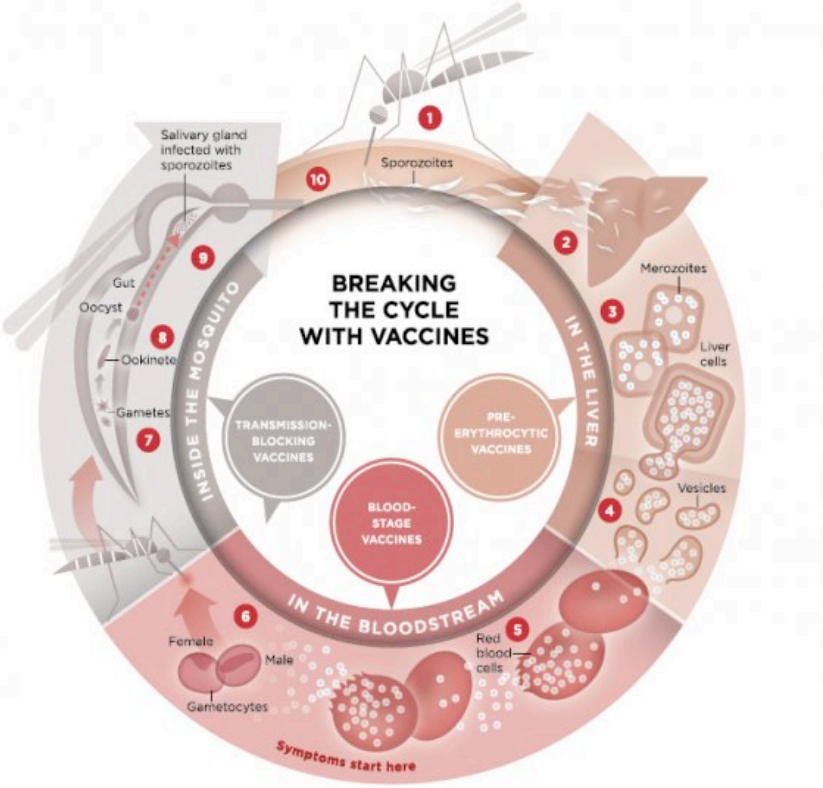

Figure 2. Parasite life cycle and pathogenesis of falciparum malaria. Courtesy of Malaria Vaccine Initiative.

The whole pre-erythrocytic stage keeps going around 5–16 days depending on the parasite species i.e. 5-6, 8, 9, 13 and 8-9 days for *Plasmodium falciparum, vivax, ovale, malariae* and *knowlesi* respectively. The pre-erythrocytic stage stays as a "quiet" stage, with no side effects and pathology as just a couple of liver cells are injured (Vaughan et al., 2008).

The merosomes containing merozoites wholly migrate from the liver and discharge merozoites into the circulatory system at the lung vessels thereby initiating the blood contamination phase (Silvie et al., 2008). In *P. vivax* and *P. ovale* malaria, some of the sporozoites may stay torpid (hypnozoites) for a considerable length of time inside the liver. The hypnozoites form into schizonts after some dormant period, usually a couple of weeks to months. Studies have suggested that hypnozoites are not genotypically identical to sporozoites that intensify the disease after a mosquito bite (Cogswell, 1992, Contamin et al., 1995, Imwong et al., 2007). The merozoites rising up out of the liver rapidly recognize and invade red blood cells (RBCs) by various receptor–ligand associations in as few as 60 seconds.

