The role of antibody mediated parasite neutralization in protective immunity against malaria

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Believe nothing, no matter where you read it, or who said it, no matter if I have said it, unless it agrees with your own reason and your own common sense

– Buddha
Summary

Malaria is the most prevalent infectious disease in the world today, in regard to morbidity and mortality, and it is mostly affecting sub Saharan Africa. High priority is put on the development of a vaccine against the malaria parasite *Plasmodium falciparum*, which due to its prevalence, virulence and drug resistance is the major cause of the high mortality. But there are many obstacles left before a rational vaccine can be developed, the major one being the limited knowledge of how the immune system is clearing a malaria infection and what protective components are involved in this context. The aim of the work presented in this thesis is to define the role of antibodies as protective components in natural *Plasmodium falciparum* infections, since definition of the isotypes and specificities of the antibodies involved are essential for designing an effective vaccine. This investigation is based on ethnic differences in susceptibility to malaria. In Mali and Burkina Faso the Fulani ethnic group shows a relative resistance to malaria as compared to other sympatric ethnic groups.

In this thesis I present studies of the antibody responses to *Plasmodium falciparum* and other pathogens in sympatric ethnic groups living in Burkina Faso and Mali. We confirm the previous findings of the different anti-malarial antibody responses between the sympatric ethnic groups. The anti-malarial IgG responses are dominated by IgG1 and IgG3, suggesting a role of these subclasses in protection, and we also suggest a protective role of anti-malarial IgM. However, we could not show any consistent differences between the ethnic groups for non-malarial antigens, nor for total IgG antibodies, suggesting the relative resistance to malaria seen in the Fulani to be pathogen specific and not due to a generally hyper-reactivity in this group. We have also analysed the impact of the Fcγ receptor IIA R131H polymorphism on IgG subclass pattern and susceptibility to malaria. Our results show that the IgG subclass pattern is different between the tribes, Fulani having a higher proportion of malaria specific IgG2 than Dogon, and we also observed a significant difference in genotype frequency, Dogon being strongly biased towards a RR or HR genotype, while Fulani present an evenly distributed genotype frequency. However we could not show any consistent results for the impact of the genotype on IgG subclass pattern. We suggest, based on our results that IgG2 is related to protection and also the HH-genotype may be related to protection.
List of Papers

This thesis is based on the following original papers, which will be referred to by their Roman numerals:

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*These authors contributed equally to this paper.

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<tr>
<td>ADCI</td>
<td>Antibody dependent cell mediated inhibition</td>
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<td>APC</td>
<td>Antigen presenting cell</td>
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<td>CRP</td>
<td>C-reactive protein</td>
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<td>iRBC</td>
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<td>ITAM</td>
<td>Immunoreceptor Tyrosin-based Activation Motifs</td>
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<td>MHC</td>
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<td>MSP</td>
<td>Merozoite surface protein</td>
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<td>NA</td>
<td>Neutrophil Antigen</td>
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<td>NK cell</td>
<td>Natural Killer cell</td>
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<td>PAM</td>
<td>Pregnancy associated malaria</td>
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<td>R</td>
<td>Arginine</td>
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<td>RBC</td>
<td>Red blood cell</td>
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<td>TCR</td>
<td>T cell receptor</td>
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<td>Th</td>
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<td>Toll like receptor</td>
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<td>VSA</td>
<td>Variant surface antigen</td>
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Introduction

The Immune system

The immune system is a highly variable and diverse component in all higher animals. It has evolved throughout the years, and its complex network of cells and molecules can distinguish between invading pathogens and the body’s own cells. Traditionally, the immune responses raised to an invading pathogen are divided into innate immune responses and adaptive immune responses.

The adaptive immunity is mediated by clonally distributed B and T cells and it exhibits specificity, diversity and memory. Small differences between pathogens can be distinguished, and a unique response will be raised against all particular antigens. Once the antigen has been recognized and responded to, an immunological memory will be developed, which by the next encounter with the same antigen will yield a faster immune response. The disadvantage with the adaptive immune system is that the primary response is delayed, due to the clonal expansion.

The innate immune system is a less specific first line of defence and it involves anatomical barriers (skin and mucosal surfaces), physiological barriers (temperature, pH and chemical mediators), endocytic and phagocytic cells (monocytes, macrophages and neutrophils), and inflammatory responses. It reacts immediately upon stimulation of pathogens and can also activate and shape the adaptive immune responses. The stimulation of the innate immune system is mainly mediated through pattern recognition receptors (PRR), which bind conserved molecular structures found in large groups of pathogens. These receptors can be secreted, be expressed on cell surfaces or in intracellular compartments. The Toll-like receptors (TLRs) are one of the most important PRR families and ten TLRs in humans are known\(^1\), of which TLR2 and TLR4 are the best characterised.

