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Analysis of the human immune response against the Merozoite Surface Protein (MSP)-1 from *Plasmodium falciparum* – a malaria vaccine candidate

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<u>Note on self-plagiarism:</u>

The text of several parts of my dissertation has been taken partially from my master thesis (Jäschke, 2012) with modifications and has been originally written by myself:

Introduction: 1.1.; 1.4.

Methods: 4.3.1./2./5.; 4.4.1./2./3.; 4.5.; 4.6.1./2.; 4.7.1./2.; 4.9.3.; 4.10.1./2.; 4.11.1./5./6.

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Acknowledgments

Summary

Malaria affects almost half of the world`s population causing more than 200 million clinical cases each year and thus, remains one of the major infectious diseases of mankind. The protozoan parasite *Plasmodium falciparum* transmitted by infected *Anopheles* mosquitoes is responsible for 99 % of deaths, of which most occur in African children. An effective vaccine is urgently required but not yet available. The malaria vaccine candidate Merozoite Surface Protein (MSP)-1 from *P. falciparum* is an essential and highly abundant protein on the surface of parasite blood stages, which has been associated with protection against malaria in various epidemiological and immunization studies and is currently tested in a clinical Phase I trial. The aim of this work is to characterize the human humoral and cellular immune response against the vaccine candidate *Pf*MSP-1D in African adults with naturally acquired immunity against malaria and after experimental infection of malaria-naïve volunteers with *Pf* wildtype sporozoites.

Antibodies against *Plasmodium* merozoites are considered as the main actors of naturally acquired immunity against malaria and can target the parasite *via* different immune mechanisms, such as direct growth inhibition or opsonization and recruitment of effector cells. By using sera from eleven semi-immune adults from Burkina Faso we show that a few sera were capable of directly inhibiting the growth of *P. falciparum* blood stages *in vitro* and MSP-1 specific antibodies partly contributed to this effect. Further antigens presumably responsible for the observed growth inhibitory activity were identified *via* western blot and mass spectrometry analysis. Furthermore, we demonstrate that serum antibodies from all African individuals could opsonize *P. falciparum* merozoites, recruit neutrophils and elicit antibody-dependent respiratory burst (ADRB). The antibody level against MSP-1 correlated with ADRB activity and MSP-1 specific antibodies, obtained by affinity-purification, induced neutrophil respiratory burst. Opsonizing antibodies effective *via* ADRB appear to be mainly cross-reactive and – by affinity purification of specific antibodies and antigen-reversal ADRB – we identified MSP-1 and its largest subunit MSP-1$_{83}$ as important targets. Additionally, we show for the first time that opsonizing antibodies can be elicited in non-human primates by immunization with recombinant *Pf*MSP-1D, suggesting that a MSP-1 based vaccine may function *via* the induction of ADRB-effective antibodies.

Since MSP-1 is initially synthesized in *Plasmodium* infected hepatocytes at late liver stage, it can be a target of the cellular immune response. Cytotoxic CD8+ T-cells secreting IFNγ are supposed to be the key players eliminating infected hepatocytes. By stimulating PBMCs from semi-immune individuals from Burkina Faso and from malaria-naïve European controls with previously identified HLA-A0201 restricted CD8+ T-cell epitopes within MSP-1D, MSP-1 specific IFNγ-secreting CD8+ T-cells were

detected in African adults *via* IFNγ ELISPOT assay. Aiming at a HLA-independent approach, potential CD8+ T-cell epitopes to frequent HLA-types were predicted within MSP-1D and the IFNγ ELISPOT assay was adapted for the use of full-length MSP-1D protein.

Furthermore, the immune response of malaria-naïve volunteers experimentally infected once with cryopreserved *P. falciparum* wildtype sporozoites during the TUECHMI I study was investigated. Antibodies against MSP-1, MSP-6 and MSP-7 were induced by *Pf* sporozoite infection as well as CD8+ T-cells, which target the cross-stage antigen MSP-1 at comparable levels as the known pre-erythrocytic antigens LSA-1 and CSP.

Overall, this work provides a detailed characterization of different immune mechanisms targeting *Pf*MSP-1D in humans with naturally acquired immunity against malaria or after single experimental infection with *Pf* sporozoites. Moreover, the established immunological assays can be employed to analyze the immune response induced by vaccination with *Pf*MSP-1D during clinical trials.

Zusammenfassung

Malaria betrifft fast die Hälfte der Weltbevölkerung, verursacht jährlich über 200 Millionen klinische Fälle und bleibt damit eine der bedeutendsten Infektionskrankheiten der Menschheit. Der einzellige Parasit *Plasmodium falciparum*, der von infizierten *Anopheles* Moskitos übertragen wird, ist für 99 % der Todesfälle verantwortlich, von denen die meisten in afrikanischen Kindern auftreten. Ein wirksamer Impfstoff wird dringend benötigt, ist aber zurzeit noch nicht verfügbar. Das Merozoiten-Oberfächenprotein-1 (MSP-1) von *P. falciparum* - ein Impfstoffkandidat gegen Malaria - ist ein lebenswichtiges und in hohem Maße vorhandenes Protein auf der Oberfläche der parasitären Blutstadien. Es wurde in verschiedenen epidemiologischen Untersuchungen und Immunisierungen mit Schutz vor Malaria assoziiert und wird derzeit in einer klinischen Phase I Studie getestet. Das Ziel dieser Arbeit besteht darin, die humane humorale und zelluläre Immunantwort gegen den Impfstoffkandidaten *Pf*MSP-1D zu untersuchen, sowohl in afrikanischen Erwachsenen mit natürlich erworbener Immunität gegen Malaria als auch nach experimenteller Infektion von Freiwilligen ohne vorherige Malaria-Exposition mit *P. falciparum* Sporozoiten.

Antikörper gegen *Plasmodium* Merozoiten gelten als die Hauptakteure bei natürlich erworbener Immunität gegen Malaria und können den Parasiten mittels verschiedener Immunmechanismen bekämpfen, z.B. durch direkte Wachstumshemmung oder Opsonisierung und Rekrutierung von Effektorzellen. Anhand von Seren von elf semi-immunen Erwachsenen von Burkina Faso wurde gezeigt, dass das Wachstum von *P. falciparum* Blutstadien *in vitro* durch Serum-Antikörper gehemmt werden kann und dass MSP-1 spezifische Antikörper teilweise daran beteiligt sind. Weitere Antigene, die vermutlich für die gemessene Wachstumshemmung verantwortlich waren, wurden mittels Western Blot und Massenspektrometrie identifiziert. Außerdem zeigen wir, dass Serum Antikörper von allen Afrikanern Merozoiten von *P. falciparum* opsonisieren, neutrophile Granulozyten rekrutieren sowie respiratorischen Burst (ADRB) auslösen konnten. Der Antikörpertiter gegen MSP-1 korrelierte mit ADRB Aktivität und MSP-1 spezifische Antikörper, die mittels Affinitätsaufreinigung gewonnen wurden, erzeugten respiratorischen Burst von Neutrophilen. Opsonisierende Antikörper, die im ADRB wirksam waren, scheinen hauptsächlich gegen kreuzreaktive Regionen gerichtet zu sein und mittels Affinitätsaufreinigung spezifischer Antikörper sowie „antigen-reversal ADRB" wurden MSP-1 und seine größte Untereinheit MSP-1$_{83}$ als wichtige Angriffsziele identifiziert. Zusätzlich zeigt diese Arbeit zum ersten Mal, dass opsonisierende Antikörper in Primaten durch Immunisierung mit rekombinantem *Pf*MSP-1D erzeugt werden können. Dies deutet darauf hin, dass ein Impfstoff basierend auf MSP-1 über die Induktion von Antikörpern, die ADRB auslösen, wirken könnte.

Da MSP-1 bereits in infizierten Hepatozyten im späten Leberstadium hergestellt wird, kann es auch von der zellulären Immunantwort angegriffen werden. Zytotoxischen CD8+ T-Zellen, die IFNγ produzieren, wird eine Schlüsselrolle bei der Eliminierung infizierter Hepatozyten zugeschrieben. Durch die Stimulierung von PBMCs von semi-immunen Personen aus Burkina Faso sowie malaria-naiven Kontrollen aus Europa mit HLA-A0201 begrenzten CD8+ T-Zell Epitopen innerhalb von MSP-1D, konnten wir mittels IFNγ ELISPOT Untersuchung MSP-1 spezifische CD8+ T-Zellen ausfindig machen, die IFNγ sekretieren. Um eine HLA-unabhängige Methode zu entwickeln, wurden potenzielle CD8+ T-Zell Epitope zu häufigen HLA-Typen vorausgesagt und die IFNγ ELISPOT Untersuchung wurde für die Nutzung des Gesamt-MSP-1D Proteins angepasst.

Außerdem wurde die Immunantwort von malaria-naiven Freiwilligen erforscht, die innerhalb der TUECHMI I Studie experimentell einmal mit kryokonservierten *P. falciparum* Wildtyp-Sporozoiten infiziert wurden. Antikörper gegen MSP-1, MSP-6 und MSP-7 wurden durch die Injektion von *Pf* Sporozoiten erzeugt, ebenso wie CD8+ T-Zellen. Diese sind gegen das phasenübergreifende Antigen MSP-1 in vergleichbarer Höhe gerichtet wie gegen die bekannten Leberstadien-Antigene LSA-1 und CSP.

Insgesamt liefert diese Arbeit eine detaillierte Untersuchung von verschiedenen Immun-mechanismen, die gegen *Pf*MSP-1D gerichtet sind, sowohl in Menschen mit natürlich erworbener Immunität gegen Malaria als auch nach einmaliger experimenteller Infektion mit *Pf* Sporozoiten. Insbesondere dienen die etablierten immunologischen Methoden als Grundlage für die Untersuchung der Immunantwort während der klinischen Studien mit rekombinantem MSP-1.

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1. Introduction

1.1. Malaria

Malaria remains one of the most important infectious diseases of mankind, affecting almost 50 % of the world`s population in 91 countries and territories, especially in the tropical and subtropical regions of Africa, Latin America and South-East Asia (WHO, 2016) (Figure 1.1). The World Health Organization estimates about 212 million malaria cases and 429 000 deaths in 2015; most of them occur in Africa. Children under the age of five in sub-Saharan Africa are mostly affected - in average every two minutes a child is killed by the disease. Furthermore, pregnant women in malaria endemic regions often suffer from placental malaria, which poses a serious problem for both mother and unborn child (WHO, 2016).

Malaria is caused by unicellular parasites of the genus *Plasmodium,* a member of the phylum Apicomplexa (class: *Sporozoa*, family *Plasmodiidae*), and is transmitted through the bites of infected *Anopheles* mosquitoes. All apicomplexan parasites share common features like the specialized apical complex which plays an important role in the invasion process of host cells. More than 150 *Plasmodium* species are known infecting various animals but only five are pathogenic to humans: *Plasmodium malariae, Plasmodium ovale, Plasmodium vivax, Plasmodium knowlesi* and *Plasmodium falciparum* (Singh and Daneshvar, 2013). The latter causes the most severe form of the disease, malaria tropica, and is responsible for 99 % of deaths (WHO, 2016).

Although the global malaria eradication campaign has eliminated malaria in some countries (Figure 1.1) and the incidence rate of malaria has been reduced worldwide by 41 % since the year 2000 (WHO, 2016), efforts to control the disease are challenging due to growing resistance of *Plasmodium* to antimalarial drugs and insecticide resistant mosquitoes (WHO, 2016). The best available treatment against malaria to date is the artemisinin-based combination therapy (ACT). However, resistance of *P. falciparum* against artemisinin has recently been detected in some Asian countries (WHO, 2016). Furthermore, the treatment with antimalarial drugs as well as the distribution of tools against malaria such as mosquito nets is often hampered by poverty and a poor infrastructure in the affected regions. Therefore, the development and delivery of an efficient malaria vaccine – which is currently not available - is considered as a key tool for sustainable malaria control, elimination or even possible eradication of the disease.
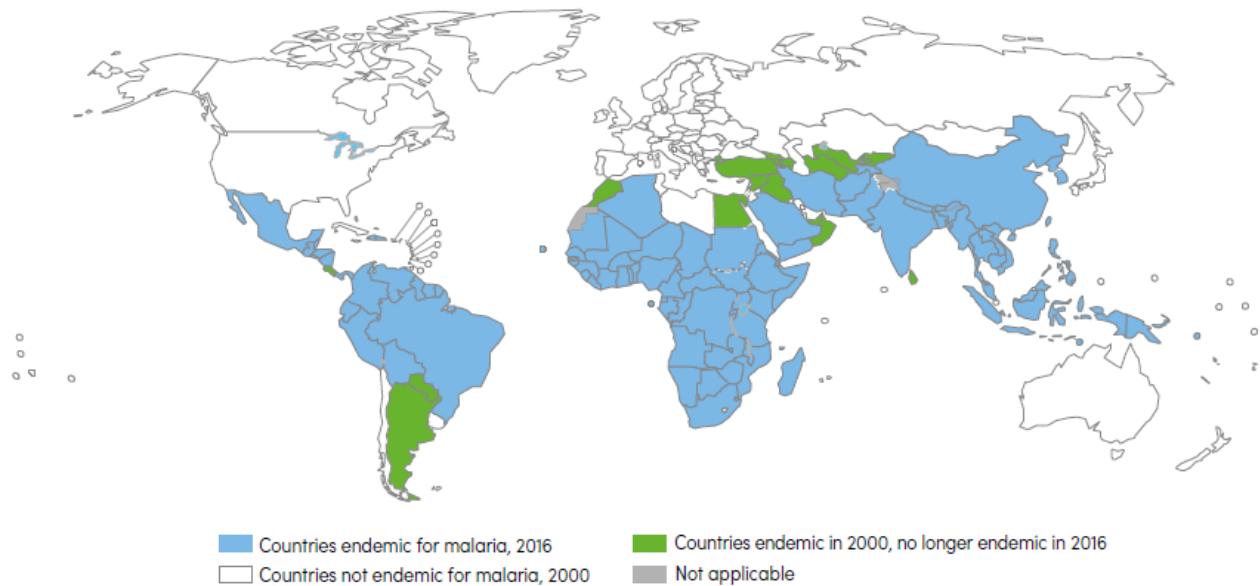
Figure 1.1: Malaria endemicity around the world. *Countries endemic for malaria in 2016 are shown in blue; 17 countries which have eliminated malaria within the last 16 years are depicted in green (WHO, 2016).*

1.1.1. *Plasmodium* life cycle

Transmission of *Plasmodium* sporozoites into the vertebrate host occurs during a blood meal of an infected female *Anopheles* mosquito. About 400 *Anopheles* species (phylum: *Arthropoda,* class: *Insecta*, family: *Culicidae*) have been recorded worldwide but only 30 play an important role for malaria transmission, especially *A. gambiae* and *A. funestus* (Tuteja, 2007). The life cycle of *Plasmodium* is very complex and involves the *Anopheles* (primary) and vertebrate (secondary) host (Figure 1.2).

Several stages of parasite development in different cell types and host tissues with sexual or asexual proliferation can be divided: Upon injection of *Plasmodium s*porozoites into the bloodstream of a human host – approximately 15 to 123 sporozoites are injected by the *Anopheles* mosquito (Frischknecht et al., 2004) - sporozoites migrate to the liver and invade hepatocytes. There, the parasite differentiates and replicates asexually for 5 to 15 days to yield a liver-stage schizont, which contains several thousand merozoites. These liver merozoites are released into the bloodstream and invade erythrocytes, thereby initiating the erythrocytic cycle (Figure 1.2).

Inside the erythrocytes, merozoites replicate asexually and develop through ring, trophozoite and schizont-stages within approximately 48 hours. Upon burst of the infected red blood cell, 8 to 32 daughter merozoites are released into the blood stream where they invade new RBCs and start another replication cycle. All clinical symptoms arise during the asexual blood stage of *Plasmodium*.

Some intraerythrocytic stages develop into sexual stages, macrogametocytes and microgametocytes, which are taken up during the next mosquito bite; inside the *Anopheles* vector they develop into gametes and form zygotes by fusion. After development of the zygote into an invasive ookinete and traversal of the midgut wall, the ookinete transforms into an oocyst, in which sporozoites arise. Upon release, these sporozoites migrate to the salivary gland of the Anopheles mosquito from where they are injected into a human host during the next blood meal and thus, the *Plasmodium* life cycle starts again (Figure 1.2).
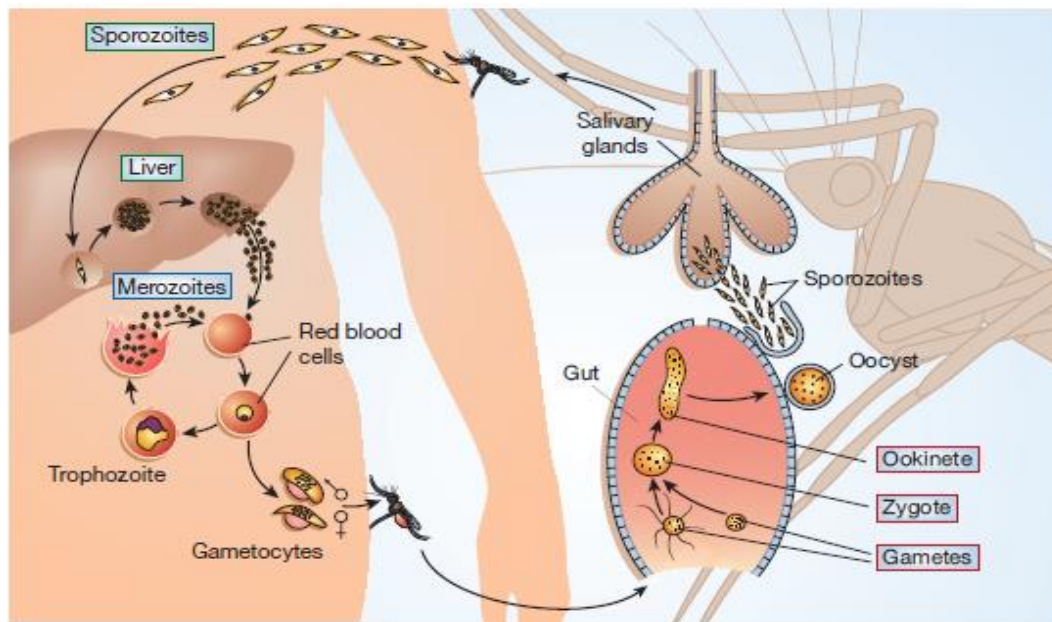
Figure 1.2: Life cycle of Plasmodium falciparum. The complex life cycle of the parasite occurs in two different species - the human host (left) and the Anopheles Mosquito (right). Details are given in the text. Picture from (Ménard, 2005).

1.1.2. Erythrocyte invasion by *Plasmodium* merozoites

Plasmodium merozoites are egg-shaped, approximately 1.5 µm long unicellular organisms (Figure 1.3 A). Their invasion into erythrocytes is a rapid, efficient and highly regulated process which occurs within minutes and can be separated into distinct phases (Farrow et al., 2011; Gilson and Crabb, 2009)(Figure 1.3 B): (1) primary contact between merozoite and host erythrocyte by low affinity and reversible interactions; (2) reorientation of the parasite towards its apical end; (3) formation of a tight junction complex between the parasite and erythrocyte membrane and invagination of the erythrocyte membrane during which the merozoite surface coat is removed and the parasitophorous vacuole (PV) is formed; (4) active invasion powered by the parasite actin-myosin motor (Baum, 2006) and sealing of the PV.

Proteins involved in the erythrocyte invasion process are either localized at the merozoite surface such as MSP-1, MSP-2, MSP-6 and MSP-7 or they are present inside specialized organelles - rhoptries, micronemes and dense granules - at the apical end of the parasite. Merozoite surface proteins are attached to the membrane either *via* GPI-anchor, as integral membrane proteins or by peripheral interactions with proteins covalently bound to the membrane. For example, the peripheral proteins MSP-3, MSP-6, MSP-7, MSPDBL-1 and MSPDBL-2 use the GPI-anchored protein MSP-1 as a platform to bind to the merozoite surface (Lin et al., 2016). Proteins maintained in apical organelles, such as Apical Membrane Antigen 1 (AMA-1) or Erythrocyte Binding Antigen 175 (EBA-175), are secreted and transported to the merozoite surface prior to RBC invasion *via* different mechanisms. Some parasite proteins have to be proteolytically processed prior to erythrocyte invasion in order to be functionally active such as MSP-1 and AMA-1 (Cowman and Crabb, 2006).

Kinetic studies of erythrocyte invasion *in vitro* show that 80 % of *Plasmodium* merozoites invade the RBC within 10 minutes while the remaining 20 % enter erythrocytes after longer time-periods post-egress (Boyle et al., 2010b). The half-life of merozoite invasive capacity was estimated to 8 minutes at 37 °C and 15 minutes at RT (Boyle et al., 2010b). However, whether these kinetics are applicable for the *in vivo* situation is currently not known (Boyle et al., 2013).
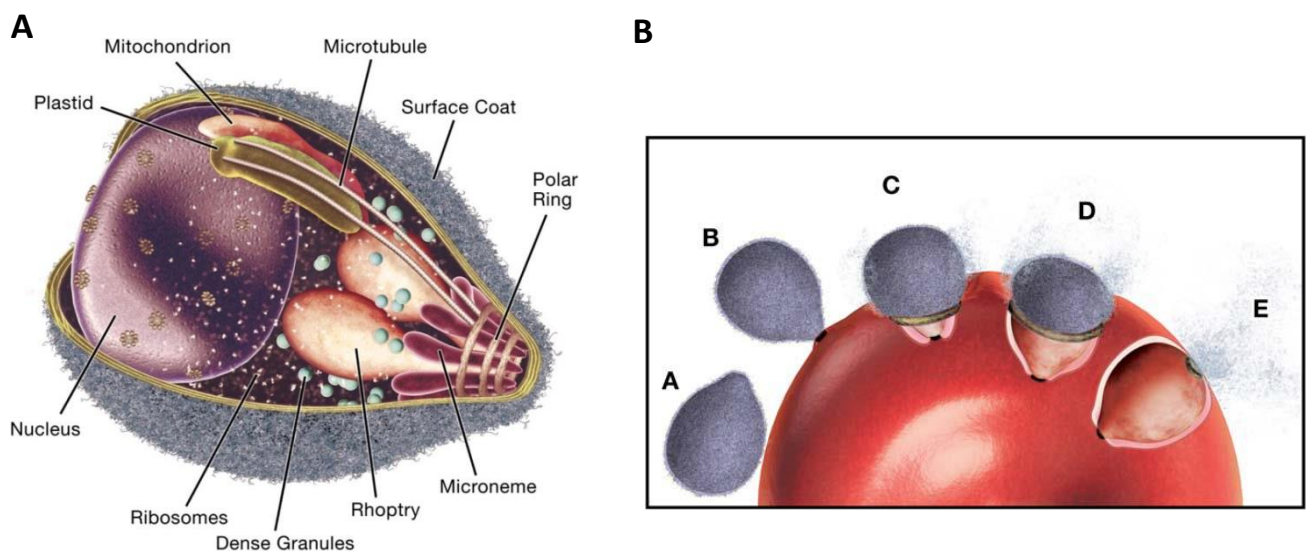
Figure 1.3: Erythrocyte invasion by Plasmodium merozoites. (A) *Morphology of a Plasmodium merozoite.* ***(B)*** *Schematic outline of the different steps during RBC invasion by merozoites: (A) Primary attachment between merozoite and RBC, (B) Reorientation of the merozoite, (C) Formation of a tight junction between the apical end of the merozoite and the RBC and start of penetration, (D) During further invasion into the RBC via the actin-myosin motor of the parasite, the merozoite surface coat is shed and a parasitophorous vacuole (PV) is formed, (E) Completion of RBC invasion, the parasite is surrounded by the PV. Picture from (Cowman and Crabb, 2006).*

1.1.3. Pathogenesis and symptomatology of malaria

The first symptoms of malaria – fever, chills, headache and vomiting – usually appear 10-15 days after infection. If not treated, *P. falciparum* malaria can progress to severe disease including anemia, respiratory distress, hypoglycaemia, multi-organ failure or cerebral malaria (WHO, 2016).

All clinical symptoms are a consequence of the asexual erythrocytic life cycle of *Plasmodium.* Different aspects of malaria pathology can be divided: (i) Rupture of infected red blood cells causes anemia and impairs oxygen transport resulting in respiratory distress. (ii) Adherence of infected red blood cells to the blood capillary endothelium after modification of the erythrocyte surface with parasite proteins such as *Plasmodium falciparum* erythrocyte membrane protein-1 (*Pf*EMP-1) (Baruch et al., 1995) in order to escape parasite elimination in the spleen. This sequestration in combination with rosetting, the agglutination of infected and non-infected red blood cells, leads to blockage of blood vessels and thus, an undersupply of affected organs, possibly resulting in multi-organ failure, cerebral malaria, coma and death. (iii) Stimulation of the host inflammatory immune response by parasite metabolites like hemozoin released during lysis of red blood cells. The overproduction of several cytokines such as tumor-necrosis-factor (TNF) α, interferon (IFN) γ or interleukin (IL)-1 is indirectly responsible for many symptoms of malaria (Clark, 1987). For instance, TNFα, whose release is triggered by malaria toxins of ruptured schizonts, is associated with severe malaria disease, especially with cerebral malaria (Clark, 1987).

1.2.   Immune response against malaria infection

So far, the human immune response against malaria infection is still not entirely understood. We observe a protective, non-sterilizing immune response against the parasite in individuals who live in malaria highly endemic regions and thus, are continuously exposed to *P. falciparum* infections during the transmission season. After multiple survived infections those individuals develop a naturally acquired immunity (NAI) against malaria, which means that they can be infected by *Plasmodium* but usually do not show any disease symptoms (Marsh and Kinyanjui, 2006; Snow et al., 1997). Antibodies against parasite blood stages are supposed to be the key players in NAI-mediated protection (Cohen et al., 1961) (Figure 1.4).

Sterile protection in humans can be induced by immunization with either radiation-attenuated sporozoites (RAS) (Clyde et al., 1973) or wildtype sporozoites under chemoprophylaxis (CPS) (Mordmuller et al., 2017; Roestenberg et al., 2009). Here, protection is mainly mediated by cellular immune mechanisms such as the elimination of infected hepatocytes by cytotoxic T-cells (Figure 1.4). A better understanding of protective immune mechanisms and the availability of suitable *in vitro* assays to measure them will be critical for the development of an effective vaccine.

1.2.1. Humoral immune response

Individuals living in malaria-endemic regions naturally acquire immunity against the disease with increasing numbers of survived infections (Aponte et al., 2007; Gupta et al., 1999; Marsh and Kinyanjui, 2006; Snow et al., 1997). However, this protection is short-lived and requires frequent *Plasmodium* infections in order to persist (Früh et al., 1991; Hoffman et al., 1987). It appears largely mediated by serum antibodies controlling levels of blood stage parasites (Cohen et al., 1961).

Merozoites are key targets of naturally acquired antibodies (Crompton et al., 2010a; Osier et al., 2008; Richards et al., 2013) and an association between antibody levels and protective human immunity has been reported for several merozoite antigens (Beeson et al., 2016; Fowkes et al., 2010; Osier et al., 2014b; Richards et al., 2013). Antibodies targeting the merozoite can function *via* different pathways such as direct growth inhibition or recruitment of immune effector cells (reviewed in (Teo et al., 2016)). However, it remains elusive which antibody mechanisms determine protection against malaria. The direct growth inhibition assay (GIA) of *Plasmodium* blood stages still remains the most commonly used functional assay for blood stage vaccine candidates and merozoite antigens (Crompton et al., 2010b; Duncan et al., 2012) although it is controversial whether direct growth inhibitory activity *in vitro* correlates with protection against clinical malaria (Duncan et al., 2012).