This brisk takeoff from the flow into the RBCs minimizes the introduction of the antigens on the surface of the parasite, in this manner shielding the merozoites from the host resistance reaction (Cowman and Crabb, 2006, Greenwood et al., 2008). Diverse mechanisms underlie the binding and entry of merozoites into RBCs. *P. vivax* utilizes Duffy restricting protein and reticulocyte homology protein to attack Duffy blood group positive RBCs. *P. falciparum* utilizes a few distinctive receptor families and exchange intrusion pathways that are exceptionally repetitive. *P.falciparum* can attack any RBC because its assortments of Duffy binding like (DBL) homologous proteins and the reticulocyte binding like homologous proteins recognize diverse RBC receptors other than the Duffy blood group or the reticulocyte receptors (Mayer et al., 2009, Weatherall et al., 2002). The merozoite secretory organelles (micronemes, rhoptries and thick granules) enhance the entry of merozoites unto RBCs. The connection between the parasite and the RBC animates a quick wave of disfigurement over the RBC layer, prompting the arrangement of a stable parasite–host cell intersection. The parasite then passes through the erythrocyte layer with the assistance of the actin-myosin apparatus, proteins of the TRAP family and aldolase and makes a parasitophorous vacuole to seal itself from the host cell cytoplasm, surviving inside the red cell. At this stage, the parasite shows up as an intracellular "ring" frame (Bosch et al., 2007, Cowman and Crabb, 2006, Haldar and Mohandas, 2007). The erythrocytic cycle happens like clockwork in the event of *P. knowlesi*, 48 h in instances of *P. falciparum, P. vivax* and *P. ovale* and 72 h in the case of *P. malariae*. With every cycle, every merozoite develops and divides inside the vacuole into 8–32 (normal 10) new merozoites, through the phases of ring, trophozoite, and schizont. Toward the end of the cycle, the parasitized red cells crack, discharging the new merozoites that then invade more RBCs.

Although asexual parasites don’t experience schizogony, they separate into sexual stage gametocytes. These nonpathogenic, extracellular gametocytes aid in disease transmission via female anopheline mosquitoes that proceed with the sexual stage of the parasitic life cycle. *P.vivax* gametocytes grow not long after merozoites arrival from the liver (Mota and Rodriguez, 2004, Mota et al., 2001).

On the contrary, *P. falciparum* gametocytes grow much later, with pinnacle densities of sexual stages occurring one week after the asexual stage (Miller et al., 2002, Pukrittayakamee et al., 2008).

1.5 THE IMMUNE SYSTEM

The main goal of the immune system is to shield the host from infectious microorganisms in its surroundings. The control and clearance of these microbes is through immune response mechanisms that recognize basic components of the pathogens and check them as unmistakably different from host cells. Such host-pathogen segregation is fundamental to allow the host to dispose of the pathogen without harming its own tissues, a phenomenon known as self-tolerance. Incompliance to self-tolerance results into autoimmune disease.

The immune response can either be innate or adaptive. In the innate immune response (primary host response) the recognizing components are extensively expressed on numerous cells and act quickly after an attacking pathogen has been experienced. The adaptive immune response occurs after the innate response and releases specific cells in small quantities for particular invading microbes. These specific cells amass to elicit an adequate immune response. The adaptive immune response produces memory cells which then elicit a more potent cognate response on the second encounter with the same pathogen (Chaplin, 2006).

1.5.1 INNATE AND ADAPTIVE IMMUNITY

The innate (non-specific) immunity is the inborn natural resistance encoded by the host's germ line genes. It includes mucociliary cover and epithelial barricades that clears ingested or breathed particles, soluble proteins and small bioactive particles found in biologic fluids (e.g. the complement proteins (Morgan et al., 2005) and defensins (Selsted and Ouellette, 2005)) or that are discharged from activated cells (including cytokines, chemokines, lipid inflammation mediators and bio active amines and biological catalysts). Finally non-specific immunity incorporates cell-surface receptors that attach to the expressed molecules of the attacking microorganism (Beutler et al., 2003).

Adaptive immunity is divided into naturally acquired and artificially acquired immunity. Whereas naturally acquired immunity results from accidental contact with a disease causing microbe, artificially acquired immunity results from intended actions like immunization.

Naturally and artificially acquired immunity can either be passive (introduction of antibodies or activated T-cells from an immune individual into another host) or active (immunity induced in the host after antigen encounter).

Active immunity is long lived as compared to passive immunity. Basing on the cells involved, adaptive immunity can be characterized as; humoral immunity attributed for by secreted antibodies, and cell mediated immunity attained through T-cells (Chaplin, 2003). The development of a huge number of antigen receptors each specific to an alternate antigen

arises from a couple of hundred germ line encoded gene components (Alam and Gorska, 2003).

The naturally inborn and adaptive immunity arms work together to bring about the immune function. The innate contributes the host first line defense and the adaptive follows a few days later after antigen specific T and B-cells clonal development. Antigen specific cell responses are augmented by the innate effector systems to achieve enhanced control of invading microorganisms. Despite the fact that the inborn and adaptive immune reactions vary in their components of activity, cooperation between the two is crucial for a viable immune reaction.

