Impact of genetic host factors on the *in vitro* growth of *Plasmodium falciparum* and the innate immune response towards infected red blood cells

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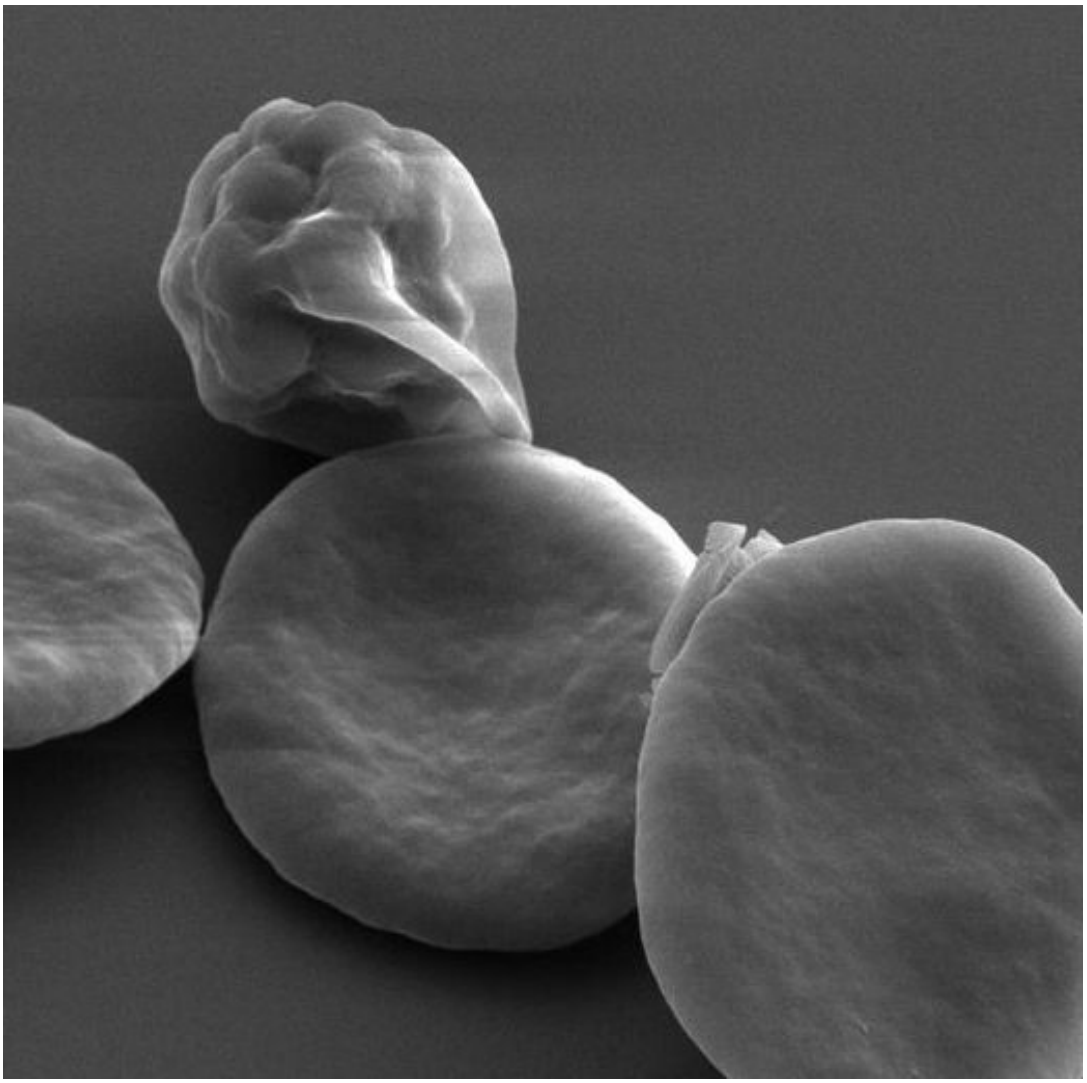

Red blood cell infected with *Plasmodium falciparum* surrounded by uninfected red blood cells. Scanning electron microscope, magnified 8 000 times. *P. falciparum* sample provided by Stefanie Meese. © eye of science

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1 INTRODUCTION

1.1 Malaria

1.1.1 Epidemiology

Malaria is one of the most common infectious diseases worldwide with almost half of the world's population living at risk. It has a broad distribution throughout all tropical and subtropical regions with the highest incidence in sub-Saharan Africa (Figure 1) (Breman et al. 2001, Snow and Marsh 1998). In 2013, an estimated 198 million cases and 584 000 deaths due to malaria were reported worldwide, with 90 % of malaria-related deaths occurring in sub-Saharan Africa, especially amongst children under the age of five years. Although malaria mortality rates dropped worldwide since 2000 by 47 %, it still constitutes a major public-health problem, predominantly in sub-Saharan Africa (WHO 2014).

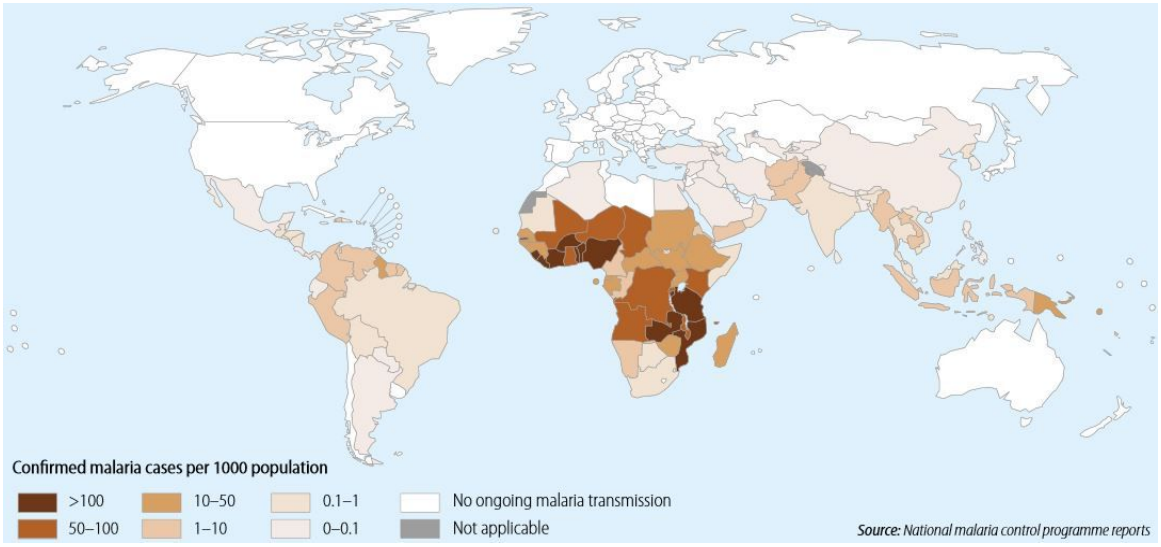

Figure 1: Countries with ongoing malaria transmission. Confirmed malaria cases per 1000 inhabitants in 2013. From World Malaria Report 2014 – World Health Organization (WHO 2014).

1.1.2 Human malaria parasites

Malaria is caused by intracellular protozoan parasites of the genus *Plasmodium*. Five species are known to infect humans, *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium ovale*, *Plasmodium malariae* and *Plasmodium knowlesi,* differing considerably in their geographic distribution, morphology and pathogenicity (Cox-Singh and Singh 2008, Garnham 1966, White 2008). Among *Plasmodium* species infecting humans, *P. malariae* and *P. ovale* only play a minor role. *P. vivax* has the widest geographic distribution, particularly in South America and Asia, and frequently causes acute febrile disease which rarely leads to death. *P. falciparum* is the most virulent species and accounts for the vast majority of severe cases and malaria-associated deaths, especially in sub-Saharan Africa (Gething et al. 2012, Snow et al. 2004, Stevenson and Riley 2004).

1.1.3 Parasite life cycle

Plasmodium parasites have a complex life cycle including a host change from a mosquito vector to a vertebrate host. Basically, it can be divided into three main stages, the sexual development inside the vector as well as the asexual liver and erythrocytic stage inside the human host. Transmission to humans only occurs through bites of female *Anopheles* mosquitoes injecting infective sporozoites into the host's skin during blood feeding (Figure 2). The majority of sporozoites is not directly injected into the blood circulation (Matsuoka et al. 2002, Medica and Sinnis 2005). They move randomly in the dermis until they encounter a blood vessel which they penetrate to enter the bloodstream (Amino et al. 2006). Once inside the bloodstream, sporozoites reach the liver, migrate through several cells before invading a hepatocyte where they undergo a first round of asexual replication (Mota et al. 2001). Inside the hepatocyte, each sporozoite multiplies and differentiates into thousands of merozoites in a process called exo-erythrocytic schizogony. Merozoites released from hepatocytes in parasite-filled vesicles, called merosomes, enter the bloodstream via the sinusoid lumen (Sturm et al. 2006). The duration of this exo-erythrocytic phase and the number of merozoites produced in each infected hepatocyte is species-specific. In the case of *P. falciparum,* about 40 000 merozoites are released five to seven days after infection (Despommier and Knirsch 2000). In *P. vivax* and *P. ovale* infections, some sporozoites remain in a latent stage that persists in hepatocytes, so-called

hypnozoites, responsible for relapses of the disease after months or years (Cogswell 1992). Released into the bloodstream, merozoites invade circulating red blood cells (RBCs) in a complex process requiring several specific molecular interactions, initiating the erythrocytic stage of malaria. Inside the RBC, another round of asexual replication starts and merozoites develop through ring (early stage), trophozoite and schizont stage (late stages). The erythrocytic cycle is completed when mature schizonts rupture and release 16-32 merozoites which continue to infect further RBCs (Cowman and Crabb 2006). Repeated cycles of intra-erythrocytic replication lead to an exponential growth of the parasite population within the human host. The duration of the erythrocytic cycle (24, 48 or 72 hours) as well depends on the parasite species; *P. falciparum* development takes approximately 48 hours (Coatney 1971). Some parasites differentiate into sexual forms, female macrogametocytes and male microgametocytes, which are ingested by *Anopheles* mosquitoes while taking a blood meal. Inside the mosquito's mid-gut, gametocytes differentiate into gametes. Male gametocytes undergo a process called exflagellation, resulting in up to eight microgametes that fertilize female macrogametes to form a zygote. The zygote differentiates into an ookinet which penetrates the mosquito's midgut wall transforming into an oocyst in which thousands of sporozoites develop. Upon oocyst rupture sporozoites are released into the hemocoel and migrate into the mosquito's salivary glands, ready to be transmitted to another human host (Despommier and Knirsch 2000, Tuteja 2007).

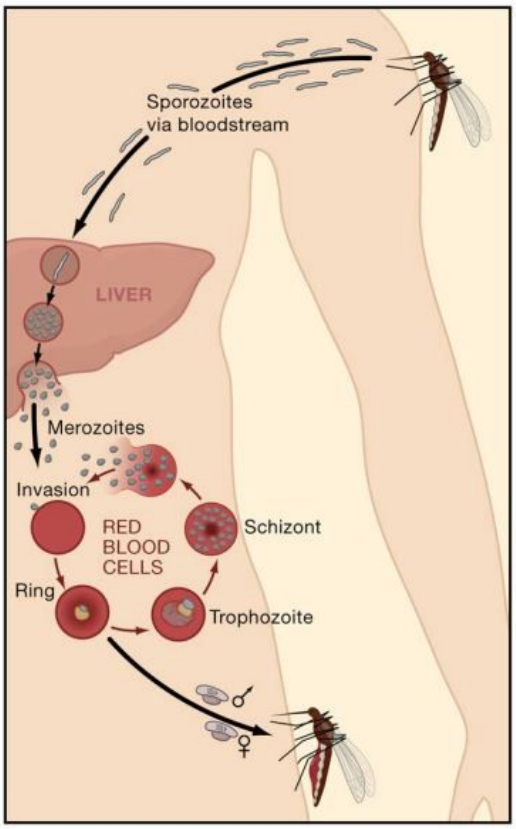

Figure 2: Schematic overview of the *P. falciparum* life cycle in the human host. Showing sporozoites being injected by an infected mosquito while taking a blood meal and the asexual replication in the liver as well as in red blood cells. From (Cowman and Crabb 2006).

1.1.4 Malaria symptoms and pathogenesis

The clinical outcome of malaria depends on various factors including parasite and host genetic factors, previous exposure and immune status of the host, but also social and geographic factors (Miller et al. 2002, Schofield and Grau 2005). Human infection with *Plasmodium* therefore leads to a wide spectrum of clinical manifestations, ranging from asymptomatic infection to severe, life-threatening disease or death. Malaria-associated symptoms and pathology are exclusively caused by the blood stages of the parasite (Haldar et al. 2007). Hence, the onset of clinical symptoms in non-immune individuals correlates with the appearance of parasites in the blood, in case of *P. falciparum* usually 6-14 days after infection (Trampuz et al. 2003). Classical malaria symptoms include cyclical patterns of fever and chills (paroxysms), headache and vomiting which correspond to high levels of pro-inflammatory cytokines in the blood circulation like tumor necrosis factor α (TNF-α), interleukin 12 (IL-12) and interferon γ (IFN-γ) (Gazzinelli et al. 2014, Schofield and Grau 2005). The periodicity of paroxysms is associated with the cyclic rupture of infected RBCs (iRBCs) and release of merozoites into the bloodstream. Fever in falciparum malaria may occur every 48 h, but is generally irregular showing no specific periodicity or is even continuous (Crutcher and Hoffman 1996, Haldar et al. 2007, Weatherall et al. 2002). Almost all severe or fatal syndromes of malaria are caused by *P. falciparum* involving severe anemia, organ failure, respiratory distress or cerebral malaria as major complications (Trampuz et al. 2003). Unlike other *Plasmodium* species infecting humans, severe pathology in *P. falciparum* infections is caused by the adherence of late-stage iRBCs to microvascular endothelium in various organs such as brain, kidney, liver, lung or placenta (Haldar et al. 2007). This process, called sequestration, avoids spleenic clearance of late parasite stages from the blood circulation being essential for parasite survival (Langreth and Peterson 1985). Sequestered parasites obstruct the blood flow causing hypoxia in the surrounding tissue and simultaneously induce systemic or local microvascular inflammation leading to enhanced leukocyte recruitment, platelet activation and endothelial cell damage (Figure 3) (Turner et al. 1994, Weatherall et al. 2002). *P. falciparum* modifies the surface of its host cell by remodeling the cytoskeletal structure and exporting parasite-encoded proteins to the RBC surface forming electron-dense knob-like protrusions about 16-18 hours after RBC invasion (Gardner J. P. et al. 1996). Cytoadhesion of iRBCs to microvascular endothelium but also to monocytes, platelets and uninfected RBCs,

forming so-called rosettes, is mainly mediated by *P. falciparum* erythrocyte membrane protein 1 (PfEMP1). PfEMP1 is a family of antigenically variant adhesion molecules expressed in association with knob-like structures on the iRBC surface interacting with a variety of receptors on host cells including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), chondroitinsulfate A (CSA), thrombospondin, CD36 and complement receptor 1 (CR1) (Horrocks et al. 2005, Newbold et al. 1999). PfEMP1 proteins are encoded by the *var* gene family which is extremely diverse and responsible for the clonal antigenic variation of *P. falciparum* (Baruch et al. 1995, Su et al. 1995). Each parasite genome contains approximately 60 distinct *var* genes encoding for PfEMP1 variants. However, only a single PfEMP1 variant is expressed on the host cell surface at a given time. The molecular basis of this mutually exclusive *var* gene expression is not yet completely understood (Chen et al. 1998, Guizetti and Scherf 2013). Switching between the expression of different *var* genes accounts for the parasite's immune evasion and also modifies the binding specificity of iRBCs (Roberts et al. 1992). Being responsible for both, cytoadhesion and antigenic variation, PfEMP1 is considered as the major virulence factor of *P. falciparum*. Although sequestration of iRBCs is associated with malaria pathology, in most cases binding of iRBCs to host endothelium does not lead to severe disease and occurs also in individuals with uncomplicated malaria. Besides adhesion, high systemic levels of pro-inflammatory cytokines have been strongly correlated with disease severity and are assumed to substantially contribute to malaria pathology. Therefore, it is widely accepted that severe

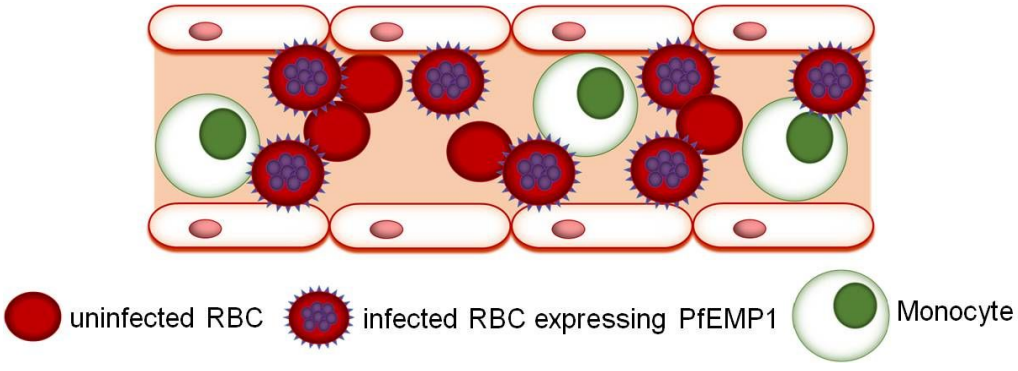

Figure 3: Schematic overview of processes involved in malaria pathogenesis. Cytoadherence of iRBCs leads to parasite sequestration in various organs such as heart, brain, liver, subcutaneous tissues and placenta. Sequestered iRBCs obstruct the blood flow and activate microvascular endothelial cells expressing enhanced levels of adhesion molecules (ICAM-1, VCAM-1) which leads to the sequestration of further iRBCs and their platelet-mediated clumping. Co-sequestration of uninfected RBCs (rosetting) and monocytes further impairs the blood flow and causes hypoxia in the surrounding tissue. Activated monocytes produce pro-inflammatory cytokines leading to local or systemic inflammation and damage of the microvascular endothelium.

malaria, at least partially, is an immune-mediated disease (Grau et al. 1989a, Kwiatkowski et al. 1990, Shaffer et al. 1991, Stevenson and Riley 2004). However, why only a minority of *P. falciparum*-infected individuals develops severe, life-threatening disease (1-2 %) and which molecular processes are involved in progression from uncomplicated infection to severe disease manifestation is largely unclear (Greenwood B. and Mutabingwa 2002, Stevenson and Riley 2004). Currently, it is thought that severe pathology is caused by a combination of several factors, rapid parasite growth leading to damage and depletion of RBCs (infected and uninfected) by spleenic macrophages, obstruction of blood vessels by sequestered parasites and excessive release of pro-inflammatory cytokines (Gazzinelli et al. 2014, Weatherall et al. 2002).

1.2 Malaria and the human immune system

The interaction of *P. falciparum* and the human immune system involves several components of the innate and adaptive immune system and is complex due to the different developmental stages of the parasite, its immune evasion inside the red blood cell and its high antigen variation (*var* genes). A protective immunity to malaria is gradually acquired only through continuous exposure and repeated infections over a period of several years and probably never results in sterile immunity. In areas with high and stable *P. falciparum* transmission, adults continue to experience infections with low parasite levels, but rarely develop severe disease and often remain asymptomatic (clinical immunity). Little is known about the mechanisms of naturally acquired immunity to malaria, but the protection seems to be primarily antibody-mediated, in particular against strain-specific PfEMP1 variants (Hviid 2005, Langhorne et al. 2008). Innate and adaptive immune mechanisms limit parasite replication and prevent severe pathology. However, they usually fail to eliminate the infection completely leading to continous low parasite densities which might persist for several months (Franks et al. 2001).

1.2.1 Innate immune response in malaria – protection or pathology?

Driven by the need to develop an effective vaccine, research has predominantly focused on the adaptive immune response in malaria, while the role of the innate immune system has been neglected for a long time. Nevertheless, it is known today that an early and adequate inflammatory innate response is essential for the initial control of parasite

blood-stage replication and the development of an effective adaptive immune response required for infection clearance (Good et al. 2005, Stevenson and Riley 2004).

Several *in vitro* studies showed that iRBCs induce a rapid TNF-α, IL-12p40, IFN-γ and IL-10 response in peripheral blood mononuclear cells (PBMCs) of malaria-naïve donors (Scragg et al. 1999, Walther et al. 2006). Live late stage-infected RBCs were found to induce higher innate responses than dead parasites or parasite lysate suggesting that either a direct contact with intact iRBCs or the process of iRBC rupture is essential for complete activation of innate immune cells (Artavanis-Tsakonas K. and Riley 2002, Hensmann and Kwiatkowski 2001). Early inflammatory responses in *P. falciparum* infections involve monocytes, macrophages, dendritic cells, γδ Tcells and NK cells (Artavanis-Tsakonas K. and Riley 2002, Stanisic et al. 2014). An early production of IFN-γ by NK cells seems to be crucial for the development of a protective immune response (Mohan et al. 1997). IFN-γ production of NK cells in *P. falciparum* infections is dependent on IL-12 and to some extent on IL-18, but also requires direct contact with iRBCs as well as with monocytes and dendritic cells (Newman et al. 2006). Mononuclear phagocytes comprising monocytes, macrophages and dendritic cells, play a central role in innate defense mechanisms in malaria. Besides the clearance of iRBCs from the bloodstream by phagocytosis, mononuclear phagocytes are able to present antigens, generate nitric oxide and are an important early source of cytokines and chemokines (Mac-Daniel and Menard 2015).

Innate cytokine responses induced by *P. falciparum* are heterogeneous and cytokine levels differ considerably among individuals (Artavanis-Tsakonas K. and Riley 2002, Walther et al. 2006). After experimental infection of malaria-naïve individuals with *P. falciparum* sporozoites, increased levels of circulating TNF-α, IL-12 and IFN-γ correlated with a faster control of parasite blood-stage replication, but on the other hand with a more rapid onset of clinical symptoms (Walther et al. 2006). Consistently, excessive production of pro-inflammatory cytokines has been clearly correlated with disease pathology in numerous epidemiological studies (Day et al. 1999, Grau et al. 1989b, Kern et al. 1989). Cerebral malaria has been linked to high levels of circulating TNF-α in particular, while severe malaria anemia has been associated with high levels of TNF-α and low levels of IL-10 (Kurtzhals et al. 1998, Kwiatkowski et al. 1990, Othoro et al. 1999). Direct effects of those pro-inflammatory cytokines are fever, impaired erythropoiesis and increased expression of adhesion molecules on endothelial cells leading to enhanced parasite sequestration (Awandare et al. 2011, Kwiatkowski et al.

1989, Udeinya and Akogyeram 1993). The anti-inflammatory and immunosuppressive properties of IL-10 might act as negative feedback by down regulating the pro-inflammatory response to *P. falciparum* (Ho et al. 1998).

Activation of innate immune cells is triggered by the binding of parasite- (pathogen-associated molecular patterns-PAMPs) and host cell-derived (damage-associated molecular patterns-DAMPs) components to receptors recognizing conserved structures, so-called pattern recognition receptors (PRRs). Three extensively studied *P. falciparum* PAMPs are, glycosylphosphatidylinositol anchors (GPI), glycolipids associated with cell membranes, plasmodial DNA motifs and hemozoin, a crystalline by-product resulting from hemoglobin digestion of the parasite during its intraerythrocytic development (Gazzinelli et al. 2014). DAMPs are endogeneous components that are released from damaged, stressed or dying cells. Only recently, uric acid, a by-product of the purine metabolism, microparticels shedding from the plasma membrane of iRBCs, and heme were identified as malaria DAMPs (Couper et al. 2010, Figueiredo et al. 2007, Gallego-Delgado et al. 2014). Besides Toll-like receptors (TLRs), cytosolic NOD-like receptors (NLRs), RIG-I-like receptors and inflammasomes (NLRP3, AIM2) are activated by parasite-derived components triggering distinct signaling pathways involved in inflammatory responses and pathogen clearance (Gazzinelli et al. 2014). However, despite numerous recent investigations, complex molecular interactions between iRBCs or parasite-derived components and innate immune cells in *P. falciparum* infections are far from being completely understood. Moreover, interactions among different innate cell populations as well as their particular role in host defense mechanisms and pathogenesis in malaria remain largely unclear. A rapid and strong pro-inflammatory response seems to be essential allowing the host to control infection until an adaptive response is generated. But at the same time excessive pro-inflammatory responses might promote progression to severe disease. Therefore, the ability to control inflammatory responses seems to be crucial. Although cytokine responses in malaria have been studied extensively, our understanding of the regulatory mechanisms required to maintain a balanced innate responses during *P. falciparum* infections is limited (Artavanis-Tsakonas K et al. 2003a, Stevenson and Riley 2004). In the light of emerging resistant *Plasmodium* strains and the lack of an effective vaccine, a better knowledge on the innate immune response to *P. falciparum* infections is crucial for novel intervention strategies.

1.2.2 Role of monocytes in malaria

Monocytes are mononuclear leukocytes, derived from myeloid progenitor cells in the bone marrow. After their release into the peripheral blood, monocytes circulate for several days, migrate into tissues such as spleen, liver, lymph nodes or subcutaneous tissue, and differentiate into resident macrophages and dendritic cells. In addition to the bone marrow, the spleen is an important reservoir for undifferentiated monocytes which are mobilized upon inflammation (Swirski et al. 2009). Monocytes have at least three major functions, phagocytosis of invading pathogens and cell debris, antigen presentation and immunomodulation through secretion of cytokines and chemokines (Dale et al. 2008). In human peripheral blood, monocytes are a heterogeneous cell population varying morphologically, in size and degree of granularity as well as functionally and constitute 5-10 % of blood leukocytes (Cohn ZA 1965, Nichols BA 1971, van Furth 1968). Monocytes are divided into two main subpopulations, $CD14^+$ monocytes, which can be further subdivided in 'classical' $CD14^+$ $CD16^+$ and 'inflammatory' $CD14^+$ $CD16^-$ monocytes, and 'patrolling' $CD14^{low}$ $CD16^+$ monocytes. $CD14^+$ monocytes are recruited to sites of inflammation and infection secreting inflammatory cytokines and migrating into tissues where they differentiate into macrophages and dendritic cells. Whereas, $CD14^{low}$ monocytes reside in the blood circulation surveying the integrity of the endothelium (Ginhoux and Jung 2014, Ziegler-Heitbrock and Hofer 2013). Being part of the innate immune system, monocytes are among the first line of defense against invading pathogens and play a crucial role during inflammation, but at the same time contribute to many pathological processes (Ginhoux and Jung 2014). During acute episodes of malaria, monocytes are a key source of pro-inflammatory mediators and crucial for the clearance of parasites from the bloodstream. However, their role in host defense mechanisms against *P. falciparum* and malaria pathogenesis still remains poorly understood.

Clearance of iRBCs through phagocytosis by circulating monocytes, besides spleenic macrophages, is important to control infection and limit initial parasite replication (Gazzinelli et al. 2014). Uptake of iRBCs is mediated either through a direct contact between iRBCs and monocytes (non-opsonic) or through complement factors and antibodies (opsonic). Antibodies specific to surface antigens of iRBCs or merozoites bind to Fcγ receptors on monocytes (CD32, CD64) (Tebo et al. 2002). RBC lysis during *P. falciparum* infection results in the release of hemoglobin and its breakdown products into the bloodstream activating the alternative complement pathway which leads to the

binding of complement component C3b to the surface of iRBCs (Pawluczkowycz et al. 2007). Phagocytosis of complement-opsonized early-stage iRBCs is mediated via complement receptor 1 (CR1) (Ayi et al. 2004, Turrini et al. 1992). The scavenger receptor CD36, one of the major receptors involved in parasite sequestration, has been identified to mediate the non-opsonic uptake of iRBCs by monocytes being particularly important in malaria-naïve individuals lacking parasite specific antibodies. This process probably involves binding of CD36 to PfEMP1 on the iRBC surface and therefore is dependent on the parasite adhesion-phenotype (McGilvray et al. 2000, Serghides et al. 2006).

Cytokine secretion is triggered by FcγR-mediated phagocytosis and the recognition of iRBCs or parasite-derived components released into the blood circulation upon iRBC rupture by pattern recognition receptors (PRRs) such as Toll-like receptors (TLRs) (Gowda D. C. 2007, Parroche et al. 2007, Zhou et al. 2012). Glycosylphosphatidylinositol (GPI) anchors of *P. falciparum* and hemozoin, were shown to induce the release of pro-inflammatory cytokines such as TNF-α, IL-1ß and MIP-1α (macrophage inflammatory protein-1α) by monocytes (Krishnegowda et al. 2005, Pichyangkul et al. 1994, Sherry et al. 1995). But at the same time, hemozoin persisting undigested in monocytes after phagocytosis, was reported to have immunosuppressive properties leading to monocyte dysfunction including impaired repeated phagocytosis, reduced expression of surface molecules (MHC II, ICAM-1) and diminished antigen presentation (Schwarzer et al. 1998, Schwarzer et al. 2001).

High serum concentrations of pro-inflammatory cytokines have been clearly associated with severe malaria, especially with cerebral malaria and severe malarial anemia (Day et al. 1999, Grau et al. 1989c, Kern et al. 1989, Lyke et al. 2004). Only recently, monocytes, besides γδ T cells, were identified as main source of those cytokines and chemokines of the early inflammatory response associated with severe disease manifestaion (Stanisic et al. 2014). The pathophysological and immunoregluatory role of monocyte-derived cytokines in *P. falciparum* infections is not yet fully understood. Though, a role of monocytes in the pathogenesis of cerebral malaria would be consistent with their intravascular accumulation in brain capillaries of cerebral post-mortem autopsies associated with sequestered iRBCs. These adherent monocytes often appear to occupy most of the capillary volume, might further reduce the already obstructed blood flow and contribute to local inflammation and vascular damage (Dorovini-Zis et al. 2011, Patnaik et al. 1994). Severe malarial anemia is marked by a pronounced decrease in the number of

RBCs (hemoglobin concentration < 8 g/dl) as a result of increased RBC loss by enhanced phagocytosis of altered RBCs (iRBCs and uRBCs) by activated mononuclear phagocytes and iRBC rupture, but also due to suppressed erythropoiesis. With their suppressive effect on erythropoiesis, monocyte-derived pro-inflammatory cytokines TNF-α, IL-6 and IL-1ß are thought to substantially contribute to the development of severe malarial anemia. Furthermore, hemozoin was also found to directly inhibit erythropoiesis and elevated levels of hemozoin-containing monocytes in the blood circulation of *P. falciparum*-infected individuals were associated with severe malarial anemia (Awandare et al. 2011, Awandare et al. 2007, Casals-Pascual et al. 2006). Although there is increasing awareness of a central role of monocytes in malaria pathogenesis, the knowledge on the monocyte phenotype as well as on molecular interactions triggering inflammatory responses of monocytes during *P. falciparum* infection is limited.

1.3 Toll-like receptors

1.3.1 Toll-like receptors in innate immunity

Toll-like receptors (TLRs) play a crucial role in the detection of pathogens by the innate immune system. To date, ten functional TLRs have been described in humans which differ in their ligand specificity, expression pattern and also in the target genes they induce. As germline-encoded, so-called pattern-recognition receptors (PRRs), TLRs recognize conserved pathogen-associated molecular patterns (PAMP) derived from various pathogens, including bacteria, viruses and parasites, and damage-associated molecular patterns (DAMPs), endogenous intracellular molecules released from damaged or necrotic cells (Janeway and Medzhitov 2002, Tsan and Gao 2004, Wagner 2006). Based on their localization TLRs can be divided into two subfamilies, intracellular TLRs localized in endosomes (TLR3, TLR7, TLR8 and TLR9) and TLRs expressed on the cell surface (TLR1, TLR2, TLR4, TLR5, TLR6 and TLR 10) (Akira et al. 2006, Medzhitov 2007). Intracellular TLRs recognize nucleic acids (DNA or RNA) from pathogens, e.g. viral double-stranded (ds) RNA (TLR3) or CpG (cytosine and guanine separated by one phosphate)-rich unmethylated DNA (TLR9), as well as nucleic acids derived from damaged autologous cells (Blasius and Beutler 2010, Kumar H. et al. 2009, Marshak-Rothstein 2006). TLRs on the cell surface mainly recognize microbial membrane components, e.g. bacterial lipopolysaccharide (LPS) (TLR4), flagellin (TLR5) or

lipopeptides (TLR2 associated with TLR1 or TLR6) (Akira et al. 2006, Kawasaki and Kawai 2014). All TLRs are type I trans-membrane proteins consisting of an ectodomain containing leucine-rich repeats mediating PAMP or DAMP recognition, a trans-membrane domain and a cytoplasmic Toll/Interleukin-1 receptor (TIR) domain that is required for downstream signaling (Hashimoto et al. 1988, Medzhitov et al. 1997, Rock et al. 1998). Upon ligand binding, TLRs form homo- or heterodimers interacting with co-receptors or accessory molecules, triggering the activation of signaling pathways leading to the induction of genes involved in inflammatory responses (Botos et al. 2011). Due to the recruitment of distinct adaptor molecules to the TIR domain of different TLRs, including MyD88 (myeloid differentiation primary response gene 88), TIRAP (TIR-associated protein), TRIF (TIR-domain-containing adaptor protein-inducing IFN-ß) and TRAM (TRIF-related adaptor molecule), individual TLRs induce different biological responses by activating different signaling pathways. MyD88 is used by all TLRs except TLR3 and induces inflammatory cytokines via NF-κB (nuclear factor κB) and MAPKs (mitogen-activated protein kinases). While TRIF, recruited to TLR3 and TLR4, activates alternative pathways leading to type I interferon and inflammatory cytokine responses mediated through the transcription factors IRF3 (interferon regulatory factor 3) and NF-κB (Akira et al. 2006, Yamamoto et al. 2002). Although TLRs are critical for host defense, it has become apparent that inappropriate TLR responses contribute to acute and chronic inflammation, as well as to systemic autoimmune diseases (Kawai and Akira 2010).

1.3.2 TLR sensing of malaria parasites

TLRs are considered as important mediators of pro-inflammatory innate immune responses in malaria. Several studies reported that TLR2, TLR4, TLR9 and recently also TLR7, recognize *P. falciparum* derived components and trigger pro-inflammatory responses.

The glycosylphosphatidylinositol (GPI) anchors of *P. falciparum* released at schizont rupture are recognized by TLR2 and to a lesser extent by TLR4 triggering the induction of pro-inflammatory responses (Figure 4) (Krishnegowda et al. 2005, Schofield and Hackett 1993, Zhu et al. 2005). GPIs are a group of glycolipids expressed ubiquitously in eukaryotes whose main role is to anchor functional proteins to the cell surface. Regarding the lipid moieties and sugar residues attached to the conserved glycan core which is

linked to phosphatidylinositol, GPIs vary widely among different species (Ferguson et al. 1999). In parasitic protozoa, GPIs are expressed at high levels, and the majority of *P. falciparum* GPIs (synthesized only at the mid to late trophozoite stage) occurs as free glycolipid in the parasite membrane without a protein anchored to it (ratio 5:1) (Naik et al. 2000a, Naik et al. 2000b).