Increasing evidence points towards an important role of opsonizing antibodies, which bind to merozoites and recruit effector cells such as monocytes (Bouharoun-Tayoun et al., 1990; Osier et al., 2014a) or neutrophils (Joos et al., 2010) *via* their Fc receptors. These eliminate the parasite, either by phagocytosis (Osier et al., 2014a) or by secretion of reactive oxygen species (Joos et al., 2010). Indeed, merozoites are mainly targeted by the cytophilic antibodies IgG1 and IgG3 (Reiling et al., 2010; Richards et al., 2010; Stanisic et al., 2009), which can bind to the Fc receptor of immune cells (Osier et al., 2014a) or fix complement factors (Boyle et al., 2015). The acquisition of opsonizing antibodies increases with age and malaria exposition (Osier et al., 2014a) and correlates with protection (Joos et al., 2010; Osier et al., 2014a). Four functional assays have been developed to measure opsonizing antibodies *in vitro*: (i) the antibody-dependent cellular inhibition assay (ADCI) (Bouharoun-Tayoun et al., 1990), (ii) the opsonic phagocytosis assay (OPA) (Hill et al., 2013; Osier et al., 2014a), (iii) the antibody-dependent complement inhibition (Ab-C) assay (Boyle et al., 2015) and (iv) the antibody-dependent respiratory burst (ADRB) assay (Joos et al., 2010). Importantly, several studies using these assays show a correlation between opsonizing antibodies and protection against malaria (Boyle et al., 2015; Chiu et al., 2015; Hill et al., 2013; Hill et al., 2016; Joos et al., 2010; Osier et al., 2014a; Tiendrebeogo et al., 2015).

A number of merozoite antigens have been identified which elicit opsonizing antibodies such as MSP-1_{19} (Boyle et al., 2015; Joos et al., 2015), MSP-1_{block2} (Galamo et al., 2009), MSP-2 (Boyle et al., 2015; Osier et al., 2014a), MSP-3 (Druilhe et al., 2005; Lundquist et al., 2006; Osier et al., 2014a), MSP-5 (Perraut et al., 2014), MSP-6 (Singh et al., 2005), MSPDBL-1/-2 (Chiu et al., 2015; Singh et al., 2009) and GLURP (Theisen et al., 2000). However, the antigenic targets of antibodies inducing complement fixation, opsonic phagocytosis or neutrophil respiratory burst might differ. So far, only MSP-1_{19}, the small conserved C-terminal part of MSP-1, has been identified as antigenic target which contributes to neutrophil respiratory burst activity (Joos et al., 2015).
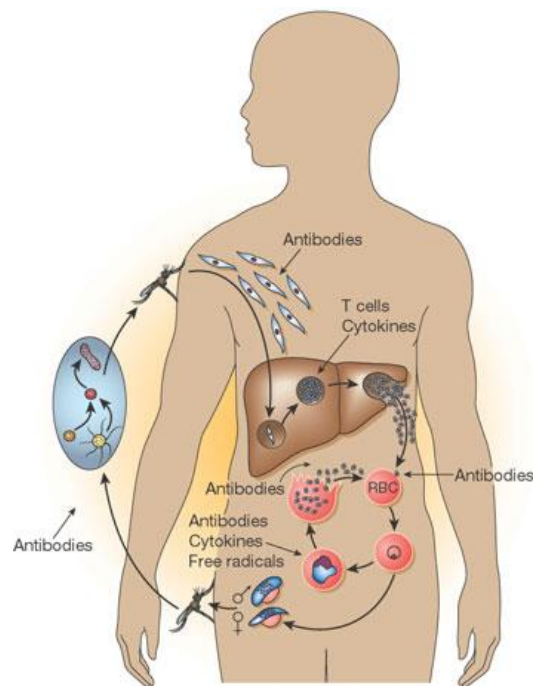
Figure 1.4: Immune mechanisms against the various developmental stages of P. falciparum. Pf sporozoites are injected into the human skin by an infected Anopheles mosquito and move via the blood-stream to the liver where they invade hepatocytes. Infected hepatocytes are targeted by T-cells, especially cytotoxic CD8+ T-cells which kill the intracellular parasite by secretion of the cytokine IFNγ. Parasites which escape the immune system develop in the liver and – upon burst of the liver schizont – several thousand merozoites enter the blood-stream and invade erythrocytes. Parasite blood stages are mainly controlled by antibodies via different mechanisms. Picture from (Richie and Saul, 2002).

1.2.2. Cellular immune response

Since erythrocytes cannot process and present parasite-derived antigens due to the lack of a nucleus and an antigen processing machinery, the T-cell response against malaria is mostly limited to the *Plasmodium* liver stages. While in a natural malaria infection some parasites are able to escape the cellular immune response in the liver and progress to blood stages, immunization of humans with *P. falciparum* RAS (Clyde et al., 1973) or CPS (Mordmuller et al., 2017) induces sterile protection against malaria mediated by T-cells (Doolan and Hoffman, 2000). T-cell priming in the liver presumably

happens by recognition of malaria-specific antigens presented *via* HLA (=MHC)-I receptors on the surface of liver-resident dendritic cells (Cockburn and Zavala, 2016; Jobe et al., 2009) or infected hepatocytes (Balam et al., 2012; Cockburn et al., 2014; Frevert and Krzych, 2015).

According to many studies performed within the last 30 years (reviewed e.g. in (Krzych et al., 2014)) cytotoxic CD8+ T-cells play the most important role in eliminating infected hepatocytes and thus, mediating protection. For example, RAS-induced sterile protection against sporozoite challenge is prevented in mice (Schofield et al., 1987; Weiss et al., 1988) and *rhesus* monkeys (Weiss and Jiang, 2012) following depletion of CD8+ but not CD4+ T-cells *in vivo*. CD8+ T-cells recognize *Plasmodium* antigens presented by infected hepatocytes (Balam et al., 2012; Cockburn et al., 2014) or other liver-resident cells *via* their HLA-1 receptor and eliminate the parasite either *via* cytolytic processes or by release of the cytokine IFNγ. The latter activates the nitric-oxide- synthase gene resulting in Nitric-oxide production, which kills the parasite inside the infected hepatocyte. The major role of CD8+ T-cells against *Plasmodium* appears to be their IFNγ production which constitutes a crucial component for protection against malaria (Ferreira et al., 1986; Imai et al., 2010; Mellouk et al., 1987; Schofield et al., 1987). Importantly, IFNγ-secreting CD8+ T-cells are also induced by immunizations of mice, monkeys and humans with radiation-attenuated sporozoites (Epstein et al., 2011; Lyke et al., 2017; Nganou-Makamdop et al., 2012; Weiss and Jiang, 2012), genetically-attenuated sporozoites (Jobe et al., 2007; Mueller et al., 2007; Tarun et al., 2007) and wildtype sporozoites under chemoprophylaxis (Brando et al., 2014; Nganou-Makamdop et al., 2012; Roestenberg et al., 2009; Roestenberg et al., 2011) and are crucial for protection against malaria (e.g. (Fernandez-Ruiz et al., 2016)). Interestingly, the application of IFNγ alone strongly inhibits the development of *Plasmodium* liver stages in mice infected with *P. berghei* and decreases the parasitemia in *P. vivax* infected chimpanzees (Ferreira et al., 1986).

In protected individuals immunized with RAS, T-cells have been detected which recognize epitopes from several *P. falciparum* proteins e.g. CSP or LSA-1 (Krzych et al., 1995) and these epitopes could induce a CD8+ T-cell response in individuals with naturally acquired immunity (Doolan et al., 1996). However, *Plasmodium* specific CD8+ T-cells show a low frequency in comparison to viral infections such as HIV (Lalvani et al., 1996; Plebanski et al., 1997). The antigenic epitopes presented to T-cells depend strongly on the HLA-subtype of each individual (Barouch et al., 1995).

1.3. Malaria vaccines

Although malaria incidence and mortality rates have been remarkably reduced within the last 15 years (Figure 1.1) by malaria control programs - such as vector control or chemoprevention (WHO, 2016) - the emergence of insecticide resistant *Anopheles* mosquitoes and multi-drug resistant parasites underline the urgent need for an effective malaria vaccine. Despite high international efforts over the last decades, a safe and efficient vaccine against *P. falciparum* malaria is still not available (Halbroth and Draper, 2015). The target population for a malaria vaccine would consist of: (i) infants and children living in regions with malaria transmission, especially sub-Saharan Africa, (ii) pregnant women, (iii) individuals migrating to malaria-endemic areas, (iv) travelers and military (Hollingdale and Sedegah, 2017). The WHO sets the requirement for vaccine efficacy against clinical disease to 75 % (Moorthy et al., 2013), the US military even to 80 % against *Plasmodium* parasites (Teneza-Mora et al., 2015).

The *Plasmodium* life cycle offers several targets for vaccine development: sporozoites, liver stages as well as sexual and asexual blood stages. Thus, four different types of malaria vaccines can be divided, respectively (reviewed in (Matuschewski, 2017)): (i) anti-sporozoite vaccines aim to induce antibodies inhibiting sporozoite invasion of the liver, (ii) whole parasite vaccine approaches which elicit mainly cytotoxic T-cell responses against *Plasmodium* liver stages, (iii) transmission blocking vaccines which aim to reduce malaria transmission by induction of antibodies against parasite sexual stages, (iv) erythrocytic vaccines which target the asexual blood stages, mostly by antibodies against merozoites.

Typical approaches are either subunit vaccines, such as recombinant antigens or viral vectors which block *Plasmodium* development and life cycle progression, or whole sporozoite vaccines aiming to induce sterile immunity against disease. It is likely that a malaria vaccine consisting of several components and acting against different *Plasmodium* life cycle stages will be most effective.

1.3.1. Anti-sporozoite subunit vaccines

Anti-sporozoite vaccines intend to decrease the initial parasite load by the induction of antibodies which inhibit sporozoite invasion of the liver. However, these antibody responses have to be long-lived (Dups et al., 2014). The RTS.S/AS01 vaccine consists of the repeat region and the thrombospondin domain from the circumsporozoite protein (CSP) - the most abundant protein on *P. falciparum* sporozoites - fused to the surface antigen S from hepatitis B virus (Cohen et al., 2010). Although RTS.S/AS01 is the most advanced vaccine candidate so far, recent results from Phase III clinical trials in African children were disappointing showing no efficacy over a 7 year period (Olotu et al., 2016). Another potential vaccine candidate on the surface of sporozoites is the Thrombospondin-

related adhesive protein (TRAP). However, a recent phase II clinical study in adults from Senegal demonstrated no efficacy against *P. falciparum* malaria (Mensah et al., 2016).

1.3.2. Whole sporozoite vaccines

Whole parasite vaccinations pursue the goal of sterile immunity elicited by T-cell mediated immunity against *Plasmodium* liver stages. Three approaches can be differentiated depending on the mode of developmental arrest (Figure 1.5): (i) radiation attenuated sporozoites (RAS): *Pf* sporozoites are exposed to γ-irradiation which induces multiple random DNA double-strand breaks and leads to an early arrest in liver stage development (Hoffman et al., 2002), (ii) genetically attenuated parasites (GAP): genes crucial for liver stage development are removed and thus, the parasite can only progress to a defined developmental stage in the liver (Mueller et al., 2005b), (iii) wildtype sporozoites are injected under simultaneous chemoprophylaxis (CPS) using different drugs such as chloroquine or azithromycin which block further parasite life cycle progression (Borrmann and Matuschewski, 2011).
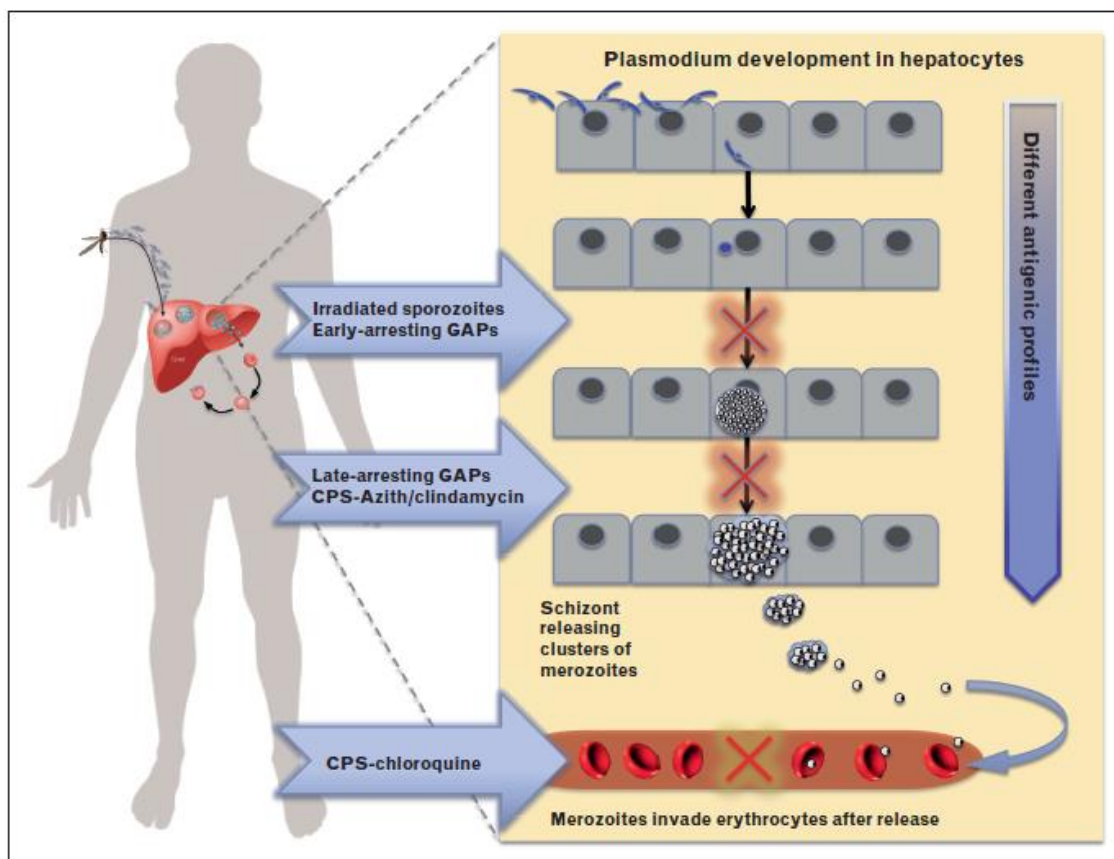
Figure 1.5: Whole sporozoite vaccination approaches and their developmental arrest. *Sporozoites attenuated by radiation (irradiated spz) and some genetically attenuated parasites (GAPs) arrest early in liver stage development. Immunization with late-arresting GAPs or chemoprophylaxis with infectious sporozoites (CPS) leads to late liver stage arrest or arrest in early blood stage and thus, a different antigenic repertoire can be presented to T-cells. Picture from (Epstein and Richie, 2013).*

Immunizations with RAS elicit a sterile immunity in rodents (Nussenzweig et al., 1967) and humans (Clyde et al., 1973) when administered by mosquito bite and also by intravenous application of cryopreserved attenuated *Pf*Spz (Lyke et al., 2017; Seder et al., 2013). However, very high *Pf*Spz doses were necessary for complete protection by intravenous injection, namely five times 135 000 *Pf*Spz (Seder et al., 2013). Furthermore, the irradiated sporozoites show a high batch-to-batch variation and – since they arrest early in liver stage development – immunity against RAS is restricted to early LS antigens (Borrmann and Matuschewski, 2011).

These restrictions were partly overcome by defined knock-out of *Plasmodium* genes important for LS development such as uis3 (Mueller et al., 2005b), uis4 (Mueller et al., 2005a) or sap1 (Aly et al., 2008). In mice, immunizations with late-arresting GAPs induced superior protection in comparison to RAS or early-arresting GAPs (Butler et al., 2011) and could even protect against blood stage challenge likely due to cross-stage antigens (Sack et al., 2015). Recently, safety and immunogenicity of a triple knockout line (*Pf* p52$^-$/p36$^-$/sap1$^-$) was assessed in 10 human volunteers and the results were encouraging (Kublin et al., 2017). Another approach is the administration of wildtype *P. falciparum* sporozoites under drug cover with chloroquine (Belnoue et al., 2004; Mordmuller et al., 2017; Roestenberg et al., 2011), azithromycin (Friesen et al., 2010) or primaquine (Putrianti et al., 2009). Depending on the used drug, the parasite arrest at late liver stage (azithromycin) or early blood stage (Chloroquine) (Figure 1.5). Importantly, CPS immunization induces sterile protection in mice (Belnoue et al., 2004) and in humans - by administration *via* mosquito bite (Roestenberg et al., 2009; Roestenberg et al., 2011) as well as by intravenous injection of cryopreserved *P. falciparum* wildtype sporozoites (Mordmuller et al., 2017).

However, several major obstacles have to be tackled before whole sporozoite vaccines could be possibly realized in malaria-endemic regions: (i) the labor-intense production and purification of *Plasmodium* sporozoites from Anopheles mosquitoes have to be automated by developing sporozoite *in vitro* culture systems; (ii) protection against heterologous *P. falciparum* strains is a requirement for all malaria vaccines; so far it is incomplete after immunization with CPS (Schats et al., 2015) or RAS (Epstein et al., 2017); (iii) the vaccine should also protect when administered by intramuscular or subcutaneous routes since intravenous injection is not an acceptable delivery route; up to date about 23-times more *Pf* sporozoites are required for injection *via* the intramuscular route compared to intravenous (Gomez-Perez et al., 2015); (iv) since the parasites need to be stored in liquid nitrogen or dry ice, vaccine delivery in remote areas of Africa seems very visionary and poses an immense logistical challenge.

1.3.3. Transmission-blocking vaccines

Transmission-blocking vaccines aim to reduce *Plasmodium* transmission by targeting the sexual forms of the parasite – gametocytes, the zygote and ookinete (reviewed in (Wu et al., 2015)). Antibodies against surface antigens of gametes and ookinetes are induced, taken up by the *Anopheles* mosquito during their blood meal and inhibit *Plasmodium* development in the mosquito midgut. Up to date, the sexual stage antigens s25 and s230 are the best available candidates but require further investigation (Kapulu et al., 2015). However, transmission-blocking vaccines will not immediately benefit the immunized individual but could prevent new parasite infections in a certain area and thus, help to eliminate malaria (Wu et al., 2015). Ideally, this vaccine should be combined with a malaria vaccine targeting the erythrocytic and/or pre-erythrocytic stages.

1.3.4. Blood stage vaccines

Blood stage (BS) vaccines either target the *Plasmodium* merozoite or the infected erythrocyte, mostly by antibodies, which play a crucial role against parasite blood stage infection and form the basis of naturally acquired immunity (Cohen et al., 1961). The objective of a BS vaccine is to reduce parasitemia and prevent clinical disease.

Plasmodium falciparum erythrocyte membrane protein 1 (*Pf*EMP-1) – a target of naturally acquired immunity - is transported to the surface of infected erythrocytes by the parasite and binds to endothelial cells *via* CD36 or to the placenta *via* chondroitin sulfate A (CSA) in order to escape splenic clearance. This cytoadhesion is mainly responsible for the pathology of severe malaria (Haldar et al., 2007) and placental malaria in primigravidae (Fried and Duffy, 2015) and thus, *Pf*EMP-1 an interesting vaccine target (reviewed in (Bull and Abdi, 2016)). However, the extreme diversity of this protein, which is encoded by over 60 var genes, pose a high obstacle to vaccine design. So far, the most advanced *Pf*EMP-1 vaccine candidate PAMVAC, based on VAR2CSA, has not entered the clinical stage (Gbedande et al., 2017).

Up to date, seven vaccine candidates targeting *P. falciparum* merozoites have been tested in clinical trials (reviewed in (Beeson et al., 2016)): apical membrane antigen 1 (AMA-1), erythrocyte binding antigen 175 (EBA-175), serine repeat antigen 5 (SERA-5), glutamate-rich protein (GLURP), and the merozoite surface proteins MSP-1, MSP-2 and MSP-3. Most of these candidates have been assessed in Phase I clinical trials as recombinant proteins in combination with adjuvant; some also used the viral vectors ChAd63 and MVA as vaccine delivery systems (Beeson et al., 2016).

AMA-1 represents the best studied BS candidate so far in 23 clinical trials and additional 9 in combination with other vaccine candidates (Beeson et al., 2016). The protein is localized within the micronemes of the parasite and – following proteolytic processing – it relocates to the merozoite

surface and plays an important role for erythrocyte invasion (Beeson et al., 2016; Yap et al., 2014). In clinical trials AMA-1 shows a good safety and immunogenicity profile with Alhydrogel as adjuvant (Dicko et al., 2008; Malkin et al., 2005) but no efficacy against malaria in a Phase IIb study in Malian children (Ouattara et al., 2010). Also a 3-4-fold increase in antibody titers by adding the adjuvant CPG7909 (Mullen et al., 2008) did not protect malaria-naïve individuals against *Pf* sporozoite challenge (Duncan et al., 2011). Supposedly, the high polymorphism of AMA-1 poses a major barrier to vaccine efficacy.

EBA-175, a microneme protein which localizes to the merozoite surface and interacts with RBCs *via* glycophorin A (Sim et al., 1994), has been tested in phase 1 trials. Antibodies were induced at high levels but demonstrated only modest growth inhibitory activity in vitro. SERA-5 is a peripherally-associated MSP which supposedly acts as protease and may be important for merozoite egress (Beeson et al., 2016). A Phase Ib trial in Uganda detected lower incidence rates of high parasitemia in vaccinated volunteers (Palacpac et al., 2013).

GLURP, a protein on the merozoite surface with unknown function (Beeson et al., 2016) has been assessed in vaccine trials in combination with MSP-3 (GMZ2 vaccine). Although three Phase I trials were successful showing good safety and immunogenicity levels in malaria-naïve individuals and malaria-exposed individuals from Gabon (Beeson et al., 2016) and even the induction of ADCI-active antibodies (Jepsen et al., 2013), a Phase IIb trial of GMZ2 in almost 2000 African children demonstrated no protective efficacy (Sirima et al., 2016). However, a vaccine based on the conserved part of MSP-3 elicited antibodies active in ADCI (Druilhe et al., 2005) and showed protective efficacy against malaria in children from Burkina Faso (Sirima et al., 2011).

The Combination B vaccine – consisting of MSP-2 (3d7 strain), the N-terminal part of MSP-1 and a part from RESA – has been tested in a Phase II clinical study in children from PNG; it could reduce parasitemia significantly (Genton et al., 2003; Genton et al., 2002). A combination of MSP-1 and AMA-1 and delivery *via* the viral vectors ChAd63-MVA in a prime-boost regiment induced high antibody levels against both proteins but protected only one out of 38 volunteers against mosquito bite challenge (Biswas et al., 2014; Sheehy et al., 2012). MSP-1$_{42}$ / ASO2A showed no protective efficacy in a Phase IIb study in 400 children from Kenya (Ogutu et al., 2009) despite the induction of high antibody levels and GIA activity *in vitro* (Beeson et al., 2016). Additionally, MSP-1$_{42}$ delivered *via* ChAd63-MVA did not protect three vaccinated volunteers against *P. falciparum* mosquito bite challenge although strong T-cell responses were detected (Sheehy et al., 2012). However, so far full-length MSP-1 has not been assessed in clinical trials.

1.4. Merozoite surface protein 1 (MSP-1)

1.4.1. MSP-1 complex & processing

MSP-1 (synonyms MSA-1, merozoite surface antigen 1; gp190) is the most abundant protein on the surface of *Plasmodium* merozoites (Gilson et al., 2006; Holder and Freeman, 1981) and is present in all *Plasmodium* species described so far. The protein - encoded by a gene on chromosome 9 with roughly 5000 bp - is initially synthesized in infected hepatocytes at late liver-stage (Haussig et al., 2011; Szarfman et al., 1988). Within the infected erythrocyte, MSP-1 is expressed as an approximately 190 kDa precursor protein in late trophozoite and schizont stages and deposited at the merozoite surface *via* glycosylphosphatidylinositol (GPI) anchor (Holder and Freeman, 1982) (Figure 1.6 A).

A

Blood stages of *P. falciparum*   Merozoite   MSP-1 complex

B
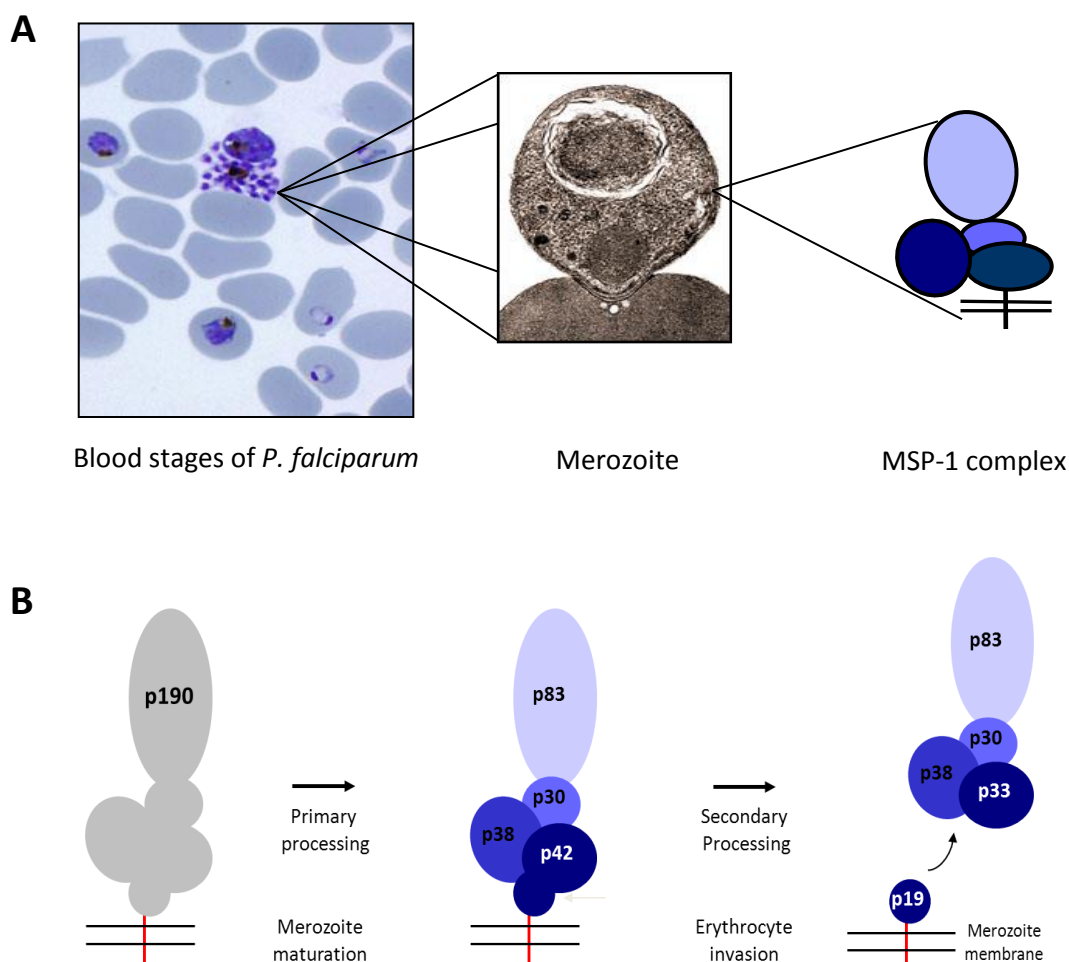
Figure 1.6: MSP-1 complex and proteolytic processing. (A) The MSP-1 complex consisting of four major subunits - MSP-1$_{83}$, MSP-1$_{30}$, MSP-1$_{38}$, MSP-1$_{42}$ – is the major protein component at the merozoite surface and is anchored via GPI. (B) The approximately 190 kDa MSP-1 precursor is proteolytically cleaved by PfSUB1 during merozoite maturation into four major subunits: MSP-1$_{83}$, MSP-1$_{30}$, MSP-1$_{38}$ and MSP-1$_{42}$. Secondary processing prior to erythrocyte invasion is mediated by PfSub2 which cleaves MSP-1$_{42}$ into MSP-1$_{33}$ and the GPI anchored MSP-1$_{19}$. The latter remains on the merozoite surface during erythrocyte invasion while all other fragments are shed.