1.5.2 B-CELLS

Upon activation, B-cells generate antibodies that play various roles including: blocking cytoadherence of microbes to host membranes, microbe opsonisation, enhancement of phagocytic action of monocytes and macrophages thereby boosting the innate immune response (Parkin and Cohen, 2001). B-cell activation can either be T-cell independent or T-cell dependent (Janeway, 2001).

1.6 MALARIA IMMUNOLOGY

Naturally acquired immunity to malaria takes as long as 10-15 years of exposure to develop (Baird, 1998) and repeated infections are necessary. This is due to high antigenic variation of parasites (Udhayakumar et al., 2001) and perhaps a suppressed immunity plus possibly masking of the parasite antigens.

People living in malaria endemic areas frequently have premonition, that is presence of parasites and antibodies without symptoms (Baird, 1998). The development of immunity is exposure related and therefore age related (Gupta et al., 1999). The severity of the disease largely depends on the species, strain of the infecting parasite and the immunological status of the person infected (Wipasa et al., 2002).

Despite the repeated infections, the acquired protective immunity is species, stage, strain as well as variant specific (Andrysiak et al., 1986). The immunity requires both acquired and innate immunity for parasite control and clearance. The acquired immunity involves the cell mediated and antibody mediated immunity. The roles of the cell mediated and humoral immunity vary according to the parasite life cycle. The humoral arm is more effective during the blood stages of the life cycle, whereas the cell-mediated immunity is important during the liver stage.

1.6.1 MALARIA INNATE IMMUNITY

There are several genetic variants of RBC, TNFα promoters and MHC which have been selected in humans to protect against malaria and these are geographically associated with the

disease (Haldane, 1949). In populations where there has been a high malaria pressure, various malaria protection factors have evolved. For example, the absence of certain blood groups like the Duffy antigen protects individuals from erythrocyte infection by *P.vivax*. *P.falciparum* uses several blood group antigens, so no negative selection for special blood group antigens is seen. However, parasites in blood group O form smaller rosettes with less binding force than blood group A, B, or AB RBC, which has led to a higher frequency of blood group O in Africa. Mutations in the hemoglobin chain cause sickle cell anemia or thalassemia (Haldane, 1949), and the two diseases alter immune recognition, impair parasite growth and rosetting. Glucose-6-phosphate dehydrogenase deficiency is an enzyme defect which impairs parasite growth and increases the RBC sensitivity to oxidative stress (Allison, 1964, Roth et al., 1983), causing hemolytic anemia. Also certain MHC class I/II haplotypes seem to protect from severe malaria (McGuire et al., 1994). Altered RBC and immune recognition as well as changed release of soluble factors have been selected in humans to protect from malaria. By understanding how these variants of the innate immunity counteract severe malaria, our knowledge of the parasite is increased and will enable us to fight the parasite more effectively.

1.6.2 ACQUIRED IMMUNITY

The developing immune response to malaria constitutes both humoral and cellular components.

A) Antibody mediated immunity in *P. falciparum* malaria

Immunity to malaria develops naturally following frequent repeated exposures. This is due to the time dependent exposure to infections. During the first six months, a child in a highly endemic area is protected by maternal antibodies passed over the placenta and in breast milk (Kassim et al., 2000), but thereafter the child is dependent on the development of its own immune system.

Elderly residents of endemic areas possess considerable immune protection to acute forms of the disease. After lifelong exposure to intense transmission, antimalarial immunity gradually develops.

Antibodies play a fundamental role in protecting the host against blood stage malaria. Some of the antibody functions include cytoadherence, blockage of parasitized erythrocytes, holding back merozoites from attacking new erythrocytes and increase of monocytes and macrophage´s phagocytic activity (Wipasa et al., 2002, Beeson et al., 2008).

Antibodies from immune sera when transferred into a host are effective in immunity against asexual blood stage malaria parasites (Perignon and Druilhe, 1994, Cohen et al., 1961, Druilhe and Perignon, 1994) responsible for malaria pathogenesis. Attaching of antibodies to parasitized red blood cells results in their opsonisation hence control of parasitemia (Gysin et

al., 1982). This mainly involves IgG1 and IgG3 antibodies (Groux and Gysin, 1990) but elevated concentrations of IgG2 binding to the Fc receptor IIa-R131H have additionally been implicated to immunity in people living in Burkina Faso (Aucan et al., 2000).