There are many different cell types involved in different stages of the immune response. The only cells capable of producing antibodies are the B cells, each B cell producing a unique specificity of their antibodies. Dendritic cells (DCs) are bridging the gap between the innate and adaptive immune responses. T cells are divided into $\alpha\beta$ T cells and $\gamma\delta$ T cells, depending on the composition of the T cell receptor (TCR). The $\gamma\delta$ T
cells, representing a relatively small part of the T cell repertoire, recognise non-peptidic antigens in a major histocompatibility complex (MHC) independent manner. The αβ T cells are further divided into CD4+ T cells, which regulate the cellular and humoral immune responses, and CD8+ T cells, that show a major cytotoxic activity toward cells infected with intracellular pathogens. The CD4+ T cells are divided into Th1/Th2 type of cells depending on the cytokines they produce. Traditionally, Th1 cells had been described to drive the type-1 pathway, the cellular immunity pathway, to fight viruses and other types of intracellular pathogens, whereas the type-2 cells has been said to drive the type-2 pathway, the humoral immunity pathway, by up-regulating antibody production to fight extra-cellular pathogens. A less characterised subset of T cells is the T-regulatory cells (Treg), which can regulate the responses by CD4+ and CD8+ T cells and NK cells. Natural killer cells have the ability to react with spontaneous cytotoxicity, without sensitisation, against a broad range of targets, and they are also one of the key producer of cytokines that will mediate the immune responses of the other immune cells. NKT cells express a TCR, and are therefore by definition T cells, however it shares some of the characteristic NK cell markers. In contrast to other T cells, NKT cells do not interact with MHC class I or II, but do interact with glycolipids presented by CD1d, a non-classical antigen presenting molecule. They can also up- or down regulate immune responses by secretion of Th1-, Th2- or regulatory cytokines.

A key function for the immune responses, is the antigen recognition and presentation, the antibodies produced by B cells can bind directly to the naive antigen, but T cells usually need to get the antigen presented as a peptide bound to a MHC molecule. Two classes of MHC molecules function in antigen presentation, MHC class I and MHC class II. The MHC class I molecules are expressed on almost all cells, they primarily present endogenous antigens (e.g. viral proteins), and it is mainly CD8+ T cells that recognise the MHC class I, leading to lysis of the cell presenting foreign peptides. The MHC class II is only expressed on the professional antigen presenting cells (APCs), which comprise B-cells, macrophages and DC. They express the MHC class II molecules together with co-stimulatory molecules that are necessary for the induction of a proper T cell response. MHC class II presents peptides from exogenous proteins, and APCs present almost exclusively to CD4+ T cells. The CD4+ T cells provide helper functions...
to stimulate specific antibody responses by B cells and specific responses by CD8+ T cells.

**Immunoglobulins**

The immunoglobulin (Ig) molecule can be found both as membrane bound on B-cells and in a secreted form that is produced by activated B-cells, the plasma cells. When bound on the surface the Ig functions as a receptor involved in differentiation, activation and apoptosis, while the secreted form can neutralize foreign antigens and recruit other effector components. The Ig consists of two large polypeptide chains, called heavy chains, and two shorter, called light chains, paired together in a Y-shape. The open upper part of the Y is the antigen binding part, and the lower part of the Y is the Fc part, which is responsible for interaction with receptors and complement. There are five different Fc parts, each corresponding to an Ig isotype, IgM, IgD, IgG, IgA and IgE, all of which can function both as receptors on the cell surface and in a secreted form.

![Figure 1: A simplified structure of the Ig molecule. Adapted from Martin (1969)](image)

IgM represents about 30% of the total serum immunoglobulins, and it is the first antibody class that encounters a new antigen. IgM can be found in two forms; membrane bound monomeric IgM and secreted pentameric IgM. The pentameric form of IgM makes it a powerful complement activator and it can up-regulate both primary and memory responses and increase affinity maturation.
Out of the total serum immunoglobulins, 0.25% is represented by IgD. It appears not to cross the placenta, and it has a weak or absent binding to normal lymphocytes, neutrophils and monocytes. Native IgD has little or no capacity to activate complement effects, whereas aggregated monoclonal IgD induces complement activation. IgD is co-expressed with IgM on most peripheral B-cells. IgD is conserved across different species and it is found in all mammals and avian species, suggesting an evolutionary advantage. The role of IgD is still not completely understood, but it seems to behave like IgM early in infections. Moreover, IgD concentrations are elevated in chronic infections, but if these specific IgD antibodies are of any clinical importance is unknown.

IgG is dominating the humoral responses in humans, around 75% of the total immunoglobulin concentration in serum being IgG. Human IgG is divided into four subclasses, IgG1-IgG4, where IgG1 is the largest subclass (66%), followed by IgG2 (24%), IgG3 (7%) and IgG4 (3%) (subclasses differ in ability to activate the complement, IgG3 and IgG1 are the most effective ones, and IgG2 is a weak activator, whereas IgG4 does not activate complement at all. The role of the different subclasses varies, IgG2 is the main antibody targeting encapsulated bacteria, IgG1 and 3 are mainly directed against protein antigens and IgG4 is common in chronic exposure to protein antigens and in allergy. IgG can also be transported through the placenta.