TLR2 is expressed on the cell surface, forming heterodimers with either TLR1 or TLR6 recognizing a broad range of microbial components, such as bacterial lipopeptides, mycobacterial glycolipids and fungal polysaccharides (Lien et al. 1999, Takeda et al. 2003). *Plasmodium* GPIs are structurally heterogeneous by having either three (intact GPI) or two (derivative) fatty acyl chains (Durai et al. 2013, Gowda D.C. 2002). Analogous to differential recognition of di- and triacylated bacterial peptides, TLR2/TLR6 and TLR2/TLR1 heterodimers preferentially recognize diacylated (*sn-2-lyso*) and triacylated (intact and Man_3) GPIs, respectively. Since bacterial lipopeptides and GPI are completely different classes of compounds the fatty acid nature of the ligand irrespective of GPI or lipopeptide seems to determine the binding to either TLR2/TLR1 or TLR2/TLR6 (Krishnegowda et al. 2005). GPIs purified from *P. falciparum* cultures were shown to be potent activators of innate immune cells *in vitro*, inducing the expression of pro-inflammatory cytokines, such as TNF-α, IL-1, IL-12 and IL-6, and also nitric oxide synthase in macrophages and endothelial cells (Krishnegowda et al. 2005, Kumar S. et al. 2012, Schofield and Hackett 1993, Tachado et al. 1996). Additionally, GPIs lead to an enhanced surface expression of ICAM-1 (intercellular-adhesion molecule 1), VCAM-1 (vascular-adhesion molecule 1) and E-selectin on endothelial cells increasing the adherence of leukocytes and iRBCs associated with malaria pathology (Schofield et al. 1996). Signaling pathways induced by plasmodial GPIs via TLR2/TLR1 or TLR2/TLR6 heterodimers lead to a MyD88-dependent activation of MAPKs and NF-κB pathways inducing the expression of pro-inflammatory mediators, such as TNF-α and nitric oxide (NO) (Krishnegowda et al. 2005). Recently, the scavenger receptor CD36 was found to act as a co-receptor for TLR2/TLR6 recognizing diacylated bacterial lipopeptides and as well seems to be involved in TLR2/TLR6-mediated activation by *Plasmodium* GPIs (Hoebe et al. 2005, Kumar S. et al. 2012, Patel et al. 2007).

Structurally unrelated PAMPs such as LPS, viral and bacterial proteins and plasmodial GPIs were identified as TLR4 ligands (Bulut et al. 2002, Chow et al. 1999, Krishnegowda et al. 2005, Kurt-Jones et al. 2000). The best characterized TLR4 ligand is bacterial LPS. Their interaction is rather complex and involves several co-factors, including LPS binding

protein (LPB), a soluble plasma protein, CD14, a GPI-linked protein expressed on the surface of phagocytes, and MD2 (myeloid differentiation factor 2) which associates with the extracellular domain of TLR4 (Shimazu et al. 1999, Wright et al. 1990). Molecular interactions of plasmodial GPIs and TLR4 are less well characterized, though, it was shown that triacylated GPIs induce higher TLR4-mediated responses than diacylated GPIs (Krishnegowda et al. 2005). Besides a MyD88-dependent pathway, GPI signaling via TLR4 also involves a MyD88-independent cell activation pathway. In the case of LPS, this signaling pathway is triggered upon internalization of the ligand receptor complex in an endosomal compartment leading to the activation of a TRIF-dependent pathway, inducing a type I interferon response (Kawai and Akira 2010). But regarding the relatively low TLR4-mediated response compared to TLR2, the contribution of this pathway to the cell activation is thought to play only a minor role (Krishnegowda et al. 2005). Besides plasmodial GPI, microparticels derived from the plasma membrane of iRBCs were shown to stimulate macrophages via TLR4 signaling (Couper et al. 2010).

Although TLR9 has been clearly associated with malaria pathogenesis, the ligand triggering TLR9 responses in *P. falciparum* infections still remains controversial (Erdman and Kain 2011). Coban et al. found that hemozoin, a crystalline breakdown product of the parasites' hemoglobin degradation, released into the blood circulation during schizont rupture, directly activates cytokine responses in a TLR9-dependent manner (Coban et al. 2005). However, others observed that hemozoin crystals purified from *P. falciparum* cultures are coated with plasmodial DNA and proteins which are actually responsible for the induction of innate responses instead of the hemozoin crystal itself (Parroche et al. 2007, Wu et al. 2010). Since the stimulatory effect of hemozoin is lost after DNase treatment, Sharma et al. suggested that hemozoin works as a carrier molecule for plasmodial DNA to an endosomal compartment where TLR9 is located. *P. falciparum* DNA being extremely AT-rich (~ 80 %, ~ 300 CpG and ~ 6000 AT-rich motifs) seems to be an unusual target for TLR9 which generally recognizes double-stranded unmethylated CpG-rich motifs found in bacterial DNA (Gardner M. J. et al. 2002, Hemmi et al. 2000, Latz et al. 2004). Despite the high AT-content, the plasmodial genome also contains several "classical" CpG motifs which were shown to trigger TLR9 responses (Parroche et al. 2007). The phagocytosed hemozoin crystal disrupts the endosome and its content including plasmodial DNA is released into the cytosol. There, the AT-rich motifs trigger a TLR9-independent type I IFN response involving an unknown cytosolic sensor linked to the STING (stimulator of interferon genes), TBK1 (Tank-binding kinase 1), and IRF3

(interferon-regulatory factor 3)/IRF7 pathway (Hornung et al. 2008, Sharma et al. 2011). The hemozoin crystal itself activates the cytosolic NLRP3 (NOD-, LRR- and pyrin domain-containing 3) inflammasome leading to the maturation and secretion of IL-1ß (Dostert et al. 2009, Shio et al. 2009). Thus, phagocytosis of the DNA-hemozoin complex leads to the activation of a TLR9-dependent response in the endosome and a TLR9-independent response in the cytosol (Figure 4) (Sharma et al. 2011). However, Wu et al. obtained controversial results showing that a protein-DNA complex is responsible for TLR9-mediated responses, while hemozoin itself does not induce any cell activation (Wu et al. 2010). Only recently, a study showed that TLR7 might also play a role in mediating early innate immune responses in malaria suggesting *Plasmodium* RNA as a potential TLR7 ligand (Baccarella et al. 2013).

The role of TLRs in malaria remains controversial, although TLRs are crucial in initiating innate responses during *P. falciparum* infections, there is growing evidence that TLR activation is involved in excessive production of pro-inflammatory cytokines leading to immunopathology and severe malaria (Ropert et al. 2008).

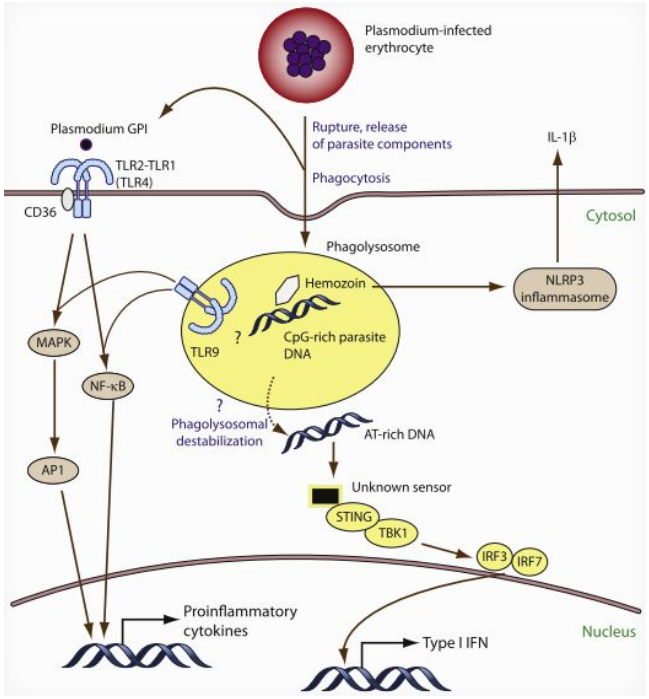

Figure 4: Innate sensing of *P. falciparum*-infected RBCs and parasite-derived components. *Plasmodium* GPI is recognized by TLR2 forming heterodimers with either TLR1 or TLR6 triggering the expression of pro-inflammatory cytokines. Phagocytosis of plasmodial DNA associated with hemozoin leads to the activation of a TLR9-dependent response in the endosome (CpG motifs) and a TLR9-independent response in the cytosol (AT-rich motifs), inducing pro-inflammatory cytokines and type I IFN responses, respectively. The hemozoin crystal activates the cytosolic NLRP3 inflammasome inducing maturation and secretion of IL-1ß. From (Erdman and Kain 2011).

1.3.3 Role of TLR polymorphisms in malaria susceptibility and outcome

Single nucleotide polymorphisms (SNPs) are variations of a given DNA sequence caused by the exchange of a single nucleotide being present at a frequency of > 1 % in a population. SNPs account for the vast majority of inter-individual genetic variation in humans. Several common SNPs in *TLR* genes were reported to alter susceptibility and outcome in various infectious and chronic inflammatory diseases (Schroder and Schumann 2005, Turvey and Hawn 2006). Their frequencies vary considerably among populations suggesting differential selection due to the exposure to distinct pathogens. Due to its pathogenicity, *P. falciparum* exerts a strong selective force on the human genome, and because of their potential role in malaria pathogenesis, genetic variations in TLRs might be under selective pressure in malaria-endemic regions (Tapping et al. 2007). In several epidemiological studies, common SNPs in TLRs being involved in the recognition of iRBCs or parasite-derived components, have been associated with malaria susceptibility and disease outcome (Leoratti et al. 2008, Mockenhaupt et al. 2006b).

A non-synonymous SNP in the intracellular TIR domain of TLR2 leading to a arginine to glutamine exchange at residue 753, *TLR2* R753Q (rs5743708, G > A), abolishes downstream signaling due to impaired tyrosine phosphorylation, TLR2/TLR6 dimerization and recruitment of adaptor proteins (Mal, MyD88) (Xiong et al. 2012). The variant *TLR2* 753Q is present only in a heterozygous state in ~ 10 % of Caucasians and is associated with increased susceptibility and disease progression in tuberculosis and pneumonia in patients with acute myeloid leukemia (Dalgic et al. 2011, Ogus et al. 2004, Schnetzke et al. 2014, Schroder et al. 2003). However, in populations from malaria endemic regions from Africa and India, the TLR2 variant was found to be completely absent. Impaired TLR2 responses might be disadvantageous in *P. falciparum* infections possibly explaining the negative selection of TLR2 variants (Bali et al. 2013, Mockenhaupt et al. 2006a, Mockenhaupt et al. 2006b).

The non-synonymous polymorphism in *TLR1* I602S (rs5743618, T > G), causing an isoleucine to serine transition, leads to a decreased cell activation upon stimulation with a synthetic TLR2/TLR1 agonist (Pam3CSK4) (Hawn et al. 2007, Johnson et al. 2007). A serine at position 602 interrupts the short trafficking motif and significantly diminishes trafficking of the receptor to the cell surface (Hart and Tapping 2012). *TLR1* I602S is the most common SNP affecting TLR signaling in Caucasians with a frequency of the 602S allele of 75 % (55 % homozygosity), but is only found in 25 % and 1 % of individuals of

African and Asian descent, respectively. The 602S allele is associated with decreased incidences of leprosy, tuberculosis and *Helicobacter pylori*-induced gastric disease (Johnson et al. 2007, Ma et al. 2007, Yang et al. 2013). In a study on malaria in pregnancy in Ghana, *TLR1* I602S was found at a very low frequency (2 %) only, possibly indicating the selective disadvantage of TLR1 deficiency in a malaria-endemic area (Hamann et al. 2010). In a Brazilian study comparing disease outcome among individuals being infected with *Plasmodium* (asymptomatic versus mild malaria), the *TLR1* 602S allele seemed to be a risk factor to develop disease (Leoratti et al. 2008). Another TLR1 variant, *TLR1* S248N (rs4833095), causing a non-synonymous amino acid change (serine to asparagine) in the extracellular domain of the receptor and leading to a decreased response upon agonist stimulation, was shown to increase the risk of placental malaria and maternal anemia (Hamann et al. 2010, Omueti et al. 2007).

The non-synonymous polymorphism *TLR6* P249S (rs5743810, C > T) encodes a proline to serine transition in the extracellular domain of TLR6 leading to conformational changes in the protein structure likely to affect binding of ligands or associated receptors (Hamann et al. 2013). Upon stimulation with a TLR2/6-agonist, the *TLR6* 249S allele shows reduced release of IL-6 and is associated with increased susceptibility to tuberculosis and asthma, but a reduced risk of atherosclerosis (Hamann et al. 2013, Ma et al. 2007, Shey et al. 2010, Tantisira et al. 2004). Concerning malaria, the *TLR6* 249S allele was associated with an increased risk to develop disease in Brazil (Leoratti et al. 2008). While the variant *TLR6* 249S allele is present in approximately 45 % of individuals of Caucasian ancestry, frequencies in African populations from malaria-endemic regions are very low (0-7 %) (Apinjoh et al. 2013, Hamann et al. 2013, Shey et al. 2010).

Two non-synonymous SNPs in *TLR4* D299G (rs4986790, A > G, aspartic acid to glycine transition) and T399I (rs4986791, C > T, threonine to isoleucine transition), causing hyporesponsiveness to LPS, have been associated with an increased risk of gram-negative bacterial infections and septic shock, but with a reduced risk of atherosclerosis (Arbour et al. 2000, Kiechl et al. 2002, Lorenz et al. 2002). Located in the extracellular domain of TLR4, the variants 299G and 399I are thought to induce structural changes resulting in a reduced affinity for ligands and/or co-receptors to TLR4 (Rallabhandi et al. 2006). While African populations show a high prevalence of 10-18 % of the *TLR4* 299G allele, the *TLR4* 399I allele is only found in 2 % of individuals. In Caucasian populations, both polymorphisms are found at frequencies of about 10 % with an extremely high level of

co-segregation (98 %) (Ferwerda et al. 2007, Schroder and Schumann 2005). In children being infected with *P. falciparum*, both TLR4 variants had no impact on the risk of infection *per se*, but were associated with severe malaria manifestation (Mockenhaupt et al. 2006b). In pregnant women with malaria, the *TLR4* 299G variant was shown to increase the risk of maternal anemia and low birth weight (Mockenhaupt et al. 2006a). The same study showed that a polymorphisms in the *TLR9* promoter region, *TLR9* T-1486C (rs1870884), increases the risk of low birth weight due to malaria during pregnancy. In patients with uncomplicated malaria, a strong association between high parasite densities and the *TLR9* -1486C variant was observed (Leoratti et al. 2008).
SNPs in *TLR* genes might influence host-pathogen interactions and affect TLR signaling leading to impaired pro-inflammatory cytokine responses in *P. falciparum* infections. A number of studies have already investigated the role of TLR pathways in malaria pathogenesis correlating TLR polymorphisms and disease susceptibility and manifestation. However, the functional role of TLRs in malaria pathogenesis as well as the underlying mechanism how TLR polymorphisms affect disease outcome still remain unclear.

1.4 Red blood cell polymorphisms and malaria

On the basis of ß-thalassemia, Haldane hypothesized more than 60 years ago that genetic variants are under positive selection due to their protection against severe disease and death caused by malaria (Haldane 1949). Today it is known that various genetic polymorphisms mediating protection against severest forms of falciparum malaria, such as hemoglobinopathies, are therefore found at high frequencies in malaria-endemic regions. Genetically-based resistance to malaria might be mediated either through impaired parasite invasion and intraerythrocytic development, modified antigen-expression on the surface of iRBCs or an altered innate and adaptive immune response of the host leading to enhanced parasite clearance (Smith et al. 2002, Taylor et al. 2013).
Hemoglobinopathies can be divided into two groups, the thalassemias, caused by a reduced production of the α- or β-globin chain, resulting in α- and β-thalassemia, respectively, and structural variants of hemoglobin (HbA) caused by the exchange of a single amino acid in the ß-globin chain, leading to hemoglobin S (HbS), HbC or HbE (Weatherall and Provan 2000). Approximately 25 % of the total variability of malaria susceptibility and disease manifestation in Africa is thought to be caused by genetic

variation of the human host (HbS and α-thalassemia account for about 2 % each) (Mackinnon et al. 2005). Sickle cell trait (HbAS) is the best-characterized genetic polymorphism associated with protection against falciparum malaria reaching prevalences of 30-40 % in sub-Saharan Africa (Rees et al. 2010). However, besides hemoglobinopathies, several other red blood cell variants were found to confer protection against malaria including genes encoding cell surface antigens, e.g. blood group O of the ABO blood group antigens, or enzymes involved in the cell metabolism, such as glucose-6-phosphate dehydrogenase (G6PD) (Bedu-Addo et al. 2014, Pathirana et al. 2005, Ruwende et al. 1995). Recently, *ATP2B4* has been described as a novel malaria protection locus and polymorphisms in *basigin* (*BSG*), a receptor essential for parasite invasion, might as well influence malaria susceptibility (Bedu-Addo et al. 2013, Crosnier et al. 2011, Timmann et al. 2012).

1.4.1 α-Thalassemia

1.4.1.1 Epidemiology and pathophysiology

α-Thalassemia is one of the most common monogenic gene disorders worldwide and is caused by a deficient synthesis of the α-globin chain of hemoglobin (Hb) (Harteveld and Higgs 2010, Weatherall DJ 1981). Like other hemoglobinopathies, α-thalassemia is highly prevalent throughout all tropical and subtropical regions and carriers are thought to be at selective advantage in areas where *P. falciparum* is or has been endemic (Flint et al. 1986, Mockenhaupt et al. 2004). α-Thalassemia affects up to 50 % of individuals in sub-Saharan Africa and reaches a prevalence of more than 80 % in Papua New Guinea and certain populations in Nepal and India (Allen et al. 1993, Fodde et al. 1988, Lell et al. 1999, Mockenhaupt et al. 2004, Modiano et al. 1991, Weatherall and Clegg 2001, Yenchitsomanus et al. 1986). In general, synthesis of α-globin is regulated by four α-globin genes, two (*HBA1* and *HBA2*) on each chromosome 16 (αα/αα). To date, about 128 molecular defects are known leading to a decreased synthesis of α-globin and α-thalassemia. Most commonly, α-thalassemia is caused by a deletion of either one (-α) or both genes (--) from the α-globin gene cluster of one chromosome, referred to as α^{+}-thalassemia or α^{0}-thalassemia, respectively. Non-deletional forms, caused by single point mutations, insertion or deletion of nucleotides are less common (Harteveld and Higgs 2010). Hemoglobin is a tetrameric molecule consisting of two α- and two β-globin chains

($\alpha_2\beta_2$, HbA). Considerably decreased α-globin production results in excess ß-globin chains which form unstable tetramers (HbH). High levels of unbound ß-globin chains associated with heme or iron induce the production of free radicals which interact with the cell membrane leading to severe oxidative damage of the cytoskeleton and the membrane in thalassemic RBCs (Rachmilewitz et al. 1985). Due to their premature depletion in the spleen caused by oxidative damage of the cell membrane, RBCs in severe α-thalassemia have a reduced half life resulting in moderate to severe hemolysis (Schrier et al. 1989).

The clinical picture of α-thalassemia depends on the molecular basis of the defect, is therefore very heterogeneous and the severity of symptoms usually correlates with the number of genes being affected. The hematological phenotype shows mild to severe anemia, a reduced mean corpuscular hemoglobin (MCH) and mean corpuscular volume (MCV) of red blood cells (Harteveld and Higgs 2010). Based on the clinical phenotype individuals are grouped into silent carriers, α-thalassemia trait and HbH disease. The silent carrier state is mostly caused by the deletion of one single α-globin gene (αα/-α) and is either symptomatically completely silent or associated with normal hemoglobin levels and moderate microcytosis (MCV $\leq$ 78 fl) and hypochromia (MCH $\leq$ 27 pg). Individuals with two deleted α-globin genes (-α/-α or αα/--) are classified as α-thalassemia trait and show mild to moderate microcytic, hypochromic anemia. HbH disease is found in individuals with only one functional α-globin gene left that usually produce less than 30 % of the normal amount of α-globin. Main characteristic of HbH are microcytic, hypochromic and hemolytic anemia with variable amounts of HbH ($ß_4$, ß-globin tetrames) and splenomegaly (enlarged spleen) (Galanello and Cao 2011, Harteveld and Higgs 2010).

In sub-Saharan Africa, a 3.7 kb deletion within the α-globin gene cluster ($-\alpha^{3.7}$) resulting in the deletion of one of the duplicated genes, is the common cause for α-thalassemia (α^+-thalassemia). Here, heterozygous individuals (αα/-α) show only slight hematological changes, but no symptoms, while homozygous individuals (-α/-α) have mild microcytic, hypochromic anemia (Mockenhaupt et al. 1999, Williams et al. 1996).

1.4.1.2 α-Thalassemia protects against severe malaria

Several epidemiological studies indicate that α-thalassemia mediates protection against severe falciparum malaria, in particular against severe malarial anemia (Allen et al. 1997, May et al. 2007, Mockenhaupt et al. 2004, Veenemans et al. 2008). Despite numerous investigations, the underlying cellular and molecular mechanisms involved still remain obscure. Impaired parasite invasion and growth in α-thalassemic RBCs, thereby limiting parasite densities, have been attributed to confer protection against severe malaria (Pattanapanyasat et al. 1999, Senok et al. 1997). Reduced growth rates were associated with a growth arrest in the schizont stage (schizont maturation arrest) and an accumulation of late parasite-stages in *P. falciparum* cultures (Senok et al. 1997). Microcytosis, enhanced production of oxidative radicals due to unpaired globin chains or increased membrane rigidity of α-thalassemic RBCs have been proposed as mechanisms responsible for defective parasite growth (Friedman 1979, Nurse 1979, Schrier et al. 1989). However, *in vitro* results on impaired parasite multiplication are contradictory (Glushakova et al. 2014, Luzzi et al. 1990, Luzzi et al. 1991a, Pattanapanyasat et al. 1999, Senok et al. 1997, Williams et al. 2002). Increased binding of malaria-specific antibodies to the surface of α-thalassemic iRBCs compared to non-variant iRBCs suggested an enhanced immune recognition and parasite clearance through phagocytosis (Luzzi et al. 1991a, Luzzi et al. 1991b, Yuthavong et al. 1988). Though, these *in vitro* findings are not consistent with epidemiological observations that α-thalassemia is not associated with reduced parasite densities *in vivo*, indicating that the trait might act through a mechanism other than affecting invasion and development of blood-stage parasites or their removal from the bloodstream (Allen et al. 1997, Mockenhaupt et al. 2004, Veenemans et al. 2008).

Further *in vitro* studies, observed impaired rosetting and cytoadherence, major factors of malaria pathology, of α-thalassemic iRBCs due to decreased expression of specific surface proteins (Carlson et al. 1994, Udomsangpetch et al. 1993). Correspondingly, Krause et al. recently proposed a reduced malaria pathogenicity in α-thalassemic individuals due to abnormal size and distribution of knob-like structures and *P. falciparum* erythrocyte membrane protein 1 (PfEMP1) expression on the surface of α-thalassemic iRBCs. Impaired surface expression of plasmodial antigens was shown to substantially diminish binding of α-thalassemic iRBCs to host cells. Cytoadherence of αα/-α and -α/-α ($-\alpha^{3.7}$) iRBCs was reduced by 22 % and 43 % to microvascular endothelial

cells (MVECs) and by 13 % and 33 % to monocytes, respectively. Impaired cytoadherence might lead to reduced microvascular inflammation and pro-inflammatory immune responses of monocytes potentially preventing severe manifestation (Figure 5) (Fairhurst et al. 2012, Krause et al. 2012). Consistent with epidemiological observations, this might explain how α-thalassemia mediates protection against severe manifestation without reducing the parasite load in *P. falciparum*-infected α-thalassemic individuals. Besides α-thalassemia, reduced cytoadherence to endotheliail cells and monocytes is also thought to mediate protection against severe falciparum malaria for further hemoglobin variants, HbS and HbC. Abnormal PfEMP1/knob expression on the surface of iRBCs might be a result of impaired surface trafficking of proteins due to increased oxidative damage of the RBC membrane or abnormalities in the actin network which is remodeled by *P. falciparum* in infected cells (Cyrklaff et al. 2011, Fairhurst et al. 2012). However, this needs to be further investigated.

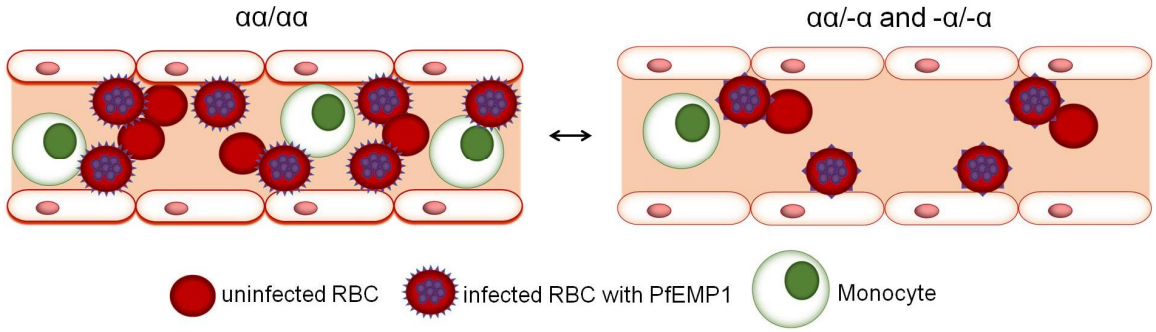

Figure 5: Model explaining how α-thalassemia might mediate protection against severe manifestations of falciparum malaria. In an individual with normal hemoglobin (αα/αα) being infected with *P. falciparum*, PfEMP1 is expressed in knob-like structures, covers the entire surface and mediates strong binding to microvascular endothelial cells. Sequestered iRBCs obstruct the blood flow and activate microvascular endothelial cells expressing enhanced levels of adhesion molecules (ICAM-1, VCAM-1) which leads to the sequestration of further iRBCs and their platelet-mediated clumping. Co-sequestration of uninfected RBCs (rosetting) and monocytes further impairs the blood flow and causes hypoxia in the surrounding tissue. Activated monocytes produce pro-inflammatory cytokines leading to local or systemic inflammation and damage of the microvascular endothelium. In α-thalassemic individuals (αα/-α or –α/-α) expression of PfEMP1 and knob-like structures on the iRBC surface appears considerably diminished leading to reduced receptor-ligand interactions. A weaker binding of iRBCs to microvascular endothelium and other blood components results in low levels of endothelial activation and inflammation and only minor obstruction of the blood flow causing uncomplicated disease (Fairhurst et al. 2012).

1.4.2 *ATP2B4*

In a genome-wide association study, *ATP2B4* encoding the ubiquitous plasma membrane Ca^{2+} ATPase type 4 (PMCA4) has recently been identified as a novel resistance locus for malaria. A SNP within *ATP2B4* (rs10900585, T > G) was strongly associated with reduced risk of severe malaria in children (Timmann et al. 2012). In addition, we could show that the *ATP2B4* variant is also protective against malaria in pregnancy as well as associated maternal anemia. The homozygous GG genotype showed a reduced risk of peripheral and also placental blood infection with *P. falciparum*, observed to a lesser extent also in heterozygous individuals (Bedu-Addo et al. 2013). However, a current multicenter study obtained controversial results with the derived G allele being a risk factor for severe malaria (MGEN 2014).

Alternative splicing of the primary transcript leads to several different isoforms of PMCA4 being expressed in a tissue-specific manner (Brini and Carafoli 2009, Strehler and Zacharias 2001). In RBCs, PMCA4 is the major calcium pump exporting Ca^{2+} and therefore defined as fine tuner of cytosolic Ca^{2+} concentrations in RBCs (Brini and Carafoli 2009, Stauffer et al. 1995). Being located in the second intron of *ATP2B4*, the SNP rs10900585 has no apparent function, but might affect mRNA splicing and hence influence protein expression and activity (Faustino and Cooper 2003). A decrease of Ca^{2+} concentrations in the parasitophorous vacuole, in which the parasite resides and develops, was shown to affect parasite maturation (Gazarini et al. 2003). Altered expression, activity or Ca^{2+} affinity of PMCA4 might change the intracellular Ca^{2+} homeostasis and therefore lead to an impaired intraerythrocytic growth and development of *P. falciparum* (Timmann et al. 2012). But, since calcium signaling plays an important role in the activation of many different cell types *ATP2B4* variants might affect malaria pathogenesis by an alternative mechanism. Modified cytosolic Ca^{2+} homeostasis might also lead to an altered activation of platelets and endothelial cells influencing platelet-mediated parasite killing and endothelial sequestration being a key factor of malaria pathogenesis (Bridges et al. 2010, McMorran et al. 2009).

1.4.3 *Basigin*

Several molecular interactions are involved in the invasion process of *Plasmodium* merozoites into host RBCs. In particular, the interaction of *P. falciparum* reticulocyte-binding homologue 5 (*Pf*RH5) and its receptor basigin (basic immunoglobulin superfamily) seems to be essential for RBC invasion of *P. falciparum,* irrespective of the parasite strain (Baum et al. 2009, Crosnier et al. 2011). Basigin (BSG), also called CD147 or leukocyte activation antigen M6, is a glycosylated transmembrane protein that belongs to the immunoglobulin superfamily and is involved in many biological processes, such as spermatogenesis and retinal development (Fadool and Linser 1993, Igakura et al. 1998). It exists in two splice isoforms, a long isoform with three immunoglobulin (Ig)-like domains (BSG-L) and a short form with just two Ig-like domains (BSG-S). Expressed on RBCs, BSG is also known as the Ok^a blood group antigen and BSG-S is thought to be the major isoform (Crosnier et al. 2011).

The BSG-*Pf*RH5 interaction was recently identified as a major determinant of *P. falciparum*-host tropism, since differential recognition of species-specific BSG variants (gorilla, chimpanzee) by *Pf*RH5 depends only on a very small number of amino acid residues (Wanaguru et al. 2013). Polymorphisms in *BSG* resulting either in reduced BSG surface expression or impaired receptor-ligand binding might therefore affect invasion efficacy of *P. falciparum* into human RBCs and consequently influence susceptibility and disease outcome in falciparum malaria. In a previous study, L90P and E92K (Ok^{a-}), two variant forms of BSG caused by non-synonymous SNPs, showed reduced binding affinities to *Pf*RH5. Additionally, parasite invasion efficacy into RBCs of donors with the E92K variant was diminished. E92K is the extremely rare Ok^{a-} blood group which to date was found in eight Japanese families only. The extreme low frequency and its restriction to Japanese individuals leads to the assumption that the E92K variant does not play a major role in malaria resistance (Crosnier et al. 2011, Spring et al. 1997). A study investigating the impact of common *BSG* polymorphisms on cerebral malaria in a Thai population, did not observe any association between BSG variants and cerebral malaria (Naka et al. 2014). However, it is likely that under the strong selective pressure of *P. falciparum* further *BSG* polymorphisms have evolved in populations of malaria-endemic regions. Only recently, a synonymous substitution in exon 2 of *BSG* (rs1803202, C > T, Asp-Asp) was found to be associated with malaria severity in an African population residing in a malaria-endemic area. The homozygous

CC genotype was described as a risk factor to develop severe malaria (Velavan TP, Institute for Tropical Medicine, University of Tübingen, data not published).

Several receptor-ligand interactions have been identified to mediate *P. falciparum* invasion of host RBCs, but so far only the interaction of BSG and *Pf*RH5 was found to be essential for RBC invasion irrespective of the *P. falciparum* strain and therefore provides a new target for therapeutic interventions (Crosnier et al. 2011).

1.5 Aims of the study

Malaria is still a major cause of disease and death in many developing countries. It remains largely ambiguous why only a minority of *P. falciparum*-infected individuals develops severe, life-threatening disease while others remain asymptomatic or show uncomplicated, febrile episodes. *P. falciparum* is thought to be the strongest selective force on the human genome in recent history. *Vice versa*, human genetic variations are considered to account for approximately 25 % of the total variability of malaria susceptibility and manifestation in Africa. Relative resistance against malaria conferred by predominantly RBC polymorphisms, is seen across endemic regions. Moreover, polymorphisms in immune-response-associated genes have been related to differential outcome of *P. falciparum* infections. However, the functional role of such genetic factors on malaria susceptibility or manifestation is largely obscure.

The present work aims to contribute to a better understanding of the impact of RBC and TLR polymorphisms on parasite growth and innate immune responses in malaria susceptibility and manifestation.
The major objectives of the investigations presented herein thus were:

(I) to ascertain whether RBC polymorphisms, namely α-thalassemia, as well as *ATP2B4* and *BSG* SNPs, affect host cell invasion and intraerythrocytic development of *P. falciparum*,

(II) to investigate whether common *TLR1*, *TLR2* and *TLR6* polymorphisms influence the activation of innate immune cells, particularly of monocytes, and their inflammatory response towards *P. falciparum*-infected RBCs (iRBCs), and

(III) to examine whether the above activation of the innate immune response is subject to variation due to the presence of α-thalassemia.

In the present work, therefore, (I) blood donors were screened for RBC polymorphisms (α-thalassemia, *ATP2B4* and *BSG* SNPs), (II) variant RBCs and control RBCs were infected with *P. falciparum*, and parasite growth was monitored over two parasite replication cycles *in vitro*. (III) PBMCs and monocytes isolated from human peripheral blood were co-cultured with iRBCs to assess whether (IV) TLR polymorphisms affect TLR signaling and the innate cytokine response to iRBCs and whether (V) α-thalassemic

iRBCs lead to an impaired activation of monocytes resulting in a reduced pro-inflammatory responses compared to normal iRBCs.

These investigations and respective results are meant to improve the understanding of the complex host-parasite interplay in malaria thereby providing the prerequisites for the development of novel interventions aimed against this deadliest parasitic disease of humans.