During merozoite maturation the MSP-1 precursor is cleaved into four major subunits - MSP-1$_{83}$, MSP-1$_{30}$, MSP-1$_{38}$, MSP-1$_{42}$ (Holder et al., 1985) - which remain non-covalently associated to the surface and interact with the processed forms of the peripheral surface proteins MSP-6 and MSP-7 (Kauth et al., 2006; Pachebat et al., 2007; Stafford et al., 1996; Trucco et al., 2001) as well as with MSP-3, MSPDBL-1 and MSPDBL-2 (Lin et al., 2016). The processing step is mediated by the subtilisin-like serine protease *Pf*SUB1 which is released by merozoite exonemes just prior to egress of daughter merozoites from the infected host cell (Koussis et al., 2009; Yeoh et al., 2007). By inhibition of *Pf*SUB1 several merozoite proteins such as MSP-1, MSP-6 and MSP-7 cannot be processed and RBC invasion is reduced, suggesting that *Pf*SUB1-mediated processing may be essential for the release of merozoites after schizont rupture and the preparation of merozoites for reinvading new RBCs (Koussis et al., 2009). Indeed, this processing step plays a crucial role for RBC rupture and parasite egress since it enables MSP-1 to bind to heparin and spectrin on the erythrocyte membrane (Das et al., 2015).

Erythrocyte invasion requires a secondary proteolytic step mediated by the membrane-bound protease *Pf*SUB2 which is released from micronemes shortly prior or during RBC invasion and cleaves MSP-1$_{42}$ into MSP-1$_{33}$ and the GPI-anchored MSP-1$_{19}$ (Blackman and Holder, 1992). This processing eventually results in shedding of the entire MSP-1/6/7 complex from the surface of *Plasmodium falciparum* except MSP-1$_{19}$ which is carried into the newly infected red blood cell (Blackman et al., 1990) (Figure 1.6 B).

1.4.2. MSP-1 structure

According to primary structure analyses of MSP-1 from different *P. falciparum* isolates, the protein structure is divided into 17 blocks corresponding to the different levels of conservation (Tanabe et al., 1987)(Figure 1.7.): While some regions are highly conserved there are two short areas of higher sequence variation (block 2 and 4). However, as the major part of MSP-1 appears dimorphic (block 5-16), all strains can be classified to one of the two allelic variants, termed K1 and MAD20 according to two representative isolates from PNG and Thailand (Tanabe et al., 1987). For instance, the laboratory strain 3d7 represents the MAD20 allele while Fcb1 is assigned as K1.

The best studied part of MSP-1 is the C-terminal MSP-1$_{19}$ fragment (block 17) which is generated during secondary proteolytic cleavage and whose sequence is highly conserved within different *P. falciparum* isolates (Holder, 2009; Miller et al., 1993). As shown by NMR spectroscopy analysis MSP-1$_{19}$ consists of two epidermal-growth factor (EGF)-like domains stabilized by disulfide bonds (Morgan et al., 1999).

The three-dimensional structure of the residual approximately 95 % of MSP-1 is currently not known - the large size of MSP-1 makes crystallization very challenging - but a structural model about the arrangements of MSP-1 subunits (Kauth et al., 2003) as well as their interaction with MSP-6 and MSP-7 (Kauth et al., 2006) has been proposed.
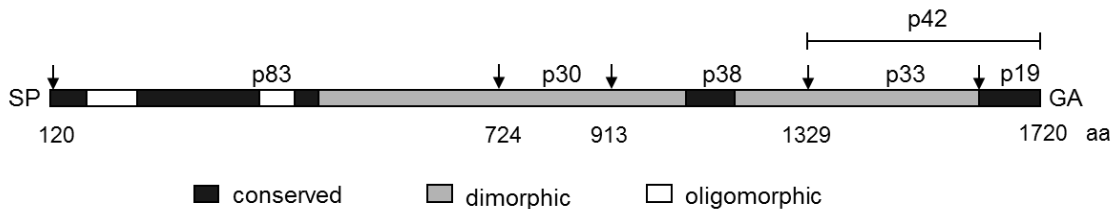
Figure 1.7: Structure of MSP-1. *The 190 kDa precursor protein consists of 1720 amino acids including a signal peptide (SP) and a GPI anchor (GA). The arrows indicate sites of primary processing of the MSP-1 precursor into the subunits MSP-1$_{83}$, MSP-1$_{30}$, MSP-1$_{38}$ and MSP-1$_{42}$. During invasion, PfSub2 cleaves MSP-1$_{42}$ into MSP-1$_{33}$ and MSP-1$_{19}$. Black, grey and white boxes represent conserved, dimorphic and oligomorphic regions, respectively.*

1.4.3. MSP-1 function

Several studies suggest that MSP-1 is essentially involved in the parasite´s life cycle and that it plays a crucial role for erythrocyte invasion and merozoite egress. Thus, attempts to knock out the *msp1* gene in *P. falciparum* blood stages by homologous recombination failed, whereas the C-terminal fragment MSP-1$_{19}$ can be replaced by the corresponding sequence of the distantly related *Plasmodium* species, *P. chabaudi* (O'Donnell et al., 2001). Different interaction partners of MSP-1 have been proposed on the erythrocyte surface and the erythrocyte cytoskeleton, suggesting a role of MSP-1 for the initial attachment to erythrocytes: Sialic acid (Perkins and Rocco, 1988), spectrin (Herrera et al., 1993), heparin-like proteoglycans (Boyle et al., 2010a), band 3 (Goel et al., 2003), glycophorin A, B and C (Hafalla et al., 2011) as well as complement receptor 1 (Hafalla et al., 2011). Interestingly, binding of the N-terminal part of MSP-1$_{83}$ to glycophorin A on erythrocytes plays a crucial role for RBC invasion (Baldwin et al., 2015). Furthermore, processing by *Pf*SUB1 enables MSP-1 to bind to heparin and spectrin, leading to RBC rupture and parasite egress (Das et al., 2015).

Interestingly, it was reported recently that several peripheral merozoite proteins – MSP-3, MSP-6, MSP-7, MSPDBL-1 and MSPDBL-2 – bind to MSP-1 independently of each other and thus, generate different MSP-1 dependent complexes on the merozoite surface with overlapping functions; complexes containing MSP-6, MSPDBL-1 or MSPDBL-2 are able to bind to human erythrocytes (Lin et al., 2016). MSP-1$_{83}$ seems to be the crucial component of all these complexes since by antibody-mediated inhibition of this subunit MSP-1 complexes cannot form and parasite growth *in vitro* is reduced (Lin et al., 2016).

Furthermore, several monoclonal antibodies targeting MSP-1$_{19}$ efficiently inhibit the invasion of red blood cells *in vitro* (Blackman et al., 1990) and prevent the secondary cleavage of MSP-1$_{42}$ by *Pf*SUB2 during erythrocyte invasion (Blackman et al., 1994). Thus, this proteolytic step resulting in the generation of the MSP-1$_{19}$ fragment appears to play a crucial role in RBC invasion. However, antibodies raised in immunized rabbits against all MSP-1 subunits (Woehlbier et al., 2006) as well as against epitopes located throughout the MSP-1, MSP-6, MSP-7 complex (Woehlbier et al., 2010) efficiently inhibit secondary processing and interfere with erythrocyte invasion and the intracellular growth of the parasite. All these studies emphasize the essential role of MSP-1 for the erythrocyte invasion process of the parasite suggesting MSP-1 as a promising candidate for malaria vaccine development.

1.4.4. MSP-1 as malaria vaccine candidate

(i) MSP-1 specific humoral immune response

Several epidemiological studies in malaria endemic regions report a positive correlation between acquired clinical immunity against malaria and serum antibody levels to different parts of MSP-1 suggesting a protective potential of this protein. Antibodies directed against a dimorphic region within the MSP-1$_{83}$ subunit are associated with protection against *P. falciparum* malaria in adolescents from Mali (Tolle et al., 1993). Furthermore, antibody titers to MSP-1$_{42}$ and MSP-1$_{19}$ have been correlated with clinical immunity against malaria in children from West Africa and Papua New Guinea (al-Yaman et al., 1996; Egan et al., 1996). Moreover, antibodies directed to the oligomorphic block 2 of MSP-1 strongly correlate with protection against *P. falciparum* disease in 337 children from Gambia (Conway et al., 2000) as well as in 280 children from Ghana (Cavanagh et al., 2004).

Interestingly, MSP-1 specific antibodies can target the parasite *via* several immune mechanisms: Direct growth inhibitory activity of *P. falciparum* blood stages *in vitro* has been reported for antibodies directed against MSP-1$_{19}$ (Blackman et al., 1990; Egan et al., 1999; O'Donnell et al., 2001), MSP-1$_{83}$ (Woehlbier et al., 2006) and the complete MSP-1 protein (Woehlbier et al., 2006). Additionally, MSP-1$_{19}$-specific antibodies inhibit the parasite *in vitro* in cooperation with complement factors (Boyle et al., 2015) and by recruitment of neutrophils *via* antibody-dependent respiratory burst (Joos et al., 2015). Antibodies to MSP-1$_{block2}$ have been reported to inhibit parasite growth *in vitro* by activation of monocytes in the antibody-dependent cellular inhibition assay (Galamo et al., 2009).

(ii)   MSP-1 specific T-cell response

Since MSP-1 is initially synthesized in infected hepatocytes at late liver-stage (Haussig et al., 2011; Szarfman et al., 1988) it may also induce a protective cellular immune response in the liver. Indeed, human T-lymphocytes from 3 out of 4 volunteers who have been vaccinated with radiation attenuated *P. falciparum* sporozoites recognize not only the pre-erythrocytic antigens CSP and LSA-1 but also the blood stage antigens SERA-1, MSP-2 and MSP-1 (Krzych et al., 1995). Moreover, CD4+ and CD8+ T-cells could be induced in mice by vaccination with MSP-1$_{42}$ using the viral vectors AdHu5 and MVA. Immunized mice were better protected against *Plasmodium* sporozoite challenge, probably due to enhanced IFNγ levels which were detected in the serum and associate with a lower liver stage parasite burden (Draper et al., 2009).

Importantly, CD8+ T-cell epitopes restricted to the HLA-A0201 receptor have been identified within MSP-1D by mass spectrometry and *via* tetramer technology (details in chapter 5.3.1)(Carralot et al., 2008; Idler, 2004), suggesting that MSP-1 induces also a cellular immune response in humans based on the activation of CD8+ cytotoxic T-cells.

(iii)   Protective potential of MSP-1 in immunization experiments

Several immunization studies in mice and monkeys have been performed with native MSP-1 or recombinant MSP-1 subunits. Mice immunized with purified *P. yoelii* MSP-1 (Holder and Freeman, 1981) or with recombinant MSP-1$_{19}$ or MSP-1$_{42}$ produced in *E.coli* (Daly and Long, 1995; Tian et al., 1997) or *S. cerevisiae* (Hirunpetcharat et al., 1997) are protected against subsequent *P. yoelii* infection. Furthermore, immunization of *Saimiri* and *Aotus* monkeys with native *Pf*MSP-1 purified from parasite cultures results in complete protection against a lethal infection with *P. falciparum* (Etlinger et al., 1991; Perrin et al., 1984; Siddiqui et al., 1987). Monkeys immunized with recombinant *Pf*MSP-1 fragments produced in *E. coli* or *S. cerevisiae* are partially protected against malaria (Cavanagh et al., 2014; Etlinger et al., 1991; Herrera et al., 1990; Kumar et al., 1995; Singh et al., 2006). Partial protection of *Aotus* monkeys has also been reported after immunization with recombinant MSP-1$_{42}$ purified from baculovirus cultures (Chang et al., 1996).

Clinical vaccine trials performed with MSP-1 in humans are summarized in chapter 1.3.4 (Blood stage vaccines). Importantly, protective efficacy of MSP-1 vaccination has only been assessed in two clinical studies so far; both used the conserved C-terminal part MSP-1$_{42}$ for immunization and were unsuccessful in conferring protection against malaria (Ogutu et al., 2009; Sheehy et al., 2012). However, several studies indicate the importance of the dimorphic and oligomorphic regions of MSP-1 for protection (Früh et al., 1991; Galamo et al., 2009; Müller et al., 1989; Siddiqui et al., 1987). Thus, full-length MSP-1 is considered as a promising vaccine candidate.

(iv)   Production of recombinant MSP-1 under GMP conditions

A major obstacle for a good immunological characterization of full-length MSP-1 and its use in immunization trials has been the difficulty of obtaining this protein in sufficient quantity and quality. The large size (nearly 5000 bp) and high AT content (~80 %) of the *msp-1* gene makes stable cloning and heterologous expression of MSP-1 very challenging. Thus, most of the immunization studies have been based on the C-terminal MSP-1 fragments MSP-1$_{42}$ or MSP-1$_{19}$. However, a re-design of the full *msp-1* gene using the human codon frequency and thereby reducing the AT content to 55 % has solved the problem (Pan et al., 1999). Due to the dimorphism of MSP-1 two different versions were synthesized (Pan et al., 1999): While the MSP-1F construct from the Fcb1 strain largely represents the K1 prototype, the MSP-1D construct from the 3d7 strain corresponds to MAD20.

By using these constructs, sufficient amounts of full-length MSP-1 and its processing products have been synthesized in *E. coli*, purified by a three-step affinity chromatography (Epp et al., 2003; Kauth et al., 2003) and used for interaction- or immunological analysis. The MSP-1 complex can be reassembled *in vitro* from the recombinant MSP-1 processing products suggesting that the conformation of all recombinant proteins is very similar to their native one (Kauth et al., 2003). Furthermore, antibodies raised against the recombinant MSP-1 subunits recognize MSP-1 on the parasite surface (Epp et al., 2003) and can inhibit parasite growth *in vitro* (Woehlbier et al., 2006).

For efficient production of MSP-1 a strategy has been developed, in which the two halves of MSP-1, MSP-1$_{83/30}$ (102kDa) and MSP-1$_{38/42}$ (90kDa), are (i) expressed separately in *E.coli* W3110-Z1, (ii) recovered as inclusion bodies, (iii) renatured and assembled to the MSP-1 heterodimer *in vitro* and (iv) purified as complete MSP-1 protein by sequential chromatography using ion-exchange matrices. Recently, recombinant full-length MSP-1D from the *P. falciparum* strain 3d7 has been successfully produced under Good Manufacturing Practice (GMP) conditions and is available for clinical studies. A Phase I trial assessing safety and immunogenicity in malaria-naïve volunteers from Germany is currently ongoing in Heidelberg. Throughout this work, recombinant full-length MSP-1 (191 kDa) from *P. falciparum* (3d7) was used, henceforth named MSP-1D.

1.5. Aim of the study

The malaria vaccine candidate MSP-1D from *P. falciparum* (3d7) has just reached clinical trials, thus it is important to accurately characterize the immune mechanisms which are elicited by the protein in humans and possibly contribute to protection against malaria. The overall aim of this work is to investigate the human humoral and cellular immune response against the vaccine candidate *Pf*MSP-1D in individuals with naturally acquired immunity against malaria and in malaria-naïve volunteers experimentally infected with *Pf* sporozoites.

MSP-1 specific antibodies, acquired by natural exposure to *P. falciparum* in eleven young adults from Nouna, Burkina Faso, will be analyzed for their capability to (i) directly inhibit the growth of *P. falciparum* blood stages *in vitro* and (ii) to induce neutrophil respiratory burst by opsonization of *Plasmodium* merozoites. Thus, the growth inhibition assay (GIA) and antibody-dependent respiratory burst (ADRB) assay will be applied, respectively. Antibody levels will be correlated with functional activity and the MSP-1 specific antibodies – purified by their affinity to recombinant MSP-1D – will be studied *in vitro* for their ability to directly inhibit parasite growth and also to opsonize merozoites and recruit neutrophils. Additionally, we aim to measure the contribution of defined antigens to GIA and ADRB activity by pre-incubation of total antibodies with recombinant proteins prior to performing the functional assays. Potentially unknown *P. falciparum* proteins present in blood stage schizonts which may mediate growth inhibitory activity *in vitro* in individuals with naturally acquired immunity, shall be identified via western blot and mass spectrometry analysis. Furthermore, purified IgG from *rhesus* monkeys immunized with recombinant MSP-1D will be analyzed for their potential to opsonize *P. falciparum* merozoites and elicit neutrophil respiratory burst.

The MSP-1 specific cytotoxic CD8+ T-cell response will be investigated in individuals with naturally acquired immunity and in malaria-naïve volunteers experiencing a single *Pf* infection by experimental sporozoite challenge. HLA-A0201 restricted CD8+ T-cell epitopes have been identified previously within MSP-1D (Carralot et al., 2008; Idler, 2004). Using these peptides semi-immune adults from Nouna and malaria-naïve controls – all have the HLA haplotype A0201 - shall be studied for their MSP-1 specific CD8+ T-cell response *via* IFNγ ELISPOT assay. Furthermore, HLA independent methods such as the use of comprehensive peptide pools or recombinant MSP-1 protein for ELISPOT analysis will be investigated. A HLA-independent approach is the prerequisite to examine large cohorts of individuals for a potential correlation between MSP-1 specific T-cell response and protection against malaria.

Besides naturally acquired immunity, we also aim to study the immune response elicited by a single *P. falciparum* wildtype infection. We will use blood samples from a controlled human malaria infection study performed in Tübingen (TUECHMI I), in which 30 malaria-naïve volunteers received different doses of cryopreserved *Pf* sporozoites by intravenous or intradermal injection (Mordmuller et al., 2015). The antibody response to the merozoite surface proteins MSP-1, MSP-6 and MSP-7 will be investigated as well as the cytotoxic CD8+ T-cell response against LSA-1, CSP and MSP-1. Overall, we examine a possible contribution of MSP-1 specific immune responses to protection against malaria.
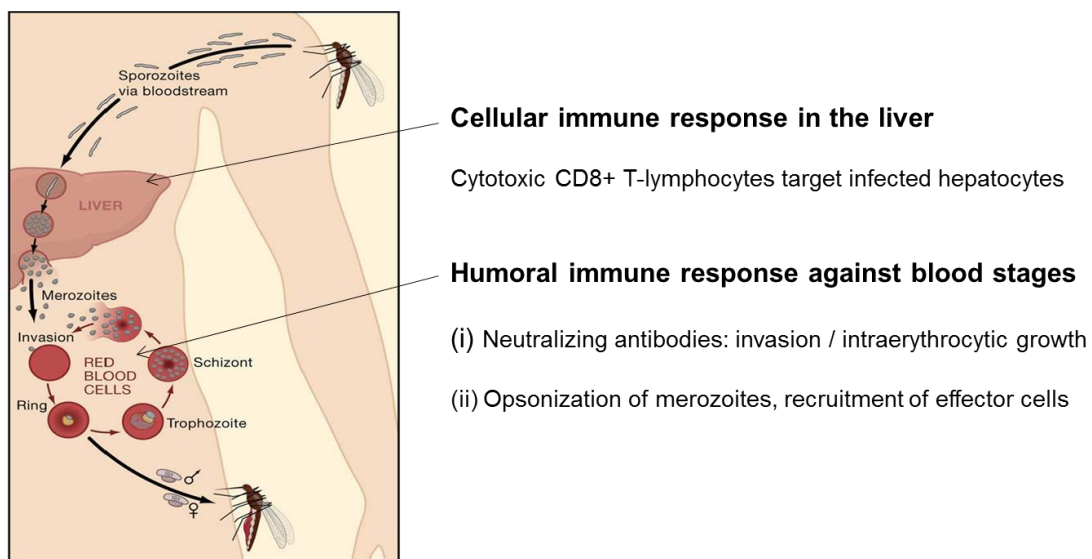
Figure 1.8: Human immune mechanisms against Plasmodium infection analyzed in this study. *A MSP-1 specific CD8+ cytotoxic T-cell response against Plasmodium liver stages will be studied in semi-immune African adults and in malaria-naïve volunteers after single Pf sporozoite infection. Plasmodium blood stages can be targeted by neutralizing antibodies, which directly inhibit Plasmodium erythrocyte invasion and/or intra-erythrocytic growth of the parasite, or opsonizing antibodies which recruit effector cells like monocytes or neutrophil granulocytes. Both mechanisms will be examined in African adults with naturally acquired immunity against malaria using the in vitro assays GIA and ADRB. Picture modified from (Cowman and Crabb, 2006).*

2. Abbreviations & Definitions

2.1. Abbreviations

α	Anti
aa	Amino acid
ACT	Artemisinin-based combination therapy
ADCI	Antibody-dependent cellular inhibition
ADRB	Antibody-dependent respiratory burst
AMA-1	Apical membrane antigen 1
AP	Alkaline phosphatase
APC	Antigen presenting cell
APS	Ammonium persulfate
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
BSA	Bovine serum albumin
°C	Degree Celsius
ChAd63	Chimpanzee adenovirus 63
CSA	Chondroitin sulphate A
CSP	Circumsporozoite protein
C-terminus	Carboxy-terminus
CTL	Cytotoxic T-lymphocytes
CV	Column volumes
Da	Dalton
DBL	Duffy-binding like
ddH$_2$O	double distilled water
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EBA	Erythrocyte binding antigen
E.coli	*Escherichia coli*
e.g.	For example (latin: exempli gratia)
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunospot assay
FCS	Fetal calf serum
FSC	Forward scatter

GIA	Growth inhibition assay
GLURP	Glutamate rich protein
GMP	Good manufacturing practice
G6PD	Glucose-6-phosphate dehydrogenase
GPI	Glycosylphosphatidylinositol
H	Histidine (His)-tag
HCl	Hydrochloric acid
HLA	Human leukocyte antigen
HRP	Horseradish peroxidase
IB	Inclusion body
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
LSA-1	Liverstage antigen-1
MHC	Major histocompatibility complex
MS	Mass spectrometry
MSP	Merozoite surface protein
MVA	Modified vaccinia Ankara virus
MW	Molecular weight
N_2	Liquid nitrogen
NA-pool	IgG pool (n=11) from African adults
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBT	Nitro blue tetrazolium chloride
N-terminus	Amino-terminus
O_2	Oxygen
OD	Optical density
ON	Overnight
OPA	Opsonic phagocytosis assay
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Pf	*Plasmodium falciparum*
*Pf*SUB	*P. falciparum* subtilisin-like protease

pH	*Potentia hydrogenii*
pI	Isoelectric point
PMN	Polymorphonuclear neutrophil
PV	Parasitophorous vacuole
r	Recombinant
RBC	Red blood cell
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT	Room temperature
SERA	Serine repeat antigen
SDS	Sodium dodecyl sulfate
SP	Signal peptide
SPAM	Secreted polymorphic antigen associated with merozoites
SSC	Side scatter
TBS	Tris-buffered saline
TBST	Tris-buffered saline + Tween 20
TEMED	Tetramethylethylenediamine
TNFα	Tumour necrosis factor α
TRAP	Thrombospondin related adhesive protein
UV	Ultraviolet
v/v	Volume to volume
Vol	Volume
WHO	World Health Organization
wt	Wildtype
ZMBH	Zentrum für Molekulare Biologie der Universität Heidelberg

2.2. Units of measurement

A	Ampere
bp	base pairs
g	gram
h	hour
kDa	kilo Dalton
l	liter
M	molar (mol/l)

38

min	minutes
rpm	rounds per minute
U	unit
V	Volt

2.3. Unit prefixes

k	kilo	10^3
m	milli	10^{-3}
µ	mikro	10^{-6}
n	nano	10^{-9}

2.4. Bases

A	Adenin
C	Cytosin
G	Guanin
T	Thymin
U	Uracil

2.5. Nomenclature of amino acids (IUPAC)

Ala	A	Alanin	Leu	L	Leucin
Arg	R	Arginin	Lys	K	Lysin
Asn	N	Asparagin	Met	M	Methionin
Asp	D	Aspartic acid	Phe	F	Phenylalanin
Cys	C	Cystein	Pro	P	Prolin
Gln	Q	Glutamin	Ser	S	Serin
Glu	E	Glutamic acid	Thr	T	Threonin
Gly	G	Glycin	Trp	W	Tryptophan
His	H	Histidin	Tyr	T	Tyrosin
Ile	I	Isoleucin	Val	V	Valin

3. Material

3.1. Laboratory equipment

Autoclaves 2540 EL & 5075ELV	Systec GmbH, Göttingen
Centrifuge	Thermo Scientific, USA
Centrifuge, Megafuge 1.0R	Heraeus Instruments, Hanau
Centrifuge, Megafuge 2.0R	Heraeus Instruments, Hanau
Centrifuge, Pico 17	Heraeus Instruments, Hanau
Chromatography system:	
Äkta Purifier 100	GE Healthcare, Freiburg
Colum corpus & auxiliary equipment	GE Healthcare, Freiburg
Electrophoresis Power supply Consort EV243	Hoefer, USA
ELISPOT reader AID	AID Diagnostika GmbH, Straßberg
FACS Calibur	Becton Dickinson, Heidelberg
FLUOstar OPTIMA microplate reader (ADRB)	BMG Labtech GmbH, Ortenberg
Freezer -20 °C comfort	Liebherr International Deutschland GmbH, Biberach an der Riss
Freezer -80 °C	Thermo Fisher Scientific, Karlsruhe
Fridge	Bosch, Stuttgart
Heating block	VWR International GmbH, Darmstadt
Ice machine, AF30	Scotsman, Vernon Hills, IL, USA
Incubator, Cell Star cytoperm 2	Heraeus Instruments, Hanau
Incubator CO_2, BBD6620	Heraeus Instruments, Hanau
Laminar Flow Cell culture hood, Hera safe	Heraeus Instruments, Hanau
Light microscope, Axiolab	Zeiss, Jena
Magnetic stirrer	VWR International GmbH, Darmstadt
Microplate reader, Multiscan FC (ELISA, GIA)	Thermo Fisher Scientific, Karlsruhe
Microwave	Zanussi, Nuremberg
Multi-channel pipette, 12-channel, 200 µl	Eppendorf AG, Hamburg
PAA gel system, MiniVE	GE Healthcare, Freiburg
pH meter, Five Easy	Mettler-Toledo GmbH, Gießen
Photometer plus	Eppendorf AG, Hamburg
Pipetman Gilson P2, P10, P20, P200, P1000	Gilson International, Bad Camberg
Pipettus® akku	Hirschmann Labortechnik, Eberstadt
Printer hp LaserJet 1300	Hewlett Packard, Heidelberg
Protein electrophoresis system, Mighty Small II	Hoefer Inc., Holliston, MA, USA