The isotype switch towards immunoglobulins of both IgG1 and IgG3 subclasses which bind to Fcγ R1 and FcγR1I receptors on human monocytes and macrophages has been proposed to play a vital role in this process (Jefferis and Kumararatne, 1990). These cytophillic antibodies act in collaboration with monocytes and macrophages in parasite killing effector responses such as opsonisation and phagocytosis, as well as antibody dependent cellular inhibition of blood stage malaria parasites (Bouharoun-Tayoun et al., 1990, Gysin et al., 1992). The function of the antimalarial antibody response may be important in the generation of protective immunity.

The levels of acquired antibodies to *P. falciparum* antigens has been shown to peak and decline rapidly after an acute malarial attack especially in children (Boutlis et al., 2003, Cavanagh et al., 1998). The antibodies tend to be higher in those with parasites than those without. A study in Kenya showed that the children with malaria had a shorter time for the antibodies to decline to 50% than the normally reported catabolic half-lives of these antibodies of the reported 21 days (Kinyanjui et al., 2007, Morell et al., 1970). Another study which showed that the half life was longer up to 52 days in those aged 4-6 years (Akpogheneta et al., 2008). The decline has been reported to be three times more in the youngest age groups as compared to the oldest group 4-6 years.

The elaborate nature and mechanisms of immune reactions that decrease or avoid malaria disease is obscure, regardless of the multiplicity of *P. falciparum* stage specific antigens. Potential candidates for development of vaccines include antigens expressed on the surface of merozoites that are involved in erythrocyte invasion and proteins that are expressed on the surface of the infected erythrocyte.

Most studies have only used ELISA to measure the usually short-lived responses of antibodies against some selected antigens, and none of the antigens tested so far has proven to be fully protective against malaria even though antibodies measured by ELISA are clearly induced. This indicates that we have to consider the function, not only the presence, of antibodies.

B) MEMORY B-CELLS AND MALARIA

Memory B-cells assume a paramount part in immunological memory to pathogens, by eliciting the immune response at subsequent encounter of the antigen. The support of serum neutralizer levels after presentation to antigen either by contamination or vaccination has been alluded to as serologic memory (Lanzavecchia et al., 2006). It has been demonstrated that generation of immune response against merozoite antigens is not long maintained after an intense malaria bout (Kinyanjui et al., 2007, Akpogheneta et al., 2008). The making of antibodies is maintained through reactivation of memory B-cells by unrelented antigens

(Ochsenbein et al., 2000) or by non-multiplying long lived plasma cells (Slifka and Ahmed, 1998).

Studies have shown that, during malaria parasite infection, there's an acute alteration in memory B-cell numbers (Achtman et al., 2005, Asito et al., 2008). A study by Dorfman et al. (Dorfman et al., 2005) based on Elispot- method showed a low reactivity of specific memory B-cells to malaria antigens compared to tenanus toxoid in the same population. The loss of malaria specific antibody reactivity may be responsible for the delay in the acquisition of immunity to malaria. Probably, this would explain the short memory and loss of immunity to malaria. There's an ongoing debate on why immunity against malaria disease is temporary.

A decrease in levels of some anti-malarial antibodies has been observed immediately after the transmission season (Riley and Greenwood, 1990). Malaria acquired immunity is dependent on periodic re-injection of malaria parasites without which antimalarial antibodies survive for a short period of time (Langhorne et al., 2008). This implies that malaria B-cell memory may be flawed or suboptimal. Nonetheless, the expansion and perseverance of B-cell memory following malaria infection has for quite some time involved level headed discussions (Struik and Riley, 2004). Animal model studies have revealed that memory B-cells have the capacity to develop and be kept up ordinarily after a malaria bout (Stephens et al., 2005, D'Imperio Lima et al., 1996), while others have found that malaria disease meddles with the improvement of memory B-cells and seemingly perpetual plasma cells (Wykes et al., 2005, Carvalho et al., 2007). In humans, a few studies have exhibited stable immune responses to malaria antigens (Udhayakumar et al., 2001, Taylor et al., 1996, Drakeley et al., 2005).