IgA is present in normal human serum at about one fifth of the IgG concentration, and it is the most abundant antibody in secretions. Mucosal surfaces are the main source of antigenic material in the body, and in the mucosal tissues the local synthesis of secretory IgA is dominating that of the other antibody classes. Secretory IgA is present in all mucosal surfaces, and is therefore an important first line of defence, and it can activate the complement system and also trigger cell-mediated events. In breast milk and colostrum, the major immunoglobulin is IgA, providing the child protection against intestinal pathogens.

IgE is the antibody class that is the least abundant in human serum. The effect of IgE is mainly known in allergy, where IgE mediates the hypersensitivity reactions responsible for the symptoms of hay fever, asthma, hives, and anaphylactic shock. IgE can up-regulate carrier-specific antibody responses, both primary and memory responses. Elevated levels of IgE have been shown for many helmintic infections and also in malaria exposed individuals.
**Fc receptors**

One receptor type that is involved in antibody recognition comprises the Fc receptors (FcR). They recognize and bind to the Fc part of the antibodies and they exist for all antibody classes, FcγR recognize and bind IgG, FcαR for IgA, FcδR for IgD, FcμR for IgM and FcεR for IgE. Signalling through the Fc receptors induces many different actions, depending on the cell carrying the receptor, e.g. phagocytosis and release of inflammatory components \(^{22}\). There are also FcRs responsible for transportation of antibodies through epithelia, they are the polymeric IgA and IgM receptors and the neonatal FcR, that mediates antibody transportation through the placenta from the mother to the child \(^{22}\). The FcRs capable of cell activation all contain intracytoplasmic activation motifs, designated immunoreceptor tyrosine-based activation or inhibiting motifs (ITAMs or ITIMs) \(^{23}\) (Fig 2). These ITAMs can be of two different types, multichain or single-chain receptors. The FcRs that lack ITAMs do not trigger cell activation, the exception is FcγRIIIB, which has no activating effect on its own, but contributes to cell signalling by associating to other FcRs \(^{22}\).

![Figure 2: Human Fc receptors. Adapted from Pleass and Woof (2001) \(^{23}\)](image-url)
Malaria

Malaria is the most prevalent infectious disease in the world, causing more than 300 million acute clinical cases and approximately 2 million deaths every year. About 90% of the deaths related to malaria occur in sub-Saharan Africa, and the disease is the leading cause of mortality (20%) in children less than five years of age. Women are also highly susceptible to so called placental- malaria (or pregnancy associated malaria, PAM) during their first and second pregnancy, which may lead to death. Moreover, malaria infections in the mother can lead to spontaneous abortion, neonatal death and low birth weight of the child. Malaria is increasingly becoming a global problem, natural disasters, agricultural projects, climate changes and the fact that people travel more are giving the parasite and the mosquito many chances to spread to non-malaria areas. The rapid development of drug-resistance amongst the malaria parasites is another problem, since several of the anti-malarial drugs available are now becoming without effect, and the drugs that still work are expensive, and many countries can not afford them as a first line treatment. However, a recent optimistic report from Malawi, presents data suggesting that the previously widely spread chloroquine resistance among the *P. falciparum* parasites in this country, seems to have disappeared, and chloroquine is again an effective anti-malarial drug. The vector has also been a target for control measures of the disease, the major ones being insecticide usage on wetlands and insecticide treated bed nets. However, the mosquito is prone to develop insecticide resistance, leaving only the insecticide treated bed nets as an effective barrier. The distribution of bed nets is ongoing, but financial issues have slowed down the progress. Today, a main goal for controlling malaria infections around the world is the development of a functional vaccine. However, this is not progressing as fast as needed. The knowledge of how the immune system is clearing a malaria infection, and what protective components that are involved in parasite neutralisation is still not defined enough for a rational vaccine design.
Malaria parasite life cycle

Malaria is caused by a protozoan parasite of the *Plasmodium* family. Although four species infect humans, *Plasmodium falciparum*, *P. vivax*, *P. malariae* and *P. ovale*, only *P. falciparum* results in high mortality as a result of its prevalence, virulence and drug resistance \(^{26}\). Transmitted by the female *Anopheles* mosquito, the sporozoites reach the liver, where they develop into merozoites. After 1-2 weeks the infected liver cells rupture, releasing thousands of merozoites, which all can invade red blood cells (RBC), and develop inside the RBC, to the so called ring-stage, trophozoite stage and schizont stage. When the schizonts rupture, many merozoites will be released and invade new RBCs. This is known as the asexual blood stage of the parasites life-cycle. Some merozoites develop into gametocytes, which are taken up by the anopheline mosquito, and the parasite starts its sexual cycle inside the mosquito midgut with sporozoites as end products \(^{26}\) (Fig 3).