Shaker (flatbed)	VWR International GmbH, Darmstadt
Vortex	VWR International GmbH, Darmstadt
Waterbath precitherm PFV	Labora Mannheim GmbH, Mannheim
Western Blot aperture, XCell SureLock, Mini Cell	Invitrogen, Karlsruhe

3.2. Software

Adobe Photoshop C5	Adobe systems Inc., USA
AID ELISPOT version 4.0	AID Diagnostika GmbH, Straßberg
Cell Quest Pro (FACS)	Becton Dickinson, Heidelberg
EndNote X7.7.1	Thomson Reuters, Philadelphia, USA
OPTIMA Data Analysis (ADRB)	BMG Labtech GmbH, Ortenberg
Microsoft Office 2010	Microsoft Corporation, WA, USA
PlasmoDB	The EuPathDB Project Team, USA
Sigma Plot 12.3	Systat Software GmbH, USA
UniCorn 5.2 (Äkta)	GE Healthcare, Freiburg

3.3. Consumables

1.5 ml tubes	Sarstedt AG & Co., Nümbrecht
15 ml tubes	Greiner Bio-One, Frickenhausen
50 ml tubes	Greiner Bio-One, Frickenhausen
5 ml polypropylene columns	Thermo Fisher Scientific, MA, USA
96-well plates, Nunc F96 Maxisorp (ELISA)	Thermo Fisher Scientific, MA, USA
96-well sterile F-bottom plates (GIA)	Greiner Bio-One, Frickenhausen
96-well white LUMITRAC plates, F-bottom, sterile (ADRB)	Greiner Bio-One, Frickenhausen
96-well multiscreen-IP filter plates, sterile (ELISPOT)	Merck Millipore GmbH, Schwalbach
24-well plates, Cellstar (ELISPOT)	Greiner Bio-One, Frickenhausen
Amicon Ultra Centrifugal Filters, 30K	Merck Millipore GmbH, Schwalbach
Cover glasses	Roth, Karlsruhe
Cuvettes	Sarstedt AG & Co, Nümbrecht
Dialysis membrane, Spectra/Por	Roth, Karlsruhe
Glass slides	Paul Marienfeld GmbH, Lauda
Gloves Latex	VWR, USA
Immobilon-P PVDF Membrane (0.45 µM)	Millipore Corp., MA, USA
NuPAGE Bis-Tris Mini gels (4-12 %)	Thermo Fisher Scientific, MA, USA
Parafilm	Pechiney Plastic Packaging, IL, USA

Pasteur pipettes, sterile & disposable	Roth, Karlsruhe
Petri dishes	Greiner Bio-One, Frickenhausen
Pipette tips, Diamond (10 µl, 200 µl,1000 µl)	Gilson International, Bad Camberg
Plastic pipettes (1 ml, 2 ml, 5 ml, 10 ml, 25 ml)	Greiner Bio-One, Frickenhausen
S-Monovette Lithium-Heparin, 7.5 ml (ADRB)	Sarstedt AG & Co, Nümbrecht
Sterile filters, Rotilabo® (0.2 µM; 0.45 µM)	Roth, Karlsruhe
Whatman TM 3MM paper	GE Healthcare, Freiburg

3.4. Chemicals

Acetic acid	Sigma-Aldrich, Taufkirchen
Acyrylamide, 30%	Carl Roth, Karlsruhe
Ammoniumdihydrogenphosphate	Carl Roth, Karlsruhe
Ammoniumsulfate	Sigma-Aldrich, USA
3-Acetylpyridine adenine dinucleotide (APAD), 90%	Sigma-Aldrich, USA
BCIP/NBT tablets (GIA & WB)	Sigma-Aldrich, USA
BCIP/NBT-plus substrate (ELISPOT)	Mabtech, Sweden
Bradford reagent	BioRad, München
Coomassie Brilliant Blue R250	Merck, Darmstadt
Dextran	Carl Roth, Karlsruhe
Diethanolamin	Carl Roth, Karlsruhe
Dimethylsulfoxid (DMSO)	Merck, Darmstadt
DL-1.4-Dithiothreitol (DTT)	Carl Roth, Karlsruhe
EDTA	AppliChem, Darmstadt
Ethanolamine	Carl Roth, Karlsruhe
Ethanol	VWR International GmbH, Darmstadt
FBS, heat inactivated	Invitrogen, Karlsruhe
Ficoll-Histopaque	Sigma-Aldrich, Taufkirchen
Gentamycin	Gibco, Karlsruhe
Giemsa stain	Carl Roth, Karlsruhe
Glycine	Sigma-Aldrich, Taufkirchen
HBSS	Thermo Fisher Scientific, MA, USA
Hydrochloric acid	Sigma-Aldrich, Taufkirchen
Hypoxanthin 10 mM	CC pro GmbH, Oberdorla
Imidazol	Merck, Darmstadt
Immersion oil (518 C)	Waldeck GmbH & Co KG, Münster

Isoluminol (4-Aminophthalhydrazide)	Santa Cruz Biotechnology, Dallas, USA
Isopropanol	Sigma-Aldrich, Taufkirchen
Kalium chloride	AppliChem, Darmstadt
L-Glutamine, 200 mM	Invitrogen, Karlsruhe
Methanol, 99.8 %	Sigma-Aldrich, Taufkirchen
Milk powder	Carl Roth, Karlsruhe
PBS	Invitrogen, Karlsruhe
Penicillin-Streptomycin, 10 000 U/ml	Invitrogen, Karlsruhe
Paraformaldehyd (PFA)	AppliChem, Darmstadt
p-Nitrophenylphosphate (pNPP) tablets (ELISA)	Sigma-Aldrich, Taufkirchen
Propidium iodide	Life Technologies, Darmstadt
Protein-A agarose (20334, Pierce)	Thermo Fisher Scientific, MA, USA
Protein-A, IgG Binding buffer (Pierce)	Thermo Fisher Scientific, MA, USA
Protein-G agarose (20399, Pierce)	Thermo Fisher Scientific, MA, USA
Protein-G, IgG Binding buffer (Pierce)	Thermo Fisher Scientific, MA, USA
RPMI 1640-Medium (Gibco)	Thermo Fisher Scientific, MA, USA
Saponin	Sigma-Aldrich, Taufkirchen
Sodium acetate	Merck, Darmstadt
Sodium azide	Sigma-Aldrich, Taufkirchen
Sodium chloride	Sigma-Aldrich, Taufkirchen
Sodium dodecyl sulfate (SDS)	Carl Roth, Karlsruhe
Sodium L-lactate	Sigma-Aldrich, Taufkirchen
Sorbitol, D-Sorbit	Carl Roth, Karlsruhe
Tetramethylethylendiamine (TEMED)	Carl Roth, Karlsruhe
Tris	Carl Roth, Karlsruhe
Triton X-100	Merck, Darmstadt
Trypan Blue	Carl Roth, Karlsruhe
Tween20	Merck, Darmstadt
UltraLink Biosupport	Thermo Fisher Scientific, MA, USA

3.5. Kits

Human IFNγ ELISPOT[Plus] kit (3420-2AW-10)	Mabtech, Sweden

3.6. Enzymes

Benzonase, 10 ku, purity > 99 % (ELISPOT)	Merck Millipore GmbH, Schwalbach
Diaphorase from *Clostridium kluyveri* (GIA)	Sigma-Aldrich, Taufkirchen
Interleukin 2, 100 000 U/ml (ELISPOT)	Kindly provided by AG Watzel
Streptavidin-Alkaline Phosphatase (ELISPOT)	Mabtech, Sweden

3.7. Antibodies

Rabbit α-AMA-1, BG98 (GIA standard, DiCo)	Dr. E. Remarque, BPRC, Rijswijk, NL
Rabbit α-MSP-1D (GIA & WB)	Confarma France SARL, Hombourg
Rabbit α-MSP-1Dp19 (mAB 5.2) (WB)	Prof. H. Bujard, ZMBH, Heidelberg
Rabbit α-gMSP-6 (3d7) (WB)	Prof. H. Bujard, ZMBH, Heidelberg
Rabbit α-gMSP-7 (3d7) (WB)	Prof. H. Bujard, ZMBH, Heidelberg
Rhesus monkey α-MSP-1D (ADRB)	Dr. E. Remarque, BPRC, Rijswijk, NL
Mouse α-human CD4-PerCP (FACS)	BD Bioscience, Heidelberg
Mouse α-human CD8-PE (FACS)	BD Bioscience, Heidelberg
Goat α-human IgG – AP conjugate (ELISA & WB)	Sigma-Aldrich, St. Louis, MO, USA
α-rabbit IgG – AP conjugate (ELISA & WB)	Sigma-Aldrich, St. Louis, MO, USA
α-human IFNγ 1D1K (ELISPOT, coating)	Mabtech, Sweden
α-human IFNγ 7-B6-1 (ELISPOT, detection)	Mabtech, Sweden
α-malaria human serum (n=11) from Nouna, BF	Dr. B. Coulibaly, Nouna, Burkina Faso
α-malaria human serum from Kisumu, Kenya (WHO)	NIBSC code: 10/198 (Bryan D, 2014)
malaria-naïve human serum (2x n=4) from Germany	Blood bank, Heidelberg

3.8 Recombinant proteins

*Pf*MSP-1D (full-length protein, 3d7 strain, *E.coli*)	BIOMEVA GmbH, Heidelberg
*Pf*MSP-1F (full-length protein, FCB1 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-1 p83 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-1 p42 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-1 p38 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-1 p30 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-6 p36 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg
*Pf*MSP-7 p22 (3d7 strain, *E.coli*)	Prof. H. Bujard, ZMBH, Heidelberg

*Pf*MSP-3 (*Pf*Sub1 processed form, 3d7, 35.6 kDa, *E.coli*)   Dr. C. Lin / Prof. A. Cowman, Australia

*Pf*MSP-9 (*Pf*Sub1 processed form, 3d7, 52.1 kDa, *E.coli*)   Dr. C. Lin / Prof. A. Cowman, Australia

*Pf*MSPDBL-1 (*Pf*Sub1 processed form, 3d7, 71.7 kDa, *E.coli*)   Dr. C. Lin / Prof. A. Cowman, Australia

Bovine serum albumin (BSA, 66 kDa)   Sigma-Aldrich, Taufkirchen

3.9. Stimuli for ELISPOT

Human anti-CD3 mAb   Mabtech, Sweden

CEF Peptide pool (3615-1)   Mabtech, Sweden

MSP-1 based peptides   Peptide Speciality Laboratories GmbH, Heidelberg

CSP based peptides   Dr. K.Heiss / Dr.AK Müller, Heidelberg

LSA-1 based peptides   Dr. K.Heiss / Dr.AK Müller, Heidelberg

*Pf*MSP-1D (full-length protein, 3d7 strain, *E.coli*)   BIOMEVA GmbH, Heidelberg

3.10. Protein marker

Color Plus Protein ladder, 10-230 kDa (prestained)   New England Biolabs, Schwalbach

Protein ladder, 10-250 kDa (unstained)   New England Biolabs, Schwalbach

3.11. Parasite strains

Plasmodium falciparum 3d7   Prof. M. Lanzer, Center for Infectious Diseases - Parasitology, Heidelberg

Plasmodium falciparum FCB1   Prof. M. Lanzer, Center for Infectious Diseases - Parasitology, Heidelberg

3.12. Buffers, solutions and media

Common buffers

10x PBS   1.4 M NaCl
27 mM KCl
100 mM Na_2HPO_4
18 mM KH_2PO_4
pH 7.4

1x PBS   100 ml 10x PBS
ad 1 L ddH_2O

1x TBS	150 mM NaCl 10 mM Tris/HCl pH 8.0

Biochemical Methods

4x Upper Tris	500 mM Tris/HCl 0.4 % SDS, pH 6.8
4 x Lower Tris	1.5 M Tris/HCl 0.4 % SDS, pH 8.8
Stacking gel, 4 % (10 ml)	330 µl PAA (30 %) 625 µl upper Tris 1.55 ml ddH$_2$O 5 µl Temed 25 µl APS (10 %)
Separation gel, 10 % (10 ml)	1.65 ml PAA (30 %) 1.25 ml lower Tris 2.1 ml ddH$_2$O 10 µl Temed 40 µl APS (10 %)
SDS-PAGE running buffer	250 mM Glycine 0.1 % SDS 25 mM Tris/HCl, pH 8.3
SDS-PAGE sample buffer (4x)	8 % SDS 50 % Upper Tris 40 % Glycerol 0.08 % Bromphenolblue
TBST buffer	0.2 % Tween 20 in TBS
Blocking buffer	2 % Skim milk powder in TBS
Western Blot transfer buffer	0.01 % SDS 25 mM Tris/HCl 192 mM Glycin 20 % methanol or ethanol
Stripping buffer	62.5 mM Tris/HCl, pH 6.8 2 % SDS 100 mM β-mercaptoethanol
Coomassie staining solution	50 % EtOH 10 % Acetic acid 40 % ddH$_2$O 0.25 % Serva Blue R

Coupling of rMSP-1D to the Ultra Link Biosupport

Coupling buffer	0.1 M NaH$_2$PO$_4$ 0.6 M C$_6$H$_5$Na$_3$O$_7$ x 2 H$_2$O pH 7.5
Quench solution	3 M Ethanolamin, pH 9

Wash solution	1 M NaCl
Storage solution	0.02 % NaN_3 in PBS

Immune affinity chromatography (α-MSP-1)

Elution buffer 1 (Glycin)	75 mM Glycin 0.5 M NaCl, pH 2.8
Elution buffer 2 (NaCl)	1 M NaCl in PBS, pH 7.4
Neutralization buffer	1 M Tris, pH 8.0

Immune affinity chromatography (Protein-G)

IgG Binding buffer (Pierce)	Thermo Fisher Scientific, USA
Elution buffer	0.1 M Glycine, pH 2.5
Neutralization buffer	1 M Tris, pH 8.0

ELISA

Coating buffer	34 mM Na_2CO_3 16 mM $NaHCO_3$ pH 10.6
TBST buffer	0.05 % Tween 20 in TBS
Blocking buffer	1 % skim milk powder in TBST
Substrate buffer	Diethanolamine (9.6 ml) 2 M $MgCl_2$ (500 µl) pH 9.5 ad 1 L ddH_2O
Substrate	p-Nitrophenylphosphate (pNPP) tablet (Sigma-Aldrich) dissolved in 20 ml substrate buffer (1 mg/ml)
Stopping solution	0.2 M NaOH

Cell culture of *Plasmodium falciparum*

Human erythrocytes, blood group 0+	Blood bank, Heidelberg
Human serum, blood group A+ (heat inactivated)	Blood bank, Heidelberg
Parasite culture medium	RPMI 1640 (500 ml) + L-Glutamine + 25 mM HEPES 0.1 mM hypoxanthin 20 µg/ml gentamycin 10 % (v/v) human serum

Freezing solution	56 % (v/v) Glycerol
	3 % (v/v) Sorbitol
	0.65 % (w/v) NaCl
Thawing solutions	1) 12 % NaCl in ddH$_2$O
	2) 1.6 % NaCl in ddH$_2$O
	3) 0.9 % NaCl + 0.2 % Glucose
Saponin solution, 1 % (10x)	0.1 g saponin in 1 ml ddH$_2$O
RIPA buffer	Sigma-Aldrich, Taufkirchen
1x SSC buffer	7.5 mM NaCl
	0.25 mM sodium citrate
	pH 7.0

GIA (*p*LDH assay)

GIA medium	RPMI 1640 (500 ml)
	+ L-Glutamine
	+ 25 mM HEPES
	0.2 mM hypoxanthin
	40 µg/ml gentamycin
	20 % (v/v) human serum
LDH buffer	10 % (v/v) 1 M Tris/HCL, pH 8.0
	2.8 g Sodium – L-Lactate
	0.25 % (v/v) Triton X-100
	Ad 500 ml ddH$_2$O
LDH substrate buffer	1 NBT tablet dissolved in 50 ml
	LDH buffer (in the dark)
APAD solution	10 mg/ml stock in ddH$_2$O
	store 50 µl aliquots at -20 °C
Diaphorase solution	50 units /ml in ddH$_2$O
	store 200 µl aliquots at -20 °C

ADRB assay

Dextran solution	3 % (w/v) Dextran in 0.9 % NaCl
	sterile filtered
Isoluminol stock	4 mg/ml in DMSO
NaCl solutions	1) 0.9 % NaCl (w/v) in ddH$_2$O
	2) 1.8 % NaCl (w/v) in ddH$_2$O
	sterile filtered

ELISPOT assay

PBMC medium	RPMI 1640
	10 % (v/v) FCS or FBS
	200 mM L-Glutamin
	1 % Pen/Strep

Peptide stocks	20 mg/ml peptide in 90 % DMSO
Wash buffer	PBS 0.05 % Tween 20
Coating Ab α-human IFNγ 1D1K	10 µg/ml in sterile PBS
Detection Ab α-human IFNγ 7-B6-1	1 µg/ml in PBS 0.5 % FCS
Streptavidin-Alkaline Phosphatase	1:1000 in PBS 0.5 % FCS
Substrate solution BCIP/NCB	Ready to use (Mabtech, Sweden) sterile filtered
Stop solution	ddH_2O

4. Methods

4.1. Animal immunizations with recombinant MSP-1D

4.1.1. *Rhesus* monkey immunizations

Rhesus immunizations and antibody preparation were carried out at the Biomedical Primate Research Centre (BPRC), Rijswijk, Netherlands in 2010. The BPRC is AAALAC accredited and compliant with recommendations of the Weatherall report on the use of non-human primates in research (Weatherall, 2006). The study was approved by an independent ethics committee at BPRC, constituted in accordance with Dutch law (DEC598) and European Acts (directive 2010/63/EU) on animal experimentation. To minimize discomfort to animals, immunization and blood sampling were all done under ketamine sedation. Animals were assigned in a manner that ensured that age, weight and sex were similar amongst groups, and treatments were randomly assigned to groups.

Captive-bred *Rhesus* macaques (n = 5, 2x female & 3x male, age: 4.6 – 12.5 years) were immunized 3 times intramuscularly (day 0, 28 and 56) with 100 µg MSP-1D per dose formulated with CoVaccine HT (Cowan et al., 2011; Mahdi Abdel Hamid et al., 2011) in 500 µl volume. From each animal 29 ml blood was collected by venous puncture at day 0, 28, 56, 70, 126 and 182. IgG from sera were purified by Protein G affinity purification and stored at -80 °C.

4.1.2. Rabbit immunizations

Rabbit immunizations were performed at Confarma France SARL, F-68490 Hombourg, in compliance with legal animal health regulations and according to EC-GMP and c-GMP guidelines. New Zealand white rabbits (n=6) were immunized intramuscularly with lyophilized full-length *Pf*MSP-1D (50 µg) and adjuvant IDRI-SE + GLA (100 µg) in a total volume of 500 µl on day 0, 28 and 56. Two weeks after the last immunization (d70) blood was sampled by heart puncture and serum was prepared and transported to Heidelberg. There, IgG were purified from serum *via* Protein A affinity chromatography and tested for growth inhibitory activity.

4.2. Production of recombinant *P. falciparum* merozoite antigens

4.2.1. MSP-1

Recombinant *Pf*MSP-1D (from 3d7 strain; 191.7 kDa) was produced in *E.coli* and purified under GMP compatible conditions by BIOMEVA GmbH, Heidelberg. In brief, the two halves of the *Pf*MSP-1 complex – p83/30 and p38/42 - were expressed separately in *E.coli* W3110-Z1, recovered as

inclusion bodies and reconstituted to the MSP-1 heterodimer *in vitro* followed by a three step purification *via* affinity chromatography. Recombinant MSP-1F (from FCB1 strain; 183.4 kDa) was produced similarly, according to a protocol developed by Kauth et al. (Kauth et al., 2003). MSP-1$_{83}$ (from 3d7 strain; 81.8 kDa) was expressed and purified as published previously (Kauth et al., 2006). All proteins except MSP-1D contain a N-terminal hexa-histidine tag.

4.2.2. MSP-3/-9/-DBL1

The *Pf*Sub1 processed forms of *Pf*MSP-3 (35.6 kDa), *Pf*MSP-9 (52.1 kDa) and *Pf*MSPDBL-1 (71.7 kDa) were kindly provided by Dr. C. Lin and Prof. A. Cowman from the Walter and Eliza Hall Institute of Medical Research in *Melbourne*, Australia. The recombinant proteins were expressed in *E.coli* and contain a N-terminal hexa-histidine tag (Lin et al., 2016).

4.3. Biochemical methods

4.3.1. Discontinuous, denaturizing polyacrylamide gel electrophoresis

SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, is a widely used technique to separate proteins in an electric field according to their molecular weight. As the charge-mass ratio of proteins is not constant due to their different amino acid compositions, the proteins are denatured by heat and their own charge is covered by binding of the ionic detergent sodium dodecyl sulfate (SDS). Additionally, disulfide bridges may be reduced by treatment with DTT. Therefore, negatively charged SDS-protein complexes with a constant charge to mass ratio are generated.

Gel electrophoresis is performed using a double-stage polyacrylamide gel, which is characterized by a pH step from the large-pored stacking gel (4 %, pH 6.8) to the narrow-pored separation gel (8-15 %, pH 8.8). Both gels are generated by radical polymerisation of acrylamide and bisacrylamide using the radical formers APS and TEMED. In the polymerized meshwork, the electrophoretic mobility of proteins decreases proportional to their size. In this thesis, the Lämmli system with a Tris-Glycine buffer was used.

- o Cast polyacrylamide gel with appropriate percentage (10 %)
- o Mix samples with SDS-sample buffer and boil for 10 minutes at 80°C. The reducing agent DTT (50 mM) was added sometimes in order to reduce disulfide bridges
- o Pipette samples in wells of the gel (12 µl)
- o Apply a voltage of 125 V in the stacking gel and 200 V in the separation gel

Alternatively, NuPAGE Bis-Tris gradient mini gels (4-12 %) were used according to the manufacturer instructions (Thermo Fisher Scientific).

4.3.2. Coomassie protein staining

After electrophoretic separation in a polyacrylamide gel, protein bands can be easily stained using Coomassie brilliant blue R-250. However, the sensitivity is only moderate with a detection limit of about 200 ng protein per band. For detection of proteins, the separation gel is incubated in coomassie staining solution for 30 minutes at RT with slight shaking. Then, the gel is unstained by boiling 5 to 6 times in fresh water.

4.3.3. Western Blot

Proteins on a polyacrylamide gel can be transferred *via* electrical current onto a PVDF membrane where they are accessible to immunodetection with specific antibodies.

Protein transfer:

- Activate PVDF membrane by incubation in 99 % ethanol for 1 min
- Soak polyacrylamide gel, PVDF membrane, whatman filter papers and blotting pads in WB transfer buffer
- Assemble all components in the transfer chamber according to the picture below (Figure 4.1.); remove air bubbles carefully
- Fill the chamber with transfer buffer ($\rightarrow$ wet blot)
- Transfer proteins at 125 mA for 2 h (self-made polyacrylamide gels) or 3 h (NuPAGE gels)
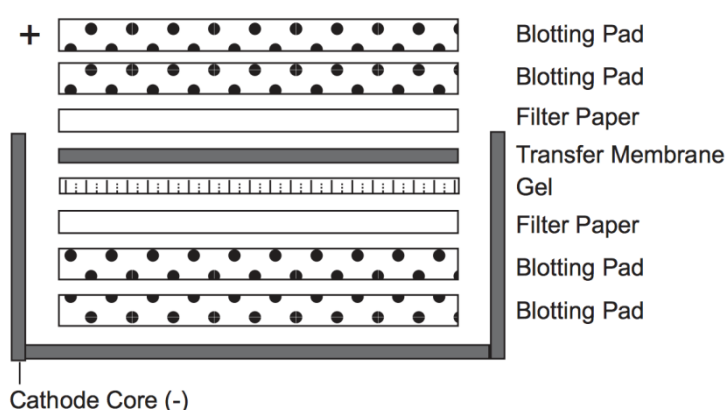
Figure 4.1: Schematic outline of the Western blot sandwich. *Proteins on the polyacrylamide gel are transferred to the PVDF membrane in the electric field.*

Immunodetection of proteins:

- o Block the PVDF membrane with blocking buffer for 1 h at RT or ON at 4 °C

- o Add primary antibody in blocking buffer. Incubate 1.5 h at RT or ON at 4 °C

- o Wash with TBST three times (5-10 min each)

- o Incubate with secondary antibody in blocking buffer for 1 h at RT (goat α-human IgG – AP conjugate; dilution 1: 30 000)

- o Wash with TBST three times (5-10 min each)

- o Add substrate (BCIP/NBT tablet dissolved in ddH$_2$O) to membrane, stop color reaction by washing with ddH$_2$O

4.3.4. Mass Spectrometry (MS)

MS analysis was performed at the ZMBH in Heidelberg. Proteins from *Pf* schizont lysate were separated on a NuPAGE Bis-Tris gradient gel, stained with Coomassie blue and protein bands of interest were cut out and digested with trypsin. The digested fragments were compared to the Swiss-Prot protein sequence database (from July 2015).

4.3.5. Bradford assay

In order to determine the concentration of protein solutions, the Bradford assay (Bradford, 1976) is a commonly used method because it is fast, easy to perform and rather unsusceptible to interference by different chemicals except SDS. However, the specificity can vary between different proteins as the dye favors alkaline and aromatic amino acids.

The test is based on binding of the acidic dye Coomassie brilliant blue G-250 to proteins resulting in a shift of the absorbance maximum from 465 nm to 595 nm. Therefore, the protein concentration can be quantified by measuring the optical density at a wavelength of 595 nm and comparing the value with a calibration curve of the protein standard BSA. The test is suitable for protein concentrations from 0.1 to 1.5 mg/ml.

- o Reaction mix: 1 ml Bio-Rad reagent (diluted 1:5 in ddH$_2$O) + 20 µl protein sample (at appropriate dilution)

- o 15 min incubation at RT

- o Determine absorbance at 595 nm (OD$_{595}$)

- o Calculate the protein concentration using a BSA calibration curve

4.4.   Cell Culture of *Plasmodium falciparum*

4.4.1. Culturing of *Plasmodium falciparum*

P.falciparum asexual blood stages can be cultured *in vitro* in petri dishes under the following conditions: Culture volume: 10 – 12 ml (small dishes) / 36 ml (large dishes)

Hematocrit:	4 % (human erythrocytes, type A)
Parasitemia:	< 5 %
Atmosphere:	37°C, 5 % CO_2, 3 % O_2, 92 % N_2, 95 % air humidity
Medium:	RPMI 1640 supplemented with 10 % human serum, 0.1 mM hypoxanthine, 20 µg/ml gentamycin

Under these conditions a parasite multiplication rate of 7 – 10 per cycle is achieved. Culture medium is replaced at least every second day. Cells are split appropriately to avoid a parasitemia of > 5 %.

4.4.2. Giemsa staining of blood smears

In order to determine parasitemia and parasite stage, blood smears are stained with Giemsa and analyzed microscopically.

- o   A drop of the parasite culture from the petri dish bottom is placed on a glass slide
- o   The blood drop is distributed to a thin smear using a second glass slide
- o   The smear is air dried and briefly fixed in 100 % methanol
- o   The smear is dried and incubated in Giemsa solution for 10 min
- o   The slide is dried and examined microscopically (100 x magnification)

4.4.3. Parasite synchronization with sorbitol

Synchronization of *P. falciparum* cultures can be achieved with sorbitol treatment since the membrane permeability of erythrocytes infected with trophozoite or schizont stages is remarkably different. Therefore, only uninfected or ring-stage infected erythrocytes survive the sorbitol treatment while the other cells burst due to osmotic processes.