Nonetheless, short-lived antibody responses have been noted (Cavanagh et al., 1998, Dorfman et al., 2005), especially in young children (Taylor et al., 1996, Akpogheneta et al., 2008). Till now, a few studies have investigated the initiation and upkeep of malaria-particular memory B-cells in humans. Dorfman et al. (Dorfman et al., 2005) were not able to recognize malaria specific B-cells in seropositive kids. However, it is indistinct whether this mirrors a non-existence of such cells or insensitivity of the methods used. Upon *P. falciparum* sporozoite vaccination, Nahrendorf et al. (Nahrendorf et al., 2014) demonstrated slow securing of memory B-cells and antibodies recognizing pre-erythrocytic and cross-stage antigens. However, the magnitude of these humoralresponses did not correlate with protection but rather reflected parasite exposure in chemoprophylaxis and sporozoites vaccination and challenge. Asito et al. (Asito et al., 2008) observed an expansion in both the aggregate memory B-cell populace and the transitional B-cell populace, occurring after a malaria episode in African youngsters. Even so, this study did not have any investigation of the specificity of B-cell reactions and involved long follow up to learn the duration of the immune response. Wipasa et al (Wipasa et al., 2010) demonstrated that even when antigen-specific antibodies were undetected in plasma, antigen-specific B-cells were observed in the blood of some individuals, suggesting that these could be maintained independent of enduring plasma cells. The greater part of these studies utilized Elispot for the identification

of antigen specific memory B-cells. While dealing with intricate antigens, flow cytometry has been shown to be a better assay option (Amanna and Slifka, 2006).

Malaria immunology can benefit from flow cytometry analysis since malaria involves a scope of parasite antigens that individually have a low number of specific B-cells. ELISA-based measures when improved can only distinguish 70% of the flow cytometry response (Amanna and Slifka, 2006). Flow cytometry is advantageous in that there's no need of cell incitement thereby expanding the odds of incorporating all cells in the reading. In order to acknowledge how Pf+ B-cells are actuated and maintained in vivo, these cells should be isolated from other B-cells (Amanna and Slifka, 2006). The rationale for the reliance of naturally acquired immunity to continued antigen exposure in residents of malaria endemic areas is still a subject of discussion. Advance in generation of a potential malaria vaccine depends on the knowledge of immunological memory development and preservation (Wipasa et al., 2010).

Whereas P. *falciparum* infections affect B-cells, there's still scanty information on B-cell homeostasis during malaria parasite infection (Asito et al., 2008). Atypical memory B-cells have been shown to expand with increase in age and cumulative malaria parasite exposure in people living in a malaria endemic area (Weiss et al., 2009). It has also been shown that HIV-malaria co-infected individuals have a higher percentage of atypical memory B-cells compared to malaria infection alone (Subramaniam et al., 2015). The role of atypical memory B-cells in malaria immunity is still unclear and needs to be investigated.

A special kind of memory B-cell sub-population that carries FcRL4 (CD307d) marker has been reported (Ehrhardt et al., 2005, Kuppers, 2008). In HIV, FcRL4 increase during the infection is of paramount importance (Jelicic et al., 2013). Therefore, there's need to investigate FcRL4+ B-cells in malaria since both HIV and malaria can persist in the body for a long time.

2 AIMS OF THE THESIS

GENERAL AIM

The general aim of this thesis is to describe how *Plasmodium falciparum* specific B-cell sub-populations and antibodies develop during infancy.

SPECIFIC AIMS

The specific aims of the work included in this thesis are;

I. To develop the technique that directly detects *Plasmodium falciparum* specific B-cells in humans

II. To evaluate the development of IgG and IgM antibody responses to *P. falciparum* during infancy and in adults

III. To ascertain the development of *Plasmodium falciparum* specific B-cell sub-populations during infancy and in adults

IV. To correlate the *P. falciparum* IgG and IgM antibody responses with *Plasmodium falciparum* specific B-cell sub-populations

3 MATERIALS AND METHODS

3.1 STUDY SITE

The studies included in this thesis were conducted at Kasanganti Health Centre (KHC). KHC is located in a peri-urban area is 20 km North-East of Kampala. Malaria is meso-endemic in Kasangati with peak transmission after the two rainy seasons (February-March and September-October) every year. Control samples used in these studies were obtained from Swedish donors at Karolinska University Hospital (KUH).