2 MATERIAL AND METHODS

2.1 Material

2.1.1 Reagents

Table 1: Reagents

Reagent	Manufacturing company, location
Agarose (LE)	Biozym Scientific, Hessisch Oldendorf, Germany
Albumax II	Gibco/Life Technologies, Darmstadt, Germany
Biocoll (1.077 g/ml, isotonic)	Biochrom AG, Berlin, Germany
BSA solution (30 %)	PAA, Pasching, Austria
CD14 MicroBeads, human	Miltenyi Biotec, Bergisch Gladbach, Germany
Citric acid monohydrate	Merck, Darmstadt, Germany
D(+)-Glucose monohydrate	Merck, Darmstadt, Germany
Dimethyl Sulfoxide (DMSO)	Sigma-Aldrich, Taufkirchen, Germany
DNA ladder (1 kb plus)	Life Technologies, Darmstadt, Germany
dNTP Mix (20 mM)	Solis BioDyne, Tartu, Estonia
D-Sorbitol	Sigma-Aldrich, Taufkirchen, Germany
Dulbecco's PBS solution (without $CaCl_2$, $MgCl_2$)	PAA, Gibco / Life Technologies
Dulbecco's PBS powder (without $CaCl_2$, $MgCl_2$)	Biochrom AG, Berlin, Germany
EDTA (0.5 M, pH 8.0)	Ambion / Life Technologies
Ethidium bromide solution (10 mg/ml)	Sigma-Aldrich, Taufkirchen, Germany
Hot FIREPol EvaGreen HRM Mix (5 x)	Solis BioDyne, Tartu, Estonia
FBS Gold	PAA, Pasching, Austria
Formaldehyde	Sigma-Aldrich, Taufkirchen, Germany
Gentamicin solution (50 mg/ml)	Sigma-Aldrich, Taufkirchen, Germany
Giemsa's azur eosin methylene blue solution	Merck, Darmstadt, Germany
HEPES	Sigma-Aldrich, Taufkirchen, Germany
HEPES buffer (1 M)	PAA, Sigma-Aldrich

Hot FIREPol DNA polymerase (5 U/µl)	Solis BioDyne, Tartu, Estonia
Human Serum Type AB (off the clot)	PAA, Pasching, Austria
Hydroethidine	Polysciences Europe, Eppelheim
Hydrogen peroxide 30 % (H_2O_2)	Merck, Darmstadt, Germany
Hypoxanthine	Sigma-Aldrich, Taufkirchen, Germany
L-glutamine (200 mM)	PAA, GE Healthcare, Freiburg, Germany
Lipopolysaccharide (*E.coli* Serotype 026:B6)	Sigma-Aldrich, Taufkirchen, Germany
Lysing buffer – BD Pharm Lyse	BD Biosciences, Heidelberg, Germany
Methanol	Carl Roth, Karlsruhe, Germany
$MgCl_2$ solution (25 mM)	Qiagen, Hilden, Germany
Pam2CSK4 (TLR2/TLR6 ligand)	InvivoGen, Toulouse, France
Pam3CSK4 (TLR2/TLR1 ligand)	InvivoGen, Toulouse, France
Penicillin-Streptomycin (10 000 U/ml)	PAA, Pasching, Austria
Q-Solution (5 x)	Qiagen, Hilden, Germany
RPMI 1640 (liquid)	PAA, GE Healthcare, Freiburg, Germany
RPMI 1640 (powder) + L-glutamine	Gibco / Life Technologies, Darmstadt, Germany
Sodium azide (NaN_3)	Merck, Darmstadt, Germany
Sodium bicarbonate ($NaHCO_3$)	Merck, Darmstadt, Germany
Sodium chloride (NaCl)	Merck, Darmstadt, Germany
Sulfuric acid (H_2SO_4)	Carl Roth, Karlsruhe, Germany
TBE buffer (10 x)	Gibco / Life Technologies, Darmstadt, Germany
Thermopol buffer (20 mM Mg^{2+})	New England BioLabs, Frankfurt a.M., Germany
TMB (3,3', 5,5'-Tetramethylbenzidine) tablets	Sigma-Aldrich, Taufkirchen, Germany
Trypan blue 0.5 % (w/v)	Biochrom AG, Berlin, Germany
Ultra-pure water (dH_20)	Biochrom AG, Berlin, Germany

2.1.2 Consumables

Table 2: Consumables

Consumables	Manufacturing company, location
BD Vacutainer, EDTA tubes, 2.0 ml (1.8 mg/ml)	BD, Heidelberg, Germany
BD Vacutainer, Heparin tubes, 10.0 ml (17 IU/ml)	BD, Heidelberg, Germany
MACS columns (MS/LS/LD)	Miltenyi Biotec, Bergisch Gladbach, Germany
Parasite gas (5 % CO_2, 5 % O_2, 90 % N_2)	Linde AG, Pullach, Germany
S-Monovette, CPDA-1, 8.5 ml	Sarstedt AG & Co., Nümbrecht, Germany

2.1.3 Antibodies

Table 3: Antibodies used for flow cytometry

Antibody	Labeling	Clone	Manufacturing company, location
Isotype control IgG1/IgG2a	FITC/PE	X40, X39	BD Biosciences, Heidelberg, Germany
mouse anti-human CD14	PE	M5E2	BD Biosciences
mouse anti-human CD40	FITC	5C3	BD Biosciences
mouse anti-human CD64	FITC	10, 1	BD Biosciences
mouse anti-human CD80 (B7-1)	FITC	L307.4	BD Biosciences
mouse anti-human CD86 (B70/B7-2)	FITC	2331 (FUN-1)	BD Biosciences
mouse anti-human TREM-1	PE	193015	R&D systems, Abingdon, UK

2.1.4 Buffers and media

Table 4: Buffers and media

Buffers and media	Components
P. falciparum culture medium	RPMI 1640 25 mM HEPES 2 mM L-glutamine 50 ng/ml gentamicin Albumax II solution (1 x)
Albumax II solution (10x) pH 7.0-7.4	5.2 g RPMI 1640 (+ L-glutamine, - $NaHCO_3$) 500 µl gentamicin (50 mg/ml) 2.98 g HEPES 1.67 g NaHCO3 1.0 g glucose 0.1 g hypoxanthine 25g Albumax II 500 ml dH_2O
PBMC co-culture medium	RPMI 1640 10 % FBS 10 mM HEPES 2 mM L-glutamine 50 U/ml penicillin-streptomycin
Monocyte co-culture medium	RPMI 1640 20 % human serum type AB 10 mM HEPES 2 mM L-glutamine 50 U/ml penicillin-streptomycin
FACS buffer	PBS 5 % FBS 10 mM sodium azide
FACS fix	PBS 10 % formaldehyde
MACS buffer	PBS 0.5 % FBS 2 mM EDTA

2.1.5 Commercial kits

Table 5: Commercial kits

Commercial kits	Manufacturing company, location
human CCL3/MIP-1α DuoSet ELISA	R&D systems, Abingdon, UK
human IL-1ß ELISA - antibody pairs	U-CyTech biosciences, Utrecht, The Netherlands
human IL-6 ELISA - antibody pairs	U-CyTech biosciences
human TNF-α ELISA - antibody pairs	U-CyTech biosciences
human IL-10 ELISA -antibody pairs	U-CyTech biosciences
old world monkey IL-12p40 - antibody pairs (with recombinant human IL12 standard)	U-CyTech biosciences
PCR Mycoplasma Test Kit I/C	PromoKine, Heidelberg, Germany
Pyrogent Plus Gel Clot LAL Single Test Kit, 0.06 EU/ml sensitivity	Lonza, Basel, Switzerland
QIAamp DNA Blood Mini Kit	Qiagen GmbH, Hilden, Germany

2.1.6 Human blood samples

2.1.6.1 Red blood cells

Donors with variant RBCs and respective non-variant controls were recruited at the Institute of Tropical Medicine and International Health Berlin within the framework of a multi-centre study on the prevalence of obesity and diabetes among Ghanaian migrants (www.rod-am.eu). A total of 158 volunteers were included in the study, 74 females (mean age ± SD, 40.7 ± 11.3 years) and 84 males (mean age ± SD, 46.6 ± 12.2 years). From all participants, 2 ml of venous blood was collected (BD Vacutainer, EDTA 1.8 mg/ml blood). Samples were stored at 4 °C and used for DNA extraction and infection with *P. falciparum* (2.2.5.1) within one week after collection. Besides α-thalassemia, SNPs in *ATP2B4* (rs10900585) and *BSG* (rs1803202), RBC donors were also screened for further RBC polymorphisms known to influence the outcome of malaria, namely, glucose-6-phosphate dehydrogenase (G6PD) deficiency, and the structural hemoglobin variants hemoglobin S and C (HbS, HbC). On the day of blood sampling, full blood counts were performed to determine hemoglobin levels and further standard hematological parameters using an Abbot CELL-DYN 1800 Hematology Analyzer. Informed consent was obtained from all participants and the study was approved by the Ethics Committee of Charité -

University Medical Center Berlin (*In vitro* immune response to malaria parasites infecting red blood cells (IREMA – EA1/018/13)).

2.1.6.2 PBMCs and monocytes

Human peripheral blood mononuclear cells (PBMCs) and monocytes were isolated either from buffy coats or fresh venous blood samples of healthy, malaria-naïve individuals. A total of 74 healthy, malaria-naïve individuals were recruited and genotyped for common SNPs in *TLR1*, *TLR2* and *TLR6* (Prof. Dr. Ralf Schumann, Institute of Microbiology and Hygiene, Charité-University Medical Center Berlin). Based on their *TLR1/TLR2/TLR6* genotype, 26 volunteers, 13 females (mean age ± SD, 33.2 ± 9.3 years) and 13 males (mean age ± SD, 34.9 ± 10.9 years), were selected to collect 60-70 ml of venous blood for PBMC and subsequent monocyte isolation (BD Vacutainer, heparin tubes, 17 IU/ml blood) (2.2.5.3.1). Informed consent was obtained from all volunteers. Ethical approval was given by the Ethics Committee of the Charité-University Medical Center Berlin. Buffy coats were obtained from the German Red Cross (DRK, Berlin), contained 70-90 ml of leukocyte enriched blood and were used to isolate monocytes for co-cultivation with *P. falciparum* cultures of α-thalassemic and control RBCs (2.2.5.3.2).

2.2 Methods

2.2.1 DNA isolation

Genomic DNA was isolated either from buccal swabs (2.1.6.2 PBMCs and monocytes) or from whole-blood samples (2.1.6.1 RBCs) using the QIAamp DNA Mini Kit or DNA Blood Mini Kit (Qiagen) according to the manufacturer's protocol.

2.2.2 Genotyping of blood donors

2.2.2.1 TLR genotyping of PBMC and monocyte donors

Screening for common functional polymorphisms in *TLR1*, *TLR2* and *TLR6* was performed with real-time PCR by measuring the melting temperature of double-stranded DNA (T_m) using two fluorescently labeled allele specific probes for signal generation. Technically, one probe is sequence-specific for the target while the other one is allele-

specific for a polymorphism. Binding of both probes is detected by fluorescence resonance energy transfer (FRET) between the two fluorochromes. Following PCR, the double-stranded (ds) DNA complex is heated and the melting temperature T_m is determined by a sharp decrease in fluorescence when the complex dissociates. Sequence matched probes melt at higher temperatures than mismatched probes and heterozygous samples show two melting peaks (Gibson 2006). *TLR1* I602S (rs5743618, T > G), *TLR2* R753Q (rs5743708, G > A) and *TLR6* P249S (rs5743810, C > T) genotyping was carried out under standard PCR conditions on a LightCycler 480 device (Roche Diagnostics) at the Institute of Microbiology and Hygiene of the Charité - University Medical Center Berlin (Prof. Dr. Ralf Schumann, Dr. Lutz Hamann). Melting curve analysis gave rise to melting peaks at 64.5 °C for *TLR1* 602I (wildtype) and 58.3 °C for *TLR1* 602S (variant), 61.8 °C for *TLR2* 753R (wildtype) and 66.3 °C for the variant *TLR2* 753Q, 61.0 °C for *TLR6* 249P (wildtype) and 52.0 °C for *TLR6* 249S (variant), respectively. Primers and probes used for TLR genotyping are listed in Table 6 (TIB MOLBIOL).

Table 6: Primers and probes used for TLR genotyping and allele-specific melting temperatures (T_m)

TLR1 I602S	rs5743618 (T > G)	(Johnson et al. 2007)	T_m (°C)
Primers:	fw	5'-TGTGACTACCCGGAAAGTTATAGA-3'	
	rev	5'-CCCAGAAAGAATCGTGCC-3'	
			TLR1 602I (T): 64.5 °C
Probes:		5'-CCATGCTGGTGTTGGCTGTGACTGTG-FL	*TLR1* 602S (G): 58.3 °C
		5'-LCRed640-CCTCCCTCTGCATCTACTTGGAT-P	
TLR2 R753Q	rs5743708 (G > A)	(Hamann et al. 2004)	
Primers:	fw	5'-AGTGAGCGGGATGCCTACT-3'	
	rev	5'-GACTTTATCGCAGCTCTCAGATTTAC-3'	*TLR2* 753R (G): 61.8 °C
			TLR2 753Q (A): 66.3 °C
Probes:		5'-CAAGCTGCAGAAGATAATGAACACCAAG-FL	
		5'-LCRed640-CCTACCTGGAGTGGCCCATGGACG-3'	
TLR6 P249S	rs5743810 (C > T)	(Hamann et al. 2013)	
Primers:	fw	5'-GAAAGACTCTGACCAGGCAT-3'	
	rev	5'-CTAGTTTATTCGCTATCCAAGTG-3'	*TLR6* 249P (C): 61.0 °C
			TLR6 249S (T): 52.0 °C
Probes:		5'-ACCAGAGGTCCAACCTTACTGAA-FL	
		5'-LCRed640- TTACCCTCAACCACATAGAAACGACTTGGA-P	

FL = fluorescein, LCRed640 = LightCycler Red-640, P = phosphate, fw = forward, rev = reverse

2.2.2.2 Screening for RBC polymorphisms

2.2.2.2.1 α-Thalassemia

A conventional PCR assay, modified after Liu et al., was applied to determine the -3.7 kb deletion type of α-thalassemia ($-\alpha^{3.7}$) which is common in West Africa (Ghana). PCR reaction was carried out in a volume of 25 µl containing 0.75 M Q-solution (5 x, Qiagen), 5 % DMSO, 0.2 mM dNTPs (20 mM, Solis BioDyne), 2 U Hotstart-Taq (5 U/µl, Solis BioDyne), 2 µl genomic DNA from RBC donors (2.2.1) in 1 x Thermopol buffer (20 mM Mg^{2+}, NEB). Primers used were *3.7 (F)* 5'-AAGTGCACCCCTTCCTTCCTCACC- 3', *3.7 (R1)* 5'-ATGAGAGAAATGTTCTGGCACCTGCACTTG-3' and *3.7 (R2)* 5' – TCCATCCCCTCCTCCCGCCCCTGCCTTTTC- 3' (TIB MOLBIOL) with a final concentration of 7.5 pmol for *3.7 (F)*, 2.5 pmol for *3.7 (R1)* and *3.7 (R2)*. The PCR reaction was performed with an initial heat activation of 5 min at 95 °C followed by 35 cycles of 94 °C for 15 sec, 63 °C for 30 sec and 72 °C for 2 min 30 sec and a final extension step of 72 °C for 10 min. PCR products were analyzed using gel electrophoresis (0.8 % agarose gel) with ethidium bromide staining. The expected fragment size for the wild-type allele (αα) was 2.2 kb and for the allele carrying the -3.7 kb deletion ($-\alpha^{3.7}$) 1.96 kb, respectively (Liu et al. 2000).

2.2.2.2.2 Further RBC polymorphisms

Genotyping of additional RBC polymorphisms was performed using either fluorescence-labeled hybridization FRET or high resolution melting dye assays followed by melting curve analysis. All assays were performed using standard PCR conditions on a LightCycler 480 platform (Roche Diagnostics). Primers and probes used for sequencing (TIB MOLBIOL) as well as the resulting melting temperatures (T_m) are given in Table 7. Hemoglobin SC (HbSC), HbCC and HbSS were grouped as sickle cell disease, while HbAC and HbAS were categorized as sickle cell trait.

For G6PD deficiency a high resolution melting dye assay was performed using unlabeled probes in combination with Eva Green (5 x Hot FIREPol EvaGreen HRM Mix, Solis Biodyne), a fluorescent dye binding to ds DNA, to identify the alleles resulting from two mutations at codon 202 (G > A) and 376 (A > G), Gd^{A-}, Gd^{A} and Gd^{B}. Donors were classified as G6PD normal (Gd^{B}, Gd^{A}, Gd^{B}/Gd^{B}, Gd^{B}/Gd^{A}, Gd^{A}/Gd^{A}), G6PD deficiency trait (heterozygous, Gd^{B}/Gd^{A-}, Gd^{A}/Gd^{A-}) and G6PD full defect (hemi-/homozygous, Gd^{A-}, Gd^{A-}/Gd^{A-}).

The synonymous SNP in exon 2 of *BSG* (rs1803202, C > T) was determined using the commercially available Light SNiP (rs1803202) BSG [D181D] assay (TIB MOLBIOL) following the manufacturer's protocol. For a 10 µl reaction mixture 1 µl of genomic DNA was added to 0.5 µl Reagent Mix (containing primers and probes), 2.0 µl LightCycler 480 Genotyping Master (Roche Diagnostics), 0.6 µl $MgCl_2$ (25 mM) and 6.9 µl of H_2O resulting in melting points (T_m) of 57.3 °C for the C and 65.6 °C for the T allele, respectively.

Table 7: Primers and probes for *ATP2B4, HbS*, *HbC* and *G6PD* genotyping giving allele-specific T_m

ATP2B4	rs10900585 (T > G)		T_m (°C)
Primers:	rs10900585 F	5'-TGGATCACTCACTGTTTTGTGTCA-3'	
	rs10900585 A	5'-AAATTAGCCACATGCCTGTAAC-3'	
			T: 64.0 °C
Probes:	rs10900585 [T]	5'-AGTCTCACTCTTTTTGCCCAG—FL	G: 56.5 °C
		5' -LC610-CAGGCTGGAGTGCAATGTGC-P	
HbS/HbC	rs334 GAG (A) > GTG (S), rs33930165 GAG (A) > AAG (C)		
LightCycler Assay (DNA926080) GAG>GTG or AAG (beta globin CD6)			
Primers:	forward	5'-AGCAACCTCAAACAGACACCA-3'	
	reverse	5'-GCCTCACCACCAACTTCATC-3'	HbA: 57.5 °C
			HbS: 64.0 °C
Probes:		5'-CTGACTCCTGTGGAGAAGTC-FL	HbC: 52.5 °C
		5'-LC640-GCCGTTACTGCCCTGTGGG-P	
G6PD 202	rs1050828 (G > A)		
Primers:	G6PD-202 F2	5'-TCTGCCCGAAAACACCTT-3'	
	G6PD-202R	5'-GAAGAAGGGCTCACTCTGTTTG-3'	G: 65.0 °C
			A: 59.0 °C
Probe:		5'-ACACCTTCATCGTGGGCTATGC-P (unlabeled)	
G6PD 376	rs1050829 (A > G)		
Primers:	G6PD-376 F2	5'-AGTACGATGATGCAGCCT-3'	
	G6PD-376 R2	5'-AGGTAGAAGAGGCGGTTG-3'	A: 66.0 °C
			G: 63.0 °C
Probe:		5'-GGGCATTCATGTGGCTGTTGAG-P (unlabeled)	

FL = fluorescein, LC610 = LightCycler Red 610, LC640 = Light Cycler Red 640, P = phosphate.
ATP2B4: (Bedu-Addo et al. 2013), HbS, HbC and G6PD: (Gahutu et al. 2012).

2.2.3 Parasites

2.2.3.1 Parasite strain

The chloroquine-sensitive *P. falciparum* laboratory strain NF54 used in this study was originally isolated in 1979 from an individual living near Schipol Airport, Amsterdam, The Netherlands (MR4-Malaria Research and Reference Reagent Resource Centre), and was kindly provided by Dr. Kai Matuschewski from the Max Planck Institute for Infection Biology, Berlin (Delemarre and van der Kaay 1979, Ponnudurai et al. 1981).

2.2.3.2 *In vitro* cultivation of *P. falciparum* blood stages

P. falciparum was maintained in continuous culture as described by Trager and Jensen, with slight modifications (Trager and Jensen 1976). Parasites were grown in human red blood cells type 0^+ (donor gave informed consent) at 5 % hematocrit in RPMI 1640 medium supplemented with 25 mM HEPES, 2 mM L-glutamine, gentamicin (50 ng/ml) and Albumax II solution (1 x) (Table 4, modified after (Moll 2013)). Culture bottles were flushed with a gas mixture of 5 % CO_2, 5 % O_2 and 90 % N_2 and incubated at 37 °C. Well-plates were incubated in boxes in which a low oxygen atmosphere was produced by a burnt out candle. The culture medium was replaced every 24 to 48 h and parasitemia was determined by examination of Giemsa-stained thin blood smears. To keep parasitemia below 5 % a corresponding volume of fresh uninfected RBCs was added. Parasite cultures were routinely shown by PCR to be free of *Mycoplasma* contamination (PCR Mycoplasma Test Kit I/C, Promokine).

2.2.3.3 Giemsa staining of blood smears and determination of parasitemia

In vitro growth of *P. falciparum* cultures was monitored with Giemsa-stained thin blood smears. Due to complexes between the dye and the parasite's nucleus which stains its DNA in bright red, Plasmodia become visible inside the nucleus-free red blood cells. Besides the determination of the parasitemia defined as percentage of red blood cells being infected with *P. falciparum*, Giemsa staining also allows the differentiation between the individual developmental blood stages of the parasite. A drop of the parasite culture was spread on a microscope slide. The thin-smear was air-dried, fixed in methanol for about 60 sec and stained with a 10 % Giemsa solution for 20 to 30 min at room temperature. After the staining, the slide was carefully rinsed under running tap water and

the air-dried slide was observed under the microscope using a 100 x objective and oil immersion (Moll 2013). At least 500 erythrocytes in five independent microscopic fields were counted. The parasitemia was calculated as follows:

$$Parasitemia\ (P) = \frac{number\ of\ infected\ red\ blood\ cells\ (\Sigma\ iRBCs)}{number\ of\ red\ blood\ cells\ counted\ in\ total\ (\sum RBCs)}\ x\ 100\ \%$$

2.2.3.4 Cryopreservation of *P. falciparum*

For long-term storage, aliquots of *P. falciparum* were kept in liquid nitrogen. A parasite culture with at least 3 % of the parasites being in ring-stage was pelleted for 5 min at 675 x g. Per 1 ml of pellet volume, 200 µl of freezing solution (28 % glycerol, 3 % sorbitol, 0.65 % NaCl in H_2O) were added drop-wise while shaking the tube gently. After adding another pellet volume of freezing solution, the mixture was transferred into cryovials and snap-frozen in liquid nitrogen where it was stored until use.
Frozen *P. falciparum* aliquots were quickly thawed in a 37 °C-water bath while shaking. First 0.2 pellet volumes of a 12 % NaCl solution and after a brief incubation 10 pellet volumes of a 1.6 % NaCl solution were added drop-wise while shaking the tube. After a centrifugation step (675 x g, 5 min) the supernatant was discarded and the pellet was washed in 10 ml of pre-warmed (37 °C) culture medium. After final centrifugation, the pellet volume was measured, fresh uninfected RBCs up to a total volume of 500 µl and 9.5 ml of fresh culture medium were added (hematocrit of 5 %). The suspension was transferred into a fresh culture bottle, gassed and kept at 37 °C.

2.2.3.5 Synchronization of *P. falciparum* blood stages

Usually, *P. falciparum* cultures contain a mixture of ring, trophozoite and schizont stages, but for different applications (e.g. invasion assays, drug tests) it is necessary that all parasites are in the same developmental stage. Therefore, different protocols are used to obtain parasite populations at a specific developmental stage.

2.2.3.5.1 Sorbitol lysis (ring-stages)

Synchronization of *P. falciparum* blood stages using sorbitol is based on the increased permeability of late-stage infected red blood cells for this sugar alcohol (Lambros and

Vanderberg 1979). This leads to a selective osmotic lysis of trophozoites and schizonts leaving uninfected and ring-stage infected erythrocytes (Hill et al. 2007).
Cultures were regularly synchronized with a 5 % sorbitol solution when parasites were mostly in the ring stage (at least 5 % parasitemia). The parasite culture was transferred into a conical tube and spun down for 5 min at 675 x g. The pellet was resuspended in 10 volumes of a 5 % sorbitol solution which was added drop-wise while shaking the tube. After 10 min incubation the suspension was centrifuged again and washed once with pre-warmed culture medium to remove remaining sorbitol. The pellet was resuspended in culture medium at a hematocrit of 5 %, transferred to a culture bottle, gassed and placed back into 37 °C. To keep the parasite culture synchronized, sorbitol treatment was performed at least once a week. The day before the isolation of late stages (MACS, 2.2.3.5.2) used for infection of variant and non-variant RBCs as well as for co-culture assays with PBMCs or monocytes, cultures were synchronized twice within an interval of 6 h.

2.2.3.5.2 MACS isolation (late-stages)

Late-stage infected erythrocytes were purified by magnetic cell sorting (MACS, Miltenyi Biotec). This technique is normally used to isolate human primary cells with specific antibodies bound to magnetic microbeads. Hemozoin, a by-product of the parasite's hemoglobin digestion, contains iron in the Fe^{3+} state which has a stronger paramagnetic effect than the iron bound in hemoglobin (Fe^{2+}). This leads to a stronger magnetic attraction of late-stage infected red blood cells, which contain large amounts of hemozoin, in a magnetic field and allows their purification (Spadafora et al. 2011, Uhlemann 2000). Cultures containing at least 5 % late stage-infected red blood cells were selected for MACS isolation. The parasite pellet was once washed in MACS buffer and loaded on a LS column to remove free hemozoin at first. The LS column was rinsed initially with 3 ml of MACS buffer before the parasite pellet diluted in MACS buffer (1:1, ~ 50 % hematocrit) was added. Free hemozoin remained in the column within the magnetic field while the flow through, containing the infected red blood cells, was applied on a LD column subsequently. The column was rinsed with MACS buffer (2 x 3 ml) until the flow through was free of red blood cells, removed from the magnetic field and the mature parasite stages were eluted into a fresh tube (Ribaut et al. 2008). The eluent was centrifuged (675 x g, 5 min) and 4 µl of the pellet were used to prepare a Giemsa-stained

thin smear. The parasitemia obtained after MACS isolation was usually > 90 % of mature-stage iRBCs.

2.2.4 Human primary cells

2.2.4.1 Isolation of human peripheral blood mononuclear cells

Human peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Biocoll separation solution (density 1.077 g/ml, Biochrom) from buffy coats or venous blood of healthy, malaria-naïve blood donors (2.1.6.2). Venous blood samples or buffy coats were diluted 1:1 in PBS (without Ca^{2+} and Mg^{2+}) and 40 ml of the diluted blood was carefully layered over 10 ml of Biocoll solution in a 50 ml conical centrifugation tube. After a centrifugation step at 840 x g for 20 min without break, the upper layer was aspirated carefully, leaving the interphase containing the mononuclear cells (lymphocytes, monocytes and thrombocytes) undisturbed. The interphase was transferred to a new conical tube and washed with PBS (470 x g, 10 min, 4 °C). After two more washes with reduced speed (275 x g, 235 x g), PBMCs were counted in a Neubauer improved hemocytometer using trypan blue (1:10 in PBS) to determine the total number of cells obtained, the concentration of the cell suspension and their viability. PBMCs were either proceeded for following monocyte isolation or resuspended in co-culture medium (RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 10 mM HEPES and penicillin-streptomycin (10 000 U/ml)) at a final concentration of 1 x 10^6 cells/ml and directly transferred to tissue culture plates for co-cultivation with iRBCs.

2.2.4.2 Isolation of human monocytes

Monocytes were purified from freshly isolated PBMCs by positive selection of $CD14^+$ cells using antibody-conjugated magnetic beads (CD14 MicroBeads, Miltenyi Biotec) according to the manufacturer's protocol. PBMCs were pelleted (275 x g, 10 min, 4 °C) and washed once with cold MACS buffer (PBS with 0.5 % FBS, 2 mM EDTA). After aspiration of the supernatant, the pellet was resuspended in 90 µl MACS buffer and 10 µl CD14 MicroBeads per 1 x 10^7 cells. After incubation of 15 min at 4 °C in the dark, cells were washed once to remove unbound beads by adding 10 to 20 ml of MACS buffer. A MACS column (MS or LS, depending on the number of total cells) was placed in a

magnetic field (MiniMACS (MS) or MidiMACS Separator (LS)), rinsed with MACS buffer (MS 500 µl, LS 3 ml) and loaded with the cell suspension. To remove unlabeled cells, the column was washed three times with MACS buffer, before $CD14^+$ cells were eluted into a fresh tube. After the isolation, cells were washed once in RPMI 1640, counted and resuspended in co-culture medium (RPMI 1640 supplemented with 20 % human serum type AB, 2 mM L-glutamine, 10 mM HEPES and penicillin-streptomycin (10 000 U/ml)) at a final concentration of 1 x 10^6 cells/ml and transferred to tissue culture plates. To determine the purity of the isolated monocyte population ($CD14^+$), an aliquot of the respective cell suspension was incubated for at least 30 min with a fluorescently-labeled CD14 antibody (Table 3) and an appropriate isotype control and was measured by flow cytometry. The purity of the isolated $CD14^+$ population was usually > 95 %.

2.2.5 Experimental assays

2.2.5.1 Infection of normal and variant RBCs with *P. falciparum*

To remove white blood cells, full blood samples (2 ml) of donors with normal and variant RBCs (2.1.6.1) were washed twice in RPMI 1640 (675 x g, 5 min). The remaining RBC pellet was resuspended in an equal volume of RPMI 1640 (~ 50 % hematocrit) and cell concentrations of RBC suspensions were determined using a Neubauer improved hemocytometer. Of each donor, 1 x 10^9 RBCs were added to 5 ml of parasite culture medium in a six-well plate (5 % hematocrit). Aliquots of enriched late parasite stages with a parasitemia ≥ 90 % (2.2.3.5 MACS isolation) were used to initiate parasite cultures with a starting parasitemia (P 0h) of 0.1 to 0.5 %. For each RBC donor, a sample with uninfected RBCs was kept in culture under the same conditions as infected RBCs and served as negative control for the flow cytometric assessment of parasite growth and the subsequent co-cultivation with monocytes. In each experiment, variant and control RBC cultures were run in parallel. Every 24 h, culture medium was changed and total parasitemia was assessed by flow cytometry using hydroethidine (2.2.5.2). In preliminary experiments, parasitemia was additionally determined by microscopy of Giemsa-stained thin smears. For microscopic analysis, > 500 erythrocytes in at least five independent microscopic fields were counted in a blinded fashion (without knowing the RBC genotype).

2.2.5.2 Flow cytometric assessment of parasitemia

To analyze the impact of RBC polymorphisms on the *in vitro* growth and development of *P. falciparum*, parasitemia was determined every 24 h using flow cytometry over two parasite replication cycles (96 h) after infection of donor RBCs. Based on the fact that RBCs do not have a nucleus, iRBCs can be easily distinguished from uRBCs using nucleic acid dyes. Hydroethidine (Dihydroethidium bromide, Polysciences), a vital stain which is converted into ethidium bromide in metabolic active cells, intercalates into DNA and gives the typical red fluorescence when excited by UV light. Due to their higher DNA content, late parasite stages show an increased fluorescence intensity compared to ring-stages which allows their differentiation by flow cytometry (Figure 6). Stock solutions of hydroethidine (10 mg/ml in DMSO) were stored at -20 °C and diluted in PBS to a working concentration of 50 µg/ml prior to use. Aliquots of 100 µl from each parasite and uRBC control culture were transferred into 96-well v-bottom shaped plates for flow cytometric staining. After an initial centrifugation (530 x g, 4 min), RBC pellets were resuspended in 50 µl of diluted hydroethidine (50 µg/ml) and incubated for 20 min at 37 °C in the dark (Jouin et al. 2004, van der Heyde et al. 1995). Cell pellets were washed twice in PBS and finally resuspended in 100 µl PBS. For flow cytometric analysis, 3 µl of this solution were diluted in 500 µl of FACS buffer. Data acquisition and analysis was performed on a FACScan using BD CellQuest Pro Software (BD Biosciences). The detectors for forward and side scatter (FSC, SSC) were set to E-00 and 350, respectively, to place the population of uRBCs in the lower left quadrant of a FL2/FSC plot (Figure 6). The FL2 detector, measuring the extinction of hydroethidine (red fluorescence), was set

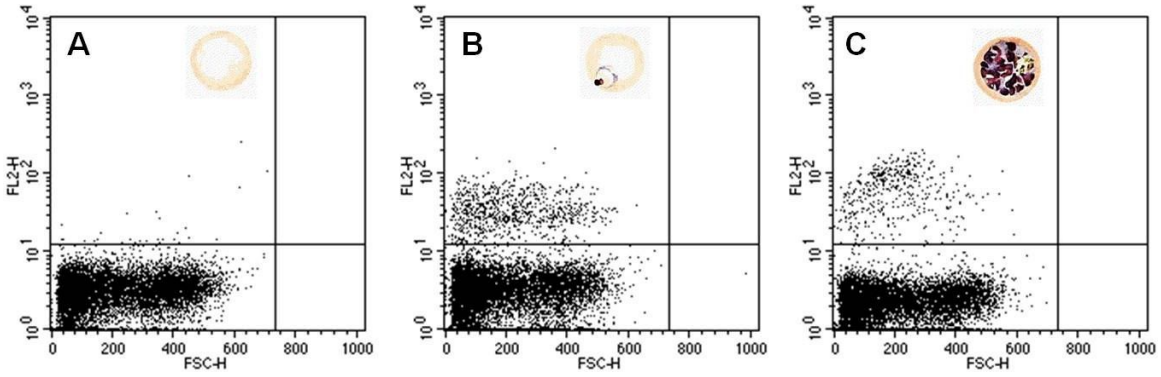

Figure 6: Flow cytometric analysis of uninfected (A), ring- (B) or late-stage infected RBCs (C) of synchronized *P. falciparum* cultures stained with hydroethidine. Cell populations of uRBCs are located in the lower left quadrant, while iRBCs are displayed in the upper left quadrant. Ring and late-stage infected RBCs are distinguished based on their fluorescence intensity. Schematic diagrams adapted from G.H. Nicholson (1971) of the corresponding parasite stage or correspondingly uRBCs are shown in the upper left quadrant of each dot plot. FSC = forward scatter (size), FL2 = fluorescence intensity.

in logarithmic scale. A total of 50 000 cells was measured and analyzed per sample. Parasites and uRBCs were distinguished based on their hydroethidine fluorescence intensity (FL2). Total parasitemia was determined as percentage of cells being positive for hydroethidine subtracted by the unspecific staining of the corresponding uRBC control (same RBC donor). Hydroethidine mean fluorescence intensities (MFIs) were given as geometric mean values of cell populations occurring in the different quadrants of the FL2/FSC plot. To overcome inter-experiment measurement variations based on the autofluorescence of uRBCs, ratios of MFI values of iRBC (upper left quadrant) and uRBC (lower left quadrant) populations of the same sample were calculated (Jouin et al. 2004). To assess parasite invasion efficacy, reinvasion rates were determined as ratios of ring-stage parasitemia at 24 h after infection (P 24h) and starting parasitemia (P 0h) (first invasion) as well as for parasitemia at 72 h (rings) and 48 h (late stages) after infection (second invasion).

2.2.5.3 Co-culture assays of mononuclear cells and iRBCs

To study the impact of genetic variants on the innate immune response, in particular of monocytes, towards *P. falciparum*-infected RBCs (iRBCs) we first had to establish an appropriate *in vitro* co-culture system to stimulate innate immune cells with a defined number of parasites and to trigger optimal, stable and reproducible cytokine responses. In preliminary experiments, co-culture conditions including optimum cell concentrations, parasite ratios, incubation time and concentration and type of serum (human or bovine) used in co-culture medium were determined. To suit the requirements of the different experimental approaches, genetic variants of both host cell (RBC) and effector cell (PBMCs and monocytes), two types of co-culture assays were performed in a slightly modified way.

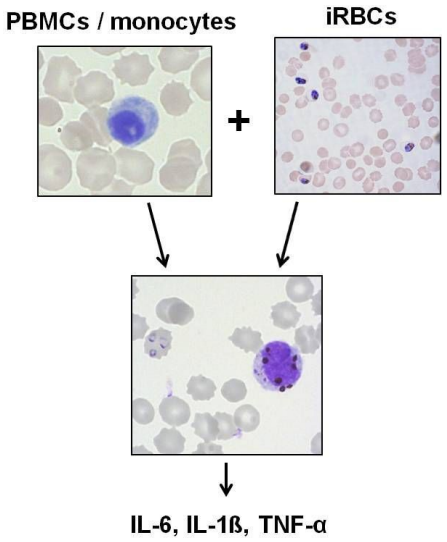

Figure 7: Co-cultivation of PBMCs or monocytes with live late-stage infected RBCs. After an incubation of 20 h cytokine concentrations in co-culture supernatants were determined by ELISA.