- o   The culture is centrifuged for 2 min at 1900 rpm. The supernatant is discarded
- o   The pellet is resuspended in 5 ml D-Sorbitol (5 %) and incubated for 5 min at 37°C
- o   Centrifugation for 2 min at 1900 rpm
- o   The erythrocyte cell pellet is resuspended in the original volume of the culture medium and the culture is plated out in a new petri dish
- o   To achieve complete synchrony, sorbitol treatment has to be repeated either 3-6 h after first synchronization or during the next cycles when parasites are in ring stage

4.4.4. Purification of *P. falciparum* merozoites

Merozoite extracts are prepared from synchronized schizont-stage cultures of about 5 % parasitemia. Schizonts are ruptured by mechanical force (pipetting) and centrifuged twice at 400 g for 20 min to remove erythrocytes (pellet). Merozoites are recovered from the supernatant by centrifugation for 15 min at 1500 g. The merozoite pellet is resuspended in RPMI (200 µl per 36 ml culture) and stored at -20 °C. The quality of the merozoite preparation is assessed *via* Giemsa-stained smears and merozoite numbers are estimated by FACS measurement. Prior to use, all merozoite preparations are tested in parallel in the ADRB assay with the NA pool and their concentration is adjusted to show the same ADRB activity.

4.4.5. Preparation of *P. falciparum* schizont extract

Proteins are extracted from a highly synchronous culture of *P. falciparum* (3d7) at late schizont-stage and with a parasitemia > 3 % in order to use them for ELISA and WB analysis.

- o Centrifuge 36 ml *P. falciparum* schizont-stage culture at 800 g for 2 min
- o Resuspend the pellet in 36 ml SSC buffer with 0.2 % saponin (RBC lysis)
- o Incubate on ice for 15 min, invert sometimes
- o Centrifuge at 2200 g for 8 min and 4 °C
- o Wash pellet 3x with ice-cold PBS
- o Resuspend pellet in 90 µl RIPA buffer + 1 mM DTT
- o Incubate on ice for 20 min (complete lysis of parasites & protein solubilization)
- o Store protein extract at -80 °C

4.5. Purification of antibodies from human sera

4.5.1. Total IgG purification by affinity chromatography (Protein A or G)

Most immunoglobulins can be purified from serum *via* Protein A or Protein G affinity chromatography with high specificity. Protein A, a bacterial cell wall component from *Staphylococcus aureus*, is used for affinity purification of immunoglobulins from rabbits; it binds strongly to rabbit total IgG. Human immunoglobulins are affinity purified *via* Protein G, a cell wall protein of group G *Streptococci*; Protein G binds with strong affinity and specificity to all human IgG subtypes (IgG 1-4) but not to IgA, IgD or IgM.

- o Column preparation: Protein A or Protein G immobilized to agarose is mixed 1:1 with storage buffer and 5 ml each are poured into a polystyrene column
- o Buffer preparation: sterile filtration

- o Serum preparation: 5 ml heat inactivated serum (30 min at 56 °C) is diluted with 10 ml binding buffer and incubated ON at 4°C. Then, the sample is centrifuged at 2400 g and 4°C for 10 min. The supernatant is transferred into a new falcon and used for chromatography

- o Equilibration of the columns with 2 CV (10 ml) binding buffer

- o Serum application (Supernatant)

- o Washing with 2 CV (10 ml) binding buffer

- o Washing with 10 CV (50 ml) PBS

- o Elution with 3 CV (15 ml) elution buffer. The pH is checked using a pH strip and as soon as it turns acidic, 13 ml eluate fractions are collected

- o Immediate neutralization of the eluate with 1.5 ml neutralization buffer

- o The Protein A or Protein G columns are washed with 3 CV PBS (15 ml) and 1 CV PBS + 0.02 % NaN$_3$ at 4°C. They are stored upright at 4°C and can be re-used several times

4.5.2. MSP-1 specific antibody purification by affinity chromatography (MSP-1D)

Prior to the chromatographic affinity purification of α-MSP-1D antibodies, recombinant MSP-1D was covalently coupled to a pre-activated resin (Ultra Link Biosupport). Details to the protocol can be found in my master thesis (Jäschke, 2012) or in the manufacturer`s instructions (Thermo Scientific). A total of 44 mg MSP-1D and 625 mg dry beads were used resulting in 5 ml resin. MSP-1 specific antibodies were affinity purified from human serum using this resin and the "Äkta Purifier 100" system at 4°C allowing a computer based real time-monitoring of OD$_{280}$, pH, conductivity, pressure and flow rate.

- o Column preparation: 5 ml Ultra Link Biosupport resin coupled to MSP-1D is poured into a column

- o Buffer preparation: sterile filtration and outgassing using a vacuum pump

- o Serum preparation: 20 ml human serum is mixed 1:1 with PBS and incubated ON at 4°C. After centrifugation the following day at 2400 g for 10 min, the supernatant is transferred to a new falcon and used for chromatography

- o Äkta preparation: Washing with (i) Ethanol, (ii) ddH$_2$O and (iii) 1x PBS. Settings: Alarm pressure 0.5 MPa, Auto Zero UV, End-timer disabled, Record on

- o Connection of the column to the "Äkta Purifier 100" without air bubbles

- o Washing of the column with 6 CV PBS (1 ml/min) to remove NaN$_3$

- o Serum application (0.5 ml/min) using either a syringe or a superloop. The flow-through is collected and used for further analysis

- o Washing with 6-8 CV PBS (1 ml/min) to remove all serum components except the α-MSP-1D antibodies bound to the matrix

- o Elution using an acidic buffer (75 mM Glycin, 0.5 M NaCl, pH 2.8) (1 ml/min). The eluate is neutralized immediately with 0.1 volumes 1 M Tris, pH 8

- o Additional elution by high salt concentration (1 M NaCl in PBS, pH 7.4). Since this step is unnecessary, it is skipped the following times

- o Washing of the column with 6 CV PBS (1 ml/min)

- o Disconnection of the column from the Äkta system. Transfer of the matrix in a falcon. Addition of 0.02 % NaN$_3$ and storage at 4°C. The matrix can be re-used

- o Cleaning of the Äkta system with (i) ddH$_2$O and (ii) ethanol

4.5.3. Concentration and dialysis of purified antibodies

Total IgG preparations purified *via* Protein A or Protein G affinity chromatography from human serum as well as the α-MSP-1D eluate from MSP-1 specific affinity chromatography are concentrated to roughly 500 µl and dialyzed into RPMI using Amicon Ultra Centrifugal filters.

- o The Amicon filters are filled with RPMI and centrifuged at 1900 g for 10 min. Discard RPMI

- o Each eluate is transferred to the Amicon filter. Centrifugation at 1900 g for 15 – 40 min at 4°C. The flow-through is discarded

- o RPMI is added to the concentrated eluate to the maximal volume. Centrifugation at 1900 g for 15 – 40 min at 4°C. The flow-through is discarded. These steps are repeated at least twice

- o Sterile filtration of the dialyzed IgG using a 0.22 µM filter

- o The protein concentration is determined *via* Bradford test and – for total IgG - adjusted to 30 mg/ml (for GIA) or 10 mg/ml (for ADRB) by addition of RPMI. 150 µl aliquots of each sample are stored at -20°C

4.6. Enzyme-linked immunosorbent assay (ELISA)

4.6.1. ELISA - Principle

ELISA is an immunological method for the qualitative and quantitative detection of antibodies binding to their antigen. Following immobilization of a certain antigen on a solid phase (Polystyrol), sera containing the antigen-specific antibodies are added in different dilutions. The antibodies binding to the immobilized antigen are then recognized by added secondary antibodies which are coupled to an enzyme enabling the visualization of the complex e.g. alkaline phosphatase. The amount of bound secondary antibody determines the strength of the signal measured at a certain OD and is used to calculate the antibody titer.

4.6.2. ELISA using recombinant proteins

o Recombinant protein is diluted in coating buffer to a concentration of 100 nM. Each well is coated with 100 µl and the plates are incubated at 4°C overnight

o Protein which has not bound to the plate is removed by two wash steps, each with 200 µl / well wash buffer (1x TBST)

o In order to prevent unspecific binding of antibodies, the plates are blocked with 100 µl blocking buffer (1 % milk powder in TBST) per well for 1 h at RT

o Wells are washed twice with 1x TBST

o 100 µl of blocking buffer is added to all wells. 100 µl of sera or purified IgG are added to the first row. Each sample is tested in duplicates. Sera or purified IgG and blocking buffer are mixed in row 1 using a multichannel pipette and 100 µl are transferred to the next row and so on. Thereby, two-fold dilutions are made from top to bottom rows with 100 µl volume in each well. One well of the plate serves as reference value for equilibration (no first antibody)

o Plates are incubated for 2 h at RT

o Wells are washed four times with 1x TBST (200 µl/well)

o Secondary antibody (goat α-human IgG-AP conjugate) was diluted 1:30 000 in blocking buffer and 100 µl are added to each well. The plates are incubated for 1 h at RT

o Wells are washed twice with 1x TBST and twice with Substrate buffer (200 µl/well)

o Substrate (pNPP, 1 mg/ml, 100 µl/well) is added and incubated for 1 h at RT in the dark

o Reaction is stopped by addition of 0.2 M NaOH (100 µl/well)

o Absorption is read at 405 nm by the microplate reader

o In order to determine the antibody titer, a linear trendline is generated using OD values of the serial dilutions in the linear range (0.01-0.2). By using the equation of the trendline, the antibody endpoint titers are determined; they represent the antibody dilution at OD_{405} = 0.2

4.6.3. ELISA using *P. falciparum* merozoites or schizont lysate

o *P. falciparum* merozoites or schizont lysate are adjusted to 5 µg/ml with 1x PBS and 100 µl/well are added to the 96 well plate. Incubation at 4°C overnight

o The following procedure is performed as described above (4.6.2.). Exception: Sera or purified IgG are not added in serial 2-fold dilutions, but with a single appropriate dilution (1:700 for *Pf* schizont lysate; 1:350 for *Pf* merozoites) resulting in OD values in the linear range

o The linear range is defined by a standard curve created with serial dilutions of IgG from malaria-exposed Kenyan individuals (WHO Standard, NIBSC 10/198 (Bryan D, 2014))

o Appropriate single-point OD values correlate well with antibody endpoint titers (Drakeley et al., 2005; Tongren et al., 2006) and thus, can represent antibody levels

4.7. Growth inhibition assay (GIA) of *P. falciparum* blood stages

4.7.1. GIA - Principle

The inhibition of both erythrocyte invasion and intraerythrocytic development of *P. falciparum* parasites by purified IgG can be measured *via* the activity of *Plasmodium* Lactate DeHydrogenase (*p*LDH). The latter is a soluble glycolytic enzyme expressed at high levels in blood-stage parasites and can convert 3-Acetylpyridine Adenine Dinucleotide (APAD) and Lactate to APADH and Pyruvate. APADH then reduces the chromogenic substrate Nitro Blue Tetrazolium (NBT) using the enzyme diaphorase. This results in the formation of Nitro Blue Formazan (NBF), a deep purple stain that can be measured at a wavelength of 650 nm.

4.7.2. Standard GIA

- o The parasitemia and parasite stage of the *P.falciparum* (3d7) culture is determined microscopically by counting 1000 erythrocytes of a Giemsa-stained blood smear. All parasites have to be in schizont stage
- o Half of the parasite culture (6 ml) is centrifuged at 1900 rpm for 2 min. The supernatant is discarded. The pellet is resuspended in 6 ml GIA medium
- o The culture is diluted to a parasitemia of 0.6 % using 4 % hematocrit
- o The purified, concentrated and RPMI-dialyzed IgG preparations (stock 30 mg/ml) are pipetted into a 96-well plate in different IgG concentrations and a final volume of 25 µl; RPMI 1640 is used for dilution. Triplicate measurements
- o 25 µl parasites (0.6 %) are added in each well
- o Controls: (i) 25 µl RPMI and 25 µl parasites (parasite growth control), (ii) 25 µl culture medium and 25 µl 4 % hematocrit (erythrocyte control), (iii) rabbit α-AMA-1 antibodies (GIA standard BG98) at a concentration of 25 % and 12.5 % in RPMI and 25 µl parasites (positive control), (iv) 25 µl pooled IgG from four malaria-naïve individuals and 25 µl parasites (negative control)
- o Incubation at 37°C for one parasite cycle (40 h)
- o Each well of the 96-well plate is filled with 200 µl sterile, cold PBS and resuspended
- o Centrifugation at 1300 g for 10 min. The supernatant is carefully discarded
- o In order to kill the parasites, the plate is frozen at -20°C for at least 45 min and thawed at RT for 45 min
- o Preparation of the substrate solution: 0.2 mg/ml NBT + 0.01 mg/ml APAD + 0.2 U/ml Diaphorase from *C. kluyveri* in LDH buffer; 10 ml are needed per plate
- o 100 µl of the solution are added rapidly in each well and the pellets are resuspended

- Centrifugation at 1800 g for 10 sec to remove potential air bubbles
- Shaking of the plate for 20-30 min in the dark
- OD values are determined at a wave length of 650 nm using the microplate reader
- Data analysis in Excel and calculation of the inhibition:

Inhibition [%] = 100 % - (((OD_{650} IgG sample − OD_{650} erythrocyte control) / (OD_{650} parasite control − OD_{650} erythrocyte control)) x 100)

4.7.3. Antigen-reversal GIA

The growth inhibitory effect of antigen-specific antibodies can be determined by pre-incubation of total antibodies with competitor antigen such as MSP-1D prior to performance of the GIA.

- 10 µl/well competitor antigen in RPMI (e.g. MSP-1D, 1 mg/ml) in two-fold dilutions ranging from 200 µg/ml to 12.5 µg/ml are pipetted in a 96-well plate
- 15 µl purified IgG in RPMI (stock concentration: 40 mg/ml for human IgG & 20 mg/ml for rabbit IgG) are added per well. Triplicate measurements
- Incubation for 1 h at 37 °C
- *P. falciparum* schizont-stage parasites are counted and diluted to 0.6 % parasitemia as described above. 25 µl parasites are added to each well
- Controls: (i) 25 µl RPMI and 25 µl parasites (parasite growth control) (ii) 25 µl culture medium and 25 µl 4 % h
- ematocrit (erythrocyte control) (iii) rabbit α-AMA-1 antibodies (GIA standard BG98) at a concentration of 25 % and 12.5 % in RPMI and 25 µl parasites (positive control), (iv) 25 µl pooled IgG from four malaria-naïve individuals and 25 µl parasites (negative control), (v) 10 µl competitor antigen at highest concentration (200 µg/ml), no IgG, 25 µl parasites (competitor control), (vi) no competitor, IgG, 25 µl parasites (IgG control)
- Incubation at 37°C for one parasite cycle (40 h)
- Development and analysis of the GIA as described above

4.8. Antibody-dependent respiratory burst (ADRB) assay

Antibodies can opsonize *P. falciparum* merozoites and induce neutrophil respiratory burst (Joos et al., 2010). The ADRB assay detects the production of reactive oxygen species by human neutrophils *via* chemiluminescence. Components of the assay are: (i) *P. falciparum* merozoites, (ii) Protein G purified IgG, (iii) freshly purified human polymorphonuclear neutrophils (PMNs), (iv) Isoluminol and (v) competitor antigen in PBS (for antigen-reversal ADRB). Two persons are needed to purify the PMNs and simultaneously perform the assay.

4.8.1. PMN purification

Whole blood samples from malaria-naïve healthy adults were collected in Lithium-Heparin tubes. Written informed consent was obtained from all participants and ethical approval was granted by the Ethical Committee of the Medical Faculty, University of Heidelberg.

Blood from 3 donors (12 ml each) was pooled, mixed 1:1 with 3 % Dextran in 0.9 % NaCl, inverted approximately 10 times, and incubated for 18 min at RT to pellet RBCs. The supernatant was centrifuged (500 g, 4 °C, 10 min) and the thin white layer of PMNs (see Figure 4.2. A) was resuspended in 0.9 % NaCl (10 ml). This suspension was layered carefully on top of Ficoll-Histopaque (3 ml) and centrifuged at 400 g, RT, for 35 min without break. The thin PMN layer above erythrocytes (see Figure 4.2. B) was resuspended in ice-cold ddH$_2$O (600 µl), incubated for 30 seconds to lyse remaining erythrocytes and neutralized with equal volume of 1.8 % NaCl. After centrifugation (500 g, 5 min, 4 °C) the pellet was washed with HBSS (800 µl), centrifuged (500 g, 5 min, 4 °C), and the PMN pellet resuspended in cold PBS (700 µl). The quality of the preparation and PMN number were determined in a hemacytometer after trypanblue staining (1:10 dilution). The following formula is used: Cells/ml = counted cell number / counted square number x dilution x 10^4. The PMN concentration was adjusted to 1.3 x 10^7/ml (ADRB) or 2.5 x 10^7/ml (antigen-reversal ADRB) with sterile PBS; purity and viability of the cells was > 95 %. PMNs were stored at 4 °C and used for ADRB within < 1 h after isolation.
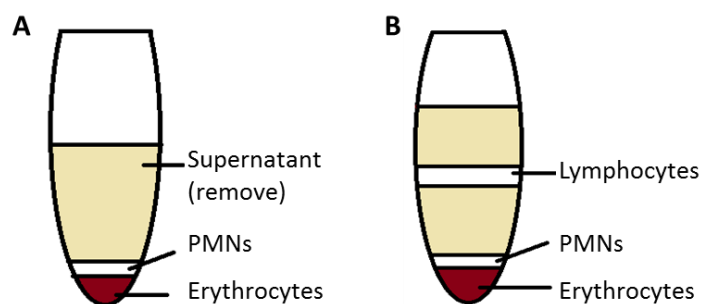
Figure 4.2: Purification of PMNs from whole blood. (A) *Schematic outline of the different blood layers after Dextran sedimentation and first centrifugation.* **(B)** *Schematic outline of the different layers after second centrifugation with Ficoll.*

4.8.2. ADRB assay protocol

- ○ 10 µl Protein G purified IgG (stock: 10 mg/ml) are pipetted to each well of an opaque white 96 well LUMITRAC microplate. To facilitate rapid handling, less than 60 wells are used. Duplicate measurements
- ○ 40 µl *P. falciparum* merozoites (about 2.5 x 10^5) are added per well

- o Controls: (i) IgG from malaria-naïve individuals (negative control), (ii) IgG from semi-immune individuals from Nouna, Burkina Faso (NA-Pool, positive control and reference), (iii) IgG from malaria-exposed individuals from Kenya (WHO 10/198 Standard (Bryan D, 2014), positive control), (iv) no IgG, no merozoites (PMN & Isoluminol control)

- o Incubation for 1 h at 37 °C

- o 100 µl/well freshly purified human PMNs in PBS (1.3×10^7/ml) are added rapidly

- o 100 µl/well Isoluminol (4 mg/ml stock in DMSO; 1:100 dilution in PBS) are added rapidly using a multichannel pipette

- o Immediate chemiluminescence detection with the FLUOstar OPTIMA microplate reader (150 min, measurements for 1 second every minute, Gain 4095)

4.8.3. Antigen-reversal ADRB assay

The contribution of antigen-specific antibodies to ADRB activity was quantified by pre-incubation of total IgG with competitor antigens such as MSP-1 prior to the ADRB assay.

- o 50 µl sterile filtered competitor antigen in PBS (0.5 µM/well; 2.5 µM stock) or 50 µl PBS per well is pipetted to an opaque white 96 well LUMITRAC microplate. Duplicate measurements

- o 10 µl Protein G purified IgG (human IgG: 5 mg/ml; *rhesus* IgG: 1.25 mg/ml) are added

- o Incubation for 1.5 h at 37 °C

- o 40 µl *P. falciparum* merozoites are added per well

- o Controls additionally to the ones described above: (i) Competitor antigen, no IgG, no merozoites (competitor control), (ii) Competitor antigen and IgG (NA-Pool), no merozoites (competitor and antibody control)

- o Incubation for 1.5 h at 37 °C

- o 50 µl/well freshly purified human PMNs in PBS (2.5×10^7/ml) are added rapidly

- o 100 µl/well Isoluminol (4 mg/ml stock in DMSO; 1:100 dilution in PBS) are added rapidly using a multichannel pipette

- o Immediate chemiluminescence detection with the FLUOstar OPTIMA microplate reader (150 min, measurements for 1 second every minute, Gain 4095)

4.8.4. ADRB data analysis

- o Transfer the chemiluminescence data for all time points in one Excel table, calculate the mean of duplicate measurements and create curves showing the raw chemiluminescence activity [Light Units (LU)] over time

- o Determine the peak of each curve within the first 30 min

- Normalize the chemiluminescence activity of a sample to the chemiluminescence activity of a reference (NA-Pool: IgG pool from eleven semi-immune individuals from Nouna). Thus, calculate the ADRB Index for each sample
- ADRB Index = (LU maximum sample / LU maximum NA-pool) x 1000

4.9. Thawing of cryopreserved human PBMCs

PBMCs are stored in liquid nitrogen (-196°C) and should be thawed quickly under sterile conditions. In order to prevent the formation of cell clumps resulting from dying cells, it could be beneficial to include benzonase in the thawing procedure.

4.9.1. Thawing of human PBMCs (without benzonase)

- Transfer the cryotubes from liquid nitrogen to ice and immediately into a 37 °C water bath. Hold the tube at the water surface for about 1 min and – with a small chunk of ice remaining – transfer the tube to a biosafety hood
- Pour the content of the cryotube into 30 ml pre-warmed PBMC medium in a 50 ml falcon tube; clean the cryotube with medium
- Centrifuge at 1400 rpm for 5 min. Discard the supernatant
- Wash the cell pellet with 12 ml PBMC medium, thereby transfer to 15 ml falcon tube
- Resuspend the cell pellet in 5 ml PBMC medium and incubate for 1-2 h at 37 °C in an incubator (atmosphere: 5 % CO_2, 3 % O_2, 92 % N_2, 95 % air humidity). The lids should be slightly opened for gas exchange
- Centrifuge at 1400 rpm for 5 min. Discard the supernatant
- Resuspend the cell pellet in 1 ml PBMC medium. Count the living cells (see 4.9.3.)

4.9.2. Thawing of human PBMCs (with benzonase)

- Transfer the cryotubes from liquid nitrogen to ice and immediately into a 37 °C water bath. Hold the tube at the water surface for about 1 min and – with a small chunk of ice remaining – transfer the tube to a biosafety hood
- Pour the content of the cryotube into a 50 ml falcon tube. Add 2 ml pre-warmed PBMC medium with benzonase (50 U/ml) drop by drop, thereby sway falcon tube gently
- Add PBMC medium to 10 ml
- Centrifuge at 1400 rpm for 7 min. Discard the supernatant
- Wash the cell pellet with 12 ml PBMC medium, thereby transfer to 15 ml falcon tube
- Continue as described above (4.9.1.)

4.9.3. Counting of PBMCs using the Neubauer hemocytometer

In order to determine the number of living cells/ml in the PBMC suspension, cells are stained with trypanblue prior to microscopic analysis. Since this dye is only absorbed by dead cells resulting in a blue color, dead (blue) and living (unstained) cells can be distinguished microscopically. Only unstained cells are counted using a Neubauer hemocytometer. This counting chamber contains a counting grid with four large squares, each divided into 16 small squares, and a depth of 0.1 mm.

- o Preparation of the counting chamber: After cleaning with ethanol the coverslip is placed over the counting area
- o 5 µl cells are mixed with 45 µl trypanblue (dilution 1:10) and pipetted under the coverslip. The area fills by capillary action
- o The number of living cells present in 2-4 large squares is determined microscopically
- o The number of cells per ml is calculated:

    Cells/ml = counted cell number/counted squares x dilution x 10^4

4.10. Flow cytometry

4.10.1. Principle of flow cytometry

Flow cytometry allows the simultaneous detection of size, granularity and fluorescence of single cells in a suspension. The method is based upon stimulation of each cell with laser light (Argon-Laser and Helium-Neon-Laser) and measurement of the light scattering in different angles, thereby determining the cell size (forward scatter, FSC) and cell granularity (sideward scatter, SSC). Furthermore, fluorophors are transferred by laser light into an excited state and can be detected *via* the wave length of their emitted energy. Therefore, cells expressing certain antigens can be identified by specific fluorophor-coupled antibodies. For instance, CD8+ and CD4+ PBMCs can be detected by flow cytometry using α-human CD4-PerCP and α-human CD8-PE antibodies.

4.10.2. Detection of CD4/CD8 positive PBMCs

- o After thawing, PBMCs are transferred into 2 ml Eppendorf tubes
- o Centrifugation at 1100 rpm for 5 min. The supernatant is discarded. The pellet is resuspended in 50 µl PBS / 2 % FCS for blocking. Incubation for 5 min on ice
- o The antibodies α-human CD4-PerCP and α-human CD8-PE are diluted 1:100 in PBS / 2 % FCS. 50 µl are transferred into each tube/ well. Controls: (i) unstained (ii) stained with α-human CD4-PerCP (iii) stained with α-human CD8-PE
- o Incubation for 20-30 min on ice in the dark

- o Washing with 1ml PBs. Centrifugation at 1100 rpm for 5 min. The supernatant is discarded and the washing step is repeated once
- o The cells are resuspended in PBS with 1 % PFA for fixation
- o Measurement using the FACS Calibur machine and the program Cell Quest Pro

4.10.3. Analysis of PBMC viability with Propidium Iodide

Propidium Iodide (PI) is a DNA binding dye which is excited at 488 nm and emits at 617 nm. Since it penetrates the cell membrane of dying or dead cells in contrast to viable cells, it can be used to determine the viability of cells.

- o After thawing, PBMCs are resuspended in PBS and transferred into Eppendorf tubes (100 µl)
- o Centrifuge at 1100 rpm for 5 min, discard the supernatant
- o Resuspend the cell pellet in 100 µl PI (1:100 dilution in PBS) or in 100 µl PBS (control)
- o Incubate for 20 min on ice in the dark
- o Add 1 ml PBS, centrifuge at 1100 rpm for 5 min, discard the supernatant
- o Wash a second time with 1 ml PBS
- o Resuspend cells in PBS and transfer them to FACS tubes
- o Analyze PI staining using the FACS Calibur machine and the program Cell Quest Pro

4.11. ELISPOT assay

4.11.1. ELISPOT Principle

The Enzyme Linked Immunospot (ELISPOT) assay is an extremely sensitive cellular assay which allows the quantification of cytokine secreting T-cells *in vitro* at the single cell level. One activated T-cell may be detected in 100 000 cells. Due to its high sensitivity and relatively easy performance, ELISPOT assays are a widely used tool to investigate specific cellular immune responses in several diseases or to develop or monitor new vaccine candidates.