The erythrocytic cycle of *P. falciparum* has a unique feature, mature trophozoites and schizonts are sequestred in the peripheral circulation, due to parasite mediated changes of the surface of the infected RBC (iRBC), causing them to adhere to endothelial cells (sequestration) and other erythrocytes (rosetting). It is an accepted theory that this adhesion is an immune escape mechanism of the parasite, and that it also may lead to a better maturation in the microaerophilic venous atmosphere \(^{27}\).
**Figure 3**: The life cycle of the *Plasmodium* parasite. The average number of *Plasmodium* parasites at each developmental stage is indicated. Adapted from Brown and Catteruccia (2006)\textsuperscript{28}

**Malaria disease**

The clinical symptoms of malaria are only presented during the erythrocytic stage of the parasites life cycle, and mild or uncomplicated malaria is characterised by fever, followed by nausea, headache, cough, diarrhoea and muscular pain. Infections by *P. vivax*, *P. ovale* and *P. malariae* usually give milder malaria as compared to that caused by *P. falciparum*. This is due to the ability of *P. falciparum* to adhere to host endothelium in combination with the high parasitemia usually seen for *P. falciparum*. WHO defines severe malaria as a parasitemic person with one or more of the following symptoms: prostration (inability to sit up without help), impaired consciousness, respiratory distress or pulmonary edema, seizures, circulatory collapse, abnormal bleeding, jaundice, hemoglobinuria or severe anaemia (haemoglobin < 50 g/L or hematocrit < 15%) \textsuperscript{26}.\textsuperscript{26}
Cerebral malaria is caused by the sequestration of *P. falciparum* infected RBCs in small blood vessels in the brain, causing blocking of the blood flow, leading to coma or other neurological phenomena, such as seizures and elevated intracranial pressure.

**Immunity to malaria**

Unlike other acute infections, malaria does not induce a long lasting immunological memory, but it rather develops gradually and requires repeated infections to persist. The developed immunity is not sterile, malaria parasites can still survive in the host but at such low levels so that the clinical symptoms do not show. The development of this semi-immunity is influenced by age, genetic background, pregnancy, coinfections and the nutritional status of the host.

**Immune responses to malaria**

The genetic influences on susceptibility to a malaria infection has been well studied and, not surprisingly, are many of the well-known malaria resistance genes related to the structure or function of the RBC, including sickle cell trait, thalassemias, enzyme deficiencies, ovalocytosis and the ABO blood groups.

In the complex immune response to malaria, both the adaptive and the innate immune responses are important. Monocytes, macrophages and NK cells are able to kill the parasite in the absence of antibodies, probably involving binding of CD36 to parasite derived surface molecules on iRBC. Human NK cells have also been shown to rapidly produce interferon (IFN) - γ, a cytokine associated with reduced susceptibility to malaria, suggesting a importance of NK cells early in the blood-stage malaria infections. The NKT cells produce large amounts of IFN-γ and IL-4, when activated through the TCR, and this rapid cytokine output may activate other lymphoid cells. It has also been shown that, in response to a *Plasmodium berghei* infection, the CD1-restricted NKT cells contribute to malarial splenomegaly, which is associated with expansion of splenic B-cells and enhanced parasite-specific antibody formation.

The role of DCs in malaria immunity is still relatively unknown, some studies show that the maturation of human DCs are suppressed, and that their ability to activate
T-cells are reduced by iRBC\textsuperscript{38, 39}. However, using animal models, it was demonstrated that DCs from infected mice are fully functional APCs\textsuperscript{40}.

During the first few days in a malaria infection, γδ T cells expand and they have been shown to have the capacity to directly inhibit the parasite growth\textsuperscript{41}. Both the CD4+ and CD8+ T cells play important roles in immunity to malaria, but at different stages. During the liver stage CD8+ T cell functions are important\textsuperscript{42}, and they also contribute to protection against severe malaria\textsuperscript{43, 44}. The CD4+ T cells are crucial in the immunity against asexual blood stage malaria. They produce cytokines which are involved in the activation of innate immune responses, and they are also required for the B cell production of anti-malarial antibodies. The immunity to blood stage malaria is dependent on the CD4+ T cells, anti-malarial antibodies and B cells\textsuperscript{45}. For other protozoan infections, the Th1/Th2 balance is crucial for the clearance of the parasites\textsuperscript{46}. In malaria, many studies, using mouse models, have shown that pro-inflammatory Th1 cytokines are crucial determinants of the outcome of the malaria disease, C57BL/6 mice, which have a predominant Th1-immune response, are more susceptible to cerebral malaria, than the Th2 biased BALB/c, which are resistant to cerebral malaria\textsuperscript{47}. And in humans, studies have shown a possible association between IL-4 and levels of anti-malarial antibodies\textsuperscript{48}. T regulatory cells are still under investigation for their role in for malaria immunity\textsuperscript{49}.