2.2.5.3.1 Mononuclear cells of donors genotyped for common TLR SNPs

Isolated PBMCs and monocytes of donors with different *TLR* genotypes were resuspended in co-culture medium (Table 4) and transferred to 96-well flat-bottom tissue culture plates (3×10^5 cells/well). Enriched late-stage-infected RBCs (iRBCs) with a parasitemia > 90 % were added in different ratios, with a multiplicity of infection (MOI) of 1, 5 and 10 iRBCs per mononuclear cell. As negative control, uninfected RBCs of the same donor kept under the same conditions as iRBCs were added at a ratio equal to a MOI of 5. Parasite culture supernatants were tested for LPS contamination using the Pyrogent Plus Gel Clot LAL (*Limulus* Amebocyte Lysate) Single Test Kit (Lonza) with a sensitivity threshold of 0.06 EU/ml. For all parasite cultures used in this study, LPS concentrations in culture supernatants were constantly below the sensitivity threshold. LPS purified from *Escherichia coli* (Sigma), Pam2CKS4 (diacylated lipopeptide, TLR2/TLR6 ligand), Pam3CSK4 (triacylated, lipopeptide, TLR2/TLR1 ligand) (Invivogen) were used as positive controls at a concentration of 10 ng/ml. Cells maintained in culture medium alone served as an additional negative control. Plates were incubated for 20 h at 37 °C in an atmosphere of 5 % CO_2. Cell-free supernatants were collected and stored at -80 °C for later cytokine analyses (2.2.5.4). In every experiment, donors with different TLR genotypes were run in parallel to overcome potential inter-experimental variations due to the use of live parasites. In total, 26 donors were tested in duplicates in eight independent experiments.

2.2.5.3.2 Stimulation of monocytes with of normal and variant iRBCs

In this study, *P. falciparum* cultures grown over four replication cycles in variant RBCs and respective controls (2.2.5.1) were used for monocyte stimulation. Monocytes isolated from buffy coats were seeded in 48-well flat-bottom tissue culture plates (5×10^5 cells/well) and iRBCs were added in a ratio of 5 iRBCs per monocyte (MOI of 5). Synchronized parasite cultures (sorbitol treatment 24 h before) and control uRBC cultures were pelleted and washed with RPMI 1640. RBC concentration and parasitemia were determined for each culture pellet and 2.5×10^6 iRBCs were added per well. As negative control, the uRBC culture of the same donor, previously kept under the same conditions as iRBCs, was added in the same volume. In all experiments, LPS at a concentration of 10 ng/ml and monocytes maintained in culture medium alone served as positive and negative controls, respectively. To overcome inter-individual variations

among monocyte donors and iRBC cultures, monocytes from two different donors were co-cultured in parallel in duplicates with *P. falciparum* cultures growing in normal and α-thalassemic RBCs. To assess the impact of α-thalassemia on the iRBC-stimulated cytokine response of monocytes, *P. falciparum* cultures from a total of 32 RBC donors were co-cultured with cells from ten monocyte donors, while *P. falciparum* cultures of 26 RBC donors and monocytes from eight donors were used to determine an effect on monocyte surface marker expression. After 20 h of incubation at 37 C°, cell-free supernatants were collected and stored at -80 °C until cytokine concentrations were determined by ELISA (2.2.5.4). Cells were harvested for surface staining for flow cytometry (2.2.5.5).

2.2.5.4 Analysis of cytokine secretion

For quantification of cytokines secreted by PBMCs and monocytes after co-cultivation with iRBCs, the enzyme-linked immunosorbent assay (ELISA) sandwich technique was used. Frozen supernatants (2.2.5.3) were thawed at room temperature, diluted and IL-1ß, IL-6, TNF-α, IL-10, IL-12p40 (40 kDa subunit of the IL-12 heterodimer) (U-CyTech) and MIP-1α (CCL3, R&D Systems) were detected using commercial ELISA kits according to the manufacturers' protocol (Table 5). Samples and serial dilutions of the standard were measured in duplicates. The read-out was based on the conversion of the substrate TMB (Tetramethylbenzidine) by horseradish peroxidase into a colored product (blue). This reaction was stopped by adding 2 M H_2SO_4 (yellow). The optical density (OD) was measured photometerically at 450 nm using a MRX Microplate Reader (Dynex Technologies). The detection limit for the IL-1ß, IL-6, TNF-α and IL-10 assay was < 2 pg/ml, for the IL-12p40 assay 4 pg/ml and for the MIP-1α assay 7.8 pg/ml. OD values of blank controls were subtracted from standard and sample OD values. Cytokine concentrations were interpolated from respective standard curves and multiplied by the dilution factor on the plate.

2.2.5.5 Analysis of monocyte surface expression

The expression of surface markers on monocytes was analyzed by flow cytometry. Cells were harvested after 20 h of incubation with iRBCs, washed once in FACS buffer (PBS with 5 % FBS and 10 mM sodium azide) and aliquots of the cell suspension were stained for different surface markers. Staining was carried out in 96-well v-bottom shaped plates

using monoclonal antibodies to CD40, CD64, CD80, CD86 (BD) and TREM-1 (R&D systems) or the appropriate isotype control (BD Biosciences). Antibodies used were labeled with FITC (fluorescein isothiocyanate) or PE (phycoerythrin) and are listed in Table 3. Cells were pelleted for 2 min at 530 x g and resuspended in 50 µl of the respective antibody diluted in FACS buffer (1:25). After an overnight incubation, remaining RBCs were lysed by adding 120 µl of 1 x Lysing buffer (BD Pharm Lyse) per well. Plates were incubated for 4 min at room temperature protected from light followed by a centrifugation at 220 x g for 5 min. After two washes with FACS wash (200 x g, 5 min), cells were resuspended in FACS fix (100 µl/well) and incubated for at least 30 min at 4 °C. For flow cytometric analysis, another 80 µl of FACS buffer per well were added and cell suspensions were transferred to FACS tubes already containing 200 µl of FACS buffer. Flow cytometry was performed with a BD FACSScan flow cytometer. Data was acquired and analyzed using BD CellQuest Pro Software (BD Biosciences). A total of 10 000 cells was measured and analyzed per sample. Median fluorescence intensity (MFI) and percentage of cells being positive for a specific surface marker were determined after subtraction of the respective isotype controls.

2.3 Protein structure analysis

The impact of the *TLR6* P249S polymorphism on the interaction of TLR6 with *P. falciparum* GPI was assessed by *in silico* analysis which was performed at the Institute of Microbiology and Hygiene of the Charité-University Medical Center Berlin (PhD Saubashya Sur, Prof. Dr. Ralf Schumann). Structures were visualized using *LigPlot* and *PyMol*.

2.4 Statistical Analysis

Statistical analysis was conducted using SPSS Statistics 22 (IBM) and STATA 12.1 (STATA Corp.). To assess normality of data we applied the Kolmogorov-Smirnov and Shapiro-Wilk test. Since most parameters did not follow a normal distribution, values are given as median along with range or interquartile range (25^{th} and 75^{th} percentile). Continuous variables of independent samples were compared using the non-parametric Mann-Whitney U and Kruskal-Wallis test, while Wilcoxon signed-rank and Friedman tests were used for correlated samples, respectively.

Additionally, the effect of RBC polymorphisms, α-thalassemia, *ATP2B4* (rs10900585, T > G), *BSG* (rs1803202, C > T), on total parasitemia and mean fluorescence intensity (MFI) of parasite cultures, was assessed in a multivariate analysis including sickle cell disease (HbS, HbC) and G6PD deficiency as covariates. After square root transformation of the data to reduce righ skewness, we used a random effects model based on the restricted maximum likelihood (REML) estimation method. Contrast analysis was used to estimate the effect of α-thalassemia, *ATP2B4* (rs10900585, T > G) and *BSG* (rs1803202, C > T) at each time point. The Wald test was used to assess significance of a single parameter and interaction terms. Association between total parasitemia (square root) with MCV and MCH was assed using a random effects model.

A two-way crossed random effects model accounting for the intra-iRBC and intra-monocyte correlation was used to assess the effect of α-thalassemia on monocyte cytokine responses to iRBCs. Because of the small number of clusters (iRBCs and monocytes), we used the REML estimation method yielding unbiaised estimates of variance components. Natural log-transformation was performed to reduce left skewness of the data. Besides α-thalassemia as main exposure, parasitemia of the *P. falciparum* culture, experimental run and G6PD deficiency were included as covariates. Sickle cell disease as confounding RBC polymorphism was excluded from the analysis due to low numbers. To assess the effect of α-thalassemia on the monocyte response for each cytokine separately, we used contrast analysis. The Wald test was used to assess significance for covariates and interaction terms.

A P value < 0.05 was considered statistically significant. Graphs were plotted using GraphPad Prism 5 (GraphPad Software).

3 RESULTS

3.1 Impact of TLR polymorphisms on cytokine response to iRBCs

3.1.1 PBMCs and monocytes predominantly release IL-6 upon iRBC contact

Previous studies have shown that PBMCs and also purified monocytes release pro-inflammatory cytokines upon stimulation with *P. falciparum* late stage-infected red blood cells (iRBCs) (Corrigan and Rowe 2010, Hensmann and Kwiatkowski 2001, Pichyangkul et al. 1994). One key factor in the induction of innate responses in malaria is *P. falciparum* GPI triggering host responses mainly via TLR2/TLR1 and TLR2/TLR6 heterodimers (Krishnegowda et al. 2005). In order to elucidate a potential modulatory effect of common functional SNPs in genes encoding for TLR2, TLR1 and TLR6 on the early innate cytokine response in malaria, PBMCs and monocytes isolated from full blood samples of 26 malaria-naïve donors with known *TLR2/TLR1/TLR6* genotype were co-cultivated with iRBCs at different parasite to mononuclear cell ratios (MOI of 1, 5 and 10). Control cells were incubated with 10 ng/ml LPS, Pam2CSK4 (TLR2/TLR6 ligand) or Pam3CSK4 (TLR2/TLR1 ligand), uninfected RBCs (uRBCs) or kept in medium alone (Figure 8). Expression levels of the pro-inflammatory cytokines IL-6, IL-1ß and TNF-α were determined for PBMCs and monocyte cultures, while

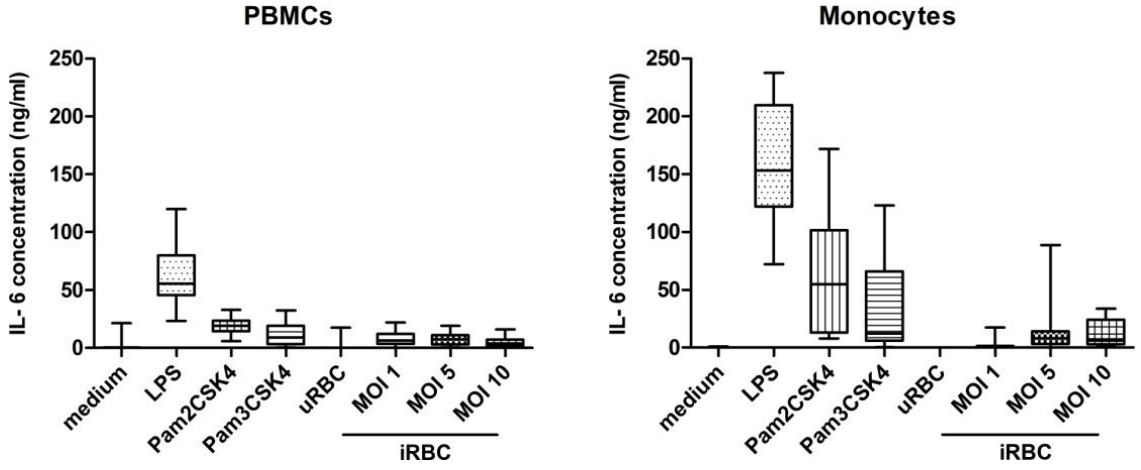

Figure 8: IL-6 response of human PBMCs and monocytes of 26 donors irrespective of their *TLR* genotype. Cells incubated in medium alone, in the presence of 10 ng/ml LPS, Pam2CSK4 or Pam3CSK4 and uRBCs were used as controls. Enriched late-stage iRBCs were added in different ratios (multiplicity of infection (MOI) of 1, 5 and 10). After 20 h of incubation supernatants were analyzed by ELISA. MOI 1: n = 22, MOI 5: n = 26, MOI 10: n = 15. Horizontal lines and boxes represent median and interquartile range (IQR), whiskers indicate data range.

concentrations of the chemokine MIP-1α (CCL3) were only assessed in supernatants of monocyte co-cultures with iRBCs and respective uninfected controls (Figure 9).

Among the 74 individuals screened for *TLR1* I602S, *TLR2* R753Q and *TLR6* P249S, only three were found to be heterozygous for the *TLR2* 753Q variant. Therefore, the *TLR2* variant was excluded from our study and only blood donors being homozygous for the *TLR2* 753R allele were selected to solely focus on the impact of *TLR1* I602S and *TLR6* P249S on the early cytokine response towards iRBCs.

Looking at cytokine expression patterns from all 26 donors irrespective of their genotype, LPS constantly induced the expression of substantial cytokine amounts in PBMCs and monocytes (data not shown). Cells kept in medium alone or with uRBCs serving as negative controls for Pam2CSK4, Pam3CSK4 and iRBC samples, respectively, generally did not secrete detectable amounts (data not shown). Compared to corresponding negative controls, cytokine levels induced by iRBCs as well as by TLR ligands, Pam2CSK4 and Pam3CSK4, were significantly upregulated in PBMC and monocyte cultures, except for IL-1ß in monocyte-iRBC co-cultures with a MOI of 1 ($p < 0.001$, Wilcoxon signed-rank test). For the subsequent analysis, basal cytokine expression in negative controls was subtracted from corresponding samples.

Upon co-cultivation with iRBCs, PBMCs and monocytes predominantly produced IL-6 and MIP-1α (ng/ml) as well as moderate to low levels of TNF-α and IL-1ß (pg/ml) (Figure 9). A ratio of one iRBC per mononuclear cell generally did not induce any detectable IL-1ß secretion. In comparison to Pam2CSK4 and Pam3CSK4, IL-6 levels induced by iRBCs were lower, in particular for monocytes, but parasites induced higher or comparable amounts of IL-1ß and TNF-α. In monocytes, higher parasite ratios gradually induced higher cytokine responses. For PBMCs, IL-6 levels decreased with increasing parasite ratios, whereas highest IL-1ß and TNF-α concentrations were induced at a MOI of 5. Overall, stimulation with iRBCs and the TLR agonists Pam2CSK4 and Pam3CSK4, induced cytokine responses with substantial interindividual variability among donors which was even more pronounced for monocytes.

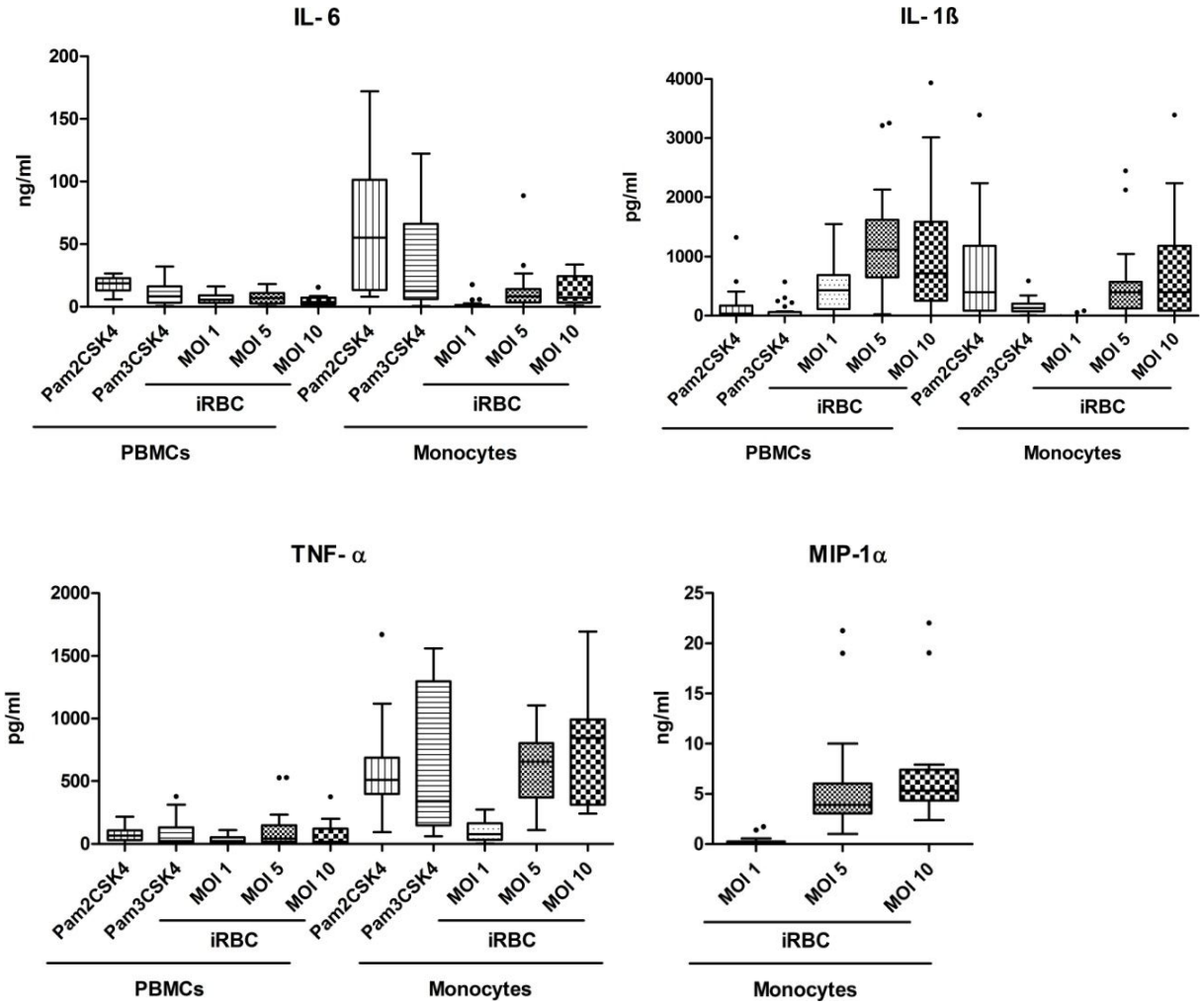

Figure 9: Variation in the cytokine response of PBMCs and monocytes stimulated with 10 ng/ml Pam2CSK4, Pam3CSK4 and different ratios of iRBCs (MOI 1, 5 and 10). After 20 h of incubation supernatants were analyzed using ELISA. Values of negative controls (cells in medium alone and uRBCs) were previously subtracted from corresponding samples. Horizontal bars and boxes display median and interquartile range (IQR, 25th and 75th percentile). The length of the whiskers is limited to 1.5 IQR. One dot represents one individual donor outside this range. Pam2CSK4, Pam3CSK4, MOI 5: n = 26, MOI 1: n = 22, MOI 10: n = 15.

3.1.2 TLR polymorphisms modulate monocyte response triggered by iRBCs

Infected-RBCs seemed to induce lower levels of IL-6, IL-1ß and MIP-1α in monocytes from donors with the *TLR1* 602S variant allele compared to *TLR1* 602I homozygous individuals, irrespective of the parasite ratio (Figure 10, Table A(Appendix)1). IL-6 levels induced by iRBCs at a MOI of 5 showed a median reduction by 52 % in co-culture supernatants of heterozygous and by 44 % in cultures of *TLR1* 602S homozygous donors relative to *TLR1* 602I homozygous individuals. IL-1ß and MIP-1α concentrations were diminished by 14 % and 35 %, and 17 % and 38 % in heterozygous and homozygous *TLR1* 602S donors, respectively. However, the observed differences among monocyte genotypes showed only a clear trend, but did not reach statistical significance. TNF-α expression of monocytes induced by iRBCs was not influenced by the *TLR1* genotype. Higher parasite ratios generally induced stronger cytokine responses with genotype-based differences being more pronounced at a MOI of 5 and 10. The inter-individual variance among monocyte responses from donors with at least one *TLR1* 602S allele was reduced in comparison with wildtype *TLR1* 602I donors, most notably for MIP-1α.

However, the observed differences in the cytokine response of monocytes due to *TLR1* I602S were not discernible on the level of PBMCs. Grouped by *TLR1* I602S genotype, PBMC cytokine expression patterns gave a less clear picture. Cytokine concentrations were generally highest in PBMC co-cultures of heterozygous individuals. Infected RBCs at a MOI of 5 induced significantly higher IL-1ß responses in PBMCs of heterozygous individuals compared to cultures of *TLR1* 602I homozygous donors ($p < 0.05$, Mann-Whitney U).

As previously reported by others, the TLR2/TLR1 ligand Pam3CSK4 induced significantly lower levels of IL-6, IL-1ß and TNF-α in homozygous *TLR1* 602S PBMCs and monocytes compared to cells of donors with at least one *TLR1* 602I allele ($p < 0.01$, Mann-Whitney U, data not shown) (Hawn et al. 2007, Johnson et al. 2007). IL-6 levels secreted by monocytes were reduced by 14 % in heterozygous individuals and by 89 % in homozygous *TLR1* 602S donors. Similarly, TNF-α and IL-1ß concentrations were decreased by 17 % and 89 %, and by 49 % and almost 100 %, respectively. As control, neither monocytes nor PBMCs stimulated with LPS showed any significant difference in cytokine levels when stratified by *TLR1* genotype (data not shown). Respective values for grouped medians with interquartile range (25^{th} and 75^{th} percentile) are given in Table A1 and Table A2 in the appendix.

TLR1 I602S:

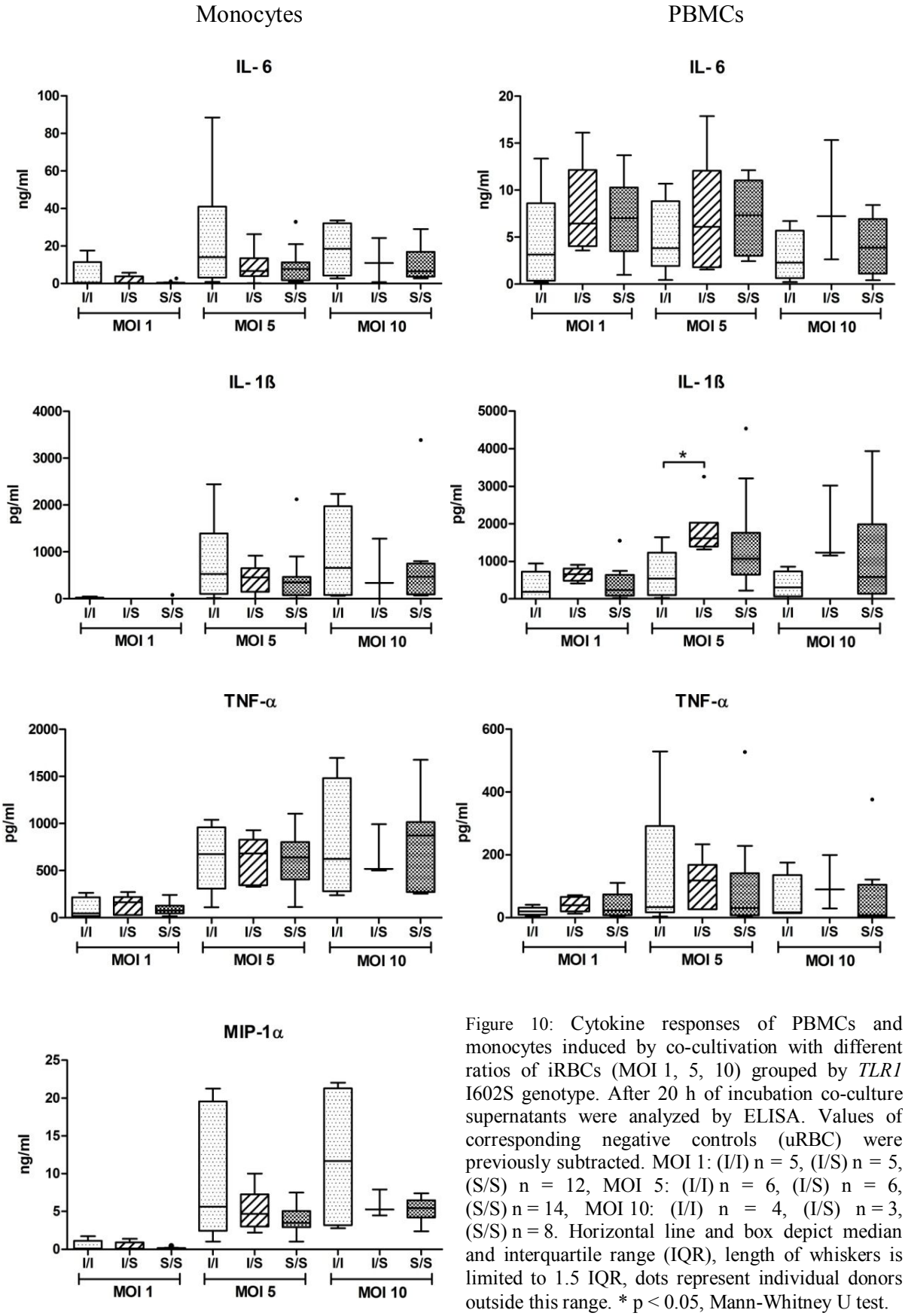

Figure 10: Cytokine responses of PBMCs and monocytes induced by co-cultivation with different ratios of iRBCs (MOI 1, 5, 10) grouped by *TLR1* I602S genotype. After 20 h of incubation co-culture supernatants were analyzed by ELISA. Values of corresponding negative controls (uRBC) were previously subtracted. MOI 1: (I/I) n = 5, (I/S) n = 5, (S/S) n = 12, MOI 5: (I/I) n = 6, (I/S) n = 6, (S/S) n = 14, MOI 10: (I/I) n = 4, (I/S) n = 3, (S/S) n = 8. Horizontal line and box depict median and interquartile range (IQR), length of whiskers is limited to 1.5 IQR, dots represent individual donors outside this range. * $p < 0.05$, Mann-Whitney U test.

Regarding *TLR6* P249S, only *TLR6* 249P and *TLR6* 249S homozygous individuals were included in our study. At a MOI of 1 and 5, monocytes of *TLR6* 249S individuals produced significantly less IL-6 compared to *TLR6* 249P monocytes ($p < 0.05$, Mann-Whitney U). IL-6 levels were reduced by 88 % and 79 % at a ratio of 1 and 5 parasites per monocyte, respectively (Figure 11, Table A3). Furthermore, IL-1ß, TNF-α and MIP-1α concentrations seemed comparatively lower in co-culture supernatants of *TLR6* 249S homozygous monoctyes, irrespective of the parasite to monocyte ratio. The amount of IL-1ß was diminished by 44 % and 22 % at a MOI of 5 and 10, correspondingly. TNF-α production was decreased by 16 %, 11 % and 32 % and MIP-1α release was reduced by 33 %, 27 % and 4 % for monocytes of *TLR6* 249S individuals as compared to *TLR6* 249P donors, at a MOI of 1, 5 and 10, respectively. The inter-individual variance among monocyte responses to iRBCs was not influenced by the *TLR6* genotype, except for a reduced heterogeneity of IL-6 responses among *TLR6* 249S donors.

Observed differences in the monocyte response could not be confirmed on the level of PBMCs. In contrast, cytokine expression patterns in PBMCs actually appeared inverse to monocytes with the *TLR6* 249S variant showing an increased expression of pro-inflammatory cytokines upon iRBC stimulation (Table A4). No significant effect of the *TLR6* P249S genotype was observed on the cytokine response of both cell subsets triggered by the TLR2/TLR6 ligand, Pam2CSK4, nor by LPS. Surprisingly, monocytes of *TLR6* 249S homozygous individuals released significantly lower cytokine levels after Pam3CSK4 stimulation ($p < 0.05$, Mann-Whitney U, data not shown). Corresponding values for grouped medians and interquartile ranges are given in Table A3 and Table A4 in the appendix.

TLR6 P249S:

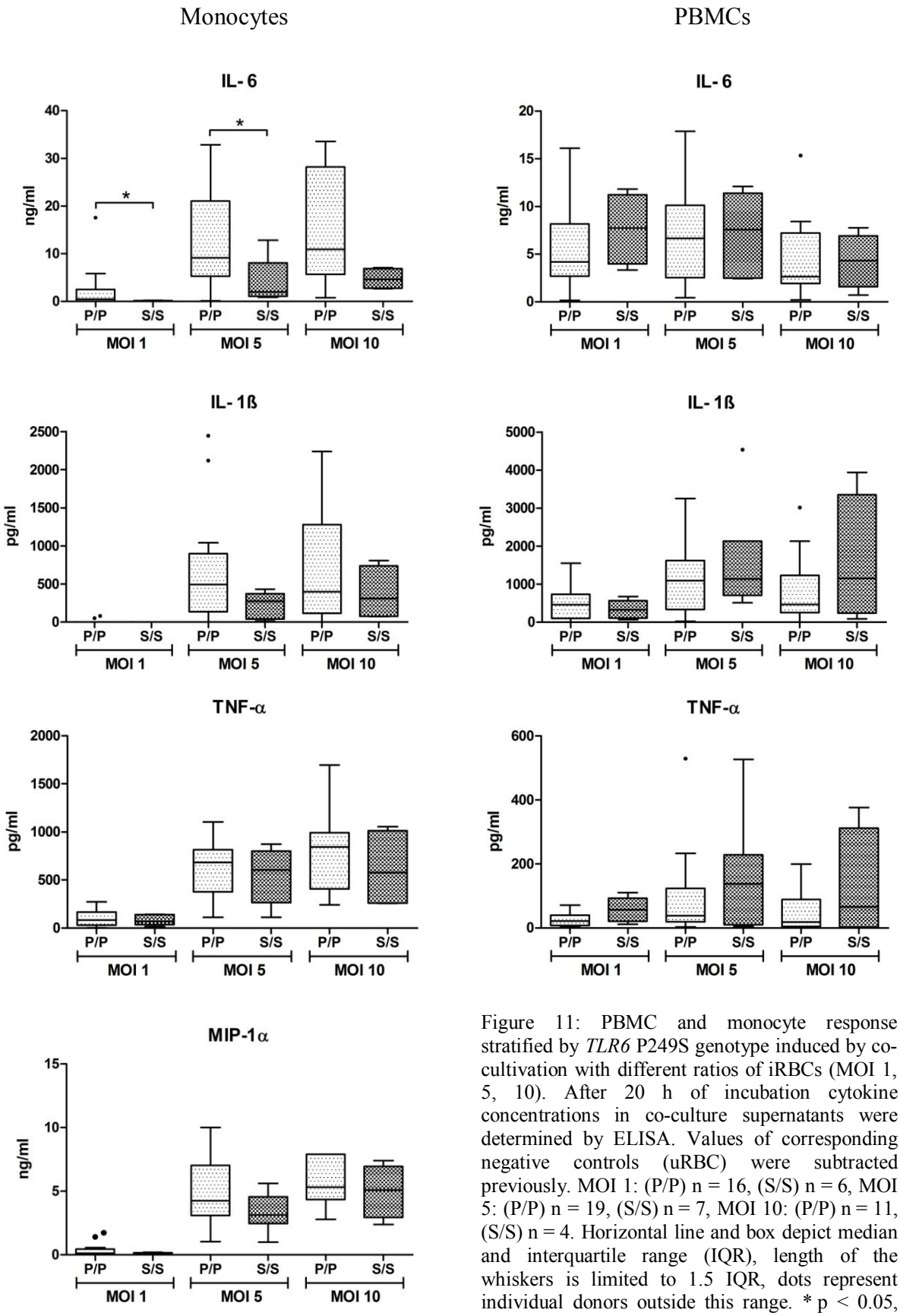

Figure 11: PBMC and monocyte response stratified by *TLR6* P249S genotype induced by co-cultivation with different ratios of iRBCs (MOI 1, 5, 10). After 20 h of incubation cytokine concentrations in co-culture supernatants were determined by ELISA. Values of corresponding negative controls (uRBC) were subtracted previously. MOI 1: (P/P) n = 16, (S/S) n = 6, MOI 5: (P/P) n = 19, (S/S) n = 7, MOI 10: (P/P) n = 11, (S/S) n = 4. Horizontal line and box depict median and interquartile range (IQR), length of the whiskers is limited to 1.5 IQR, dots represent individual donors outside this range. * $p < 0.05$, Mann-Whitney U test.

Finally, we analyzed how different *TLR1* I602S and *TLR6* P249S genotype combinations jointly affect the pro-inflammatory cytokine response towards iRBCs. We restricted the combined analysis to monocyte co-cultures. According to their *TLR1* I602S and *TLR6* P249S genotype we defined three groups, donors being homozygous for both wildtype alleles (I/I, P/P), donors with at least one variant *TLR1* 602S allele (I/S or S/S) combined with the *TLR6* 249P wildtype allele (P/P), and individuals being homozygous for both variant alleles (*TLR1* 602S/*TLR6* 249S) (Figure 12, Table A5). In accordance with the previous results, cytokine levels gradually decreased in variant genotypes and were lowest for monocytes from donors carrying both variant alleles, *TLR1* 602S and *TLR6* 249S (S/S, S/S), except for TNF-α. At a MOI of 5, the IL-6 response decreased from double wildtype (*TLR1* I/I / *TLR6* P/P) to donors with at least one variant *TLR1* 602S allele (I/S, S/S / P/P) and further to double variant allele donors (S/S / S/S). Statistical significance was present only for the difference between the two latter groups ($p < 0.05$, Mann-Whitney U). IL-6 response were 42 % lower for monocytes of donors with at least one variant *TLR1* 602S allele (I/S, S/S / P/P) and reduced by 86 % for monocytes homozygous for both variant alleles, as compared to wildtype monocytes. A similar pattern was observed for IL-1ß and MIP-1α, although not statistically significant, but not for TNF-α (Figure 12).