3.1.1 LABORATORY INVESTIGATIONS:

The experimental assays were conducted at Karolinska Institutet, MTC and Makerere University, Biomedical Cross Cutting Laboratory.

3.2 STUDY POPULATION

3.2.1 PAPER I

Malaria endemic (n=57) and non-endemic (n=25) samples were obtained from blood donors at KHC and KUH respectively. All samples from KHC were examined with malaria rapid diagnostic test (mRDT). The parasitaemia for the malaria positive samples was calculated using microscopy according to the WHO guidelines (WHO, 2000).

3.2.2 PAPER II

Study participants (n=131 mothers) were recruited in their last trimester from KHC between March of 2012 to July of 2013. The study included mothers who had normal deliveries with healthy newborns, and who agreed to come to the study clinic for follow up at 10 weeks, 6 and 9 months of the child's age. The selection of the study participants was random (patients were selected sequentially as they came to the clinic unless they failed the inclusion criteria). The negative control samples were obtained from Swedish donors at KUH.

3.2.3 PAPER III

The study was conducted from November 2010 to January 2011 after the September-October rains. A total of 362 malaria patients were recruited into the study, of whom 19% were early diagnosed people living with HIV/AIDS (PLWHA). Eligibility criteria included uncomplicated malaria as described by WHO (Organization, 2003).

3.3 ETHICAL CONSIDERATION

Ethical approvals for the human participants in Paper I-III included in this thesis were obtained from the ethical research committees in Uganda, and Sweden respectively. Written informed consents were obtained from adult participants and mothers/guardians of all children.

3.4 MALARIA PARASITE'S IN VITRO CULTURES

P. falciparum parasite lab isolates (FCR3S1.2) used in Papers I-III were cultivated using standard methods (Beeson JG et al, 1999). In summary, the parasite isolates were cultivated in a gas mixture of 90% NO2, 5% O2 and 5% CO2, at 37 degrees Celsius with constant shaking. Parasites were kept synchronized using 5 % sorbitol (w/v) treatment.

3.5 IMMUNOPHENOTYPING OF *P. falciparum* SPECIFIC B-CELLS

P. falciparum specific B-cells were immunophenotyped using B-cell markers including; FITC, CD 19 PE CF594, CD20 V450, FcRL4 APC fluorochrome-conjugated mouse anti-human mAb, and carboxyl quantum dot ghost infected RBC. Analysis was done on a LSRII flow cytometer and data was processed using FLOWJO software.

3.6 MEASUREMENT OF TOTAL IgG AND IgM BY ELISA

Antibody responses to *P. falciparum* schizont extract antigens in patient plasma were assessed using enzyme linked immunosorbent assay (ELISA). Total IgG and IgM levels against schizont extract and synthetic peptides including MSP3, GLURP, and Histidine Rich Peptide-II (HRPII) were measured using previously published ELISA protocols (Dodoo et al., 2000).

3.7 MEASUREMENT OF CD4 + T CELL COUNTS

The FACS Counter (Becton Dickinson, San Jose, CA, USA) was used to measure CD4+ T-cell counts.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Novel flow cytometry technique for detection of *Plasmodium falciparum* specific B-cells in humans: Increased levels of specific B-cells in ongoing malaria.

Antibody immune response assumes a critical part in malaria control, however information of the principle of sustenance and regular boosting of immunity is exceptionally constrained. Previously, it has not been conceivable to research malaria particular B-cells specifically in flow cytometry, making it hard to know the exert amount of B-cell immune response against malaria parasites, or what amount is because of other immunological stimulators.

In prior studies, Elispot measures have been utilized to attempt and gauge quantities of *P. falciparum* particular B-cells. During the Elispot assays, there's need for the B-cells to withstand the exploratory environment that brings about B-cell activation, and not all the cells adhere to this treatment. More so clashing results pertaining to antigen particular IgG levels and memory B-cell frequencies have been reported (Dorfman et al., 2005, Fievet et al., 1993, Migot et al., 1995). Nahrendorf and others (Nahrendorf et al., 2014) demonstrated that Elispot measures of malaria particular B-cells does not anticipate security from malaria disease. Hence there may be troubles in the strategies that have been previously utilized.