The role of TLRs is still not fully understood, but it is known that they can recognise malaria parasites or their metabolites. The glycosylphosphatidylinositol (GPI) anchors of the \textit{P. falciparum} antigens have been shown to mediate signals, mainly through TLR2 and to a lesser extent by TLR4\textsuperscript{50}. Furthermore, hemozoin, a parasite heme metabolite, is recognised by TLR9\textsuperscript{51}. Common polymorphisms in TLR4 may be associated to the clinical outcome of a malaria infection\textsuperscript{52}, and polymorphisms in TLR4 and TLR9 have been shown to increase the risk of low birth weight in \textit{P. falciparum} infected pregnant women as well as the risk of maternal anemia\textsuperscript{53}. However, none of these polymorphisms were found to affect the prevalence and parasite density of the \textit{P. falciparum} infection\textsuperscript{53}. 
The importance of antibodies in protection against malaria

Innate immune mechanisms involving mononuclear phagocytes and NK cells play an important role early in malaria infections. However, the importance of antibodies in protective immunity against *P. falciparum* infection was demonstrated by the classical experiments of Cohen and McGregor \(^{54}\), in which passive transfer of IgG from adults had curative effects in children. Furthermore, in Thai individuals, passive transfer of human IgG with a high content of cytophilic antibodies was associated with protection \(^{55}\). The *in vitro* effect of antibodies on *P. falciparum* isolates have been studied by different methods using whole sera or Ig fractions from individuals that have experienced malaria \(^{56}\). Antibodies can neutralize the parasite either by inhibition of merozoite invasion, by neutralization of the free merozoites or by interference with the merozoite invasion process. Antibodies may also react with parasite-derived antigens expressed on the surface of infected RBC, thereby inhibiting the intraerythrocytic development of the parasite \(^{56}\). The leaky membrane of infected erythrocytes just prior to merozoite release may give the antibodies access to the intraerythrocytic parasite, and they may interfere with merozoite dispersal. Another pathway for antibody attack may be the possible parasitophorous duct, which forms a connection for direct access of serum macromolecules to the parasite \(^{56}\). Studies on antibody-mediated inhibition of the growth of parasite-isolates from different regions, showed significantly better inhibition by sera/Ig coming from the same area as the parasites, than by those from remote areas \(^{56}\). However, the growth inhibition is not always the result from the action of antibodies, but rather from some other, as yet undefined, serum factors \(^{56}\). Furthermore, some studies have shown that certain sera or Ig-fractions may enhance the growth of the parasite, instead of inhibiting the growth \(^{56}\). Even though antibodies can inhibit parasite invasion/growth on their own, the main effects of the malaria specific antibodies are to induce antibody dependent cell-mediated inhibition (ADCI) \(^{57}\) and the secretion of monocyte-derived mediators \(^{58}\). The main players in this type of killing are the Fc receptors on the surface of the effector cells, which will bind the Fc part of the antibodies, while the Fab part of the antibody is bound to antigens on the surface of merozoites \(^{55}\) or late stage infected RBC \(^{59}\).

Several studies have shown that high titres of malaria specific IgG are related to protection from severe malaria and seroepidemiological studies in different endemic
areas have demonstrated the association of IgG antibodies of the cytophilic subclasses IgG3 and IgG1 with protection against *P. falciparum* malaria. This association is, however, quite inconsistent when considering antibody responses to single malaria antigens. IgG3 is the major subclass in responses against *P. falciparum* antigens showing a high degree of diversity, e.g. merozoite surface protein 2 (MSP-2), while responses against more conserved antigens, e.g. the C-terminal part of MSP-1, are dominated by IgG1. Interestingly, in some populations, IgG2 is related to protection, so it is not clear what IgG subclass profile that is the most protective.

The most important antigens that are being targeted by the antibodies are mainly expressed during the merozoite stage or the later trophozoite stage (Fig 4). At the merozoite stage, the MSP antigens, antigens present in the apical complex organelles of the merozoites (EBA-175, Rhop 1-3, RAP 1-3 and AMA-1) and Pfl55/RESA, all have been shown to be targets to antibodies with the capacity to inhibit merozoite invasion. Also, the recently identified SURFINs are found in this stage. There are several antigens synthesised during the trophozoite development (e.g. GLURP, SERA, ABRA, PfEMP1, Pf332, RIFINs, STEVORs), and antibodies to several of these antigens have been shown to have a high capacity to inhibit parasite growth or invasion.

Immunity to malaria is parasite and strain specific, and clonal antigenic variation is common in *P. falciparum*. The mechanism behind this antigenic variation is still not clear, but one very likely hypothesis is that there is a frequent ongoing switching of variant surface antigens (VSA) in the parasite population, and an outgrowth of one of these subpopulations would occur when antibodies are being raised towards the other VSA presented. The importance of VSAs in immunity can be shown by correlating the range of different anti-VSA antibodies to protection. The pregnancy associated malaria is caused by accumulation of iRBCs in the placenta. These parasites express a specific VSA that binds to chondroitin sulphate A (CSA), and the immune responses that are induced are sex specific and parity dependent. Some important antigenically variable antigens are PfEMP1, RIFINs, STEVORs and SURFINs.
Polymorphisms in Fcγ receptors and malaria protection