Combined *TLR1* I602S / *TLR6* P249S genotypes:

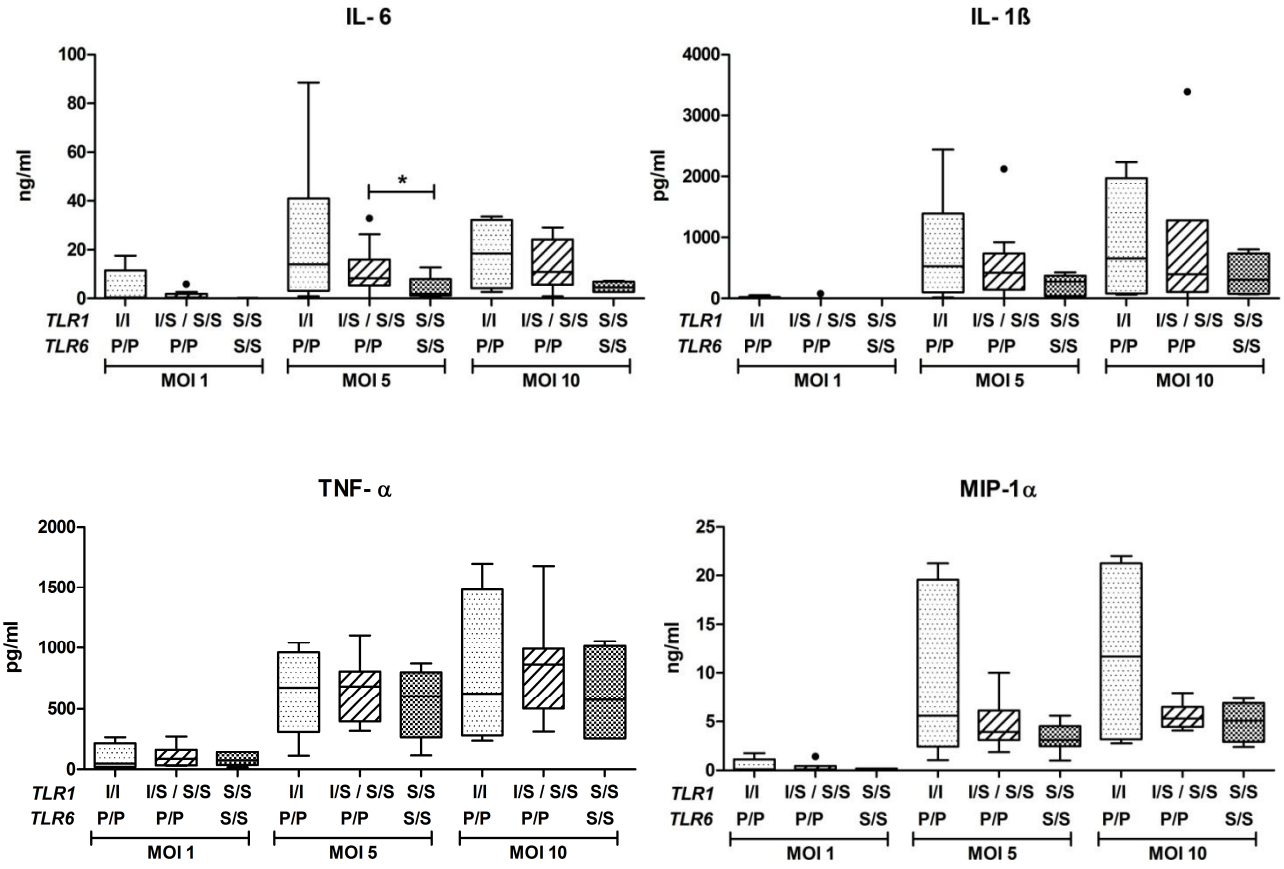

Figure 12: Cytokine responses of monocyte donors stratified by combined *TLR1* I602S and *TLR6* P249S genotypes. Horizontal line and box depict median and interquartile range (IQR), length of the whiskers is limited to 1.5 IQR, dots represent individual donors outside this range. MOI 1: *TLR1*(I/I), *TLR6* (P/P) n = 5, *TLR1* (I/S, S/S), *TLR6* (P/P) n = 11, *TLR1* (S/S), *TLR6* (S/S) n = 6, MOI 5: *TLR1*(I/I), *TLR6* (P/P) n = 6, *TLR1* (I/S, S/S), *TLR6* (P/P) n = 13, *TLR1* (S/S), *TLR6* (S/S) n = 7, MOI 10: *TLR1*(I/I), *TLR6* (P/P) n = 4, *TLR1* (I/S, S/S), *TLR6* (P/P) n = 7, *TLR1* (S/S), *TLR6* (S/S) n = 4. * $p < 0.05$, Mann-Whitney U test.

3.1.3 *TLR6* P249S modifies TLR6 interaction with *P. falciparum* GPI

The *TLR6* P249S polymorphism encodes a proline to serine transition in the extracellular domain of TLR6. Hamann et al. recently investigated the effect of the non-synonymous SNP on the TLR6 protein structure by *in silico* analysis (Hamann et al. 2013). They observed an altered surface topography of major clefts and cavities as well as a decreased number of functional pockets in the variant TLR6 249S protein indicating potential conformational changes which might affect binding of ligands or associated receptors. Furthermore, flexibility of the TLR6 249S variant was substantially reduced compared to the TLR6 249P protein form likely to interfere with ligand-induced conformational changes and hence TLR6 protein functionality (Hamann et al. 2013).

We assessed an impact of the *TLR6* 249S variant on the interaction of TLR6 with *P. falciparum* diacylated (*sn-2-lyso*) GPI, preferentially recognized by TLR2/TLR6, by *in silico* analysis. The TLR6 249S variant showed altered binding of *sn-2-lyso* GPI compared to the wildtype form of the TLR6 receptor. Differences regarding amino acid residues located in binding pockets interacting with *sn-2-lyso* GPI were observed. The main binding residue of the TLR6 249P form linking *sn-2-lyso* GPI to the TLR6 receptor, an aspartic acid at position 340 (Asp 340), was not situated in a pocket itself, but sandwiched between amino acid residues situated in binding pockets. Additionally, glutamine (Gln) 321, isoleucine (Ile) 344, proline (Pro) 342, phenylalanine (Phe) 317 and Ile 312, amino acid residues found in binding pockets, supported the binding of *sn-2-lyso* GPI to the wildtype form of the receptor. Whereas in the TLR6 249S variant, the major binding residues interacting with *sn-2-lyso* GPI, Phe 319 and Pro 342, were found in pockets. Supporting amino acid residues, situated in binding pockets of the TLR6 249S protein form, were Phe 343, Phe 317, Ile 344, Gln 321 and serine (Ser) 320 (Figure 13, Figure 14). Due to substantial differences in the tertiary structure between the TLR6 variants affecting binding pockets, clefts and cavities, binding of *sn-2-lyso* GPI to the TLR6 249S variant differed markedly from the wildtype form of the receptor. Altered binding might affect the affinity of the receptor-ligand interaction and eventually also ligand-induced TLR2/TLR6 heterodimerization. However, this is speculative and needs to be further investigated.

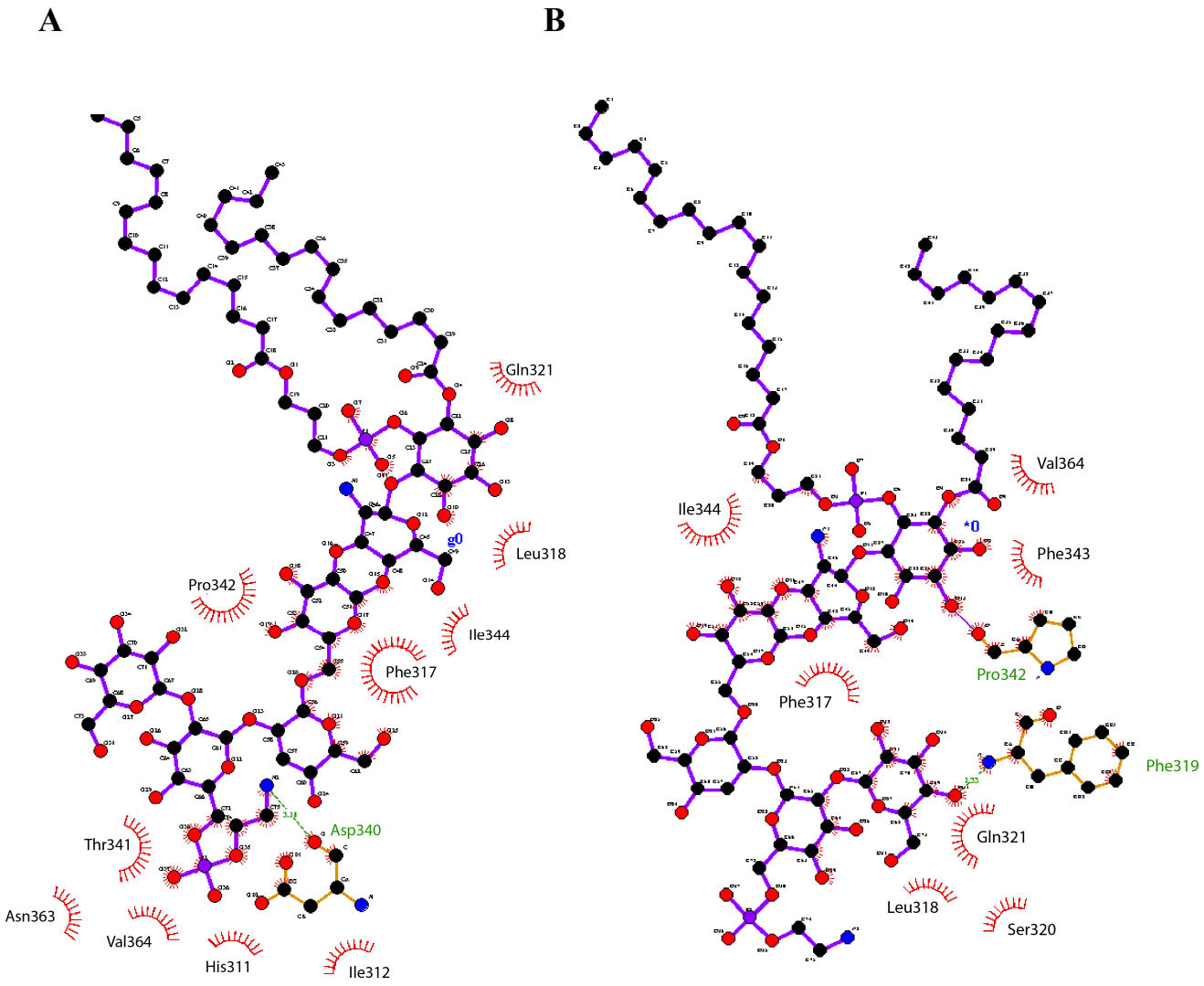

Figure 13: Schematic 2D model of TLR6 249P (A) and TLR6 249S (B) interacting with *P. falciparum* diacylated GPI (*sn-2-lyso*) generated using LIGPLOT. Bold lines represent bonds of GPI, while thin lines belong to the hydrogen bonded residues of TLR6. Interactions shown are those mediated by hydrogen bonds, green dashed lines, and hydrophobic contacts, indicated by an arc with spokes radiating towards the ligand atom which are marked with spokes radiating back.

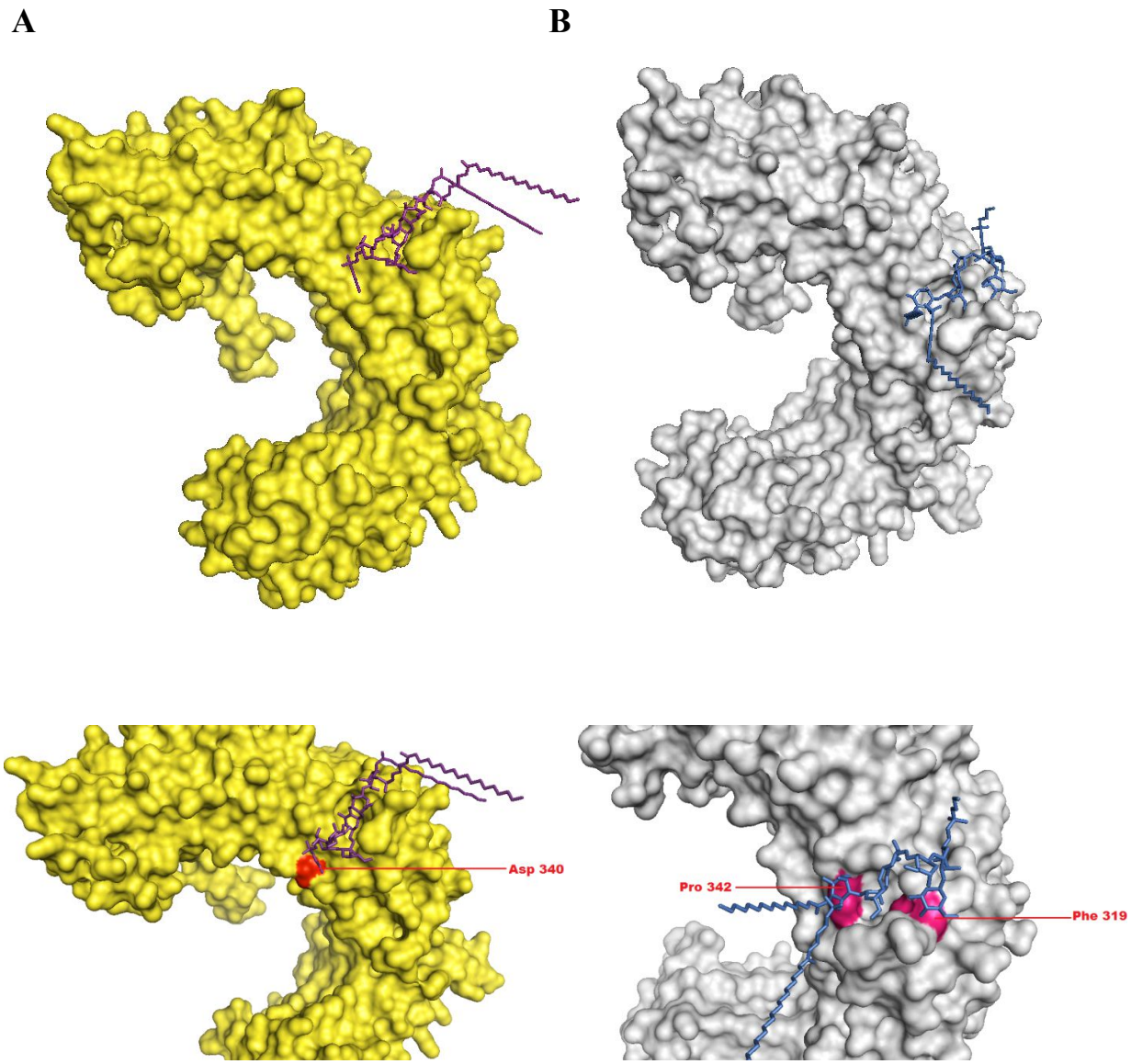

Figure 14: 3D model on TLR6 249P (A, yellow) and TLR6 249S (B, grey) interaction with *P. falciparum* diacylated GPI (*sn-2-lyso*) (purple, blue) generated using PyMol. Main amino acid residues interacting with *P. falciparum* GPI are indicated in red and pink, Asp 340 of TLR6 249 is binding to GPI and Phe 319 and Pro 342 of the TLR6 249S variant, respectively.

3.2 Prevalence of RBC polymorphisms and hematological parameters

A total of 158 red blood cell donors of Ghanaian origin, 74 females (mean age ± SD, 40.7 ± 11.3 years) and 84 males (mean age ± SD, 46.6 ± 12.2 years), were screened for the common West African -$\alpha^{3.7}$ deletion type of α-thalassemia as well as for SNPs in *ATP2B4* (rs10900585) and *BSG* (rs1803202). Regarding α-thalassemia, about one quarter (26.6 %, 42) of the screened blood donors was heterozygous (αα/-α), while 3.2 % (5) were homozygous (-α/-α) for the -$\alpha^{3.7}$ deletion type of α-thalassemia (Table 8). Approximately half of the donors were heterozygous (48.1 %, 76) and about one quarter was homozygous (25.9 %, 41) for the variant *ATP2B4* allele (T > G). In the case of *BSG* (C > T), 3.8 % (6) of the donors were homozygous, whereas 30.4 % (48) were heterozygous for the variant T allele. The observed genotype frequencies were all in Hardy-Weinberg equilibrium and corroborated with frequencies of sub-Saharan African populations reported in previous studies.

To adjust for their potential effect on parasite growth and inflammatory monocyte responses induced by iRBCs in the subsequent analysis, RBC donors were additionally screened for G6PD deficiency, hemoglobin S and C (HbS, HbC) also known to influence malaria susceptibility. Sickle cell disease (HbSC, HbCC and HbSS) was present at a frequency of 2.5 % (4) in the screened cohort, while sickle cell trait (HbAC and HbAS) was confirmed in 29.1 % (46). Regarding the X-linked G6PD deficiency, donors were classified as G6PD normal, G6PD deficiency trait (heterozygous) and full G6PD deficency (hemi-/homozygous). Heterozygous G6PD deficiency was present in 12.0 % (19), while a full defect was confirmed in 13.9 % (22) of individuals.

Full blood counts were performed and parameters assessed included RBC count, hemoglobin levels, mean corpuscular volume (MCV) and mean corpuscular hemoglobin (MCH) of RBCs. Values grouped by RBC genotype are given in Table 8.

α-Thalassemia showed clear associations with declining hemoglobin levels, MCV and MCH as well as with increasing numbers of RBCs (Muller et al. 2015). MCV and MCH were significantly reduced in heterozygous and homozygous (αα/-α and –α/-α) α-thalassemic individuals compared to individuals with normal hemoglobin, and furterhmore in homozygous related to heterozygous α-thalassemic RBCs. The number of RBCs was significantly higher in heterozygous and homozygous α-thalassemic individuals, while hemoglobin levels were significantly reduced only in homozygous

compared to donors with normal hemoglobin ($p < 0.05$, Mann-Whitney U). *ATP2B4* and *BSG* genotype showed no significant influence on hematological parameters. Individuals with sickle cell disease showed significantly reduced hemoglobin levels, MCH and MCV compared to individuals with sickle cell trait and normal hemoglobin. The latter parameter was also significantly decreased in individuals with sickle cell trait ($p < 0.05$, Mann-Whitney U). Concerning G6PD deficiency, only hemoglobin levels in donors with G6PD trait were significantly reduced compared to individuals with normal G6PD. In general, RBC counts, hemoglobin levels and MCH of female blood donors were significantly lower ($p < 0.05$, Mann-Whitney U, data not shown).

Table 8: Prevalence of red blood cell polymorphisms among 158 Ghanaian blood donors with hematological parameters grouped by genotype

	Prevalence % (n)	**RBCs** x 10^{12} / l	**Hemoglobin** g / dl	**MCV** fl	**MCH** pg
Reference values		4.6 [3.4-5.8]	13.1 [9.8-16.0]	87.0 [72.0-97.0]	28.6 [22.6-33.5]
α-thalassemia (3.7kb deletion)					
αα/αα	70.3 (111)	4.8 [3.3-6.1]	14.5 [9.7-20.3]	90.0 [73.4-103.3]	30.3 [23.8-43.5]
αα/-α	26.6 (42)	5.1 * [3.8-6.7]	13.8 [8.9-17.9]	84.2 * [64.9-95.6]	27.7 * [20.9-32.2]
-α/-α	3.2 (5)	5.4 * [5.0-6.3]	12.2 * [10.5-16.3]	67.2 * [63.9-75.9]	21.8 * [19.7-25.8]
ATP2B4 rs10900585 (T > G)					
G/G	25.9 (41)	4.9 [3.7-6.4]	14.3 [11.8-20.3]	88.5 [67.2-103.3]	29.5 [21.8-37.2]
G/T	48.1 (76)	4.9 [3.3-6.7]	13.9 [8.9-20.1]	89.1 [63.9-100.6]	29.4 [19.7-43.5]
T/T	25.9 (41)	4.8 [3.9-5.9]	14.3 [11.0-18.0]	88.3 [70.0-95.6]	30.0 [22.6-32.9]
BSG rs1803202 (C > T)					
C/C	65.8 (104)	4.9 [3.3-6.7]	13.9 [8.9-20.3]	88.5 [63.9-103.3]	29.6 [19.7-37.2]
C/T	30.4 (48)	4.9 [3.9-6.3]	14.4 [11.4-17.7]	88.8 [72.8-100.6]	29.8 [25.8-43.5]
T/T	3.8 (6)	4.7 [4.4-5.6]	13.8 [13.2-16.3]	90.1 [86.8-94.9]	29.5 [28.8-30.8]

MCV = mean corpuscular volume, MCH = mean corpuscular hemoglobin. Values given represent median and range (square brackets). Reference values from 625 healthy adults from central Ghana (Dosoo et al. 2012). * $p < 0.05$, Mann-Whitney U test, compared to corresponding wildtype.

3.3 Impact of RBC polymorphisms on parasite growth and development

Normal and variant RBCs of a total of 158 blood donors were infected with *P. falciparum* and parasite growth was followed over two erythrocytic replication cycles *in vitro* (96 h). One representative dot plot series demonstrating the flow cytometric analysis of *P. falciparum* growth in one parasite culture (one RBC donor) over 96 h is shown in Figure 15. Levels of parasitemia obtained by flow cytometry were similar to those initially assessed by microscopy of Giemsa-stained thin blood smears (data not shown, total parasitemia at 96 h: Spearmann rank correlation coefficient $r = 0.53$, $p = 0.001$). Besides total parasitemia values, additional information on invasion efficacy (reinvasion rates) and changes in parasite development (MFI) were obtained for each parasite culture based on the acquired flow cytometry data.
We then examined associations between genotypes of α-thalassemia, *ATP2B4* (rs10900585) and *BSG* (rs1803202) with total parasitemia, mean fluorescence intensity and reinvasion rate.

For each time point, total parasitemia was determined as percentage of cells being positive for hydroethidine subtracted by the unspecific staining of the corresponding negative control (same RBC donor). In general, total parasitemia increased over time and reached median values of approximately 1 % of infected cells after 96 h of *in vitro* culture. The increase in parasitemia was higher from 0 h to 24 h and substantially higher from 48 h to 72 h compared to other time intervals correlating with the completion of erythrocytic replication cycles within these time intervals.

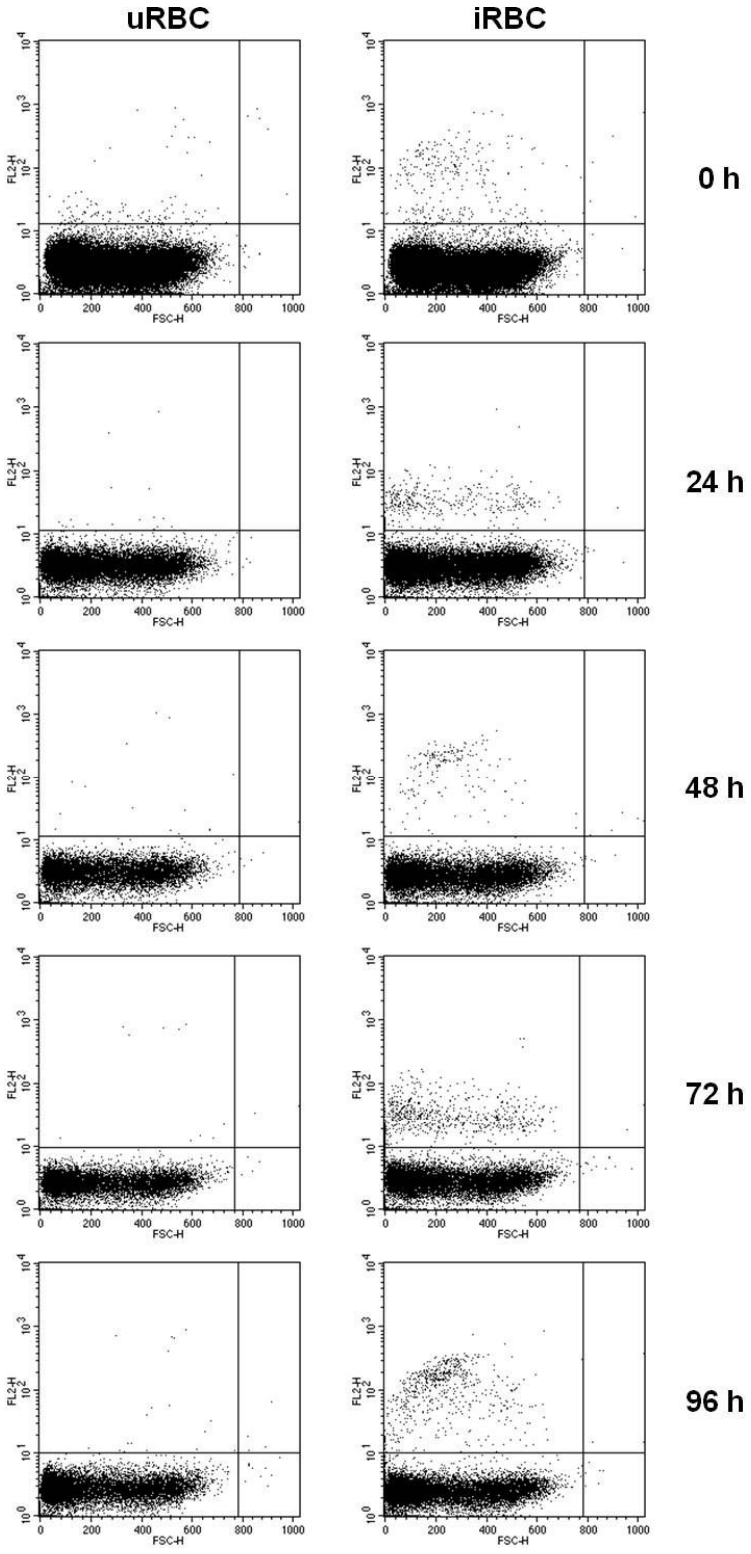

Figure 15: Dot plots of flow cytometric analysis of one representative parasite culture at different time points followed over 96 h. Infected and uninfected RBCs were distinguished based on their hydroethidine fluorescence (FL2). Lower left quadrant shows uRBCs, while upper left quadrant represents iRBCs. Total parasitemia was determined as percentage of cells being positive for hydroethidine (right column) subtracted by the unspecific staining of the corresponding negative control (left column). Due to their higher DNA content, late parasite stages showed an increased fluorescence intensity compared to ring-stages (e.g. 96 h versus 72 h). A total of 50 000 events were analyzed for each sample.

Cultures of heterozygous α-thalassemic RBCs (αα/-α) showed a significantly lower parasitemia after 96 h of *in vitro* culture as compared to cultures with non-variant RBCs ($p < 0.01$, Mann-Whitney U). RBCs of heterozygous individuals yielded a parasite multiplication which was only 56 % of control cultures with normal hemoglobin. However, this effect was not discernible for the small group of cultures with homozygous α-thalassemic (-α/-α) RBCs (Figure 16, Table A6). The *ATP2B4* genotype showed no significant influence on *P. falciparum* growth. As for *BSG*, parasite levels gradually decreased in *P. falciparum* cultures containing RBCs with the variant *BSG* allele and were lowest in cultures with homozygously variant RBCs (Figure 16). Parasitemia in heterozygous RBC cultures was significantly reduced 24 h post-infection ($p < 0.05$, Mann-Whitney U). Parasite cultures with homozygous variant RBCs showed significantly lower parasite densities after 48 h and 72 h reaching only 44 % and 42 % of the parasitemia in non-variant RBCs, respectively. Additionally, parasitemia after 72 h was also significantly reduced compared to parasite cultures containing heterozygous (C/T) RBCs ($p < 0.05$, Mann-Whitney U).

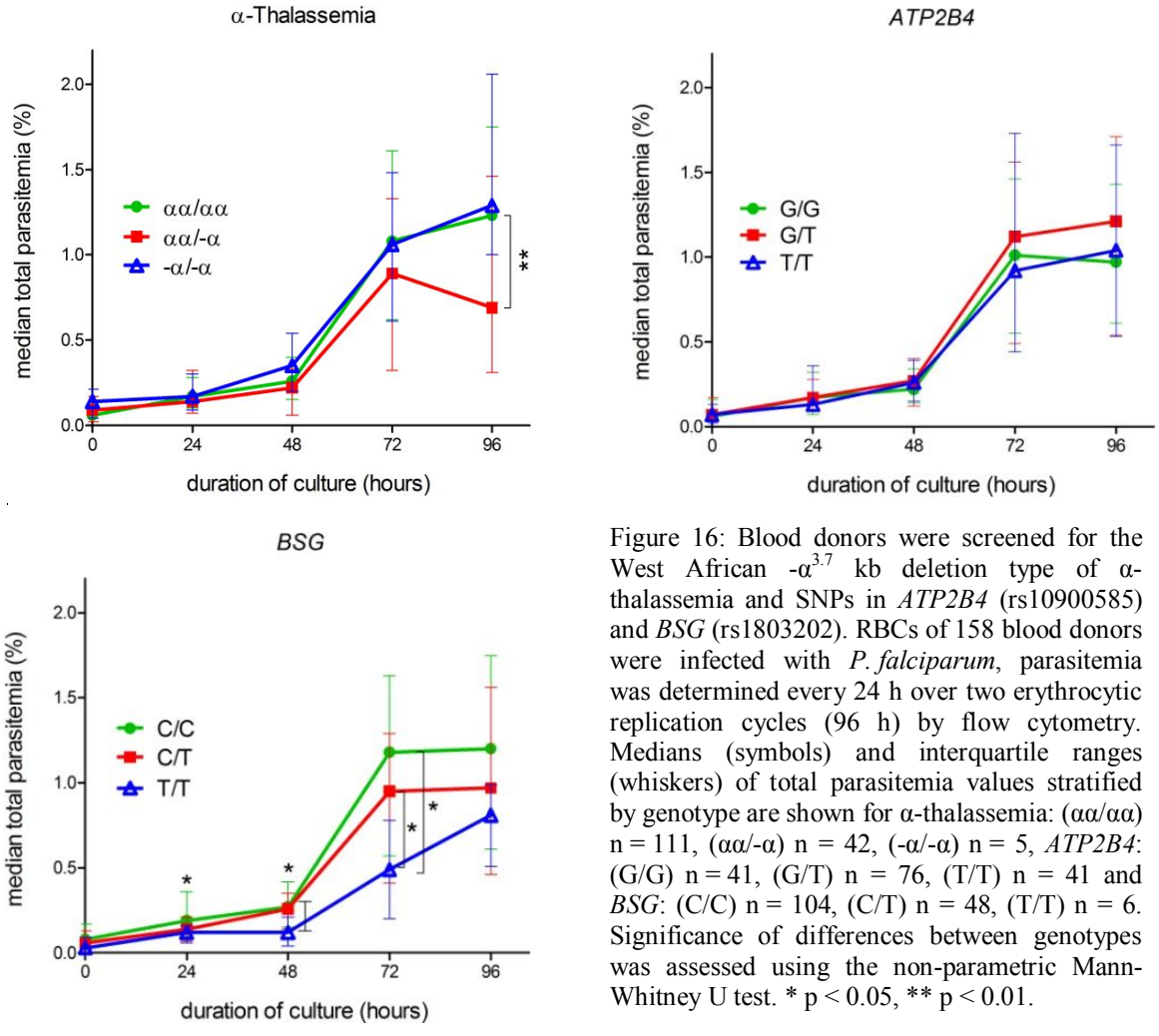

Figure 16: Blood donors were screened for the West African -$\alpha^{3.7}$ kb deletion type of α-thalassemia and SNPs in *ATP2B4* (rs10900585) and *BSG* (rs1803202). RBCs of 158 blood donors were infected with *P. falciparum*, parasitemia was determined every 24 h over two erythrocytic replication cycles (96 h) by flow cytometry. Medians (symbols) and interquartile ranges (whiskers) of total parasitemia values stratified by genotype are shown for α-thalassemia: (αα/αα) n = 111, (αα/-α) n = 42, (-α/-α) n = 5, *ATP2B4*: (G/G) n = 41, (G/T) n = 76, (T/T) n = 41 and *BSG*: (C/C) n = 104, (C/T) n = 48, (T/T) n = 6. Significance of differences between genotypes was assessed using the non-parametric Mann-Whitney U test. * $p < 0.05$, ** $p < 0.01$.

Given the fact that the amount of nucleotide-binding fluorochrome taken up by the parasite increases according to its DNA content, *P. falciparum* late-stage iRBCs show elevated hydroethidine fluorescence intensities compared to ring-stage parasites (Jacobberger et al. 1983, van der Heyde et al. 1995). Therefore, fluorescence intensity allows to determine the developmental stage of a *P. falciparum* culture as well as changes in the intraerythorcytic parasite development such as growth delay or arrest.

Fluorescence intensities of late-stage parasites (0 h, 48 h and 96 h) were about 3-4 fold greater than those of ring-stages (24 h and 72 h). Hence, monitoring the development of *P. falciparum* cultures over two replication cycles resulted in a serrated graph with alternating high and low fluorescence intensities (Figure 17, Table A7).

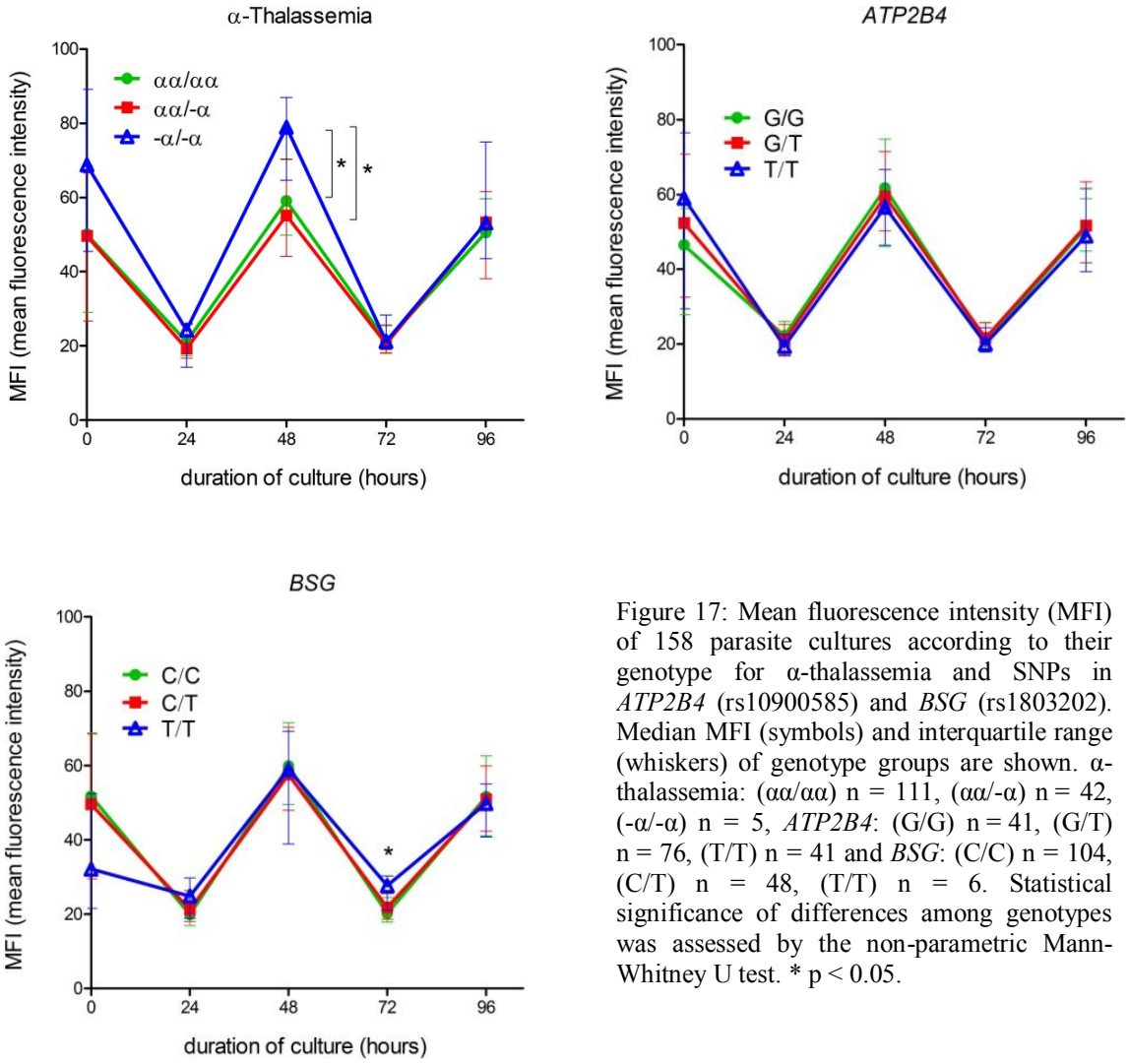

Figure 17: Mean fluorescence intensity (MFI) of 158 parasite cultures according to their genotype for α-thalassemia and SNPs in *ATP2B4* (rs10900585) and *BSG* (rs1803202). Median MFI (symbols) and interquartile range (whiskers) of genotype groups are shown. α-thalassemia: (αα/αα) n = 111, (αα/-α) n = 42, (-α/-α) n = 5, *ATP2B4*: (G/G) n = 41, (G/T) n = 76, (T/T) n = 41 and *BSG*: (C/C) n = 104, (C/T) n = 48, (T/T) n = 6. Statistical significance of differences among genotypes was assessed by the non-parametric Mann-Whitney U test. * $p < 0.05$.