One of the most common ELISPOT assays is based on the detection of IFNγ. In this thesis, the ELISPOT[Plus] kit for human IFNγ from Mabtech AB (Sweden) was used. Whereas in the *ex vivo* ELISPOT the PBMCs are directly transferred to the ELISPOT plate after thawing, a prior cultivation period of 9 days or 24 h in presence of the stimuli of interest is required in the cultured or short-term cultured ELISPOT assay, respectively.
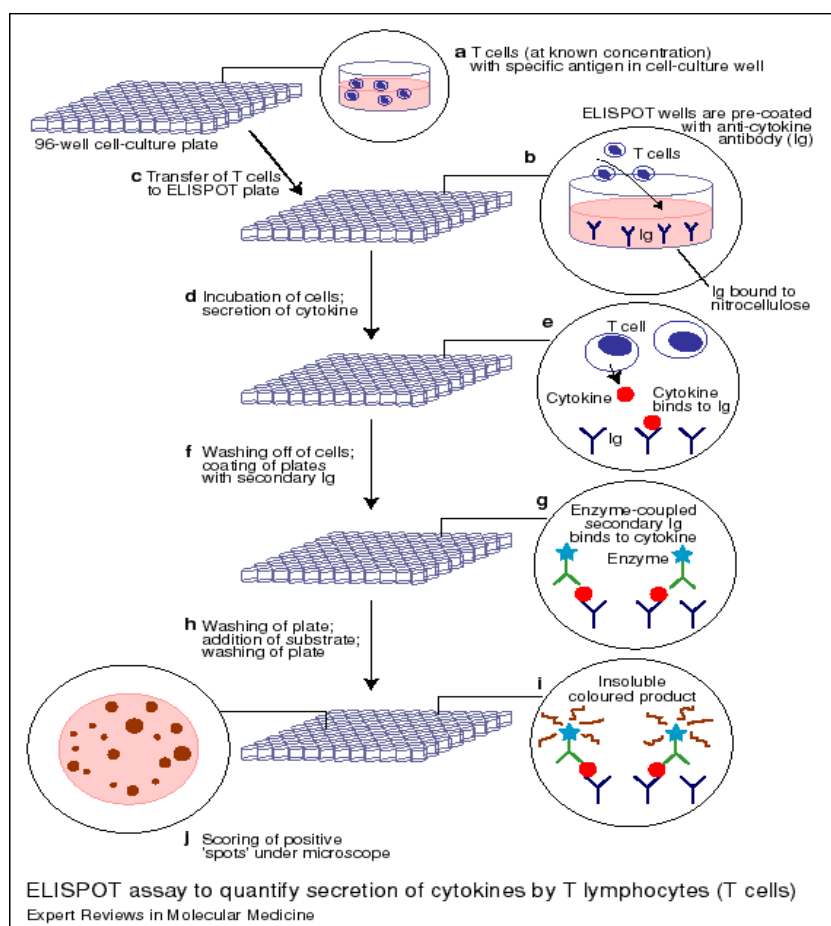
Figure 4.3: Procedure of a cytokine ELISPOT assay. *T-cells are added to a 96-well ELISPOT plate coated with a cytokine specific monoclonal antibody. During the incubation period of 20-24h activated T-cells secrete certain cytokines e.g. IFNγ which bind to the capture antibody. After removal of the cells, the enzyme-coupled detection antibody is added which recognizes another epitope of the cytokine of interest. Following addition of substrate the enzymatic reaction visualizes the position of cytokine secreting T-cells as colored spots. The picture was taken from (Caulfield, 2004).*

4.11.2. Overview of the stimuli used for ELISPOT experiments

Peptide stocks at a concentration of 20 mg/ml were prepared in 90 % DMSO and sterile filtrated. Aliquots containing 1 µl peptide(s) were stored at -80 °C until their use for PBMC stimulation. The used concentration (2x) for all peptides was 20 µg/ml, thus 1 ml PBMC medium was added to 1 µl peptide(s) (1:1000 dilution). Since stimuli and PBMCs are combined 1:1 in the ELISPOT assay, stimuli are always prepared with the 2-fold concentration.

Protein stimuli: Full-length *Pf*MSP-1D, produced under GMP-compatible conditions by BIOMEVA GmbH, Heidelberg, was available at a concentration of 1 mg/ml in PBS. 20 µl, 10 µl and 2 µl aliquots were prepared and stored at -80 °C until usage. Upon addition of 1 ml PBMC medium, used concentrations (2x) of 20 µg/ml, 10 µg/ml and 2 µg/ml were derived.

Controls: Cells without stimulus served as negative controls. The α-CD3 mAb from Mabtech AB served as positive control (stock concentration 100 µg/ml; 2x used concentration 0.2 µg/ml).

Epitope	Aa Sequence	From Protein	Reference	HLA restriction
674	KLKEFIPKV	*Pf*MSP-1	(Carralot et al., 2008; Idler, 2004)	A0201
291	GLHHLITEL	*Pf*MSP-1	(Idler, 2004)	A0201
374	SLLTELQQV	*Pf*MSP-1	(Idler, 2004)	A0201
437	VIYLKPLAGV	*Pf*MSP-1	(Idler, 2004)	A0201
J	ERRAKEKLQEQQRDLEQRKADTKK	*Pf*LSA-1	(Luty et al., 1999)	N/D
S6	KPIVQYDNF	*Pf*LSA-1	(Luty et al., 1999)	N/D
T1	LTMSNVKNVSQTNFKSLLRNLGVS	*Pf*LSA-1	(Krzych et al., 1995)	N/D
1	GLIMVLSFL	*Pf*CSP	?	A02
2	YLNKIQNSL	*Pf*CSP	?	A2.1
3	ILSVSSFLFV	*Pf*CSP	?	A2.1
CMV	NLVPMVATV	control		A0201

Table 4.1: Overview of the peptide stimuli used for ELISPOT experiments

4.11.3. Pre-cultivation of PBMCs & Coating of the ELISPOT plate (Day 1)

- After thawing and counting of PBMCs (4.9.), the cell number is adjusted to 10^6 cells in 300 µl
- Stimuli are prepared two-fold as described above (4.11.2.)
- 300 µl PBMCs (10^6 cells) and 300 µl stimulus (2x) are combined in a 15 ml falcon tube
- Incubation for approximately 24 h at 37 °C in a CO_2 incubator (atmosphere: 5 % CO_2, 3 % O_2, 92 % N_2, 95 % air humidity). The falcons are placed diagonal into a rack and the lids are slightly opened for gas exchange

- Coating of the ELISPOT 96-well filter plate: the coating antibody (IFNγ, 1-D1K) is diluted 1:100 in sterile PBS (Invitrogen); for 1 plate 5 ml PBS and 50 µl antibody is needed. 50 µl (10 µg/ml) are added to each well using a multichannel pipette
- Seal the plate with parafilm and store it ON at 4 °C

4.11.4. Transfer of Stimuli & PBMCs to the ELISPOT plate (Day 2)

o   Wash the ELISPOT plate 3x with 200 µl/well sterile PBS

o   Block the ELISPOT plate with PBMC medium (200 µl/well) for at least 1 h

o   Prepare a 96-well "masterplate" for restimulation of PBMCs allowing a fast and easy transfer of the stimuli to the ELISPOT plate with the multichannel pipette. For each condition (either unstimulated or one of the stimuli used for pre-cultivation) add each stimulus in triplicates and a fourth well with the α-CD3 mAb (positive control). The stimuli are prepared two-fold concentrated as described above (4.11.2.). If the same stimulus is needed for several donors or different time-points, multiply the stimuli volume accordingly (~70 µl per donor and time-point are required)

o   Transfer the stimuli (50 µl/well) from the "masterplate" to the ELISPOT plate using a multi-channel pipette. The ELISPOT plate must not dry out

o   Centrifuge the PBMCs in the 15 ml falcons for 5 min at 1400 rpm. Discard the supernatant and resuspend the pellet in 250 µl PBMC medium

o   Transfer the PBMCs to the ELISPOT plate (50 µl/well, estimated 150 000 - 200 000 cells)

o   Incubate the ELISPOT plate for 22 -24 h at 37 °C

o   Determine the exact number of living PBMCs transferred to the ELISPOT plate in each falcon tube: stain the cells with trypanblue (1:10 dilution) and count viable cells as described above (4.9.3.). This step is important since some of the PBMCs die during the pre-cultivation period or are lost during transfer

		Donor 1			Donor 2				Donor 3			
	1	2	3	4	5	6	7	8	9	10	11	12
A	unstim			CD3	unstim			CD3	unstim			CD3
B	MSP-1 peptide mix			CD3	MSP-1 peptide mix			CD3	MSP-1 peptide mix			CD3
C	MSP-1D protein (10 µg/ml)			CD3	MSP-1D protein (10 µg/ml)			CD3	MSP-1D protein (10 µg/ml)			CD3
D	MSP-1D protein (1 µg/ml)			CD3	MSP-1D protein (1 µg/ml)			CD3	MSP-1D protein (1 µg/ml)			CD3
E	LSA-1 peptide mix			CD3	LSA-1 peptide mix			CD3	LSA-1 peptide mix			CD3
F	CSP peptide mix			CD3	CSP peptide mix			CD3	CSP peptide mix			CD3
G												
H												

Figure 4.4: Example of a pipetting plan for the ELISPOT assay. *Each stimulus is added in triplicates and the fourth well contains the positive control α-CD3 mAb. An unstimulated control is used per donor and time point in triplicates.*

4.11.5. Development of the ELISPOT assay

o Dumping of the cells

o Washing of the plates 5x with PBS / 0.05 % Tween 20 (200 µl/well)

o 50 µl /well biotinylated detection antibody (1 µg/ml; 7-B6-1) in PBS / 0.5 % FCS is added. Incubation for 2 h at RT

o Washing of the plates 5x with PBS / 0.05 % Tween 20 (200 µl/well)

o Streptavidin-Alkaline Phosphatase is diluted 1:1000 in PBS / 0.5 % FCS and 50 µl are added per well. Incubation for 1 h at RT

o Washing of the plates 5x with PBS / 0.05 % Tween 20 (200 µl/well)

o Substrate solution BCIP/NBT-plus is sterile filtrated before use and stored in the dark. 50 µl are pipetted to each well

o As soon as blue-colored spots are clearly visible (after 5 – 20 min) the reaction is stopped by dipping the ELISPOT plate 10x in ddH_2O

o The plate is air-dried and stored in the dark at RT

4.11.6. Analysis of the ELISPOT assay

o The ELISPOT plate is photographed and analyzed using the AID ELISPOT reader and the AID ELISPOT software version 4.0. The reader counts the spots in each well. All plates are analyzed with the same settings (Intensity: 20-255; Size: 50-5000; Gradient: 5-90)

o The number of activated cells secreting IFNγ (spot forming units: SFU) per 10^6 PBMCs is calculated in Excel. The number of living cells transferred to the ELISPOT wells – as determined by cell counting of each falcon tube - is included in the calculations. For each condition, the spot number of the unstimulated wells is subtracted from the spot number of the stimulated wells

o Net SFU/ 10^6 cells = ($\emptyset_{\text{spots in stim. wells}} - \emptyset_{\text{spots in unstim. wells}}$) / cells per well x 10^6

4.12. Statistical analyses

Sigma Plot version 12.3 (Systat Software) was used for data analysis. Antibody levels (ELISA), GIA levels and ADRB activity were compared by Spearman`s rank correlation. Paired data was analyzed for statistical significance using paired t-Test (for two samples) or One Way RM Anova (for > 2 samples); unpaired data was examined by t-Test (for two samples) or One Way Anova (for > 2 samples). Significances: $P \leq 0.05$ (*), $P \leq 0.01$ (**), $P \leq 0.001$ (***).

5. Results

5.1. Overview of the semi-immune individuals from Burkina Faso

Blood samples were obtained from eleven healthy young adults (18 – 31 years) from Nouna, an area with high seasonal malaria transmission in Burkina Faso, West Africa. Blood (10 ml) from 40 individuals was taken who (i) had no fever episodes within the last 15 days (ii) were not vaccinated within the last month (iii) received no medication within the last two weeks and (iv) were HIV, HBV and VDRL negative. The HLA-type of each individual was determined in the Institute for Immunology, University of Heidelberg; the HLA-A0201 genotype - the most common allele in Africa (~30%) – was detected in 11 from 40 individuals. A further blood donation of 100 ml each was taken from these 11 individuals with the HLA-A0201 haplotype at the end of the dry, non-malaria transmission season (March 2004). PBMCs and sera were prepared *via* Ficoll centrifugation and transported to Heidelberg. Additionally, PBMCs from malaria-naïve individuals from Heidelberg carrying the HLA-A0201 allele were prepared as controls. This work was done by the laboratory of Prof. Hermann Bujard (ZMBH, Heidelberg) and Dr. Boubacar Coulibaly (Centre de Recherche en Santé de Nouna (CRSN), Burkina Faso). Ethical approval was granted by the Ethical Committee of the Medical Faculty, University of Heidelberg, and Prof. B. Kouyaté, former Director of the CRSN.

Donor	Age	Sex	HLA	Location	Sample date	PIN
1	28	M	A 0201	Nouna	March 04	549
2	31	M	A 0201	Nouna	March 04	494
3	18	F	A 0201	Nouna	March 04	501
4	19	M	A 0201	Nouna	March 04	504
5	20	F	A 0201	Nouna	March 04	513
6	22	M	A 0201	Nouna	March 04	545
7	17	M	A 0201	Nouna	March 04	528
8	19	M	A 0201	Nouna	March 04	497
9	19	M	A 0201	Nouna	March 04	534
10	21	M	A 0201	Nouna	March 04	533
11	22	M	A 0201	Nouna	March 04	548

Table 5.1: Overview of the eleven semi-immune individuals from Nouna. Shown are their age, sex, HLA-subtype and PIN for exact identification. All samples were taken in Nouna (Burkina Faso) in March 2004 by Dr. Boubacar Coulibaly (CRSN, Burkina Faso).

5.2. Humoral immune response against *Pf*MSP-1 in individuals with naturally acquired immunity

5.2.1. Antibody levels to *Pf*MSP-1 and *Pf* blood stages in Nouna donors

Sera from eleven healthy young adults from Nouna, Burkina Faso were analyzed for antibodies directed towards *P. falciparum* specific antigens. Sera from malaria-naïve Europeans and a WHO reference reagent for anti-malaria (*P. falciparum*) human serum, containing a serum pool from malaria-exposed individuals from Kisumu, Kenya (NIBSC code: 10/198; (Bryan D, 2014)), served as controls. There was a high correlation between antibody levels to MSP-1 and to *P. falciparum* schizont lysate or merozoites (Spearman`s correlation coefficients r=0.899 (***) and r=0.886 (***), respectively) (Figure 5.1).
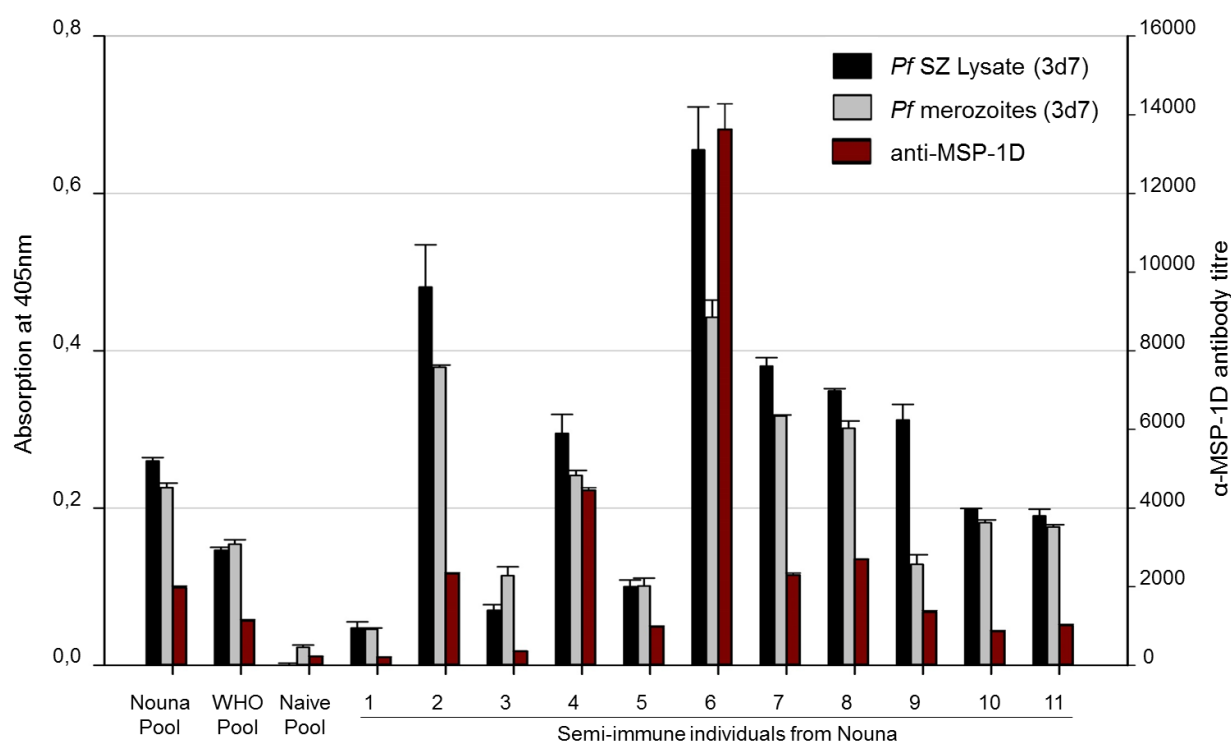
Figure 5.1: Antibody profiles of 11 semi-immune individuals from Burkina Faso. *Antibody levels determined by ELISA against P. falciparum 3d7 schizont lysate (black), merozoites (grey) and MSP-1D (red). Spearman`s rank correlation coefficients between antibody levels to MSP-1 and to P. falciparum schizont lysate or merozoites were r=0.899 (***) and r=0.886 (***), respectively. Nouna Pool (n=11): IgG from eleven individuals from Nouna; WHO Pool: IgG from malaria-exposed Kenyan adults (NIBSC code: 10/198; (Bryan D, 2014)); Naïve Pool: IgG from malaria-naïve European individuals (n=4).*

Furthermore, antibody levels to each of the four processing fragments of MSP-1D - MSP-1$_{83}$, MSP-1$_{30}$, MSP-1$_{38}$ and MSP-1$_{42}$ - were determined in the eleven semi-immune individuals from Nouna. The MSP-1$_{83}$ and MSP-1$_{42}$ fragment showed the highest immunogenicity in all Nouna donors while much lower antibody titers were detected to MSP-1$_{30}$ and MSP-1$_{38}$ (Figure 5.2).
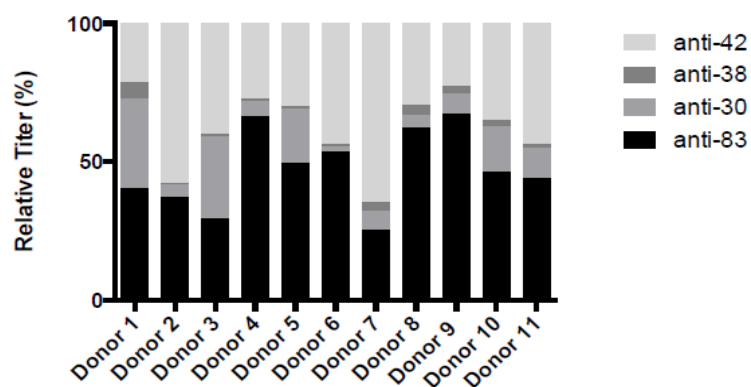
Figure 5.2: MSP-1 specific antibody profile of 11 semi-immune individuals from Burkina Faso. *Antibody levels were determined by ELISA against the four MSP-1 processing fragments p83, p30, p38 and p42; shown is the relative titer [%] of each MSP-1 subunit.*

5.2.2. Growth inhibitory potential of MSP-1 specific antibodies

Sera from eleven healthy young adults from Nouna, Burkina Faso were analyzed for their potential to inhibit parasite growth *in vitro*. Antibodies from four semi-immune individuals directly inhibited the growth of *P. falciparum* blood stage parasites *in vitro* (Figure 5.3 A). In order to quantify the contribution of MSP-1 specific antibodies to growth inhibitory activity *in vitro*, an antigen-reversal GIA was established in which antigen-specific antibodies are bound to their respective antigen, such as MSP-1, prior to performance of the classical GIA assay.

By this approach, the growth inhibitory activity of purified α-MSP-1 antibodies from rabbits immunized 3x with MSP-1D and adjuvant (IDRI-SE + GLA), could be gradually reduced with increasing concentrations of MSP-1D from about 45 % without antigen to 0 % in presence of 200 µM MSP-1D (Figure 5.3 B). Interestingly, GI activity of purified α-MSP-1 antibodies from immunized rabbits could also be reduced in presence of MSP-1F or MSP-1_{42}, albeit not as good as with MSP-1D (Figure 5.4). Thus, growth inhibitory MSP-1 specific antibodies in immunized rabbits mainly target cross-reactive regions within MSP-1 (~70 %) and these are partially localized in the MSP-1_{42} subunit.

Total IgG from the two Nouna donors with highest GI activity (Donor 6 & 8) were analyzed for the contribution of MSP-1D specific antibodies to growth inhibitory activity using the antigen-reversal GIA. While MSP-1D had no effect on IgG from donor 8, the growth inhibitory activity of IgG from donor 6 was diminished by about 50 % upon addition of 200 µM MSP-1D (Figure 5.3 B). Interestingly, donor 6 had the highest antibody titer to MSP-1D (Figure 5.1) suggesting that high antibody concentrations are required in GI assays. Thus, naturally acquired MSP-1 specific antibodies can contribute to growth inhibitory activity *in vitro*.

A

B
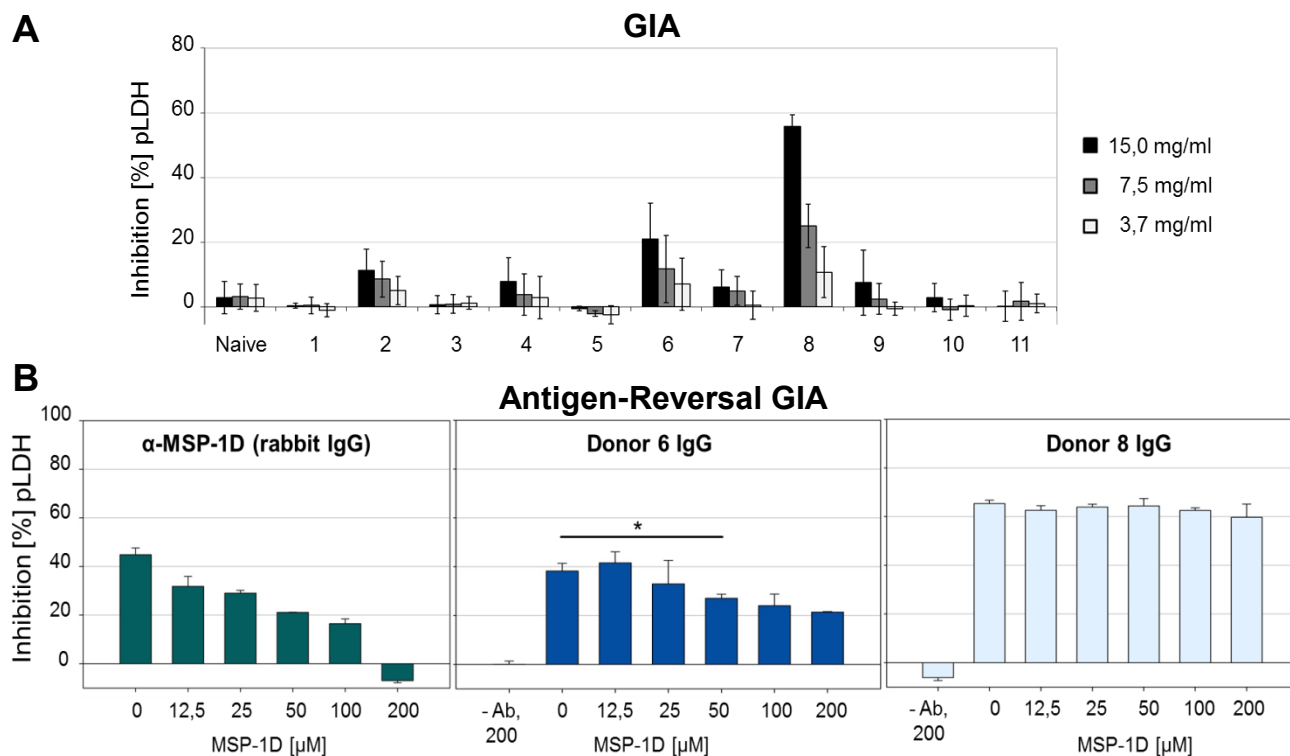
Figure 5.3: Antibodies to MSP-1 partly contribute to growth inhibitory activity. (A) *Growth inhibitory activity of Protein G purified IgG from 11 semi-immune donors from Nouna and from a malaria-naïve pool (n=4), 3 independent experiments. (B)* *Antigen-Reversal GIA with increasing concentrations of MSP-1D. P. falciparum (3D7) parasites are cultured in presence of purified IgG and increasing concentrations of competitor antigen MSP-1D for one parasite cycle (40 h). Readout is the activity of Plasmodium Lactate Dehydrogenase (pLDH). Mean values are shown with standard deviations; statistical differences were calculated using paired t-Test (Donor 6, P=0.024).*
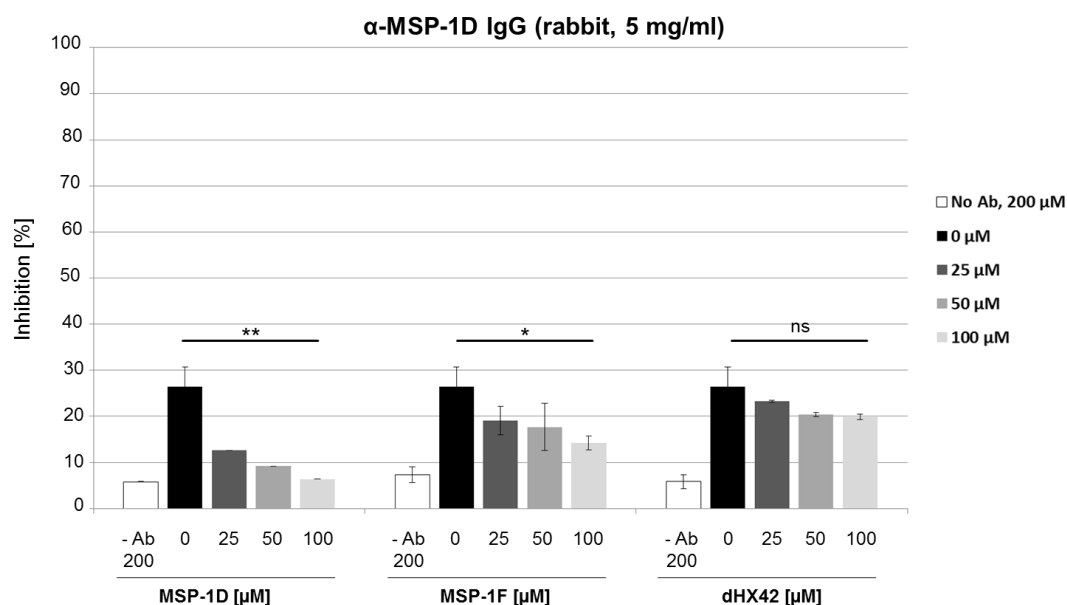
Figure 5.4: Antigen-Reversal GIA with α-MSP-1D antibodies from immunized rabbits. Protein A purified IgG from rabbits immunized 3x with recombinant PfMSP-1D and adjuvant were pre-incubated with increasing concentrations of competitor antigen MSP-1D, MSP-1F or MSP-1₄₂. P. falciparum (3d7) parasites were added and incubated for 40 h (one parasite cycle). Readout is the activity of Plasmodium Lactate Dehydrogenase (pLDH). Statistical differences were calculated using paired t-Test.