In this study, we developed a novel flow cytometry technique based on quantum dots and parasitized erythrocytes ghosts in order to study *P. falciparum* particular B-cells. With a specific end goal to gauge immune reactions against merozoites and infected red blood cell surface particles, the study considered schizont extract. The benefit of this is sufficiently high levels of B-cells can be found. Contrary very low amounts of B-cells would be detected if one particular (recombinant) antigen is searched for. A blend of antigens could be a good way forward in immunization research, where particular immune responses are explored.

More so with this new technique, there's no cell activation, and antigen particular memory B-cells are recognized because of their affinity/avidity for cognate antigen making it more specific and sensitive method for detecting malaria specific B-cells.

In this study we established clear-cut differences in *P. falciparum* specific B-cells from immune (22.3%), non-immune (1.7%) and people with an ongoing infection (27.9%). This is very intriguing since before it's been difficult to study malaria specific B-cells.

This technique can ideally be utilized in vaccine research that is dependent on either a particular (recombinant) antigen or whole infected RBCs that can conjugate with carboxyl quantum dots. Furthermore this method can be used in naturally acquired immunity development investigations.

4.2 PAPER II

Development of Plasmodium falciparum specific naïve, atypical, memory and plasma B-cells during infancy and in adults in an endemic region.

B-cells are essential for development of immunity against malaria, but which kind of B-cells that are important is still largely unknown. This study investigated the development of *P. falciparum* specific B-cell sub-populations using the quantum dot flow cytometry technique for detection of *P. falciparum* specific (Pf+) B-cells.

For the first time we were able to measure the development of Pf+ B-cell (CD19+) phenotypes in Ugandan babies from birth up to nine months, and in their respective mothers. The proportions of Pf+ plasma cells/blasts, atypical MBCs, and IgG MBCs increased in babies in the first 9 months of life as compared to their mothers. Even though the levels of these B-cell sub-sets increased during the 9 months, the proportions were still lower than in their mothers, indicating that immunity is not yet reached.

In the mothers, there was no notable difference in the proportions of Pf+ IgG MBCs, Pf+ non-IgG+ MBCs, atypical MBCs, plasma cells/blasts, and naïve B-cells at birth and 9 months later, suggesting that the number of these circulating Pf+ B-cell sub-sets remains stable during adulthood. Pf+ non-IgG+ MBCs and naïve B-cells binding to Pf were found to be higher in the babies compared to their mothers suggesting that these B-cell subsets are of significance in targetting *P. falciparum* in newborns.

The high proportions of Pf+ atypical MBCs (18%) and non-IgG+ MBCs (12%) as compared to long-term IgG MBCs (5%) in mothers indicate that atypical and non-IgG+ MBCs need to be considered in future studies of malaria immunity

In ELISA, there was an increase in IgG and IgM antibodies over time in babies, and stable levels in mothers. This affirms that the population in the study area is routinely exposed to malaria and that full immunity is not attained by the first 9 months of life.

The study also compared mother -baby parasitaemia with the respective antibody ELISA ODs and B-cell subpopulations. An increase in parasitemia was associated with low IgG antibody OD values (p=0.009), whereas there was no significant correlation between parasitaemia and IgM antibody OD values. Similarly, there was no significant correlation between parasitaemia and the different proportions of Pf+ specific CD19+ B-cell subsets. However the small numbers of Pf+ samples (10.7%) available might have contributed to this observation.

The analysis of primigravidae (27%) and mutigravidae (73%) mothers revealed significant differences in proportions of Pf+ IgG MBCs and naïve B-cells at birth. Pf+ IgG MBCs were higher in multigravidae than in primigravidae mothers ($p<0.05$), whereas Pf+ naïve B-cells were higher in primigravidae compared to in multigravidae ($p<0.05$). However, after 9 months, there was no notable difference in the proportions of these cell populations. It has been documentated before that primigravidae are more vulnerable to malaria, and from this study results, it is assumed more advantageous to have Pf+ IgG MBCs compared to Pf+ naïve B-cells, which affirms the general assumption of how protective memory against a disease is formed.