Already at the time of infection, *P. falciparum* cultures in homozygous α-thalassemic (-α/-α) RBCs yielded greater fluorescence intensities which were significantly increased after 48 h of *in vitro* culture compared to cultures in heterozygous (αα/-α) RBCs and RBCs with normal hemoglobin (αα/αα) ($p < 0.05$, Mann-Whitney U test). Irrespective of the *ATP2B4* genotype MFI values were almost equal showing parasite cultures growing with a high level of synchronity. The *BSG* genotype did not have a major influence on fluorescence intensities of parasite cultures. Only MFI values of homozygous variant RBC cultures were significantly higher compared to parasite cultures in RBCs of homozygous wildtype individuals at 72 h ($p < 0.05$, Mann-Whitney U test). However, the observed deviations in cultures with homozygous α-thalassemic and variant *BSG* RBCs might be explained by a great inter-experimental variation and low numbers of RBC donors homozygous for those variants. Overall, no obvious impact on intraerythrocytic parasite development in terms of growth delay or arrest was observed for any of the RBC polymorphisms.

The susceptibility of RBCs for merozoite invasion is reflected by an increased parasitemia after the completion of one erythrocytic replication cycle. (Figure 18, Table A8). To assess parasite invasion efficacy, reinvasion rates were determined as ratios of ring-stage parasitemia at 24 h after infection (P 24h) and starting parasitemia (P 0h) (first invasion) as well for parasitemia at 72 h (rings) and 48 h (late stages) post-infection. In general, invasion efficacy for the second merozoite invasion at 72 h was almost doubled compared to corresponding values at 24 h. Furthermore, the observed variances regarding reinvasion among parasite cultures of the same genotype was lower for the second invasion regardless of the genotype. Comparison of reinvasion rates of normal and α-thalassemic RBCs showed a gradual decrease for heterozygous and homozygous thalassemic RBCs at 24 h and 72 h (αα/αα > αα/-α > -α/-α). Invasion efficacy of parasites in homozygous thalassemic RBCs (-α/-α) at 72 h was significantly lower compared to RBCs with normal hemoglobin (αα/αα) ($p < 0.05$, Mann-Whitney U). *ATP2B4* and *BSG* homozygous variant RBCs showed slightly increased parasite reinvasion rates at 24 h compared to the other genotypes. However, a clear effect on invasion efficacies due to different *ATP2B4* and *BSG* genotypes was not evident.

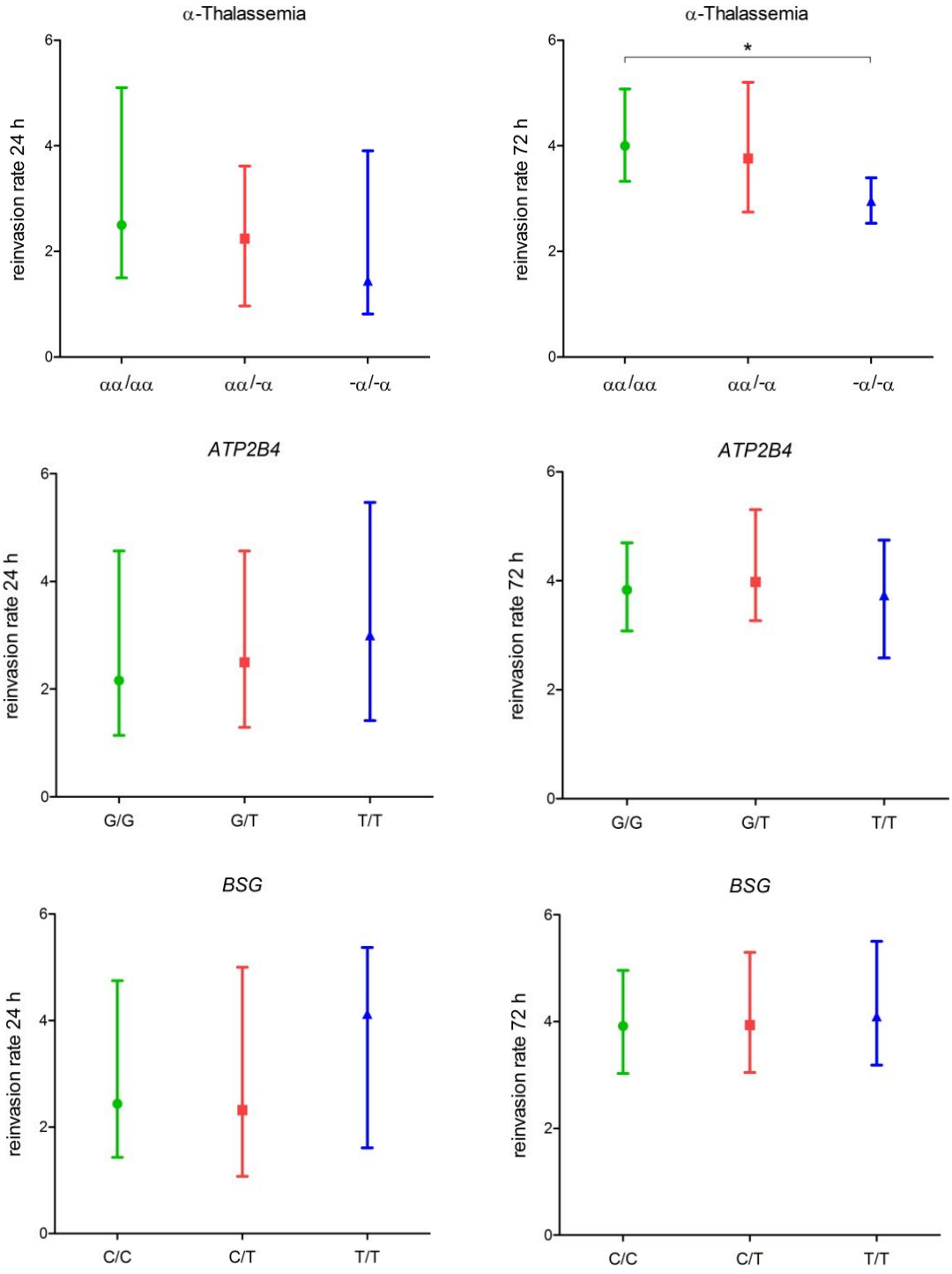

Figure 18: Reinvasion rate of *P. falciparum* parasites at 24 h and 72 h according to the RBC genotype. Symbols indicate grouped medians of reinvasion rates, whiskers represent interquartile range. α-thalassemia: (αα/αα) n = 111, (αα/-α) n = 42, (-α/-α) n = 5, *ATP2B4*: (G/G) n = 41, (G/T) n = 76, (T/T) n = 41 and *BSG*: (C/C) n = 104, (C/T) n = 48, (T/T) n = 6. Statistical significance of differences between genotypes was assessed by the non-parametric Mann-Whitney U test. * $p < 0.05$.

Some of the RBC donors had sickle cell disease and G6PD deficiency as potentially confounding RBC polymorphisms. To exclude interactions regarding total parasitemia and mean fluorescence intensities between α-thalassemia, *ATP2B4* (rs10900585), *BSG* (rs1803202), sickle cell disease and G6PD deficiency, a multivariate analysis was performed. Due to the low frequency of the homozygous state for α-thalassemia and the variant *BSG* allele, corresponding heterozygous and homozygous *P. falciparum* cultures were pooled for the subsequent analysis. Adjusted for polymorphisms in *ATP2B4* and *BSG*, sickle cell disease and G6PD deficency, *P. falciparum* cultures in α-thalassemic RBCs showed significantly reduced levels of parasitemia after 72 h and 96 h of *in vitro* culture compared to cultures in non-variant RBCs ($p = 0.007$ and $p = 0.0005$, respectively, Wald test). There was strong evidence of linear increase in the effect over time, meaning that α-thalassemic RBCs became less susceptible to *P. falciparum* with increasing duration of the *in vitro* culture ($p < 0.0001$, Wald joint test). After adjusting for covariates, there was still no evidence for differences in parasite growth due to the *ATP2B4* genotype. Adjusted for covariates, parasite densities in *P. falciparum* cultures containing variant *BSG* RBCs were significantly lower compared to cultures with non-variant RBCs after 72 h and 96 h of *in vitro* culture ($p = 0.003$ and $p = 0.007$, respectively, Wald test). In conclusion, the effect of reduced parasite multiplication observed for α-thalassemia and *BSG* (rs1803202) are indepdent from each other as well as from other confounding RBC polymorphisms included in our model. As for mean fluorescence intensities, after adjusting for sickle cell disease and G6PD deficiency, there was no evidence for differences between different genotypes for none of the three RBC polymorphisms tested.

To identify whether altered RBC properties associated with α-thalassemia or sickle cell disease affect parasite multiplication, we examined a potential influence of MCV and MCH (3.2) on total parasitemia of *P. falciparum* cultures irrespective of the RBC genotype. At the time of infection, after 24 h and 48 h of *in vitro* culture, there was no evidence of association between total parasitemia (square root) and MCV or MCH. There was some evidence of association between MCV and MCH ($p = 0.02$ and $p = 0.03$, respectively, Wald test, data not shown) with parasite densities after 72 h and weak evidence of association for both parameters with total parasitemia after 96 h of *in vitro* culture ($p = 0.07$ and $p = 0.08$, respectively, Wald test). Concluding that in prolonged *in vitro* cultures *P. falciparum* was multiplying better in larger RBCs containing high levels of hemoglobin than in smaller RBCs with less hemoglobin (Table 8).

3.4 Effect of α-thalassemia on the monocyte response towards iRBCs

To investigate whether the reduced cytoadherence of α-thalassemic iRBCs to monocytes reported by others (Krause et al. 2012), consequently leads to an impaired activation and a curbed inflammatory monocyte response, we co-cultivated monocytes with α-thalassemic and normal iRBCs and compared cytokine responses as well as the expression of monocyte surface markers. Due to the low frequency of the homozygous state of α-thalassemia in our RBC donor cohort, monocyte responses induced by heterozygous and homozygous α-thalassemic iRBCs were grouped. Parasitemia of *P. falciparum* cultures varied from 0.9 to 7.3 % (median: 3.2%) at the time of co-culture inoculation.

3.4.1 α-Thalassemia curbs cytokine response towards iRBCs

Infected RBCs induced substantial amounts of IL-6 and MIP-1α, and moderate to low levels of IL-1ß, TNF-α and IL-12p40 (40 kDa subunit of the IL-12 heterodimer). Concentrations of the anti-inflammatory cytokine IL-10 were below the detection limit for most samples. Monocytes kept in medium alone or with uRBCs generally did not secrete any or only very low cytokine amounts (data not shown). The presence of LPS triggered high levels of IL-6, IL-1ß, TNF-α, IL-12p40 and MIP-1α, but only little IL-10. Cytokine amounts induced by LPS were several magnitudes higher than those triggered by iRBCs (data not shown). In the presence of iRBCs and LPS, cytokine expression was significantly upregulated compared to corresponding controls ($p < 0.01$, Wilcoxon signed-rank test). For the subsequent analysis, basal cytokine expression in negative controls (uRBCs) was subtracted from iRBC samples. Of the 32 *P. falciparum* cultures used for co-cultivation with monocytes, 17 were growing in RBCs with normal hemoglobin and 15 in RBCs of α-thalassemic donors (13 heterozygous, two homozygous).

In general, cytokine responses induced by α-thalassemic RBCs were substantially lower compared to responses induced by iRBCs with normal hemoglobin (Figure 19, Table 9). However, iRBC-stimulated IL-10 was hardly detectable in both subsets, and was therefore excluded from further analysis. A pronounced effect of reduced monocyte activation due to α-thalassemia was observed for IL-1ß and IL12-p40 ($p < 0.05$, Mann-Whitney U test). Compared to iRBCs with normal hemoglobin, median IL-1ß and IL-

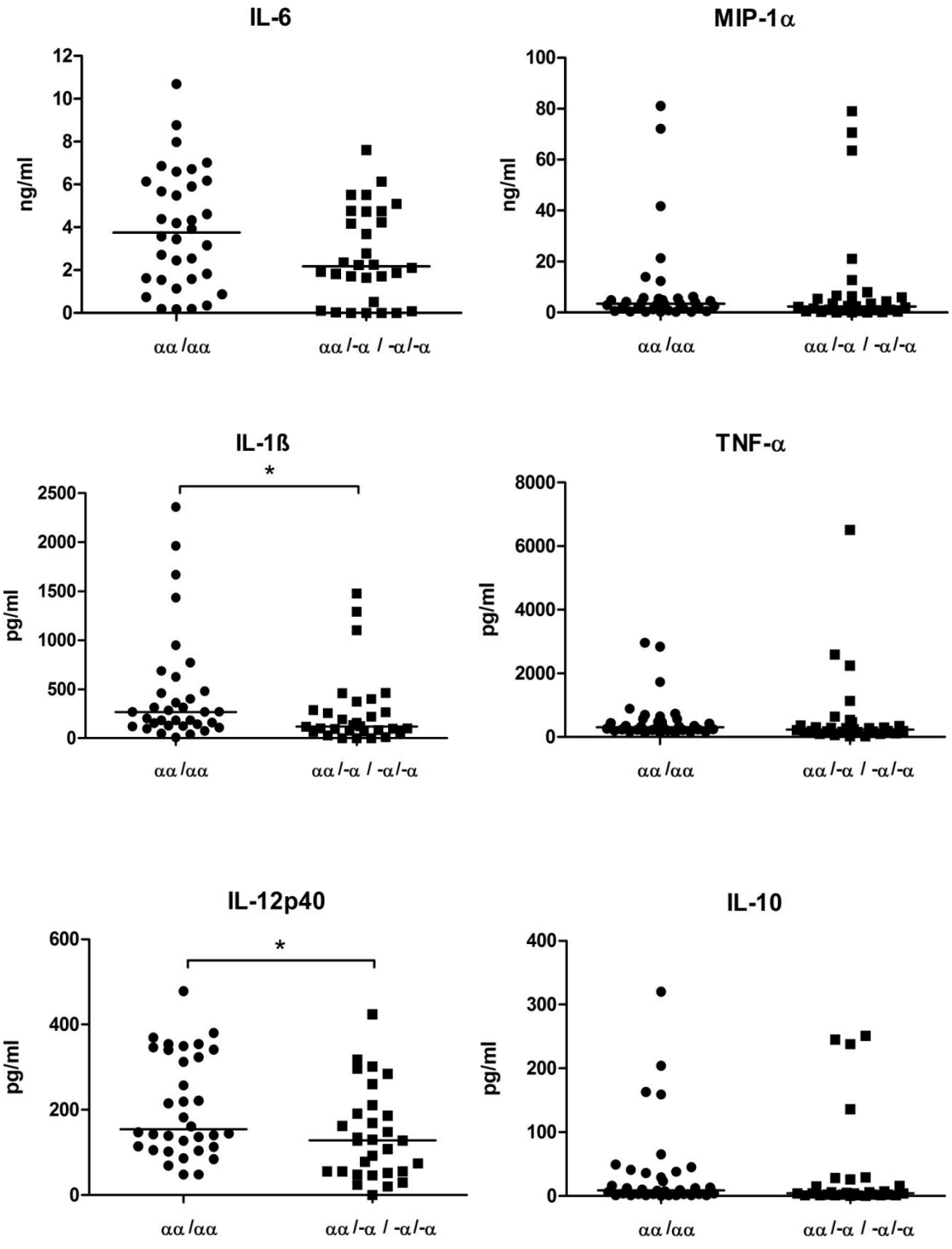

Figure 19: Monocytes were co-cultivated with *P. falciparum* infecting α-thalassemic and non-thalassemic RBCs. 20 h of incubation supernatants were analyzed by ELISA. Co-cultures were run in parallel with monocytes from two different donors. One dot represents one iRBC/monocyte co-culture. Lines indicates groupwise median. αα/αα = 17, αα/-α /-α/-α = 15, monocyte donors = 10. * $p < 0.05$ Mann-Whitney U test.

12p40 release of monocytes after co-cultivation with α-thalassemic iRBCs was reduced by 57 % and 17 %, respectively, compared to iRBCs with normal hemoglobin. Moreover, the iRBC-stimulated IL6-production appeared considerably reduced by 42 % in co-cultures with α-thalssemic iRBCs compared to non-variant iRBCs. Except for some outliers, heterogeneity of monocyte responses was similar after co-cultivation with *P. falciparum* cultures growing in non-variant and α-thalassemic RBCs. Only IL-1ß responses towards α-thalassemic iRBCs showed a slightly reduced variance.

Table 9: Cytokine expression of monocytes to *P. falciparum*-infecting normal and α-thalassemic RBCs

Genotype	IL-6	MIP-1α	IL-1ß	TNF-α	IL-12p40	IL-10
	ng/ml	ng/ml	pg/ml	pg/ml	pg/ml	pg/ml
αα/αα	**3.75** [1.57, 6.15]	**3.41** [1.11, 5.53]	**277** [128, 516]	**303** [209, 567]	**154** [111, 340]	**10** [3, 39]
αα/-α / -α/-α	**2.17** [0.42, 4.74]	**2.30** [0.54, 6.46]	**119** * [67, 310]	**232** [154, 394]	**128** * [54, 196]	**4** [0, 19]

Cytokine production of monocytes induced by *P. falciparum* cultures growing in RBCs with normal hemoglobin (αα/αα) and α-thalassemic (αα/-α, -α/-α) RBCs (MOI 5). Co-cultures were run in parallel with monocytes from two different donors. Corresponding negative controls (uRBCs) were previously subtracted. Values displayed are grouped medians with interquartile range (25^{th} and 75^{th} percentile) given in square brackets. Monocyte donors = 10, αα/αα = 17 , αα/-α /-α/-α = 15. * $p < 0.05$, Mann-Whitney U test.

We further assessed whether confounding RBC polymorphisms and non-genetic factors, such as parasitemia and experimental run, affect the observed association between α-thalassemia and reduced monocyte responses to iRBCs, simultaneously accounting for the inter-individual variance among RBCs and monocytes. Due to low numbers, sickle cell trait, present in only three RBC donors, was neglected and G6PD deficiency trait and G6PD full defect were pooled (9 RBC donors). Consistent with previous results, iRBCs from donors with α-thalassemia induced considerably lower monocyte responses for all cytokines irrespective of parasitemia and experimental run. Multivariate analysis revealed a significant effect of α-thalassemia on reduced IL-1ß and IL-6 responses of monocytes ($p < 0.02$, Wald test). G6PD deficiency showed no evidence of interaction with α-thalassemia.

3.4.2 Infected RBCs induce phenotypic alterations of monocytes

In order to assess whether contact with *P. falciparum*-infected RBCs leads to phenotypic alterations of monocytes, we investigated the expression of the Fcγ receptor CD64, CD40, CD80 and CD86, co-stimulatory molecules involved in T cell activation and TREM-1 (triggering receptor expressed on myeloid cells 1) enhancing monocyte-mediated inflammatory responses, after 20 h of co-culture with iRBCs (Figure 21).

Flow cytometric analysis indicated that significantly more cells expressed CD40, CD86 and TREM-1 after co-cultivation with iRBCs compared to cells incubated with uRBCs or kept in medium alone. Contrarily, a significant lower percentage of cells expressed CD64 in the presence of iRBCs relative to monocytes incubated with uRBCs only ($p < 0.005$, Wilcoxon signed-rank test) (Table 10, Figure 20). The presence of non-autologous uRBCs, RBCs originating from a different donor than monocytes, already induced slight phenotypic alterations compared to cells kept in medium alone. Generally, monocytes did not express any CD80 and it was only induced in the presence of LPS (Table 10, Figure 21). Furthermore, LPS stimulation gave rise to a significant larger percentage of cells expressing CD40, CD86 and TREM-1 compared to unstimulated monocytes ($p < 0.005$, Wilcoxon signed-rank test). LPS had no influence on CD64 expression of monocytes.

Concomitantly, increased numbers of monocytes expressing the activation markers CD40, CD86 and TREM-1 upon co-cultivation with iRBCs, correlated with significantly higher median fluorescence intensitiy (MFI) values. That means, individual cells expressed higher levels of respective surface molecules upon contact with iRBCs (Table 11). In the case of CD64, in accordance with reduced numbers of CD64 positive cells, MFI values for CD64 were also significantly decreased after co-cultivation with iRBCs ($p < 0.001$, Wilcoxon signed-rank test). Presence of LPS induced MFI values comparable to iRBCs which were significantly higher in comparison with MFI values of cells kept in medium alone ($p < 0.005$, Wilcoxon signed-rank test). However, the allover changes of median MFI values triggered by iRBCs and LPS, except for TREM-1, were only minor.

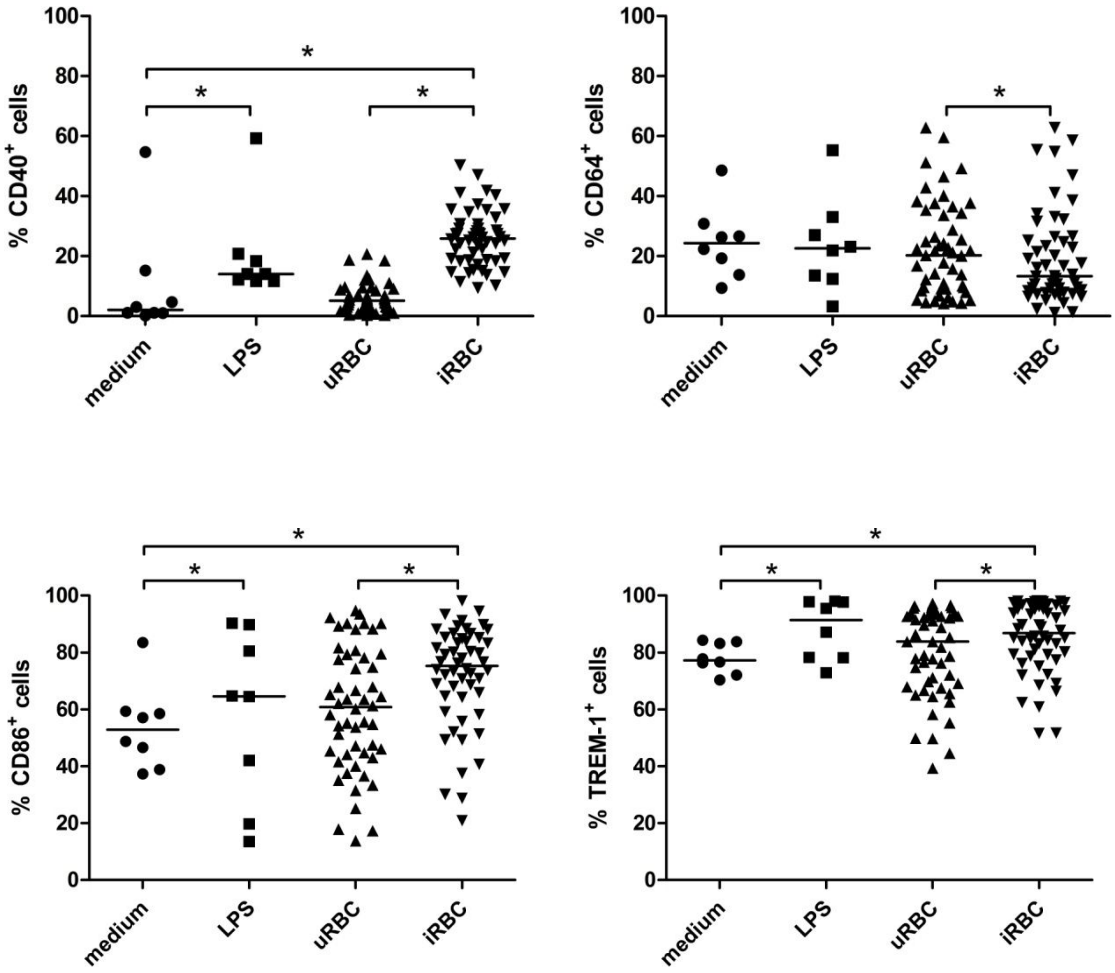

Figure 20: Expression of surface molecules by monocytes after co-cultivation with iRBCs irrespective of the RBC genotype. Medium, monocytes cultured in medium alone; LPS, stimulated with LPS (10 ng/ml); iRBC, monocytes co-cultivated with *P. falciparum*-infected RBCs; uRBC, monocytes incubated with uninfected RBCs of respective donors. Cells were stained with fluorsecently-labelled antibodies against CD40, CD64, CD86 and TREM-1, expression of surface markers was analyzed by flow cytometry. Values of corrseponding isotype controls were subtracted. Line represents median percentage of positive cells of eight independent experiments (eight monocyte donors, 26 RBC donors/*P. falciparum* cultures). * $p < 0.05$, Wilcoxon signed-rank test.

Table 10: Expression of surface molecules by monocytes after co-cultivation with iRBCs

Surface molecule	medium	LPS	uRBC	iRBC
CD40	2 [1, 13]	14 * [12, 20]	5 [3, 8]	26 * [19, 30]
CD64	24 [15, 30]	23 [13, 32]	20 [9, 34]	13 * [8, 26]
CD80	0 [0, 1]	6 * [5, 9]	0 [0, 1]	0 [0, 1]
CD86	53 [41, 59]	65 * [25, 88]	61 [44, 79]	75 * [64, 85]
TREM-1	77 [73, 84]	91 * [78, 98]	84 [68, 93]	87 * [79, 96]

Median percentage of positive cells and interquartile range (in square brackets) determined in eight independent experiments are given. Values of corresponding isotype controls were previously subtracted. * $p < 0.05$, Wilcoxon signed-rank test, compared to corresponding negative controls, LPS compared to cells kept in medium alone and iRBCs to uRBCs.

Table 11: MFIs of surface molecules expressed by monocytes after co-cultivation with iRBCs

Surface molecule	medium	LPS	uRBC	iRBC
CD40	2 [1, 4]	4 *[3, 9]	2 [2, 4]	6 * [4, 8]
CD64	12 [11, 14]	13 * [10, 15]	11 [10, 12]	9 * [8, 11]
CD80	0 [0, 1]	1 * [1, 3]	0 [0, 1]	0 [0, 1]
CD86	19 [14, 22]	22 * [11, 34]	20 [14, 24]	23 * [19, 28]
TREM-1	34 [29, 50]	84 * [54, 96]	52 [40, 60]	60 * [41, 84]

MFI values are given as medians of eight independent experiments (eight monocyte donors). Values in square brackets are the 25^{th} and 75^{th} percentile (interquartile range), respectively. MFIs of isotype controls were previously subtracted. * $p < 0.05$, Wilcoxon signed-rank test compared to corresponding negative controls, LPS compared to cells kept in medium alone and iRBCs to uRBCs, correspondingly.

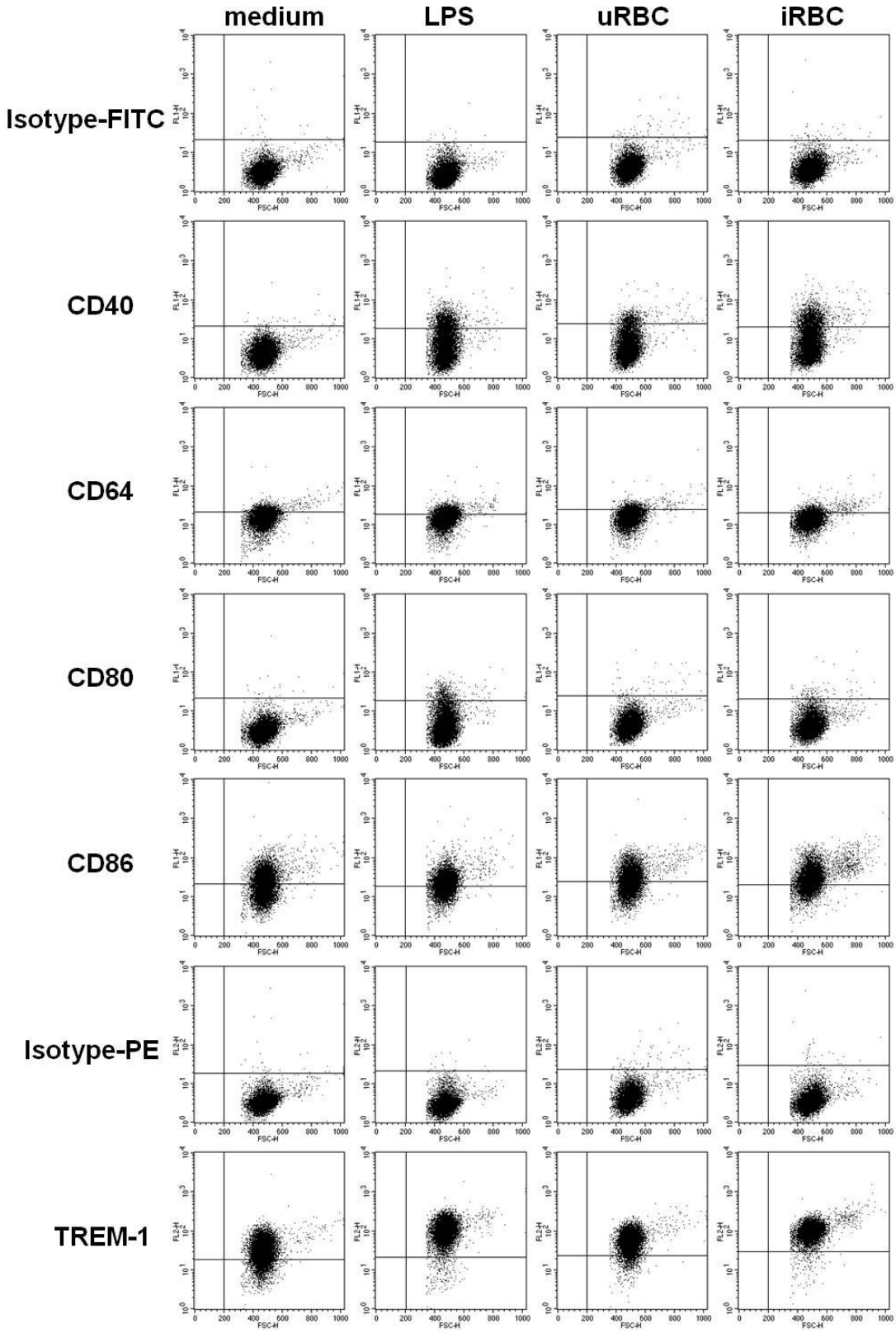

Figure 21: Expression of surface molecules by monocytes after co-cultivation with *P. falciparum*. Monocytes were co-cultivated with iRBCs for 20 h at a MOI of 5. Cells incubated with uRBCs of the same donor, with LPS (10 ng/ml) or growth medium alone served as controls. Monocytes were stained with fluorescently-labeled antibodies against CD40, CD64, CD80, CD86 (FITC) and TREM-1 (PE). Expression of surface markers on monocytes was measured by flow cytometry. Data shown represents one of eight independent experiments (eight monocyte donors) co-cultivated with iRBCs from one RBC donor.

3.4.3 α-Thalassemia has no impact on monocyte phenotype

To investigate whether α-thalassemic iRBCs trigger a differential expression of surface activation markers by monocytes compared to iRBCs with normal hemoglobin, we analyzed the obtained flow cytometry results based on the RBC genotype of *P. falciparum* cultures. Twelve of the 26 parasite cultures were growing in α-thalassmic RBCs (eleven heterozygous, one homozygous).

To compare the expression of monocyte surface markers induced by normal and α-thalassemic RBCS, we first subtracted the percentage of monocytes being positive for a specific surface marker in the presence of uRBCs from the respective percentage induced by iRBCs to compare the obtained deviations grouped by RBC genotype only.

No significant difference in the expression of monocyte surface markers triggered by iRBCs due to α-thalassemia was observed for none of the analyzed surface molecules (Table 12, Figure 22). MFI values were processed and analyzed in the same way and neither showed any alteration due to the RBC genotype of the parasite culture (Table 12).

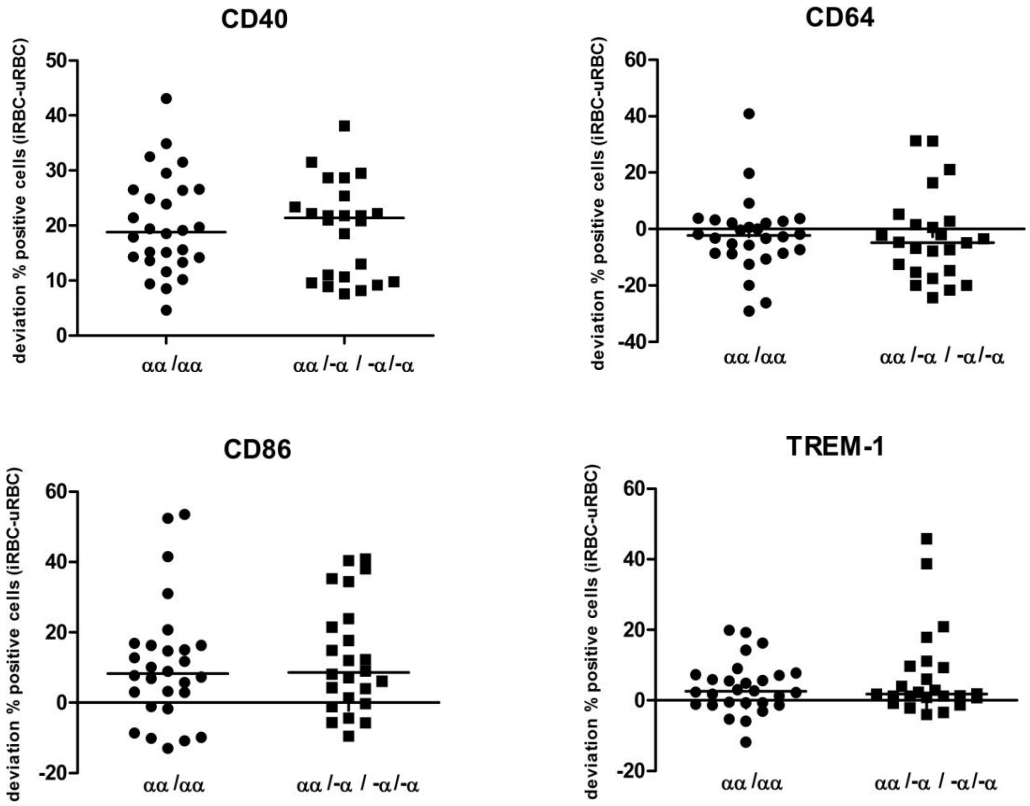

Figure 22: Expression of surface molecules by monocytes after co-cultivation with α-thalassemic and non-thalassemic iRBCs. Cells were stained with fluorsecently-labelled antibodies against CD40, CD64, CD86 and TREM-1. Expression of surface markers was analyzed by flow cytometry. Corresponding negative controls (uRBCs) were previously subtracted. Lines represent median deviation of positive cells (iRBC-uRBC) of eight independent experiments (eight monocyte donors). αα/αα = 14, αα/-α /-α/-α = 12.