5.2.3. Search for new malaria antigens responsible for growth inhibitory activity in semi-immune individuals by mass spectrometry

The finding that antibodies from the semi-immune Nouna donors 2 and 8 show growth inhibitory activity of *P. falciparum* blood stages *in vitro* independent of MSP-1 specific antibodies (Figure 5.3 B and Figure 5.13 C) prompted us to search for the target antigens responsible for the observed growth inhibition. Thus, *P. falciparum* (3d7) schizont lysate was separated by SDS-PAGE, transferred to a nitrocellulose membrane and stained with the different fractions of purified IgG from Donor 2 and 8 received *via* MSP-1 specific affinity purification (Figure 5.13 A and B). While we detected in the α-MSP-1D eluate only bands from full-length MSP-1 and processing fragments thereof, several other bands were visible in the flow-through (FT) fractions of Donor 2 and 8 (Figure 5.5). Since these antigens might be responsible for the observed growth inhibitory activity of the FT fractions (Figure 5.13 C), we aimed to identify them by Mass Spec analysis.
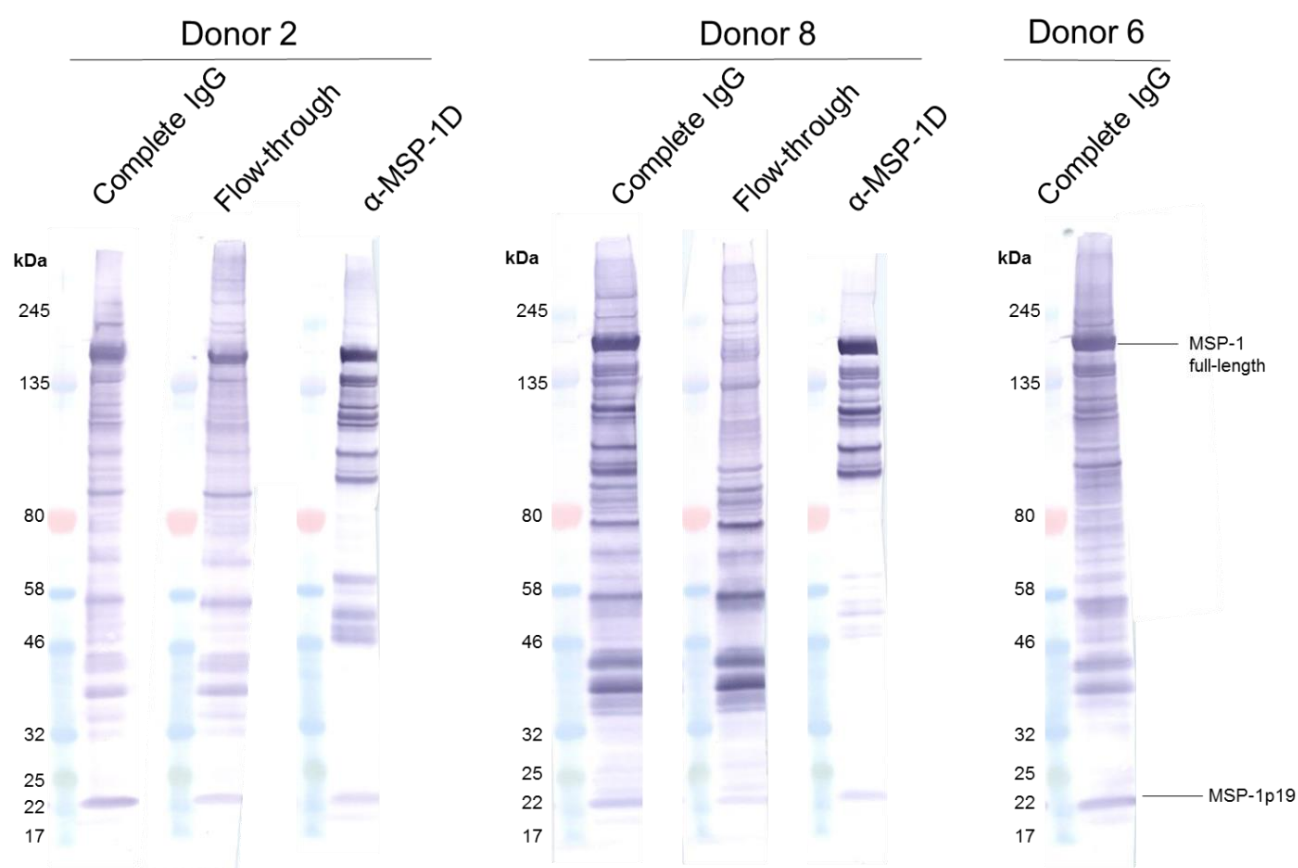
Figure 5.5: Western blot of Pf schizont lysate with different IgG preparations from Nouna donors. *Pf schizont lysate (3d7) was separated on a NuPage gradient gel, transferred to a nitrocellulose membrane and immuno-stained with either Protein G purified complete IgG, flow-through IgG (complete IgG without α-MSP-1 antibodies) or affinity purified α-MSP-1D antibodies from Donor 2 and 8; from Donor 6 only complete IgG is available. The location of full-length MSP-1D and MSP-1$_{19}$ – as identified using α-MSP-1D IgG from immunized rabbits – is indicated.*

Another *P. falciparum* (3d7) schizont lysate preparation was separated *via* SDS-PAGE and either used for WB analysis using antibodies from Donor 2 (FT) and 8 (FT) or stained with coomassie blue. Six bands of interest were identified which were visible at the same location by both staining with coomassie and immuno-staining with antibodies from the two donors by WB (Figure 5.6 B). Mass spectrometry analysis of the 6 bands - performed at the MS facility in the ZMBH in Heidelberg – revealed two proteins from *P. falciparum*: MSP-9 (band 3 with 15 % sequence coverage) and MSP-2 (band 5 and 6 with 28 and 12 % sequence coverage, respectively) (Figure 5.6 A). Strikingly, many proteins from human erythrocytes were detected, such as Spectrin α-chain (band 1, 61 % sequence coverage) and β-chain (band 2, 65 % sequence coverage), Flotillin (band 5, 60 % sequence coverage) or Erythrocyte band 7 protein (band 6, 55 % sequence coverage) (Appendix, Figure 7.3). However, in WB analysis using antibodies from donor 2 (FT) and 8 (FT) on lysed human erythrocytes (ery ghosts) no bands were detected (Figure 5.6 C). Thus, we can exclude the possibility that the two semi-immune donors from Nouna might have antibodies against human erythrocytes.
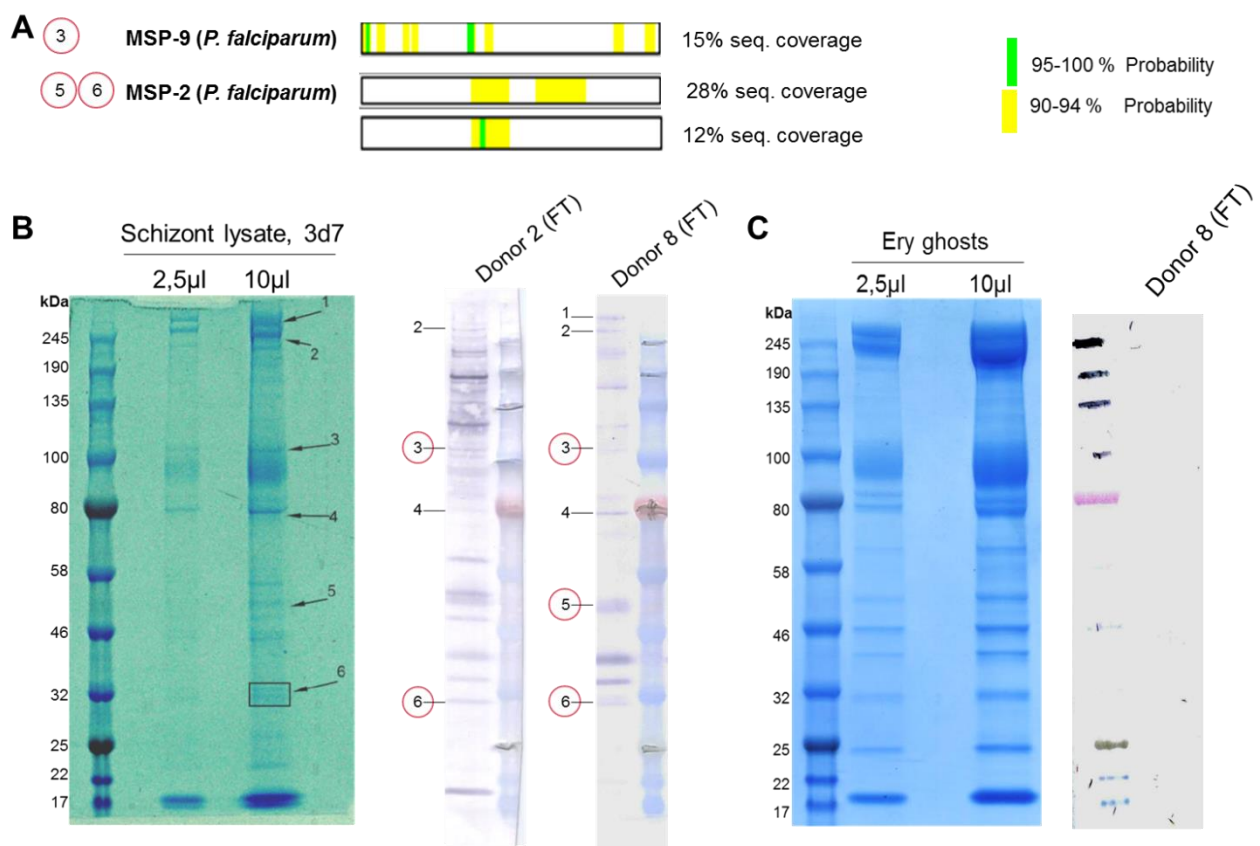
***Figure 5.6: P. falciparum proteins identified by Mass Spec analysis. (A)** Two P. falciparum proteins were identified by MS: PfMSP-9 (band 3, 15 % sequence coverage) and PfMSP-2 (band 5 & 6, 28 % and 12 % sequence coverage, respectively). **(B)** Localization of the six bands analyzed by MS. P. falciparum schizont lysate (3d7) was separated on a gel via SDS-PAGE and stained with either Coomassie blue or transferred to a nitrocellulose membrane and immuno-stained with flow-through IgG (FT, no α-MSP-1 antibodies) from Donor 2 (dilution 1: 11000) and 8 (dilution 1:5000). Six bands were detected by both approaches and potential proteins were identified by Mass Spec at the MS facility in the ZMBH (Heidelberg). **(C)** Lysed erythrocytes (Ery ghosts) were analyzed via Coomassie-staining following SDS-PAGE and western blot immuno-stained with IgG from Donor 8 FT (dilution 1:5000).*

5.2.4. Establishment of the ADRB assay in our laboratory

Antibody-dependent cellular immunity was analyzed *via* respiratory burst (ADRB) assay (Joos et al., 2010), based on killing of parasites by reactive oxygen species (ROS) from recruited neutrophils (Figure 5.7). The ADRB assay, in which oxygen radicals are measured by chemiluminescence using Isoluminol, was established in our lab. A clear chemiluminescence signal was only detected if all assay components - *P. falciparum* merozoites, purified IgG from malaria exposed individuals, freshly purified PMNs and Isoluminol - were present. Only background activity was obtained with IgG from malaria-naïve individuals (Figure 5.8). The ADRB Index was calculated as previously described (Joos et al., 2010; Joos et al., 2015; Perraut et al., 2014) using the chemiluminescence maximum of the curve. Interestingly, this readout correlated perfectly with the total area under curve within the first 60 min (Pearson correlation coefficient R = 0.996) (Figure 5.9). Furthermore, the ADRB assay showed a remarkable reproducibility even if different *P. falciparum* merozoite and PMN preparations were used (intra-assay CV < 6 %, inter-assay CV < 12 %; Figure 5.10). Similar results for ADRB activity were obtained with Protein A or Protein G affinity-purified human immunoglobulins (Figure 5.11 A).
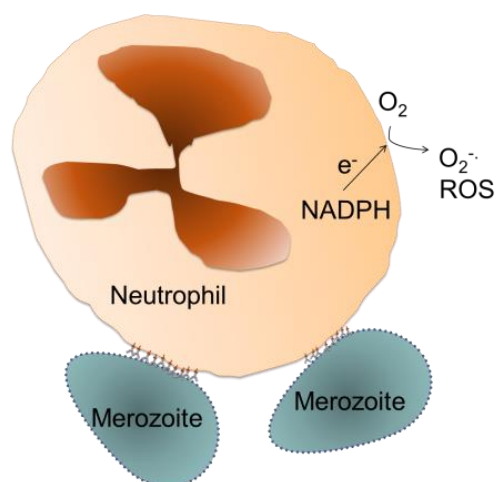
Figure 5.7: Mechanism of the Antibody-dependent Respiratory Burst (ADRB). *Antibodies opsonize merozoites, bind to the Fc receptors of neutrophils and cross-link them. Thereby, the neutrophil gets activated and its NADPH oxidase produces reactive oxygen species (ROS) by electron transfer to molecular oxygen. ROS can be measured via chemiluminescence.*
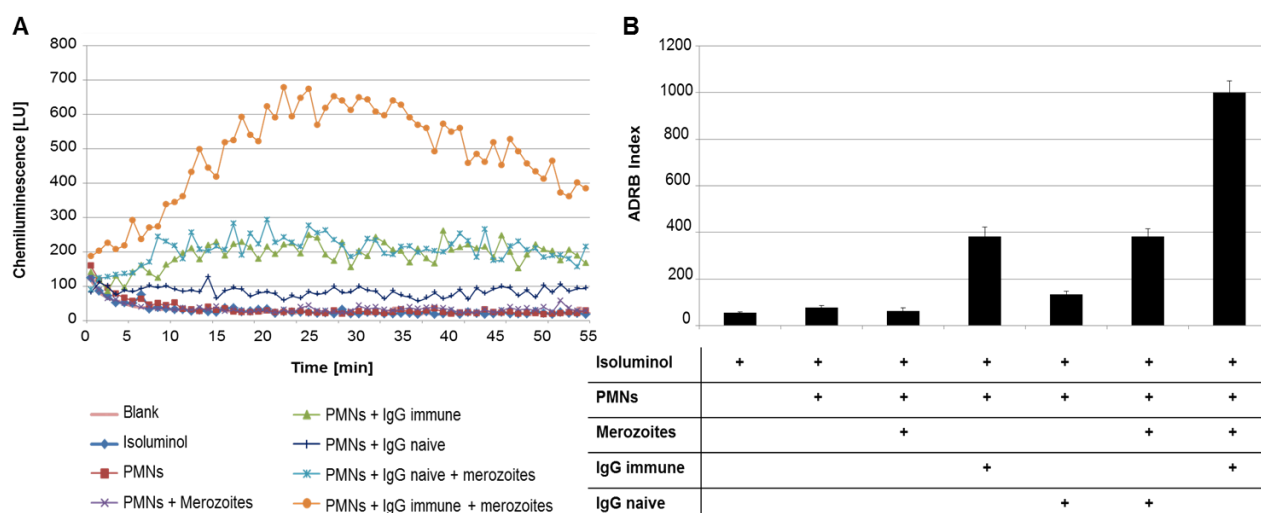
Figure 5.8: Controls for the ADRB assay. (A) *Chemiluminescence raw data of a semi-immune IgG pool opsonizing P. falciparum 3d7 merozoites (orange line) and different controls. Each dot represents the mean of duplicate measurements. **(B)** Calculation of the ADRB Index for all samples shown in A using the maximum value of the chemiluminescence curve within the first 30 min. ADRB Index = LU maximum sample / LU maximum semi-immune IgG pool x 1000. Shown is the mean with SEM. LU: Light units; PMNs: Polymorphonuclear neutrophils; IgG naïve: Protein G purified IgG from a malaria-naïve pool (n=4); IgG immune: Protein G purified IgG from semi-immune individuals (n=11).*
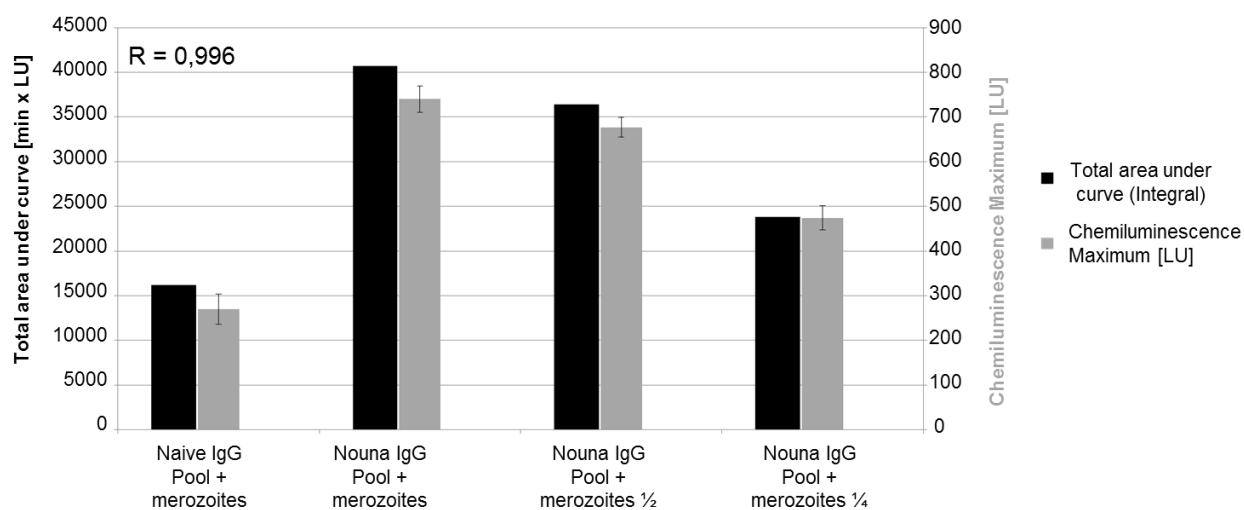
Figure 5.9: Comparison of possible readouts of ADRB data. *ADRB raw data are analyzed by either calculating the total area under curve (isoluminol as baseline, 0-60min) or by using the chemiluminescence maximum value (0-30min). As an example, the ADRB activity of a malaria-naïve IgG Pool (n=4) and a semi-immune IgG Pool (n=11, Nouna Pool) against different concentrations of P. falciparum 3d7 merozoites was analyzed using both methods; these show a very good correlation (Pearson correlation coefficient R = 0,996).*
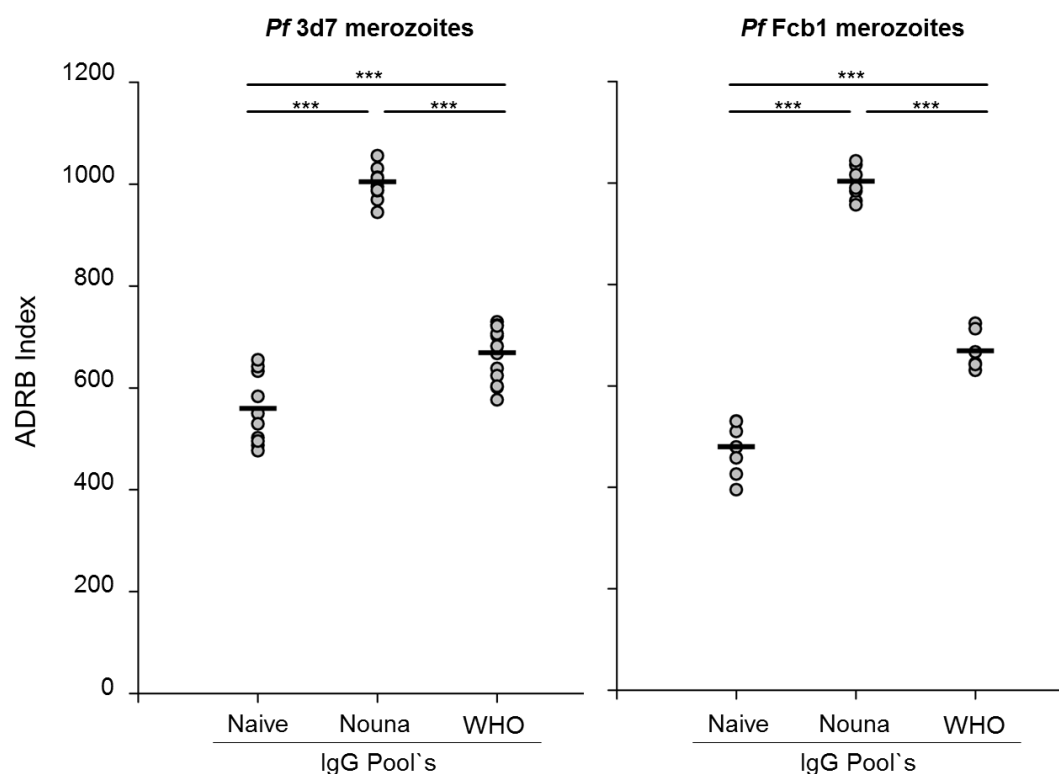
Figure 5.10: Precision of the ADRB assay. *ADRB activity against P. falciparum 3d7 and FCB1 merozoites was determined in 5 (3d7) or 4 (FCB1) independent experiments with two different analysts. Intra-assay CV was < 6 %, inter-assay CV was < 12%. Statistical differences were assessed by One Way Anova. Naïve: Protein G purified IgG from malaria-naïve individuals (n=4); Nouna: Protein G purified IgG from semi-immune individuals (n=11) from Burkina Faso; WHO: Protein G purified IgG from malaria-exposed Kenyan adults (NIBSC code: 10/198; (Bryan D, 2014)).*
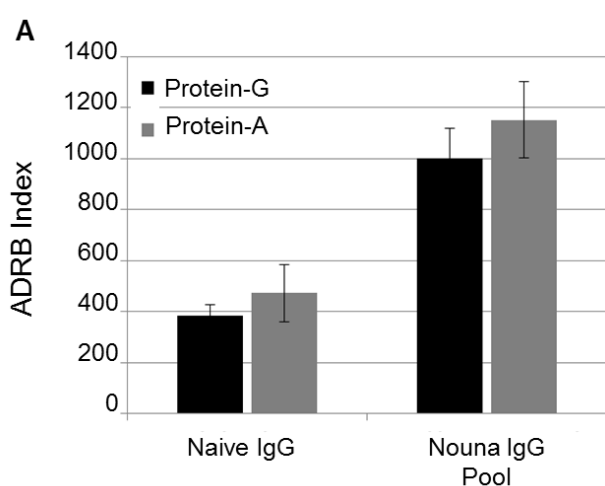
	Protein A	Protein G	Opsonization
IgG1	++	++	+++
IgG2	++	++	
IgG3	-	++	++
IgG4	++	++	+
IgA	+	-	+
IgD	+	-	-
IgE	+	-	-
IgM	+	-	+

Figure 5.11: ADRB activity of IgG purified by affinity chromatography via Protein A or G. (A) *ADRB activity of IgG from a malaria-naïve European pool (n=4) and from a malaria-exposed African pool (Nouna Pool, n=11) was determined against P. falciparum 3d7 merozoites. IgG were affinity-purified either by Protein G (black bars) or Protein A (grey bars); no significant difference was determined between the purification methods. Shown is the mean of duplicate measurements with SEM. **(B)** Overview of human immunoglobulins isolated via Protein A or Protein G affinity purification and their importance for opsonization. Table from (Murphy, 2014).*

5.2.5. Correlation of MSP-1 antibody titer with ADRB activity

Purified IgG from all eleven semi-immune individuals from Burkina Faso can mediate the activation of neutrophils and release of ROS (Figure 5.12 A) and, remarkably, ADRB activity of these donors was higher than the activity measured with the WHO IgG Pool prepared from malaria-exposed Kenyan adults (NIBSC code: 10/198; (Bryan D, 2014); Figure 5.14 A). The level of ADRB activity correlated well with antibody levels to MSP-1D (Spearman`s rho r= 0.758, P=0.001) (Figure 5.12 B), MSP-1F (r=0.615, P=0.024) and also to *P. falciparum* schizont lysate (r=0.767, P<0.001) or merozoites (r=0.855, P<0.001) (Figure 5.12 C). Thus, MSP-1 specific antibodies may opsonize merozoites and activate neutrophils.
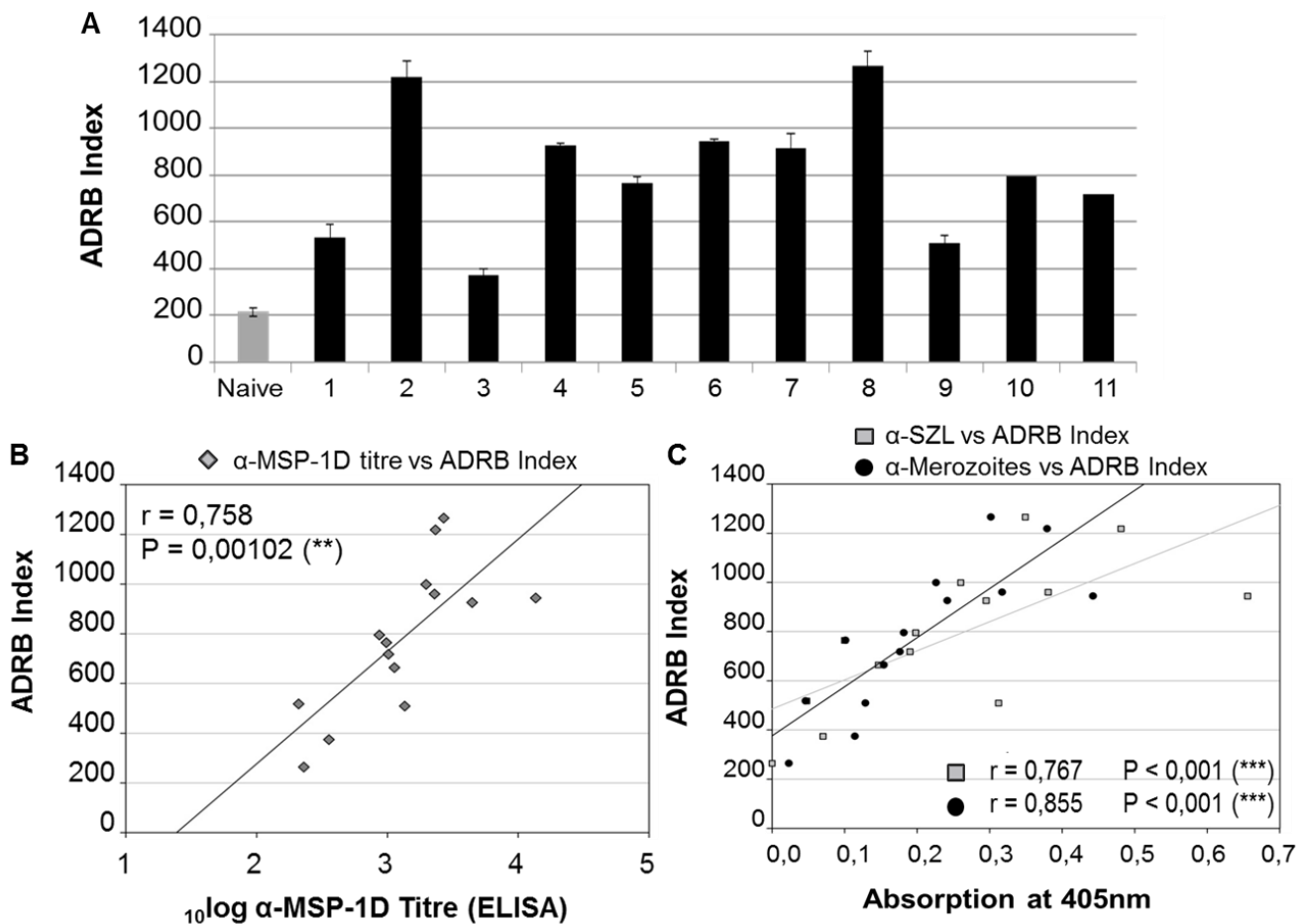
Figure 5.12: Correlation of ADRB activity with MSP-1 antibody titer. (A) *ADRB activity of 11 semi-immune individuals and a malaria-naïve pool (n=4) against P. falciparum 3d7 merozoites. Shown is the mean of duplicate measurements with SEM.* ***(B)*** *MSP-1 antibody titer in semi-immune sera correlates with ADRB activity (Spearman`s rank correlation coefficient r=0.758).* ***(C)*** *Antibody levels to P. falciparum 3d7 schizont lysate (α-SZL) and merozoites correlate with ADRB activity (Spearman`s rank correlation coefficients r=0.767 and r=0.855, respectively).*