In humans there are three families of Fc-receptors binding IgG, FcγRI (CD64), -RII (CD32) and -RIII (CD16). FcγRI is a high-affinity receptor that binds monomeric IgG, FcγRII and -RIII are low-affinity receptors only binding complexed or aggregated IgG. Polymorphisms in the Fcγ receptors critically affect their binding of different IgG subclasses. Accumulating evidence suggests a relevance of these polymorphisms for susceptibility to disease. FcγRIIa has two codominantly expressed allotypes, differing at position 131R/H. FcγRIIa 131H is the only human FcγR that efficiently binds IgG2. FcγRIII has two isoforms, FcγRIIIα exhibits a dimorphism at position 158F/V with different affinity to IgG1 and IgG3. FcγRIIIb occurs in two allotypes, neutrophil
antigen 1 (NA1) and NA2, where the FcγRIIIb-NA2/NA2 genotype has a lower capacity for phagocytosis 75.

Only a few studies on FcγR polymorphisms in relation to malaria have been performed, most of them are associating the FcγRIIa 131R/R genotype with protection against malaria and the FcγRIIa 131H/H genotype with susceptibility to the disease 76. A study in Western Kenya showed that infants carrying the FcγRIIa 131R/R genotype had a significantly lower risk for high-density *P. falciparum* infection than 131R/H genotype carriers 57. In the same region, the FcγRIIa 131H/H genotype was found associated with enhanced susceptibility to placental malaria in HIV-positive, but not in HIV-negative women 77. Furthermore, a study in Thailand showed that the FcγRIIa 131H/H genotype and the FcγRIIIb NA2 allele were associated with susceptibility to cerebral malaria 78, while the 158F/V polymorphism in FcγRIIIa had no effect in this respect 79. Similarly, the FcγRIIa 131H/H genotype was significantly associated with susceptibility to severe malaria in a study performed in The Gambia 80. A study in Burkina Faso indicates the impact of the genotype of FcγRIIa on protective immunity; a significant correlation was demonstrated between the levels of anti-malarial IgG2 antibodies and the incidence of malaria, in a population where the IgG2 binding FcγRIIa 131H allele was predominant 81. Transfected phagocytic cells, expressing the FcγRIIa 131R allotype, tended to show higher phagocytosis of *P. falciparum* infected erythrocytes following opsonisation with IgG1-containing sera, in contrast to the 131H allotype, that showed the highest phagocytosis with IgG3-containing sera 82.

**Ethnic groups in West Africa showing differences in susceptibility to malaria**

Several studies have demonstrated differences in susceptibility to malaria between different ethnic groups. In East-Africa, the Fulani showed a higher frequency of splenomegaly and lower incidences of malaria than other sympatric groups, despite the same exposure to malaria and no differences in socio-cultural circumstances 83. This finding was later confirmed by various studies, showing that the Fulani have a lower parasite prevalence and density and have a more prominent spleen enlargement compared
to other ethnic groups \(^8^4,^8^5\). Moreover, the Fulani have generally higher anti-malarial antibody responses, covering antigens from both the liver stage \(^8^6,^8^7\) and the blood stage \(^8^7,^8^8\), as well as the crude \textit{P. falciparum} extract \(^8^5\). HLA analyses have shown that the Fulani are genetically distinct from other African tribes \(^8^9\), and regarding established genetic malaria resistance factors, the haemoglobin S and C, \(\alpha\) thalassemia, G6PDA and HLA B, have been shown to occur in a lower frequency in the Fulani than in their sympatric neighbours \(^9^0^8^5\). The proportion of individuals not having any of these protective alleles was more than 3-fold greater in the Fulani, as compared to the other ethnic groups \(^9^0\). IL-4 levels and polymorphisms have been suggested as contributing factors to this lower susceptibility in the Fulani \(^4^8,^9^1\), but these findings are not enough to explain this ethnic differences, so many studies are ongoing, covering a wide range of possible effector functions, e.g. cytokine expression, TLR expression, cell activation and receptor functions.

**General aim of the study**

The aim of this study was to define the role of antibodies as protective components in the complex immune responses to natural \textit{P. falciparum} infections. Definition of the isotypes and specificities of antibodies in relation to their anti-parasitic activities is essential for a rational development of a vaccine to malaria. The differences in susceptibility to malaria between the sympatric living Fulani and their neighbours, give a unique opportunity to study the importance of anti-malarial antibodies and their role in protection against severe outcome of the disease.

**Material and Methods used in the study**

The methodologies, study areas and study populations of the included studies are described in detail in the corresponding paper.
Results and Discussion

Antibody responses to *P. falciparum* and other pathogens in ethnic groups living in sympatry in West Africa (Study I)

The well known relative resistance to malaria seen in the Fulani, as compared to other sympatric tribes, is related to their higher concentrations of anti-malarial antibodies. The ability of the Fulani to mount stronger immune response has been suggested to be at least in part genetically regulated. However, if these inter-ethnic differences can be ascribed a generally more activated immune system or specifically enhanced anti-malarial immune responses in the Fulani is still unknown.