Table 12: Monocyte expression of surface molecules induced by iRBCs grouped by RBC genotype

	% positive cells		MFI	
Surface molecule	**αα/αα**	**αα/-α / -α/-α**	**αα/αα**	**αα/-α / -α/-α**
CD40	+ 18.8 [13.7, 26.5]	21.4 [10.0, 24.9]	3.3 [2.2, 5.8]	3.2 [1.65, 4.6]
CD64	- 2.3 [-8.6, 2,5]	- 4.8 [-15.1, 2.5]	-1.6 [-2.2, -0.8]	-1.6 [-2.0, -0.8]
CD80	+ 0.3 [0.1, 0.7]	+ 0.2 [-0.3, 0.5]	0.1 [-0.2, 0.2]	0 [-0.2, 0.2]
CD86	+ 8.3 [-0.1, 16.3]	+ 8.6 [0.2, 23.3]	2.6 [0.3, 4.6]	2.3 [0.2, 4.6]
TREM-1	+ 2.5 [-1.0, 7.3]	+ 1.9 [0.8, 9.6]	6.7 [1.4 21.6]	9.7 [-0.3, 17.8]

Monocyte expression of surface molecules after co-cultivation with α-thalassemic and non-thalassemic iRBCs. Values given are calculated by subtracting corresponding negative controls (uRBCs) from expression values triggered by iRBCs. Medians of processed values for the percentage of cells being positive for a certain marker with corresponding MFI values of eight independent experiments are displayed (eight monocyte donors). IQR is given in square brackets. αα/αα = 14, αα/-α /-α/-α = 12.

4 DISCUSSION

Malaria is still a major cause of disease and death in many developing countries. It remains largely ambiguous why only a minority of *P. falciparum*-infected individuals develops severe, life-threatening disease while others remain asymptomatic or show uncomplicated, febrile episodes. Due to its pathogenicity, *P. falciparum* is thought to be the strongest selective force in the recent history of the human genome. *Vice versa*, human genetic factors are considered to strongly determine malaria susceptibility and manifestation. Besides red blood cell polymorphisms involving classic protective factors such as sickle cell trait, G6PD deficiency and α-thalassemia, genetic variations of immune-response-associated mediators including TLRs, TNF-α and IFN-γ receptors, have been identified to affect susceptibility and outcome of falciparum malaria (Aidoo et al. 2002, Aitman et al. 2000, Bedu-Addo et al. 2013, Bienzle et al. 1972, Koch et al. 2002, Mockenhaupt et al. 2004, Mockenhaupt et al. 2006b). We recently added to the list of protective traits or confirmed their role as malaria resistance loci, blood group O, the *ATP2B4* polymorphism rs10900585 and a common matrix metalloproteinase-9 variant (Apoorv et al. 2015, Bedu-Addo et al. 2013, Bedu-Addo et al. 2014). The underlying functional mechanisms, however, remain largely obscure. Therefore, we investigated in the present study the impact of α-thalassemia and two novel RBC polymorphisms, *ATP2B4* (rs10900585) and *BSG* (rs1803202), as well as of TLR polymorphisms either on parasite invasion and multiplication or on the innate immune response induced by iRBCs. The current findings contribute to an improved understanding of the complex host-parasite interplay in *P. falciparum* infections and the impact of host genetic polymorphisms on malaria susceptibility and manifestation.

4.1 Innate cytokine responses towards iRBCs are heterogeneous

In the present study we investigated the impact of genetic variants on the innate cytokine response induced by iRBCs. In our experiments, we used *P. falciparum* late-stage-infected RBCs (iRBCs) to stimulate mononuclear cells. During co-cultivation, iRBCs ruptured releasing merozoites and parasite-derived components. The rupture of parasitized RBCs induces the release of substantial amounts of pro-inflammatory cytokines into the blood circulation associated with fever in malaria patients (Kwiatkowski and Nowak 1991, Kwiatkowski et al. 1989). Previous studies have shown

that peripheral blood mononuclear cells (PBMCs) and also purified monocytes rapidly release pro-inflammatory cytokines upon incubation with iRBCs *in vitro* (Artavanis-Tsakonas K. et al. 2003b, Corrigan and Rowe 2010, Hensmann and Kwiatkowski 2001, Pichyangkul et al. 1994, Scragg et al. 1999). In the present study, iRBCs induced substantial amounts of IL-6 and MIP-1α and moderate to low levels of TNF-α and IL-1ß in PBMC and monocyte co-cultures depending on the parasite-effector cell ratio. Hence, the presence of iRBCs was sufficient to induce an adequate activation of monocytes without the need of additional signals from accessory cells. A ratio of five parasites per mononuclear cell (MOI 5) induced stable responses in both cell subsets and seemed to be the optimum parasite concentration for the activation of PBMCs and monocytes consistent with clinical observations that the probability to develop clinical malaria is significantly increased with a parasite-leukocyte ratio higher than two (Greenwood B. M. et al. 1987, Trape et al. 1985).

Cytokine expression patterns induced by iRBCs in mononuclear cells differed from those triggered by the TLR agonists, LPS, Pam2CSK4 and Pam3CSK4. In contrast to LPS isolated from *E. coli* and the synthetic lipopeptides Pam2CSK4 and Pam3CSK4, specific ligands for TLR4, TLR2/TLR6 and TLR2/TLR1, respectively, live *P. falciparum*-infected RBCs operate as multicomponent immunostimulatory mediators. Binding of several parasite- (PAMPs) and host cell-derived (DAMPs) components to different PRRs (TLRs, NOD-like receptors, RIG-I-like receptors, inflammasomes) simultaneously trigger the activation of distinct signaling pathways mediating inflammatory responses (Gazzinelli et al. 2014). Not all molecular interactions involved in the induction of innate response in *P. falciparum* infections have been identified yet. As reported by others, iRBC-stimulated cytokine expression was several magnitudes lower compared to responses induced by LPS (Scragg et al. 1999).

Consistent with previous reports, cytokine responses induced by iRBCs were heterogeneous and cytokine levels differed considerably among individual donors (Artavanis-Tsakonas K. and Riley 2002, Walther et al. 2006). Except for IL-1ß inter-individual heterogeneity was even more pronounced in monocyte co-cultures compared to PBMCs. Considerable heterogeneity was also observed among cytokine responses induced by Pam2CSK4 and Pam3CSK4 indicating that differential responses to iRBCs originate rather from genetic differences among donors than inter-experimental variance due to the use of live parasites.

4.2 *TLR1* I602S and *TLR6* P249S impair monocyte response to iRBCs

TLRs are considered to play a crucial role in mediating inflammatory innate responses in malaria. GPIs purified from *P. falciparum* cultures were shown to be potent activators of innate immune cells inducing the expression of pro-inflammatory cytokines and chemokines, mainly through TLR2/TLR1, TLR2/TLR6 and to a lesser extent through TLR4 (Krishnegowda et al. 2005, Kumar S. et al. 2012, Schofield and Hackett 1993, Tachado et al. 1996). Several genetic polymorphisms of the TLR signaling pathways have been associated with malaria susceptibility and manifestation (Leoratti et al. 2008, Mockenhaupt et al. 2006b, Schroder and Schumann 2005). However, the underlying mechanisms largely remain unclear. In this study, we investigated polymorphism of genes encoding for TLRs involved in the recognition of *P. falciparum* GPI, *TLR2* R753Q, *TLR1* I602S and *TLR6* P249S. The latter two, *TLR1* I602S and *TLR6* P249S, have been associated with an increased risk to develop malaria (Leoratti et al. 2008). In our donor cohort, the frequency of the *TLR2* R753Q variant was very low and was therefore excluded. To our knowledge, this is the first study showing a direct impact of TLR polymorphisms associated with differential malaria outcome on the innate cytokine response to *P. falciparum*-infected RBCs.

In response to iRBCs, monocytes from malaria-naïve donors with variant *TLR1* I602S and *TLR6* P249S alleles showed reduced levels of IL-6, IL-1ß and MIP-1α. Differential cytokine expression due to *TLR1* I602S and *TLR6* P249S was more prominent at higher parasite densities. Homozygous *TLR6* 249S monocytes revealed a pronounced decrease of iRBC-stimulated IL-6 production and in contrast to *TLR1* 602S also showed a reduced release of TNF-α. Correspondingly, IL-6, IL-1ß and MIP-1α responses to iRBCs were lowest for monocytes from donors carrying both variant alleles.

The specificity of our findings regarding genotype-based differential cytokine responses to iRBCs were supported by the results of the included controls. Analogous to differential recognition of bacterial peptides with two or three fatty acyl groups, di- (*sn-2-lyso*) and triacylated (intact and Man_3) *P. falciparum*-derived GPIs are preferentially recognized by TLR2/TLR6 and TLR2/TLR1 heterodimers, respectively. In accordance with previous studies, *TLR1* I602S conferred a decreased cytokine production of PBMCs and monocytes in response to Pam3CSK4 (Hawn et al. 2007, Johnson et al. 2007). Unexpectedly, the *TLR6* P249S polymorphism was associated with altered cytokine expression after stimulation with Pam3CSK4, but not Pam2CSK4. However, the same

findings were previously reported by another study investigating the impact of *TLR6* polymorphisms on cytokine responses induced by bacterial peptides (Shey et al. 2010). Whereas, cytokine production after stimulation with the TLR4 agonist LPS was not associated with differential cytokine expression due to *TLR1* and *TLR6* polymorphisms.

The more prominent effect of *TLR6* P249S compared to *TLR1* I602S on differential cytokine responses to iRBCs might be explained by the use of 20 % human serum in our monocyte co-culture medium. Phospholipase A_2 and phospholipase D present in human serum, but also on the surface of monocytes, have been found to degrade *P. falciparum*-derived GPIs by removing fatty acid residues (Davitz et al. 1987, Hoffmann et al. 1987). One in vitro study showed that after exposure to monocytes in culture medium with or without serum, the majority of plasmodial GPIs was degraded mainly into inactive forms lacking phosphatidylinositol while a smaller proportion was converted into *sn-2-lyso* GPIs suggesting a mode of partial regulation of GPI activity by the host's immune system (Krishnegowda et al. 2005). Therefore, a large proportion of *P. falciparum*-derived GPIs in our co-culture system is potentially degraded into inactive or *sn-2-lyso* GPIs preferentially recognized by TLR2/TLR6.

The more pronounced effect of differences in the monocyte activation due to *TLR1* I602S and *TLR6* P249S induced by Pam3CSK4 and Pam2CSK4 compared to iRBCs might be explained by the nature of iRBCs as multicomponent mediator of inflammatory responses in contrast to Pam3CSK4 and Pam2CSK4, triggering only one specific signaling pathway. Parasites or parasite-derived components released upon schizont rupture trigger multiple inflammatory pathways besides TLR2 heterodimers, involving TLR4, TLR9, NOD-like and RIG-I-like receptors as well as cytoplasmic inflammasomes (Gazzinelli et al. 2014). However, the use of live, late-stage-infected RBCs instead of synthetic or *P. falciparum*-derived GPI allowed us to study the impact and relevance of the polymorphisms on the early inflammatory response triggered by iRBCs as a whole instead of looking at an isolated receptor-ligand interaction.

TLR1 I602S is the most common SNP among Caucasians affecting TLR function identified to date (Johnson et al. 2007). An isoleucine to serine transition at position 602 interrupts the trafficking motif inhibiting cell surface expression of TLR1. Monocytes and macrophages isolated from individuals homozygous for the *TLR1* 602S variant were shown to be hyporesponsive to soluble TLR1 agonists due to lack of cell surface TLR1 (Hart and Tapping 2012, Hawn et al. 2007, Johnson et al. 2007).

The polymorphism *TLR6* P249S (rs5743810, C > T) encodes a proline to serine transition in the extracellular domain of TLR6 leading to conformational changes in the protein structure involving a lower number of functional pockets and a substantially reduced flexibility likely to affect binding of ligands or associated receptors and interfere with ligand-induced conformational changes (Hamann et al. 2013). Due to the considerable conformational changes caused by *TLR6* P249S affecting binding pockets, clefts and cavities, *in silico* modelling revealed that binding of *sn-2-lyso* GPI to the TLR6 249S differed substantially from the wildtype form of the receptor (Figure 13, Figure 14). Altogether, the different tertiary structure, the reduced flexibility of the TLR6 249S and the altered interaction with GPI might affect the affinity of the receptor-ligand interaction and eventually also ligand-induced TLR2/TLR6 heterodimerization and abolish signaling. However, this is speculative and needs to be further investigated.

Concomitantly to reduced cytokine levels, heterogeneity of iRBC-stimulated responses among monocytes isolated from individuals with the variant *TLR1* I602S allele was markedly reduced. A genome-wide association study recently identified the *TLR1/6/10* locus as dominant genetic factor controlling inter-individual variance in cytokine responses to TLR2/TLR1 agonists. Common genetic variants of *TLR2* and genes of the intracellular signaling pathway such as *TIRAP*, *MyD88* and *IRAK* only appear to play a minor role in TLR2/TLR1 response variation (Mikacenic et al. 2013). It seems that intracellular TLRs (TLR3, TLR7, TLR8, TLR9) have undergone a stronger purifying selection than cell-surface TLRs and therefore show a lower genetic variance which might also apply to further intracellular PRRs such as NOD-like and RIG-I-like receptors (Barreiro et al. 2009). Therefore, inflammatory responses to iRBCs in *TLR1* I602S and *TLR6* P249S homozygous individuals might be limited to alternative signaling pathways due to impaired TLR2/TLR1- and TLR2/TLR6-mediated signaling and thus result in lower heterogeneity.

The observed differences among monocyte responses due to *TLR1* I602S and *TLR6* P249S were not discernible on the level of PBMCs. Expression levels of TLR2 forming heterodimers with TLR1 or TLR6 are higher in monocytes than in any other cell subset of PBMCs (Flo et al. 2001, Hornung et al. 2002). Proportions of the different PBMC subpopulations including lymphocytes (B cells, T cells and NK cells), monocytes and dendritic cells vary widely among individuals. Due to their high expression levels, monocytes appear as the most appropriate PBMC subpopultation to investigate the impact of genetic variants of TLR2 heterodimers. Varying monocyte proportions among PBMCs

from different donors might explain the lack of *TLR1* I602S and *TLR6* P249S genotype-specific cytokine expression patterns in PBMC co-cultures compared to monocytes.

Our results show that *TLR1* I602S and *TLR6* P249S lead to a decreased production of pro-inflammatory cytokines of monocytes towards *P. falciparum*-infected RBCs suggesting a functional role of the polymorphisms in malaria pathogenesis. A tightly controlled balance between pro- and anti-inflammatory cytokines seems to be essential to clear an infection with *P. falciparum* without inducing host immunopathology (Stevenson and Riley 2004). IL-6, IL-1ß and TNF-α are essential for the induction of the acute-phase response. Their early production together with other inflammatory mediators is crucial for rapid parasite clearance. Whereas, MIP-1α attracts macrophages and other leukocytes to sites of inflammation (Lee et al. 2000). In particular, low levels of IL-6 have been linked to hyperparasitemia in *P. falciparum* infected children (Kremsner et al. 1995, Lyke et al. 2004, Walther et al. 2006). On the one hand, severe malaria has been clearly associated with elevated levels of inflammatory cytokines, on the other hand, curbed early innate responses may also promote the development of clinical disease. Lowered responses might fail to limit the initial phase of parasite replication and concomitantly affect adaptive responses essential for infection clearance. High parasite densities have been correlated with disease severity of *P. falciparum* infections previously (Idro et al. 2006, Phillips et al. 2009).

In contrast to high prevalences in Caucasians, *TLR1* I602S and *TLR6* P249S variants are remarkably scarce in populations of malaria-endemic regions (Apinjoh et al. 2013, Hamann et al. 2010, Hamann et al. 2013, Shey et al. 2010). Furthermore, functional *TLR2* SNPs are reportedly absent indicating that, likely due to *P. falciparum* pathogenicity, genetic variations of *TLR1*, *TLR2* and *TLR6* have been under selective pressure in malaria-endemic regions (Bali et al. 2013, Hise et al. 2003, Mockenhaupt et al. 2006b). Besides *TLR1* I602S and *TLR6* P249S, another genetic variant of *TLR1*, *TLR1* S248N (rs4833095) has been identified as a risk factor for malaria (Hamann et al. 2010, Leoratti et al. 2008, Omueti et al. 2007).

Taken together, our findings provide relevant information on the functional role of the *TLR1* I602S and *TLR6* P249S polymorphisms in malaria pathogenesis. This is the first study to describe a differential responsiveness of innate immune cells to *P. falciparum*-infected RBCs due to TLR polymorphisms. Together with epidemiological data, our results emphazise the functional relevance of TLR2/TLR1- and TLR2/TLR6-mediated innate responses in *P. falciparum* infections and that differential innate immune responses

might contribute to malaria pathology. However, further investigations are needed to disentangle the effect of *TLR1* I602S and *TLR6* P249S on innate responses to *P. falciparum in vivo* and to determine to which extent differential monocyte activation influences disease outcome in malaria. Such knowledge would allow to deduce intervention strategies by modulating *P. falciparum* infection towards less pathology.

4.3 A polymorphism of *ATP2B4* has no impact on parasite development

Recent studies have identified ATP2B4, an ubiquitous plasma membrane Ca^{2+} ATPase (PMCA4), as a novel resistance locus for malaria (Bedu-Addo et al. 2013, MGEN 2014, Timmann et al. 2012). The intronic polymorphism of *ATP2B4* rs10900585 might affect mRNA splicing, thereby impair protein expression or function leading to a modified cellular Ca^{2+} homeostasis. Decreased Ca^{2+}concentrations of the parasitophorous vacuole have been shown to affect parasite maturation (Gazarini et al. 2003). Given that PMCA4 is the major Ca^{2+} pump of RBCs, *ATP2B4* rs10900585 was proposed to mediate protection against severe malaria by impaired intraerythrocytic development of *P. falciparum* due to a modified Ca^{2+} homeostasis (Timmann et al. 2012). We therefore investigated an impact of the polymorphism on parasite growth also to account for such an influence when analyzing the respective effect of α-thalassemia, but our results clearly showed that the *ATP2B4* polymorphism has no impact on intraerythrocytic development and growth of *P. falciparum*. Neither absolute parasite density nor parasite development and invasion efficacy was altered due to the *ATP2B4* variant which is consistent with our epidemiological data revealing no correlation between parasite densities and *ATP2B4* genotypes (Bedu-Addo et al. 2013). Our results therefore suggest that an alternative mechanism is responsible for differential malaria manifestation due to the *ATP2B4* variant. PMCA 4 is expressed in different splice isoforms, in a tissue-specific manner, in RBCs, endothelial cells, platelets and many other cells (Brini and Carafoli 2009, Strehler and Zacharias 2001).

Therefore, modified cytosolic Ca^{2+} homeostasis in cells other than RBCs, such as platelets and endothelial cells, might play a role in the protective mechanism of the polymorphisms against severe pathology. Ca^{2+} signaling is essential for many cellular processes including regulation of gene transcription and cell activation (Feske 2007). Moreover, modified activation of platelets, including intraerythrocytic parasite killing and their involvement in iRBC sequestration, as well as endothelial cells, affecting iRBC

cytoadherence, were proposed as alternative mechanisms by which altered Ca^{2+} homeostasis might affect malaria pathology (Bridges et al. 2010, McMorran et al. 2009, Timmann et al. 2012). Since Ca^{2+} signaling plays a crucial role in the differentiation and activation of several effector mechanisms of various immune cells, modified Ca^{2+} homeostasis might also alter immune responses in *P. falciparum* infections and thereby malaria manifestation (Feske 2007). Our study is the first addressing the underlying mechanism of *ATP2B4* rs10900585 on differential malaria manifestation. Further functional studies are required to identify the responsible mechanism confering protection against severe malaria.

4.4 A polymorphism of *BSG* impairs *P. falciparum* growth

Several receptor-ligand interactions have been identified to mediate *P. falciparum* invasion into host RBCs, but so far only the interaction of BSG and *Pf*RH5 was found to be essential for RBC invasion irrespective of the parasite strain. Two naturally occurring genetic polymorphisms of BSG, L90P and E92K (Ok^{a-} blood group), showed reduced affinity to *Pf*RH5 and lower parasite invasion efficacy for RBCs of donors with the E92K variant (Crosnier et al. 2011). The extreme low frequency of E92K and its restriction to individuals of Japanese origin indicate that this polymorphism does not play a major role in malaria resistance (Crosnier et al. 2011, Spring et al. 1997). However, it is likely that under the strong selective pressure of *P. falciparum* further *BSG* polymorphisms have evolved in populations of malaria-endemic regions.

A synonymous substitution in exon 2 of *BSG* (rs1803202, C > T, Asp-Asp) was recently found to be associated with malaria severity in an African population residing in a malaria-endemic area (Gabon). The variant allele of *BSG* rs1803202 was found to reduce the risk of severe malaria (Velavan TP, Institute for Tropical Medicine, University of Tübingen, data not published). Synonymous SNPs (sSNPs) do not result in a change of the amino acid in the translated protein and it has long been assumed that they do not have any functional consequences. However, there is growing evidence that sSNPs affect protein confirmation and function and substantially contribute to human disease susceptibility (Sauna and Kimchi-Sarfaty 2011). It was shown that synonymous mutations can cause abnormal RNA splicing which might affect RNA stability or protein confirmation and thereby protein expression and activity (Cartegni et al. 2002, Kimchi-Sarfaty et al. 2007, Nackley et al. 2006).

To determine whether the observed protection against malaria conferred by *BSG* rs1803202 is mediated by an impaired invasion and intraerythrocytic growth of *P. falciparum* and to account for such an influence when analyzing the respective effect of α-thalassemia, we assessed parasite growth in *BSG* variant and non-variant RBCs over two replication cycles. Parasitemia of *P. falciparum* cultures containing *BSG* variant RBCs gradually decreased over time and was lowest for cultures with homozygous variant RBCs. Comparing univariate analysis investigating solely the effect of the *BSG* genotype and multivariate analysis including confounding RBC polymorphisms, parasite densities were significantly reduced due to *BSG* rs1803202 at different time points. As for univariate analysis, parasitemia in heterozygous RBC cultures was only observed at the time of the first RBC invasion (24 h), while cultures with homozygous variant RBCs showed considerably lower parasite densities only after 48 h and 72 h. Grouping of *P. falciparum* cultures of heterozygous and homozygous variant *BSG* RBCs for multivariate analysis revealed that parasite densities in *P. falciparum* cultures containing variant *BSG* RBCs were significantly lower compared to cultures with non-variant RBCs after 72 and 96 h. Nonetheless, apart from differences regarding time points both analysis clearly showed that the variant *BSG* allele is less supportive for intraerythrocytic *P. falciparum* growth.

Given the assumption that the *BSG* variant abrogates RBC invasion of *P. falciparum*, we would have expected a significant attenuated invasion efficacy accompanied by a drop in parasitemia at the time of merozoite invasion, at 24 h and 72 h. However, susceptibility of RBCs to merozoite invasion did not differ between genotypes contradicting with the substantially decreased parasite densities observed in *P. falciparum* cultures containing *BSG* variant RBCs at 24 h and 72 h. Determination of invasion efficacy by merly calculating the ratio of total parasitemia measured at the time of invasion and 24 h before (24 h/0 h, 72 h/48 h) might not be the optimal method to assess accurate reinvasion rates. Comparing total parasite densities without taking into account that parasite cultures might contain distinct developmental stages due to the loss of synchronous growth over time, might lead to false results and alternative methods might be more appropriate. Furthermore, we did not observe any evidence of developmental delay or growth arrest due to *BSG* genotypes.

Functional consequences of the synonymous polymorphism *BSG* rs1803202 as well as the exact mode of impaired of *P. falciparum* growth in variant *BSG* RBCs need to be further investigated. It is likely that additional genetic variants of *BSG* providing

protection against malaria are found in populations residing in endemic areas. Due to the proposed dependence on the BSG-*Pf*RH5 interaction for *P. falciparum* irrespective of the strain, respective insights might provide a novel target for intervention strategies.

4.5 α-Thalassemia impairs parasite growth and dampens early inflammatory response of monocytes

Like other hemoglobinopathies, α-thalassemia is found at particularly high frequencies in malaria-endemic regions and was shown to provide protection against severe falciparum malaria, in particular against severe malarial anemia. Clinical studies suggest that the protective effect of α-thalassemia acts on the level of disease manifestation rather than on parasite denisities or infection *per se* (Allen et al. 1997, May et al. 2007, Mockenhaupt et al. 2004, Veenemans et al. 2008). Despite numerous investigations, the underlying cellular and molecular mechanisms involved still remain obscure.

We assessed *P. falciparum* growth in α-thalassemic and non-variant RBCs over two replication cycles *in vitro*. After completion of the first replication cycle, poor reinvasion rates were observed for α-thalassemic RBCs (at 72 h) accompanied by substantially reduced levels of total parasitemia suggesting that the trait might confer protection against severe falciparum malaria by limiting parasite densities. Our results are consistent with previous findings on diminished parasite growth and reduced reinvasion in α-thalassemic RBCs in the second or third replication cycle (Pattanapanyasat et al. 1999, Senok et al. 1997). With increased culture duration, reduced growth rates in α-thalassemic RBCs were found to be associated with a growth arrest at the schizont stage (schizont maturation arrest) and an accumulation of late parasite-stages with morphological changes due to cellular degeneration (Senok et al. 1997, Senok et al. 2006). However, we did not observe any evidence of developmental delay or growth arrest at a mature schizont stage due to α-thalassemia. A recent study investigating the intraerythrocytic multiplication factor (IMF) of *P. falciparum* by quantifying the number of merozoites released from individual schizont-infected RBCs *in vitro*, found that IMFs are lower in α-thalassemic compared to normal RBCs (Glushakova et al. 2014). The release of lower numbers of merozoites from α-thalassemic iRBC coincides with our findings that reinvasion rates and parasite densities drop after the first round of replication without involving a developmental arrest or cellular degeneration. Furthermore, we observed an association between the RBC

parameters, MCH and MCV, and total parasitemia levels after completion of the first replication cycle irrespective of the RBC genotype. Correspondingly, MCH and MCV were previously found to strongly influence IMFs with *P. falciparum* producing more merozoites in larger RBCs with higher levels of hemoglobin (Glushakova et al. 2014). *P. falciparum* uses hemoglobin as a major nutrient source during blood-stage development (Goldberg et al. 1990). Microcytic α-thalassemic RBCs with reduced hemoglobin concentrations might therefore provide suboptimal conditions for parasite development leading to lowered parasite densities. However, an effect of MCV and MCH on parasite multiplication implies that altered RBC parameters might also affect parasite growth in other hemoglobinopathic RBCs with modified RBC indices, such as HbS RBCs. Therefore, it needs to be further investigated whether altered RBC parameters are responsible for impaired parasite growth in α-thalassemic RBCs or whether a mechanism independent of MCV and MCH specific for α-thalassemia leads to reduced parasite multiplication.

Further, we investigated whether α-thalassemic iRBCs induce an impaired monocyte activation compared to non-variant iRBCs. Recent evidence has indicated a role of impaired cytoadherence as protective mechanism in α-thalassemia, but also in further hemoglobinopathies such as HbS and HbC (Fairhurst et al. 2012, Fairhurst et al. 2005, Krause et al. 2012). A previous study, showed substantially reduced cytoadherence of *P. falciparum*-infected α-thalassemic RBCs to monocytes and microvascular endothelium cells. Reduced adhesion was associated with an abnormal display of fewer and enlarged knobs on the iRBC surface correlating with a reduced expression of PfEMP1 (Krause et al. 2012). Our results showed that monocytes produced significantly less IL-1ß and IL-12p40 when exposed to *P. falciparum* infecting α-thalassemic RBCs compared to control iRBCs. Adjusting for confounding RBC polymorphisms, the effect of curbed monocyte activation due to α-thalassemia was pronounced for IL-1ß and IL-6.

An early production of inflammatory cytokines such as IL-6, IL-12, TNF-α and IFN-γ is crucial for rapid parasite clearance, however, excessive production of pro-inflammatory cytokines has been strongly associated with malaria pathology. Decreased inflammatory responses in α-thalassemia might be sufficient to control the concomitantly lowered parasite densities thereby preventing host immunopathology and severe malaria as a result of excessive pro-inflammatory responses. A major complication of severe falciparum malaria is severe malarial anemia which is caused by the loss and the immune-mediated destruction of iRBCs in addition to an excessive removal of uninfected RBCs

and an insufficient compensation due to impaired erythropoesis (Haldar and Mohandas 2009). Monocyte-derived pro-inflammatory cytokines IL-6, IL-1ß, TNF-α and IFN-γ are thought to substantially contribute to the development of severe malarial anemia due to their suppressive effect on erythropoiesis (Awandare et al. 2011, Chang and Stevenson 2004, Haldar and Mohandas 2009, Weatherall et al. 2002). IL-6, IL-1ß and TNF-α were shown to directly inhibit the renal production of erythropoietin as well as erythroid cell differentiation (Buck et al. 2009, Faquin et al. 1992, Vannucchi et al. 1994). Furthermore, IL-6 incudes the production of hepcidin, a key regulator of iron metabolism, controlling the systemic iron homeostasis by diminishing the iron availability for erythropoesis (Nemeth et al. 2003, Wrighting and Andrews 2006). IL-23 and IL-12 share the IL-12p40 subunit. Therefore, decreased levels of IL-12p40 induced by α-thalassemic iRBCs would affect both cytokines. IL-12 and IL-23 share common properties, but apparently also have distinct immunological functions. In immuno-epidemiological studies, elevated levels of IL-23, but low levels of IL-12 were associated with an increased risk of severe malaria (Ong'echa et al. 2008, Perkins et al. 2000). Thus, reduced levels of IL-12p40 might limit circulating IL-23 thereby mediating protection against anemia. However, the divergent roles of IL-12 and IL-23 in malaria anemia need to be further investigated. IL-10 seems to play an important role in counteracting pro-inflammatory response in *P. falciparum* infections preventing immunopathology and ratios of TNF-α or IL-6 and IL-10 seemed to be good predictors of malaria outcome (Day et al. 1999, Ho et al. 1998, May et al. 2000). However, after 20 h of co-culture IL-10 was not present in most samples indicating that either a prolonged incubation or additional signaling from accessory cells is required for the monocyte expression of IL-10. The chemokine MIP-1α attracts macrophages and other leukocytes to sites of inflammation and was also found to inhibit proliferation of hematopoetic progenitor cells (DiPietro et al. 1998, Lee et al. 2000, Owen-Lynch et al. 1998). An extensive activation of macrophages is thought to considerably contribute to malaria pathology through the secretion of high levels of pro-inflammatory cytokines as well as through their enhanced phagocytosis of infected and uninfected RBCs (Biemba et al. 1998). Reduced levels of MIP-1α leading to a moderate activation of macrophages and a modest impact on hematopoesis might therefore be beneficial in protection against severe disease and malarial anemia. Curbed innate responses could thus contribute to the observed mildened courses of disease in α-thalassemic patients, in particular to the reduced risk to develop severe malaria anemia.

In addition to cytokine release, we observed monocyte activation based on the surface expression of activation markers. The presence of iRBCs enhanced the expression of CD40, CD86 and TREM-1 in our monocyte population. CD40 and CD86 are co-stimulatory molecules important for T cell activation, predominantly expressed on antigen-presenting cells such as monocytes, macrophages, DCs and B cells. Together with antigens, pro-inflammatory cytokines were shown to synergistically promote expression of CD40 and CD86 (Steinbrink et al. 2000). The observed elevated levels of CD40 and CD86 might be a consequence of monocyte exposure to iRBCs and pro-inflammatory cytokines in co-cultures. An upregulation of CD40 expression by monocytes after incubation with iRBCs has previously been reported (Newman et al. 2006). However, a study investigating a differential CD86 expression induced by *P. falciparum*-infected RBCs observed a downregulation of the surface marker which is contradictory to our results (Cordery et al. 2007). TREM-1 enhances the secretion of pro-inflammatory cytokines during acute inflammation and is assumed to play an important role in regulating the function of activated neutrophils, monocytes and macrophages (Nathan and Ding 2001, Schenk et al. 2007). Consistent with our findings, monocytes from individuals infected with *P. falciparum* were reported to show elevated expression of TREM-1 (Chimma et al. 2009). However, the role of TREM-1 in human malaria still remains unclear. In contrast to CD40, CD86 and TREM-1, upon contact with iRBCs CD80 expression was not induced and a significantly lower percentage of monocytes expressed CD64. CD64 is a high-affinity receptor for IgG involved in antibody-opsonized phagocytosis of iRBCs (Tebo et al. 2002). A drop in CD64 could explain the impaired repeated phagocytosis of iRBCs or hemozoin by monocytes observed by others (Schwarzer et al. 2003). In contrast to, impaired cytokine responses to α-thalassemic iRBCs, we did not observe any differences in the expression of monocyte activation markers between monocytes co-cultivated with α-thalassemic and normal iRBCs. This might indicate an involvement of different surface markers or the restriction of the α-thalassemic effect to early cytokine production.

Whether the abnormal PfEMP1/knob display on the surface of α-thalassemic iRBCs observed by others and a therby weakend interaction of CD36 expressed on monocytes with PfEMP1 leads to an impaired activation of monocytes remains unclear. CD36 mediates the non-opsonic phagocytosis of iRBCs by mononuclear cells but its role in the induction of inflammatory responses in malaria is controversial (McGilvray et al. 2000, Serghides et al. 2006). Several lines of evidence suggest that CD36-mediated

phagocytosis alone does not trigger the production of pro-inflammatory cytokines but that CD36 rather presents plasmodial antigens such as *P. falciparum* GPI to TLR2 (Erdman et al. 2009, Patel et al. 2007). Alternatively, upon schizont burst, microcytic, hypochromic α-thalassemic RBCs might release less parasite-derived components and metabolic waste products, such as uric acid, as well as hemoglobin and free heme triggering the activation of innate immune cells (Figueiredo et al. 2007, Gallego-Delgado et al. 2014). Reduced levels of respective PAMPs and DAMPs released from α-thalassemic iRBCs might therefore lead to an impaired activation of monocytes. However, the functional reason of impaired monocyte activation by α-thalassemic iRBCs needs to be further investigated. Our findings suggest that α-thalassemia confers protection against severe *P. falciparum* malaria by a combination of reduced parasite multiplication limiting the rapid development of high parasite densities and a curbed activation of host cells. We show for the first time that pro-inflammatory responses of monocytes to malaria parasites growing in α-thalassemic RBCs are impaired potentially thereby preventing severe manifestation. However, the exact mode of diminished monocyte activation in α-thalassemia remains obscure. Severe malarial anemia is a leading cause of morbiditiy and mortality in children. Monocyte-derived pro-inflammatory cytokines directly suppress erythropoiesis and thereby promote the development of severe malarial anemia. Lower levels of circulating inflammatory cytokines together with the modest loss of total hemoglobin due to elevated numbers of microcytic, hypochromic RBCs might prevent the development of severe malarial anemia in individuals with α-thalassemia (Fowkes et al. 2008). Although it is well known that monocytes are a major source of inflammatory cytokines in early *P. falciparum* infection, little is known about the monocyte phenotype during acute malaria and whether monocyte activation might determine disease manifestation. Our findings confirm their role as key players of innate responses in *P. falciparum* infections and malaria pathology and provide additional information on their phenotype during acute infection. In contrast to the potentially deleterious curbed innate responses due to TLR polymorphisms, decreased monocyte responses in α-thalassemia might be sufficient to control the lowered parasite multiplication while being beneficial in terms of not inducing excess damage to the host. Concomitantly, a reduced cytoadherence of α-thalassemic iRBCs to microvascular endothelial cells, a major contributor to disease pathology in *P. falciparum* infections, might lead to an reduced activation of endothelial cells thereby preventing microvascular inflammation and endothelial damage (Krause et al. 2012).