4.3 PAPER III

Parasite Specific Antibody Increase Induced by an Episode of Acute *P. falciparum* Uncomplicated Malaria

There is no affirmed malaria vaccine, and exactly how the human humoral immune response against malaria parasite antigens is formed, remains ineffectively characterized. This study explored antibody anamnestic or memory immune response elicited by one *P. falciparum* episode. This study was based on 362 malaria patients with an age range of 6 months to 60 years. 19% of this populace had HIV/AIDS (PLWHA). A measure and comparison of; the parasite density, $CD4^+$cell count, and antibodies specific to *P. falciparum* synthetic peptides including GLURP, HRPII and MSP3 was made on the day of malaria diagnosis and 42 days later.

We showed that IgG antibodies against GLURP, MSP3 and HRP II peptides were available in the blood of 75%, 41% and 60% of patients, respectively.

After 42 days, most of the patients had boosted their serum IgG antibody more than 1.2 fold. The expansion in level of IgG immune response against the peptides was not influenced by parasite density at diagnosis.

Furthermore there was no statistical difference between the median $CD4^+$ cell counts of PLWHAs and HIV negative individuals, and a similarity in the post-infection median increment in anti-peptide IgG was noted in both groups of patients.

From this study, we inferred that an episode of *P. falciparum* infection boosts at least 20% expansion in level of anti-malaria parasite IgG, and this boost is not influenced by parasite density on the day of malaria diagnosis, or by HIV status.

5 CONCLUSION AND FUTURE PROSPECTIVES

Study aim I: To develop the technique that directly detects *Plasmodium falciparum* specific B-cells in humans.

From our results it is possible to successfully detect *P.falciparum* specific B-cells in humans using the flow cytometry technique based on quantum dots and schizont extract made from ghosts of infected erythrocytes.

This technique can largely be explored in vaccine research and investigations looking at development of naturally acquired immunity.

Study aim II: To evaluate the development of IgG and IgM antibody responses to *P. falciparum* during infancy and in adults.

The development of plasma IgG and IgM antibodies in the babies against schizont extract antigens increased over time as expected in individuals living in a malaria endemic area whereas stable levels were noted in adults. These studies were performed as a preparation for aim IV.

Study aim III: To ascertain the development of *Plasmodium falciparum* specific B-cell sub-populations during infancy and in adults.

There was an increase in Pf+ IgG memory B-cells (MBCs), atypical MBCs, and plasma cells/blasts during infancy, however the extent of these cell proportions were still lower than in the adults who showed stable levels.

Pf+ non-IgG+ MBCs and naïve B-cells binding to Pf antigens were higher in the babies compared to the mothers.

The high percentages of atypical and non-IgG+ MBCs in both babies and adults demonstrate that these populaces of cells ought to be considered in future investigations of malaria immunity. Whether atypical MBCs are positive or negative to have early on in the development of immunity, is still open for further investigations.

Study aim IV: To correlate the *P. falciparum* IgG and IgM antibody responses with *P. falciparum* specific B-cell sub-populations.

In babies, low proportions of FcRL4+ Pf+ B-cell sub-populations including non-IgG+MBCs and naïve B-cells were associated with high levels of schizont specific plasma IgG whereas high levels of FcRL4- non-IgG+ MBCs were associated with high levels of schizont specific plasma IgG at birth. At 6 and 9 months high levels of FcRL4+ IgG MBCs, FcRL4+ plasma cells/blasts and FcRL4-plasma cells/blasts were associated with high levels of Pf+ plasma IgG respectively.

In adults, low proportions of Pf+ B-cell sub-populations including FcRL4+ non-IgG+ MBCs (at birth and 9 months), and FcRL4+ IgG MBC at 9 months were associated with high levels of Pf+ plasma IgG.

For IgM, high proportions of FCRL4- Pf+ atypical MBCs and FcRL4- IgG MBCs were associated with high levels of schizont binding plasma IgM for babies at birth and mothers at 9 months, respectively. However, low levels of FCRL4- Pf+ atypical MBCs were associated with high levels of IgM antibodies in mothers at 9 months.

In conclusion, the general pattern indicates that low proportions of FCRL4+ cells are associated with high levels of IgG and IgM antibodies. Since it is assumed that high levels of antibodies is good, we could speculate that high levels of FCRL4+ cells is a sign telling us that immunity is not yet reached. However, whether it is a necessary step towards developing the best possible immunity still needs further investigation.

In order to develop a successful anti malaria vaccine, it is of great importance to understand the underlying role of antibodies as well as B-cells, and how they are sustained in vivo in the development of protective immunity against malaria parasites.

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