In this study, we investigated the isotypic distribution of malaria specific antibodies to crude *P. falciparum* antigen, the total IgG and IgM concentrations and concentrations of IgG antibodies, reactive with a panel of non-malarial antigens in Fulani individuals from Burkina Faso and Mali, and compared them to sympatric individuals from ethnic groups with a different genetic background, in order to clarify if the relative resistance seen in Fulani is malaria specific or a general hyperreactivity in this tribe.

Despite a difference in transmission intensity, Fulani from Mali showed similar levels of *P. falciparum* specific IgG, IgM and IgG subclasses as the Fulani from Burkina Faso. Fulani from both Burkina Faso and Mali had higher levels of all malaria-specific antibodies when compared with those of the respective sympatric tribes. Also, total IgM levels were shown to be higher in Fulani than in the non-Fulani, but for total IgG we could not show any difference between the tribes. For the non-malarial antigens included in the study, some showed the same pattern as for malarial antigen, with Fulani having higher levels of specific antibodies, while some other antigens showed no such difference.

The higher levels of anti-malarial IgG and IgM in the Fulani groups, suggest a role of these antibodies in the lower susceptibility to malaria seen in Fulani. This is in line with previous studies, suggesting a role of malaria specific IgG and IgM in the defense against malaria. It has been shown that memory IgM⁺ B cells can persist long after the malaria transmission seasons, which could be further supported in our result by the consistently higher total concentrations of total IgM in Fulani as compared
to non-Fulani groups. However, ethnic differences in persistence of these IgM+ B lymphocytes has to be further studied before any conclusion of that kind can be made.

IgG subclass antibodies with specificity to malaria antigens have been shown to be important in protection against malaria. In particular, antibodies of the IgG1 and IgG3 subclasses have been related to protection\textsuperscript{94}. The suggested mechanism by which these subclasses are protective, involves their binding to the Fc receptors on monocytes, leading to antibody dependent cell mediated inhibition of parasite replication\textsuperscript{94, 95}. This is supported by our results, since the two most predominant IgG subclasses in this study are IgG1 followed by IgG3.

The results for the included non-malarial antigens showed higher levels of IgG against measles and \textit{T. gondii} antigens in the Fulani compared to the other ethnic groups. Only the Malian Fulani showed higher antibody levels against \textit{M. tuberculosis} (PstS-1) compared to their sympatic tribe, while no difference was seen in Burkina Faso for this antigen. For Rubella and \textit{H. pylori}, no differences between the ethnic groups were seen. The higher levels of anti-mycobacterial antibodies in Fulani of Mali can be an indication of a higher prevalence of the disease or more frequent vaccinations in this group as compared to their neighbouring tribe. The responses to measles and \textit{T. gondii} in the Fulani may be explained by a possible cross-reaction with \textit{P. falciparum}, which has also been shown to signal through TLR 9\textsuperscript{96-99}. Thus, maybe polymorphisms in TLR9\textsuperscript{100} can be a contributing factor for the differences in anti-malaria response seen between Fulani and their sympatic neighbours.

In conclusion, this study supports the previously reported higher anti-malarial responses seen in Fulani as compared to sympatic tribes. Also, we show that the response is dominated by IgG1 and IgG3, suggesting a role of these IgG subclasses in protection against malaria. We also suggest a protective role of anti-malarial IgM, and the fact that only some of the non-malarial antigens showed the same inter-ethnic differences as malaria antigens, indicates that Fulani is not immunological hyper-reactive to all pathogens.
Several studies report a protective effect of a polymorphism in amino acid 131 in Fcγ receptor IIa to malaria. The wild type allele, arginine (R), has been shown to be associated with protection against malaria, while the mutated allele, histidine (H), was linked to susceptibility. The H allele is the only FcγR that efficiently binds IgG2, which has been suggested to inhibit the anti-malarial effects of IgG1 and IgG3. There are a few studies suggesting that IgG2 is important in protection, and this conflict in results could be due to the FcγRIIa polymorphism.

Since we have suggested that the relative resistance in Fulani against malaria is not due to a general hyper immunological reactivity, we wanted to confirm these results by comparing the total IgG subclass levels between Fulani and Dogon in Mali. We also analysed the malaria specific IgG subclass antibodies in relation to Fcγ receptor IIa polymorphism in order to investigate if this polymorphism could be a contributing factor for the protection seen in Fulani.

Our results show that Fulani are less parasitized, have fewer parasites clones and have higher spleen rate than Dogon. This is in line with what has been previously reported.

For the total IgG subclasses, we could not find any consistent difference between the two ethnic groups, only IgG4 was slightly increased in Dogon as compared to Fulani. This confirms our suggestion that the relative resistance in Fulani as compared to sympatric tribes is pathogen specific. We can also confirm the findings that the Fulani have higher anti-malarial IgG subclasses than the Dogon, however the pattern of distribution differed between the two groups. While the Dogon and many of the Fulani had a similar pattern to that previously described in many studies (IgG1>IgG3>IgG2>IgG4), some Fulani individuals showed higher IgG2 levels than IgG3, making the order IgG1>IgG2>IgG3>IgG4. This difference was more obvious when looking at the ratios between IgG1:IgG2, the Fulani showing more IgG2 than the Dogon, the ratios being 17.5:1 for Dogon and 6.3:1 for Fulani. The IgG1 and IgG3 subclasses have been given the most attention as protective antibodies, however there are
some reports suggesting IgG2 to be protective \textsuperscript{81, 101} and our results confirms these results, since Fulani are supposed to be relatively more resistant to malaria.