Concluding, we suggest a multifactorial protection against severe malaria in α-thalassemia mediated by a combination of impaired parasite multiplication, curbed monocyte activation and reduced microvascular inflammation. Further investigations are needed to elucidate the molecular mechanisms responsible for impaired monocyte activation by α-thalassemic iRBCs, to disentangle the interplay of the different factors involved in the protection against severe malaria and their compliance with *in vivo* observations.

4.6 Conclusion

The present work provides novel insights into the complex host-pathogen interaction in human *P. falciparum* infections and contributes to the understanding of the underlying functional mechanisms of RBC and TLR polymorphisms influencing malaria susceptibility and manifestation.

Our results show that *TLR1* I602S and *TLR6* P249S which have been associated with increased malaria risk correspond to a decreased monocyte production of pro-inflammatory cytokines towards *P. falciparum*-infected RBCs suggesting a functional role of *TLR1* I602S and *TLR6* P249S in malaria pathogenesis. Together with epidemiological data on prevalence and impact on clinical outcome, our results emphazise the relevance of TLR2/TLR1- and TLR2/TLR6-mediated responses in *P. falciparum* infections.

Like other hemoglobinopathies, α-thalassemia is found at particularly high frequencies in malaria-endemic regions and was shown to provide protection against severe falciparum malaria, in particular against severe malarial anemia. We observed reduced reinvasion rates for α-thalassemic RBCs accompanied by substantially decreased levels of total parasitemia and impaired pro-inflammatory responses of monocytes to malaria parasites growing in α-thalassemic RBCs. Our findings therefore suggest that α-thalassemia confers protection by a combination of reduced parasite multiplication limiting the rapid development of high parasite densities in infected α-thalassemic individuals accompanied by an impaired activation of host cells.

The implications of *ATP2B4* (rs10900585) and *BSG* (rs1803202) conferring protection against malaria and their underlying mechanisms need to be further investigated.

Taken together, our results suggest that besides RBC polymorphisms conferring relative resistance to malaria by limiting parasite densities, variance in the ability to induce an

adequate inflammatory response due to genetic factors substantially contributes to differential malaria outcome. Inflammatory innate responses are essential to limit the initial phase of parasite replication until specific adaptive responses are acquired to clear the infection. Curbed innate responses induced by impaired TLR signaling might therefore lead to an uncontrolled parasite replication and high parasite densities associated with severe pathology. Whereas, decreased inflammatory responses in α-thalassemia might be sufficient to control the concomitantly lowered parasite densities thereby protecting from immunopathology as a result of excessive pro-inflammatory responses.

High mortality rates of falciparum malaria resulted in a positive selection of genes that confer relative resistance. Our *in vitro* findings therefore help to explain high prevalences of α-thalassemia in malaria-exposed populations and the virtual absence of *TLR1* I602S and *TLR6* P249S.

The present findings contribute to a better understanding of malaria pathology which is essential for the development of novel intervention strategies against this deadliest parasitic disease of humans. Due to the lack of an efficient vaccine and emerging resistance to antimalarial drugs novel intervention strategies are urgently needed. The development of novel anti-parasitic approaches to modulate host-mediated immunopathology, such as corticosteroids or monoclonal antibodies to TNF-α or plasmodial GPI, used in combination with antimalarial treatment might substantially reduce disease severity and malaria mortality.

5 ABSTRACT

Malaria is one of the most important infectious diseases worldwide and is still a major cause of disease and death in many developing countries. *Plasmodium falciparum* is considered as the strongest selective force in the recent history of the human genome. *Vice versa*, genetic host factors play an important role in susceptibility to and manifestation of falciparum malaria. Relative resistance conferred predominantly by red blood cell (RBC) polymorphisms against malaria is seen across endemic regions. Further understanding of the human genetic factors determining disease outcome will help to unravel the complex host-pathogen interplay in *P. falciparum* infections and may reveal potential novel intervention strategies.

We investigated the impact of Toll-like receptor (TLR) polymorphisms on innate immune responses to *P. falciparum*-infected red blood cells (iRBCs) as well as the role of RBC polymorphisms on *P. falciparum* growth and innate immune responses to iRBCs to deduce insights in malaria susceptibility and pathophysiology.

TLRs are crucial mediators of innate immune responses in *P. falciparum* infections and single nucleotide polymorphisms (SNPs) of TLRs involved in the recognition of iRBCs or parasite-derived components have been linked to differential malaria manifestation. *P. falciparum* glycosylphosphatidylinositol (GPI) induces pro-inflammatory responses mainly *via* TLR2 forming heterodimers with TLR1 or TLR6. We co-cultivated iRBCs with human mononuclear cells from malaria-naïve donors with different TLR genotypes, and found monocytes with variant *TLR1* I602S and *TLR6* P249S alleles to produce decreased levels of inflammatory cytokines in response to iRBCs. Curbed, TLR-mediated innate responses might promote the development of high parasite densities and, consequently, severe disease suggesting a functional role of the polymorphisms in malaria pathogenesis. Together with epidemiological data, our results emphasize the relevance of TLR2/TLR1- and TLR2/TLR6-mediated responses in *P. falciparum* infections as well as the role of differential innate immune responses in malaria pathology.

Next, we assessed the impact of several RBC polymorphisms on RBC invasion and intraerythrocytic parasite development. RBCs with and without genetic variants were infected with *P. falciparum* and parasite growth was followed over two erythrocytic replication cycles. A recently described malaria-protective RBC polymorphism of

ATP2B4, encoding the major erythrocytic calcium pump, showed no impact on intraerythrocytic development and growth of *P. falciparum*. Conversely, a variant allele of *Basigin*, a receptor essential for RBC invasion by *P. falciparum*, was associated with significantly reduced intraerythrocytic parasite growth.

As main focus of the study, we investigated the effect of α-thalassemia, an extremely common RBC polymorphism in malaria endemic areas known to protect against severe, life-threatening falciparum malaria. So far, the cellular and molecular mechanisms involved in protection against malaria remain largely obscure. In the present study, we observed significantly reduced *P. falciparum* reinvasion rates accompanied by substantially decreased levels of total parasitemia in parasite cultures containing α-thalassemic RBCs. In addition, co-cultivation of monocytes with normal and α-thalassemic RBCs infected with *P. falciparum*, revealed that monocytes produced markedly less inflammatory cytokines when exposed to α-thalassemic iRBCs. Our findings therefore suggest that α-thalassemia confers protection by a combination of reduced parasite multiplication limiting the rapid development of high parasite densities accompanied by a curbed activation of host cells, in particular of monocytes, thereby preventing pathophysiology induced by excessive pro-inflammatory responses.

The present work contributes to a better understanding of the complex host-parasite interplay in malaria by providing novel insights into underlying mechanisms of RBC and TLR polymorphisms associated with differential malaria manifestation. A better knowledge on the molecular basis of malaria pathology may promote the development of novel approaches for anti-malarial intervention.

6 ZUSAMMENFASSUNG

Die Malaria ist weltweit eine der bedeutendsten Infektionskrankheiten und noch immer eine führende Todesursache in vielen Entwicklungsländern. Es wird angenommen, dass die durch den Erreger *Plasmodium falciparum* hervorgerufene Erkrankung den stärksten Selektionsdruck in der jüngeren Geschichte des humanen Genoms ausgeübt hat. Umgekehrt spielen genetische Wirtsfaktoren eine zentrale Rolle bei der Anfälligkeit gegenüber der Infektion mit *P. falciparum* und dem Krankheitsverlauf. Vor allem erythrozytäre Polymorphismen vermitteln relative Resistenz gegenüber der schweren und potentiell tödlichen Malaria und sind in endemischen Gebieten folglich häufig. Ein verbessertes Verständnis der humangenetischen Faktoren, die die komplexe Wirt-Pathogen-Interaktion und Pathophysiologie beeinflussen, ist die notwendige Grundlage neuer Ansätze zur Bekämpfung oder Eindämmung der Malaria.

Im Rahmen dieser Arbeit wurde der Einfluss von Polymorphismen der Toll-like Rezeptoren (TLR) auf die angeborene Immunantwort gegen *P. falciparum*-infizierte Erythrozyten ermittelt. Zudem wurde untersucht, inwiefern ausgewählte erythrozytäre Polymorphismen das Wachstum von *P. falciparum* beeinflussen und ob die angeborene Immunantwort gegenüber *P. falciparum* durch α-Thalassämie, eine in Malaria-Endemiegebieten extrem häufige Erythrozytenvariante, verändert wird, um letztlich neue Einblicke in die Pathophysiologie der Malaria zu erlangen.

TLRs sind an der Induktion angeborener Immunantworten gegenüber *P. falciparum* beteiligt, und *TLR*-Varianten sind mit veränderten Risiken für die Infektion oder Manifestation der Malaria assoziiert. *P. falciparum* Glycosylphosphatidylinositol (GPI) induziert pro-inflammatorische Antworten hauptsächlich über TLR2, welcher Heterodimere mit TLR1 oder TLR6 bildet. Mononukleäre Zellen von Malaria-naiven Spendern unterschiedlicher TLR-Genotypen wurden mit infizierten Erythrozyten ko-kultiviert. Dabei zeigte sich, dass *TLR1* I602S und *TLR6* P249S mit einer verminderten monozytären Zytokinantwort einhergingen. Eine abgeschwächte angeborene Immunantwort kann mutmaßlich zur Entstehung hoher Parasitendichten und schweren Krankheitsverläufen beitragen. Zusammen mit den Erkenntnissen epidemiologischer Studien unterstreichen unsere Ergebnisse die funktionelle Rolle von TLR2/TLR1- und TLR2/TLR6-vermittelten Antworten bei Infektionen mit *P. falciparum* und weisen darauf

hin, dass Unterschiede in der angeborenen Immunantwort den Malariaverlauf beeinflussen können.

Desweiteren untersuchten wir den Einfluss verschiedener erythrozytärer Polymorphismen auf das Eindringen und das Wachstum von *P. falciparum* innerhalb der Wirtszelle. Dazu wurden Erythrozyten mit und ohne genetische Varianten mit *P. falciparum* infiziert, und das Parasitenwachstum wurde über zwei erythrozytäre Replikationszyklen verfolgt. Ein kürzlich als protektiv beschriebener Polymorphismus in *ATP2B4*, welches die primäre erythrozytäre Calciumpumpe codiert, zeigte keinerlei Einfluss auf die intraerythrozytäre Entwicklung von *P. falciparum*. Im Gegensatz dazu war ein variantes Allel von Basigin, ein für die Invasion von *P. falciparum* essentieller Oberflächenrezeptor, mit signifikant vermindertem parasitärem Wachstum assoziiert.

Ein Schwerpunkt der Arbeit lag auf der α-Thalassämie, eine in Malaria-Endemiegebieten extrem häufige Erythrozytenvariante, die vor schwerer, lebensbedrohlicher Malaria schützt. Bislang sind die zellulären und molekularen Mechanismen der relativen Resistenz weitgehend unklar. In der vorliegenden Arbeit zeigten sich verminderte Reinvasionsraten in α-thalassämischen Erythrozyten und damit erheblich verringerte Parasitendichten. Die Ko-Kultivierung von Monozyten mit α-thalassämischen infizierten Erythrozyten zeigte im Vergleich zu den Ergebnissen mit normalen Erythrozyten eine signifikante Drosselung der Monozytenantwort in Form inflammatorischer Zytokine gegenüber *P. falciparum*. Unsere Ergebnisse legen folglich eine Kombination zweier α-thalassämischer Schutzmechanismen gegenüber der Malaria nahe: Die reduzierte Parasitenvermehrung im varianten Erythrozyten verhindert die rasante Entstehung hoher Parasitendichten, während die verminderte Aktivierung von Wirtszellen, insbesondere von Monozyten, durch infizierte α-thalassämische Erythrozyten der Pathophysiologie infolge einer exzessiven pro-inflammatorischen Immunantwort entgegenwirkt.

Die vorliegende Arbeit trägt zu einem besseren Verstehen der komplexen Wirt-Parasit-Interaktion bei der Malaria bei, indem sie neue Erkenntnisse zu den funktionellen Mechanismen genetischer Erythrozytenvarianten und TLR-Polymorphismen liefert, die ihrerseits mit veränderter Malaria-Anfälligkeit und Manifestation assoziiert sind. Ein besseres Verständnis der molekularen Ursachen, die zum Krankheitsverlauf beitragen, kann neue Ansatzpunkte für die Bekämpfung der Malaria aufzeigen.

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8 PUBLICATIONS

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9 CURRICULUM VITAE

For reasons of data protection, the curriculum vitae is not included in the online version.

10 APPENDIX

10.1 Supplementary data tables

10.1.1 Impact of *TLR1* I602S and *TLR6* P249S on innate responses to iRBCs

TLR1 I602S:

Table A1: Cytokine production of monocytes stratified by *TLR1* I602S genotype induced by different stimuli

	***TLR1* I602S genotype**	**n**	**IL-6** ng/ml	**IL-1ß** pg/ml	**TNF-α** pg/ml	**MIP-1α** ng/ml
iRBC (MOI 1)	**I/I (wt)**	**5**	**0.49** [0.25, 11.50]	**0** [0, 26]	**46** [19, 217]	**0.12** [0.05, 1.15]
	I/S (het)	**5**	**0.35** [0.10, 3.89]	**0**	**164** [31, 220]	**0.11** [0.01, 0.93]
	S/S (hom)	**12**	**0.09** [0.05, 0.46]	**0**	**78** [46, 128]	**0.09** [0.04, 0.16]
iRBC (MOI 5)	**I/I (wt)**	**6**	**14.06** [3.11, 40.92]	**532** [105, 1392]	**676** [310, 961]	**5.65** [2.46, 19.58]
	I/S (het)	**6**	**6.74** [3.94, 13.58]	**457** [151, 653]	**683** [344, 829]	**4.70** [3.02, 7.25]
	S/S (hom)	**14**	**7.85** [1.78, 11.36]	**348** [80, 469]	**641** [405, 805]	**3.51** [2.93, 5.06]
iRBC (MOI 10)	**I/I (wt)**	**4**	**18.57** [4.30, 32.25]	**658** [84, 1 974]	**626** [282, 1 483]	**11.70** [3.18, 21.29]
	I/S (het)	**3**	**10.93** [-]	**338** [-]	**517** [-]	**5.25** [-]
	S/S (hom)	**8**	**6.55** [3.75, 16.89]	**465** [93, 756]	**873** [274, 1 014]	**5.48** [4.21, 6.48]
Pam3CSK4	**I/I (wt)**	**6**	**71.57** [15.82, 107.30]	**598** [488, 862]	**1 437** [1 186, 2 380]	nd
	I/S (het)	**6**	**61.66** [10.50, 79.96]	**305** [124, 668]	**1 189** [988, 2 944]	nd
	S/S (hom)	**14**	**7.65** * [1.34, 11.54]	**2** * [0, 23]	**163** * [113, 260]	nd
LPS	**I/I (wt)**	**6**	**176.73** [134.76, 221.44]	**6 861** [4 270, 9 679]	**5 298** [3 316, 7 463]	nd
	I/S (het)	**6**	**151.84** [109.38, 210.65]	**3 843** [2 390, 5 955]	**4 085** [2 934, 6 239]	nd
	S/S (hom)	**14**	**153.21** [117.95, 197.36]	**4 061** [3 283, 6 000]	**4 337** [2 973, 8 073]	nd

Cytokine production of monocytes induced by different stimuli grouped by *TLR1* I602S genotype. n = number of donors. nd = not determined. Values of corresponding negative controls were previously subtracted. Values displayed are grouped medians, interquartile range (25^{th} and 75^{th} percentile) is given in square brackets. * $p < 0.05$, Mann-Whitney U test, compared to wildtype (I/I) or wildtype and heterozygous (I/S) (Pam3CSK4 samples).

Table A2: Cytokines secretion of PBMCs induced by different stimuli grouped by *TLR1* I602S genotype

	***TLR1* I602S genotype**	**n**	**IL-6** ng/ml	**IL-1ß** pg/ml	**TNF-α** pg/ml
iRBC (MOI 1)	**I/I (wt)**	**5**	**3.14** [0.34, 8.59]	**188** [0, 724]	**20** [9, 31]
	I/S (het)	**5**	**6.43** [4.05, 12.17]	**662** [487, 808]	**39** [20, 66]
	S/S (hom)	**12**	**6.99** [3.48, 10.28]	**234** [87, 639]	**23** [7, 74]
iRBC (MOI 5)	**I/I (wt)**	**6**	**3.81** [1.91, 8.81]	**542** [106, 1231]	**33** [17, 291]
	I/S (het)	**6**	**6.08** [1.80, 12.07]	**1 615** * [1 394, 2 027]	**118** [27, 168]
	S/S (hom)	**14**	**7.34** [3.00, 11.05]	**1 072** [649, 1 762]	**30** [7, 141]
iRBC (MOI 10)	**I/I (wt)**	**4**	**2.27** [0.63, 5.69]	**305** [63, 732]	**16** [14, 136]
	I/S (het)	**3**	**7.22** [-]	**1 230** [-]	**89** [-]
	S/S (hom)	**8**	**3.88** [1.12, 6.92]	**587** [139, 1 991]	**7** [3, 105]
Pam3CSK4	**I/I (wt)**	**6**	**17.90** [13.60, 26.41]	**64** [22, 179]	**118** [67, 204]
	I/S (het)	**6**	**15.49** [9.08, 30.08]	**126** [13, 370]	**164** [50, 248]
	S/S (hom)	**14**	**3.56** * [1.79, 5.55]	**0** *	**4** * [0, 16]
LPS	**I/I (wt)**	**6**	**55.14** [35.74, 74.75]	**2 018** [1 530, 3 615]	**546** [236, 1 001]
	I/S (het)	**6**	**49.83** [35.60, 95.66]	**2 561** [848, 5 505]	**580** [128, 829]
	S/S (hom)	**14**	**55.52** [46.68, 80.77]	**2 019** [1 677, 2 869]	**407** [270, 530]

Cytokine production of PBMCs induced by different stimuli grouped by *TLR1* I602S genotype. n = number of donors. nd = not determined. Values of corresponding negative controls were previously subtracted. Values displayed are grouped medians, interquartile range (25^{th} and 75^{th} percentile) is given in square brackets. * $p < 0.05$, Mann-Whitney U test, compared to wildtype (I/I) or wildtype and heterozygous (I/S) (Pam3CSK4 samples).

TLR6 P249S:

Table A3: Cytokines secretion of monocytes triggered by different stimuli stratified by *TLR6* P249S genotype

	***TLR6* P249S genotype**	**n**	**IL-6** ng/ml	**IL-1ß** pg/ml	**TNF-α** pg/ml	**MIP-1α** ng/ml
iRBC (MOI 1)	**P/P (wt)**	**16**	**0.48** [0.09, 2.53]	**0**	**84** [32, 167]	**0.12** [0.04, 0.44]
	S/S (hom)	**6**	**0.06** * [0.04, 0.14]	**0**	**71** [35, 140]	**0.08** [0.02, 0.17]
iRBC (MOI 5)	**P/P (wt)**	**19**	**9.12** [5.25, 21.04]	**493** [134, 900]	**684** [376, 816]	**4.26** [3.10, 7.03]
	S/S (hom)	**7**	**1.96** * [1.09, 8.07]	**277** [40, 372]	**606** [265, 801]	**3.12** [2.46, 4.56]
iRBC (MOI 10)	**P/P (wt)**	**11**	**10.93** [5.70, 28.19]	**397** [113, 1 280]	**844** [407, 993]	**5.32** [4.35, 7.90]
	S/S (hom)	**4**	**4.63** [2.79, 6.87]	**309** [76, 737]	**578** [259, 1014]	**5.09** [2.93, 6.96]
Pam2CSK4	**P/P (wt)**	**19**	**52.41** [13.29, 106.67]	**130** [39, 198]	**520** [353, 699]	nd
	S/S (hom)	**7**	**57.27** [9.82, 99.76]	**115** [81, 241]	**500** [459, 684]	nd
LPS	**P/P (wt)**	**19**	**155.24** [121.94, 209.74]	**5 449** [3 449, 8 304]	**4 954** [3 116, 7 920]	nd
	S/S (hom)	**7**	**151.18** [76.44, 216.04]	**3 938** [2 720, 4 159]	**4448** [3 031, 7 871]	nd

Table A4: Cytokine response of PBMCs induced by different stimuli grouped by *TLR6* P249S genotype

	***TLR6* P249S genotype**	**n**	**IL-6** ng/ml	**IL-1ß** pg/ml	**TNF-α** pg/ml
iRBC (MOI 1)	**P/P (wt)**	**16**	**4.12** [2.70, 8.17]	**460** [104, 735]	**21** [8, 40]
	S/S (hom)	**6**	**7.74** [3.99, 11.23]	**326** [106, 561]	**57** [20, 93]
iRBC (MOI 5)	**P/P (wt)**	**19**	**6.65** [2.53, 10.13]	**1 095** [334, 1 618]	**38** [19, 123]
	S/S (hom)	**7**	**7.58** [2.48, 11.41]	**1 137** [705, 2 127]	**138** [10, 228]
iRBC (MOI 10)	**P/P (wt)**	**11**	**2.62** [1.92, 7.22]	**463** [252, 1 230]	**18** [4, 89]
	S/S (hom)	**4**	**4.31** [1.58, 6.92]	**1 148** [241, 3 348]	**66** [3, 312]
Pam2CSK4	**P/P (wt)**	**19**	**17.28 [**10.16, 23.48]	**31** [0, 196]	**53** [30, 104]
	S/S (hom)	**7**	**19.32** [16.99, 21.76]	**41** [0, 96]	**77** [24, 153]
LPS	**P/P (wt)**	**19**	**56.64** [39.93, 80.51]	**2 127** [1 777, 3 585]	**489** [236, 602]
	S/S (hom)	**7**	**53.33** [46.49, 63.41]	**1 852** [1 291, 2 203]	**439** [300, 569]

Cytokine production of PBMCs and monocytes induced by different ratios of iRBCs (MOI 1, 5 and 10), 10 ng/ml of Pam2CSK4 and LPS grouped by *TLR6* P249S genotype. n = number of donors. nd = not determined. Values of corresponding negative controls were previously subtracted. Values displayed are grouped medians with interquartile range (25^{th} and 75^{th} percentile) shown in square brackets. * $p < 0.05$, Mann-Whitney U test, compared to wildtype (P/P).

TLR1 I602S / *TLR6* P249S combinations:

Table A5: Monocyte response towards iRBCs stratified by combined *TLR1* I602S/*TLR6* P249S genotype

iRBC	*TLR1* I602S genotype	*TLR6* P249S genotype	n	IL-6 ng/ml	IL-1ß pg/ml	TNF-α pg/ml	MIP-1α ng/ml
MOI 1	I/I	P/P	5	**0.49** [0.25, 11.50]	**0**	**46** [19, 217]	**0.12** [0.05, 1.15]
	I/S / S/S	P/P	11	**0.35** [0.08, 1.95]	**0**	**84** [31, 165]	**0.11** [0.03, 0.43]
	S/S	S/S	6	**0.06** [0.04, 0.14]	**0**	**71** [35, 140]	**0.08** [0.02, 0.17]
MOI 5	I/I	P/P	6	**14.06** [3.11, 40.92]	**532** [105, 1392]	**676** [310, 961]	**5.65** [2.46, 19.58]
	I/S / S/S	P/P	13	**8.19** [5.31, 15.96]	**421** [146, 739]	**684** [398, 806]	**3.92** [3.12, 6.13]
	S/S	S/S	7	**1.96** * [1.09, 8.07]	**277** [40, 372]	**606** [265, 801]	**3.12** [2.46, 4.56]
MOI 10	I/I	P/P	4	**18.57** [4.30, 32.25]	**658** [84, 1974]	**626** [282, 1482]	**11.70** [3.18, 21.29]
	I/S / S/S	P/P	7	**10.93** [5.70, 24.23]	**397** [113, 1280]	**861** [502, 993]	**5.32** [4.46, 6.52]
	S/S	S/S	4	**4.63** [2.79, 6.87]	**309** [76, 737]	**578** [259, 1014]	**5.09** [2.93, 6.96]

Cytokine production of monocytes induced by different ratios of iRBCs (MOI 1, 5 and 10) grouped by combined *TLR1* I602S/*TLR6* P249S genotype. n = number of donors. Values of corresponding negative controls were previously subtracted. Values displayed are grouped medians with interquartile range (25^{th} and 75^{th} percentile) given in square brackets. * $p < 0.05$, Mann-Whitney U test, compared to (*TLR1* I/S, S/S combined with *TLR6* P/P).

10.1.2 Impact of RBC polymorphisms on parasite growth and development

Table A6: Total parasitemia of parasite cultures stratified by RBC genotype for α-thalassemia, *ATP2B4*, *BSG*

Total parasitemia (%)	*n*	Duration of culture (hours) 0 h	24 h	48 h	72 h	96 h
α-thalassemia (3.7kb deletion)						
αα/αα	**111**	**0.06** [0.03, 0.14]	**0.17** [0.10, 0.28]	**0.26** [0.15, 0.40]	**1.08** [0.62, 1.61]	**1.23** [0.72, 1.75]
αα/-α	**42**	**0.09** [0.02, 0.17]	**0.14** [0.07, 0.32	**0.22** [0.06, 0.33]	**0.89** [0.32, 1.33]	**0.69** ** [0.31, 1.46]
-α/-α	**5**	**0.14** [0.04, 0.21]	**0.17** [0.09, 0.30]	**0.35** [0.20, 0.54]	**1.06** [0.61, 1.48]	**1.29** [1.00, 2.06]
ATP2B4 rs10900585 (T > G)						
G/G	**41**	**0.06** [0.03, 0.16]	**0.17** [0.07, 0.32]	**0.22** [0.14, 0.34]	**1.01** [0.55, 1.46]	**0.97** [0.61, 1.43]
G/T	**76**	**0.07** [0.03, 0.17]	**0.17** [0.10, 0.28]	**0.27** [0.12, 0.40]	**1.12** [0.49, 1.56]	**1.21** [0.54, 1.71]
T/T	**41**	**0.07** [0.02, 0.13]	**0.13** [0.09, 0.36]	**0.26** [0.15, 0.39]	**0.92** [0.44, 1.73]	**1.04** [0.53, 1.66]
BSG rs1803202 (C > T)						
C/C	**104**	**0.08** [0.03, 0.17]	**0.19** [0.10, 0.36]	**0.27** [0.15, 0.42]	**1.18** [0.57, 1.63]	**1.20** [0.61, 1.75]
C/T	**48**	**0.06** [0.02, 0.13]	**0.14** * [0.07, 0.21]	**0.26** [0.14, 0.35]	**0.95** [0.41, 1.29]	**0.97** [0.46, 1.55]
T/T	**6**	**0.03** [0.02, 0.09]	**0.12** [0.06, 0.16]	**0.12** * [0.04, 0.21]	**0.49** * [0.20, 0.78]	**0.81** [0.51, 0.99]

Values for total parasitemia are given as median percentage for each genotype group with interquartile range (25th and 75th percentile) in square brackets. * $p < 0.05$, ** $p < 0.01$, Mann-Whitney U test, compared to respective wildtype.

Table A7: MFI of *P. falciparum* cultures stratified by genotype for α-thalassemia, *ATP2B4* and *BSG*

MFI	n	Duration of culture (hours) 0 h	24 h	48 h	72 h	96 h
α-thalassemia (3.7kb deletion)						
αα/αα	**111**	**51** [29, 69]	**21** [17, 26]	**59** [50, 70]	**21** [18, 26]	**51** [44, 60]
αα/-α	**42**	**50** [27, 67]	**19** [17, 23]	**55** [44, 70]	**21** [18, 26]	**53** [38, 62]
-α/-α	**5**	**69** [46, 89]	**24** [14, 26]	**79** * [65, 87]	**21** [19, 28]	**53** [44, 75]
ATP2B rs10900585 (T > G)						
G/G	**41**	**47** [28, 56]	**22** [18, 26]	**62** [46, 75]	**21** [18, 26]	**51** [45, 59]
G/T	**76**	**52** [33, 71]	**21** [17, 25]	**59** [50, 72]	**22** [18, 26]	**52** [42, 63]
T/T	**41**	**59** [30, 77]	**19** [17, 23]	**57** [46, 67]	**20** [18, 24]	**49** [39, 62]
BSG rs1803202 (C > T)						
C/C	**104**	**52** [30, 69]	**20** [17, 24]	**60** [50, 72]	**20** [18, 25]	**52** [41, 63]
C/T	**48**	**50** [30, 68]	**21** [18, 26]	**58** [48, 70]	**22** [19, 26]	**51** [42, 60]
T/T	**6**	**32** [22, 52]	**25** [19, 30]	**59** [39, 69]	**28** * [21, 30]	**50** [41, 55]

Values given are grouped medians and interquarile ranges (squared brackets) of processed MFI values.
* $p < 0.05$, Mann-Whitney U test, compared to the corresponding wildtype.

Table A8: Reinvasion rates of *P. falciparum* stratified by genotype

		Reinvasion rate	
	n	**24 h**	**72 h**
α-thalassemia (3.7kb deletion)			
αα/αα	**111**	**2.5** [1.5, 5.1]	**4.0** [3.3, 5.1]
αα/-α	**42**	**2.3** [1.0, 3.6]	**3.8** [2.7, 5.2]
-α/-α	**5**	**1.5** [0.8, 3.9]	**3.0** * [2.5, 3.4]
ATP2B4 rs10900585 (T > G)			
G/G	**41**	**2.2** [1.1, 4.6]	**3.8** [3.1, 4.7]
G/T	**76**	**2.5** [1.3, 4.6]	**4.0** [3.3, 5.3]
T/T	**41**	**3.0** [1.4, 5.5]	**3.7** [2.6, 4.7]
BSG rs1803202 (C > T)			
C/C	**104**	**2.4** [1.4, 4.8]	**3.9** [3.0, 5.0]
C/T	**48**	**2.3** [1.08, 5.0]	**3.9** [3.1, 5.3]
T/T	**6**	**4.1** [1.6, 5.4]	**4.1** [3.2, 5.5]

Values given are medians and IQRs (squared brackets).
* $p < 0.05$, Mann-Whitney U test, compared to respective wildtype.

10.2 List of abbreviations

A	adenine
Asp	aspartic acid
BSA	bovine serum albumin
BSG	basigin
C	cytosine
C	carbon
°C	degree centigrade
Ca	calcium
CCL3	chemokine (C-C motif) ligand 3
CD	cluster of differentiation
Cl	chlorine
CPDA	citrate phosphate dextrose adenine
CpG	cytosine and guanine separated by one phosphate
CR1	complement receptor 1
CSA	chondroitinsulfate A
DAMP	damage-associated molecular pattern
d	deci
D	aspartic acid
dH_2O	distilled water
DMSO	dimethyl sulfoxide
DNA	desoxyribonucleic acid
ds	double-stranded
DPBS	Dulbecco's phosphate buffered saline
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EU	European unit
F	forward
FACS	fluorescence activated cell sorting (flow cytometry)
FBS	fetal bovine serum
FcγR	Fc-gamma receptor
Fe	iron
FITC	fluorescein isothiocyanate

fl	femtoliter
FL	fluorescein
FRET	fluorescence resonance energy transfer
FSC	forward scatter
fw	forward
g	gram
g	gravity
G	guanine
G	glycine
γδ T cells	gamma delta T cells
Gln	glutamine
G6PD	glucose-6-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
h	hour
H	hydrogen
Hb	hemoglobin
HE	hydroethidine
HEPES	hydroxyethyl-piperazineethane-sulfonic acid buffer
I	isoleucine
ICAM-1	intercellular adhesion molecule 1
IFN	interferon
Ig	immunoglobulin
IL	interleukin
Ile	isoleucine
iRBC	infected red blood cell
IRF	interferon regulatory factor
IU	international unit
l	liter
LAL	*Limulus* amebocyte lysate
LBP	LPS binding protein
LC	LightCycler
LPS	lipopolysaccharide
µ	micro
M	molar (mol/l)

MACS	magnetic cell separation
MAPK	mitogen-activate protein kinase
MCH	mean corpuscular hemoglobin
MCV	mean corpuscular volume
MD2	myeloid differentiation factor 2
MFI	mean fluorescence intensity
Mg	magnesium
mg	milligram
µg	microgram
MHC	major histocompatibility complex
min	minutes
MIP-1α	macrophage inflammatory protein-1α
ml	milliliter
µl	microliter
MOI	multiplicity of infection
mRNA	messenger RNA
MVEC	microvascular endothelial cell
MyD88	myeloid differentiation primary response gene 88
N	asparagines
N	nitrogen
Na	sodium
NaCl	sodium chloride
NF-κB	nuclear factor κB
ng	nanogram
NK cells	Natural killer cells
NLR	NOD-like receptor
NLRP3	NOD-, LRR- and pyrin domain-containing 3
nm	nanometer
NO	nitric oxide
O	oxygen
OD	optical density
p	pico
P	proline
P / PH	phosphate

PAMP	pathogen-associated molecular pattern
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PE	phycoerythrin
P. falciparum	*Plasmodium falciparum*
*Pf*EMP1	*P. falciparum* erythrocyte membrane protein 1
*Pf*RH5	*P. falciparum* reticulocyte-binding homologue 5
pg	pictogram
Phe	phenylalanine
PMCA4	plasma membrane Ca^{2+} ATPase type 4
Pro	proline
PRR	pattern recognition receptor
Q	glutamine
R	arginine
R	reverse
RBC	red blood cell
rev	reverse
RNA	ribonucleic acid
RPMI	Rosewell Park Memorial Institute
rs	RefSNP
S	serine
S	sulfur
sec	second
Ser	serine
SD	standard deviation
SNP	single nucleotide polymorphism
sSNP	synonymous single nucleotide polymorphism
SSC	side scatter
STING	stimulator of interferon genes
T	thymine
T	threonine
T_m	melting temperature of ds DNA
TBE	Tris-borate EDTA

TBK1	Tank-binding kinase 1
TIR	Toll/Interleukin-1 receptor
TIRAP	TIR-associated protein
TLR	Toll-like receptor
TMB	tetramethylbenzidine
TNF	tumor necrosis factor
TRAM	TRIF-related adaptor molecule
TREM-1	triggering receptor expressed on myeloid cells 1
TRIF	TIR-domain-containing adaptor protein-inducing IFN-ß
U	units
UK	United Kingdom
uRBC	uninfected red blood cell
VCAM-1	vascular cell adhesion molecule 1

10.3 List of Figures

10.4 List of Tables