Regarding the Fcγ receptor IIa R131H polymorphism, our results are not confirming the idea of the R-allele being associated with protection from malaria. We demonstrated a significant difference between the genotype frequencies in Fulani and Dogon, with RR homozygotes being more common in Dogon than Fulani, and the HH genotype occurring at higher frequency in the Fulani. Some studies have suggested the HH genotype to be mildly associated to protection \textsuperscript{81, 102}, and our results confirm these findings. Interestingly, the frequencies of the genotype distribution are for the Fulani very similar to what has been reported for Caucasians \textsuperscript{103, 104}, this suggests that the allelic change is not driven by malaria pressure in Fulani, but rather is a reflection of their genetic background \textsuperscript{89}. However, the impact of Fcγ receptor IIa 131 R/H polymorphism on malaria protection has been shown in many studies, so if the HH genotype can be shown to be a protective factor, then the relative resistance seen in the Fulani could, at least in part, be explained by this genetic predisposition. The proposed protection of the R-allele could come from a difference in IgG subclass distribution as compared to the H-allele, and in Fulani individuals we show higher IgG3 levels among R-allele carrier than among HH individuals. However, no such trend was seen in the Dogon. Interestingly, for IgG2 the R-allele seemed to be related to higher IgG2 levels in the Dogon tribe. Since the receptor is less effective in binding IgG2 when the R-allele is present, it is possible that more IgG2 will be present in the serum of individuals with the R-allele than those with the H-allele. However, for the Fulani, the higher IgG2 levels were not found in individuals with the RR genotype, but rather in those with the H-allele, i.e. the total opposite result from that found in Dogon. These conflicting results may suggest that this polymorphism is not of high importance in malaria protection, or that the importance of this polymorphism lies elsewhere and not in the IgG subclass distribution. Importantly, although the levels of antibodies to the crude malaria antigen are associated with relative resistance from malaria, their importance in protective immune mechanisms remains to be defined. Based on our results, we suggest that the FcγRIIa 131HH genotype is associated to a lower susceptibility to malaria, and that IgG2 may be important in susceptibility to malaria. We also suggest, that the FcγRIIa R131H polymorphism could
influence the IgG subclass responses, and also be a contributing factor to the lower susceptibility to malaria seen in the Fulani as compared to their sympatric neighbours.

**Future projects**

So far, our results have shown a possible relation between FcγRIIa and a lower susceptibility to malaria. We have also been able to show, that the proposed protected IgG subclass pattern, maybe should include IgG2. However, the transmission intensity may influence these findings, so it is important to extend the studies on FcγR and IgG subclass distribution and their relevance for protection against malaria in other areas with different malaria transmission intensity. I will also investigate the effect of transmission intensity on other factors known be related to FcγR, such as C-reactive protein.

C-reactive protein (CRP) is an acute-phase serum protein that belongs to the pentraxin protein family, and it plays a regulating role in infections and inflammations. CRP can bind to Fcγ receptor I and II, with an allele-specific binding to FcγRIIa. Studies on *P. falciparum* malaria show a relation between high CRP concentrations and parasite density and severity of the *P. falciparum* infection. Recently, a three allelic single nucleotide polymorphism in the promoter of CRP (-286 C>T>A) were strongly associated with the plasma concentration of CRP, predominantly in patients with coronary heart disease (CHD). We are interested in analysing the CRP levels in Fulani and the sympatric ethnic groups and relate the CRP concentrations to the genotypes of the tri-allelic polymorphism in the promoter region. Our hypothesis is that higher concentrations of CRP are a contributing factor to malaria outcome, since CRP may compete with IgG subclasses in the binding to the FcγIIa receptor. Hence, high CRP levels may lead to a general activation of the immune system instead of a malaria specific one. We will also correlate the levels of CRP with the levels of IgG subclasses and FcγRIIa R131H genotypes. The study population is the same as in study 2.

In order to understand the importance of the different IgG subclass antibodies in protection, and the impact the FcγRIIa R131H polymorphism has on protection, analyses on the parasitic growth inhibitory capacity of the IgG subclasses on field isolates in intra-
and inter ethnic combination among Fulani and their sympatric neighbours will be done, in relation to the donor’s FcγRIIa genotype and IgG subclass pattern. Ideally, monocytes from the participating individuals will be genotyped and used in the assays as effector cells. If this turns out to be too time consuming, or other practical difficulties makes it impossible, monocytic cell lines, expressing the three different genotypes, will be used instead. This study will be performed during the malaria transmission season in 2007 at a study site still to be confirmed.
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