5.2.6. Affinity-purified MSP-1 antibodies opsonize *P. falciparum* merozoites

To further examine opsonizing MSP-1 specific antibodies, sera from two semi-immune individuals (Donor 2 & 8) were fractionated by antigen affinity purification with MSP-1D. A preparation of MSP-1 specific antibodies was obtained (Figure 5.13 A and B) while the flow-through (FT) material was efficiently depleted, as shown by ELISA (Figure 5.13 B). Importantly, ADRB activity was reduced substantially in the FT fraction compared to total IgG and MSP-1 specific antibodies could opsonize merozoites and recruit neutrophils (Figure 5.13 D). Interestingly, the same eluate fractions containing MSP-1 specific antibodies were not active in the GI assay while MSP-1 depleted sera showed undiminished activity (Figure 5.13 C). Thus, MSP-1 specific antibodies from Donor 2 and 8 - though not active in the GI assay – were capable of activating neutrophils *via* opsonized merozoites.
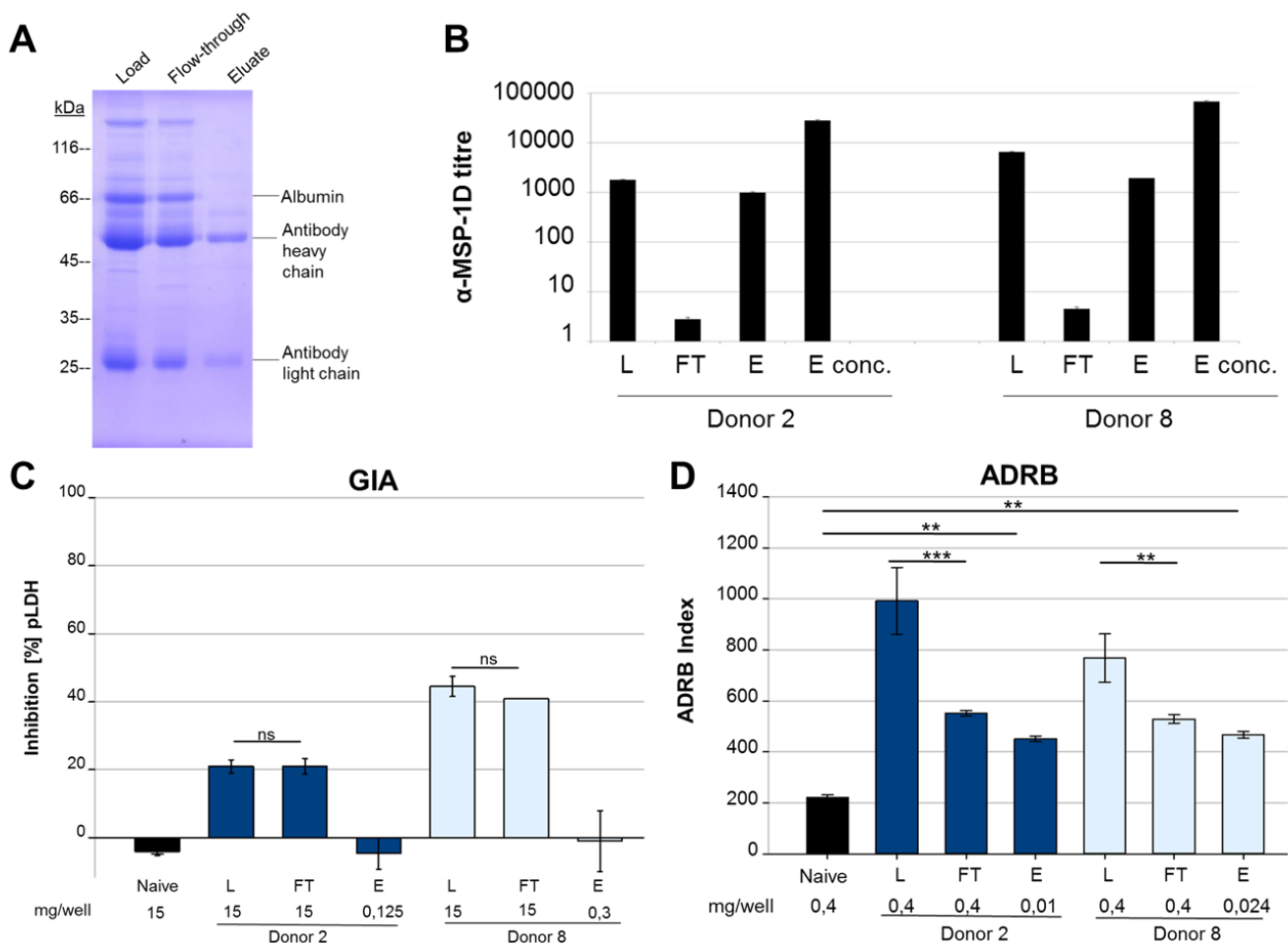
Figure 5.13: MSP-1 as target of opsonizing antibodies. *Sera from two semi-immune individuals were fractionated by antigen affinity purification to MSP-1D. The fractions were analyzed by Coomassie staining **(A)** and ELISA **(B)**. MSP-1 antibody titers are strongly reduced in MSP-1 depleted IgG (flow-through, FT) and enriched in the eluate fraction (E and E conc.). **(C)** Affinity-purified MSP-1 antibodies do not neutralize the parasite. Affinity purified MSP-1 antibodies (E) from donors 2 and 8 did not inhibit parasite growth in vitro while MSP-1 depleted sera (FT) showed undiminished activity similar to total IgG (L). **(D)** Affinity-purified MSP-1 antibodies opsonize merozoites. ADRB activity is reduced in MSP-1 depleted IgG (FT) compared to the total IgG fraction (L). MSP-1 specific IgG (E) can recruit neutrophils. Statistical differences were assessed using One Way RM Anova.*

5.2.7. Establishment of the antigen-reversal ADRB

An antigen-reversal ADRB assay was established in order to (i) assess if MSP-1 specific antibodies play a role in opsonization in all eleven semi-immune individuals from Burkina Faso, (ii) examine potential cross-reactivity of the opsonizing antibodies between different *P. falciparum* strains and (iii) identify the MSP-1 processing fragments eliciting opsonizing antibodies. By pre-incubation of serum antibodies with recombinant antigens such as MSP-1 the contribution of antigen-specific antibodies to ADRB activity could be quantified. Prior to their use in antigen-reversal ADRB, all recombinant antigens were pre-tested for their chemiluminescence activity in presence of PMNs and isoluminol but in absence of merozoites; all competitors showed only background chemiluminescence activity (Figure 5.14 B). Furthermore, pre-incubation of human IgG from semi-immune individuals together with control proteins such as BSA did not reduce ADRB activity (Figure 5.14 A) indicating that a potential decrease of ADRB activity by pre-incubation of antibodies with MSPs is specific. After identifying an appropriate IgG concentration for the antigen-reversal ADRB assay (200 µg/ml for human IgG) (Figure 5.15), we determined an adequate competitor antigen concentration by pre-incubation of IgG from semi-immune individuals with increasing concentrations of MSP-1D followed by ADRB. Neutrophil respiratory burst activity decreased substantially even at very low MSP-1D concentrations (2 nM) and followed a hyperbolic decay curve (Figure 5.16); to ensure that nearly all antigen-specific antibodies are removed, a competitor antigen concentration of 500 nM was chosen.
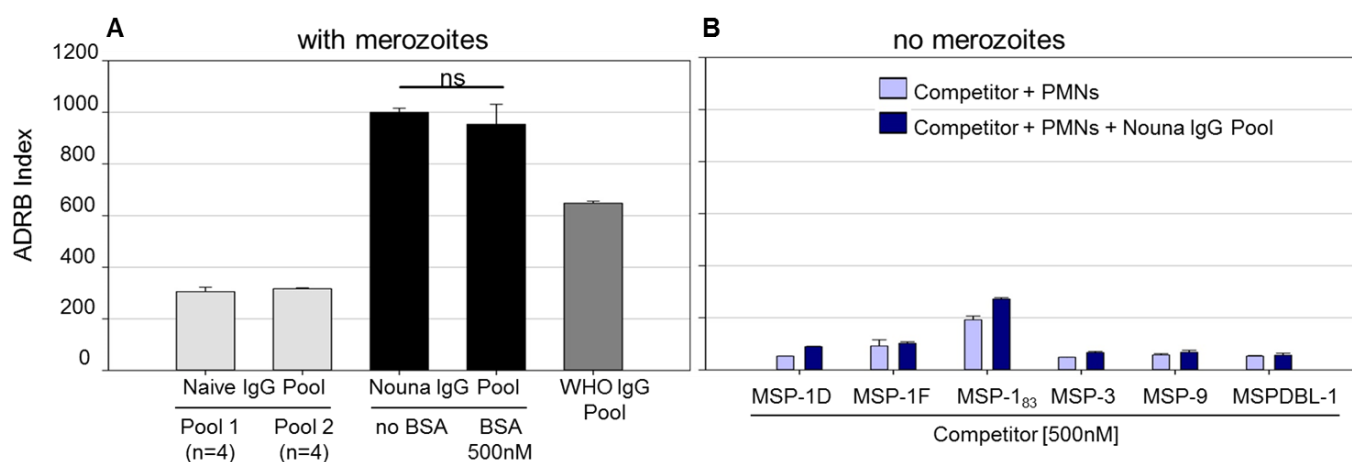
Figure 5.14: Controls for Antigen-Reversal ADRB. (A) *ADRB activity of IgG from 2 malaria-naïve European pools (each n=4) in comparison to malaria-exposed donors from Burkina Faso (Nouna Pool, n=11) and from Kenya (WHO pool, NIBSC code: 10/198; (Bryan D, 2014)) was determined against P. falciparum FCB1 merozoites. Presence of 500 nM BSA does not reduce ADRB activity of semi-immune donors (Nouna IgG Pool, black bars).* ***(B)*** *ADRB activity of the indicated competitor antigens (500 nM) alone and in combination with purified IgG from 11 semi-immune donors (Nouna pool) but in absence of merozoites was assessed; only background activity was detected. Mean values of duplicate measurements are shown; statistical differences were calculated using paired t-test.*
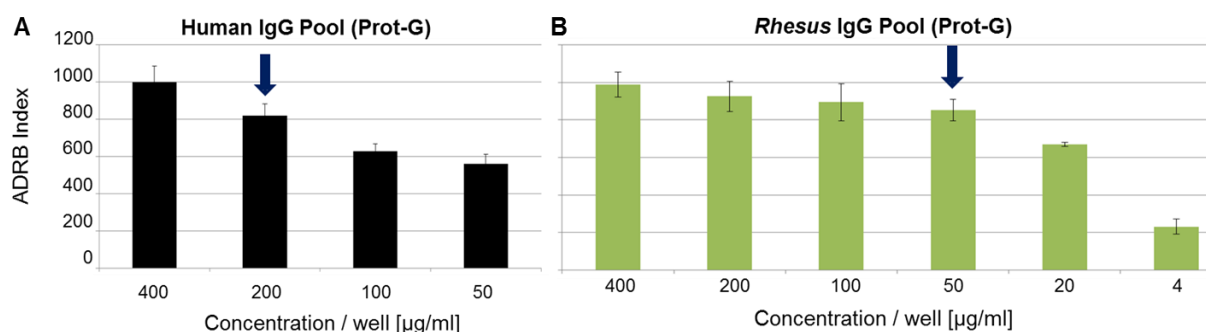
Figure 5.15: Finding an appropriate IgG concentration for Antigen-Reversal ADRB. *ADRB activity of Protein G purified IgG from 11 semi-immune individuals from Burkina Faso **(A)** and from rhesus monkeys (n=5) immunized 3x with rMSP-1D and adjuvant (d70) **(B)** was analyzed against P. falciparum 3d7 merozoites using different IgG concentrations. For antigen-reversal ADRB we used the following IgG concentrations per well: 200 µg/ml for human IgG and 50 µg/ml for α-MSP-1D IgG from immunized rhesus monkeys.*
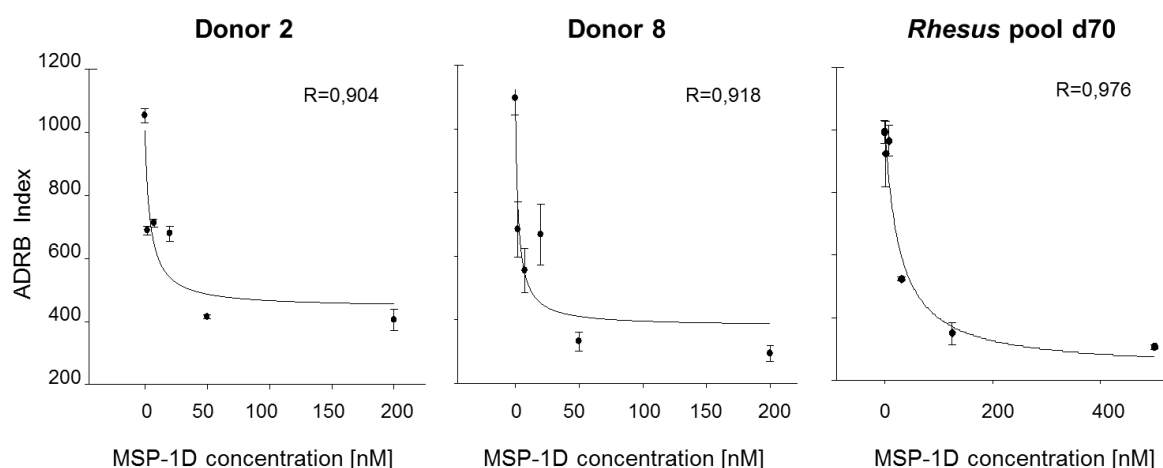
Figure 5.16: Antigen-Reversal ADRB with increasing concentrations of MSP-1D. *Protein G purified IgG from two semi-immune donors and rhesus α-MSP-1D antibodies (d70) were tested against P. falciparum 3d7 merozoites in presence of increasing concentrations of MSP-1D. ADRB activity strongly decreases even at low MSP-1D concentrations. The data points fit well (R > 0.9) to a hyperbolic decay curve with 3 parameters.*

5.2.8. MSP-1 and MSP-1$_{83}$ are major targets of opsonizing antibodies

In presence of 500 nM MSP-1D antigen, ADRB activity was strongly reduced in all eleven semi-immune donors from Burkina Faso. The mean reduction was 58.6 % using *P. falciparum* (3d7) merozoites and 51.7 % using *P. falciparum* (FCB1) merozoites (Figure 5.17 A and B), indicating a strong cross reactivity of MSP-1 specific opsonizing antibodies. Antibody levels to the partially conserved MSP-1D processing fragments MSP-1$_{83}$ and MSP-1$_{42}$ were comparable in the eleven individuals from Burkina Faso (Figure 5.2). Interestingly, pre-incubation of antibodies from the semi-immune individuals with 500 nM MSP-1$_{83}$ highly reduced ADRB activity; the mean reduction was 50.2 % (Figure 5.17 C). Thus, MSP-1 and especially its processing fragment MSP-1$_{83}$ is a major target of opsonizing antibodies in individuals with naturally acquired immunity.
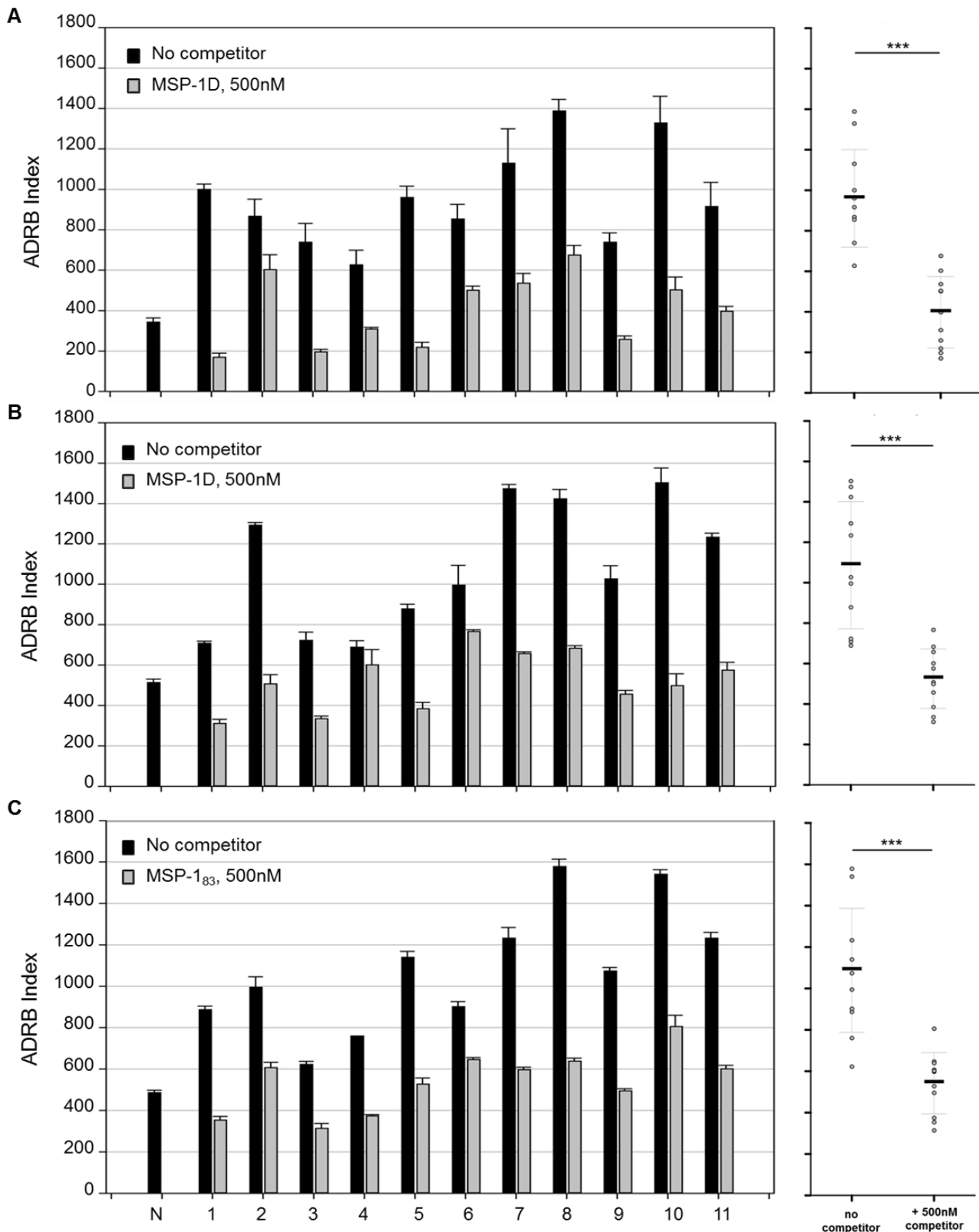
Figure 5.17: MSP-1 as target of opsonizing antibodies (Antigen-Reversal ADRB) and importance of conserved regions. *ADRB activity of Protein G purified IgG from 11 semi-immune donors was analyzed against P. falciparum 3d7 **(A,C)** and FCB-1 **(B)** merozoites in the absence and presence of MSP-1D **(A,B)** or its processing fragment MSP-1$_{83}$ **(C)**. Mean values of duplicate measurements are shown as individual columns and as vertical point plot; statistical differences were calculated using paired t-test. Average reduction of mean in presence of competitor: 58.6% (MSP-1D, 3d7), 50.2% (MSP-1$_{83}$, 3d7), 51.7% (MSP-1D, FCB1). N: IgG from malaria naïve European donors (n=4).*

5.2.9. Comparison of *Pf*MSPs for their opsonizing activity

Having established an important role for MSP-1 in eliciting opsonizing antibodies, we aimed to compare its contribution to ADRB activity with other merozoite surface antigens. We thankfully received the recombinant proteins MSP-3, MSP-9 and MSPDBL-1 from *P. falciparum* (3d7) produced in *E.coli* from our cooperation partners in Melbourne, Australia (Dr. Clara Lin & Prof. Alan Cowman). In absence of merozoites but presence of PMNs, isoluminol and the NA pool, these antigens showed only background chemiluminescence activity (Figure 5.14 B) and thus, can be used for antigen-reversal ADRB experiments.

Strikingly, ADRB activity against *P. falciparum* (3d7) merozoites was strongly reduced in all eleven semi-immune donors from Burkina Faso in presence of MSP-3 or MSPDBL-1 and – to a lower extent – also of MSP-9. The average reduction of mean in presence of 500 nM competitor antigen was 48.2 % for MSP-9, 83.8 % for MSP-3 and 89.2 % for MSPDBL-1 (Figure 5.18). Thus, besides MSP-1 also other merozoite surface antigens such as MSP-3, MSP-9 and MSPDBL-1 elicit opsonizing antibodies which are able to activate neutrophils and cause respiratory burst.
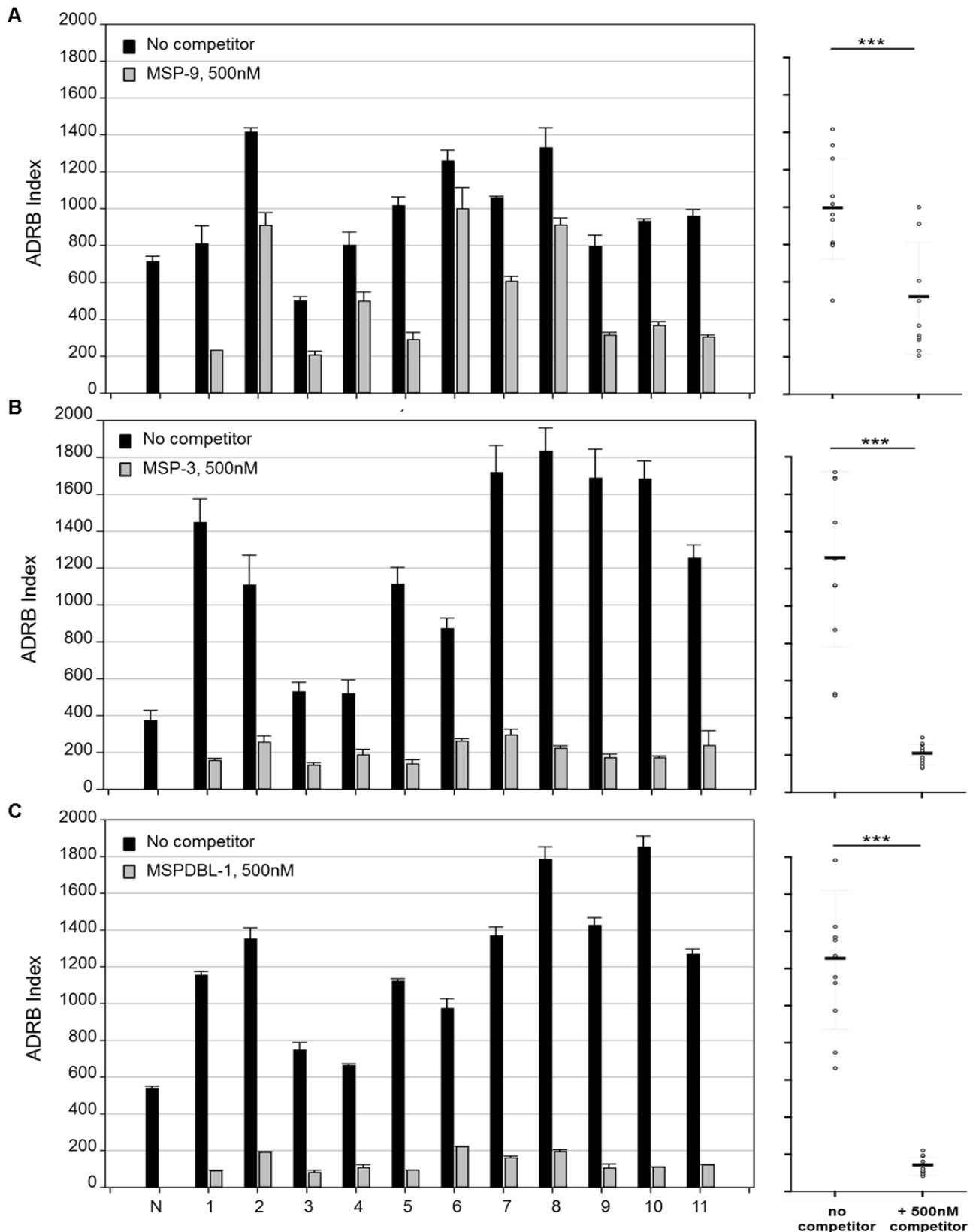
Figure 5.18: Other MSPs as target of opsonizing antibodies (Antigen-Reversal ADRB). *ADRB activity of Protein G purified IgG from 11 semi-immune donors was analyzed against P. falciparum 3d7 merozoites in the absence and presence of MSP-9 **(A)**, MSP-3 **(B)** and MSPDBL-1 **(C)**. Mean values of duplicate measurements are shown as individual columns and as vertical point plot; statistical differences were calculated using paired t-test. Average reduction of mean in presence of competitor: 48.2 % (MSP-9), 83.8 % (MSP-3), 89.2 % (MSPDBL-1). N: IgG from malaria naïve European donors (n=4).*

5.2.10. Immunization with recombinant MSP-1D induces opsonizing antibodies in *rhesus* monkeys

Purified IgG from *rhesus* monkeys (n=5), immunized three times with recombinant MSP-1D and adjuvant (CoVaccine HT), were examined by antigen-reversal ADRB assay for opsonizing antibodies directed against MSP-1D and MSP-1$_{83}$. Sera were sampled two weeks (d70) and > 4 months (d182) after third immunization. Antibody levels against MSP-1D were comparable in immunized *rhesus* monkeys (d70) and the NA pool (Appendix, Figure 7.2). Interestingly, the observed high ADRB activity induced by immunization with MSP-1D was similar to the activity found for the NA pool (Figure 5.19) and was efficiently competed out by pre-incubating the IgG with 500 nM MSP-1D; a substantial decrease was also observed after pre-incubation with 500 nM MSP-1$_{83}$ (Figure 5.20). Comparable results were obtained with *P. falciparum* 3d7 or FCB1 strains. Remarkably, ADRB activities were similar at d70 and d182 (Figure 5.19) indicating that immunization of non-human primates with MSP-1D apparently induces a long-lasting antibody response capable of opsonizing *P. falciparum* merozoites.

In summary, both natural exposure against malaria and immunization of *rhesus* monkeys with recombinant MSP-1D elicits antibodies against MSP-1 and its processing fragment MSP-1$_{83}$, which are effective in merozoite opsonization and neutrophil activation.
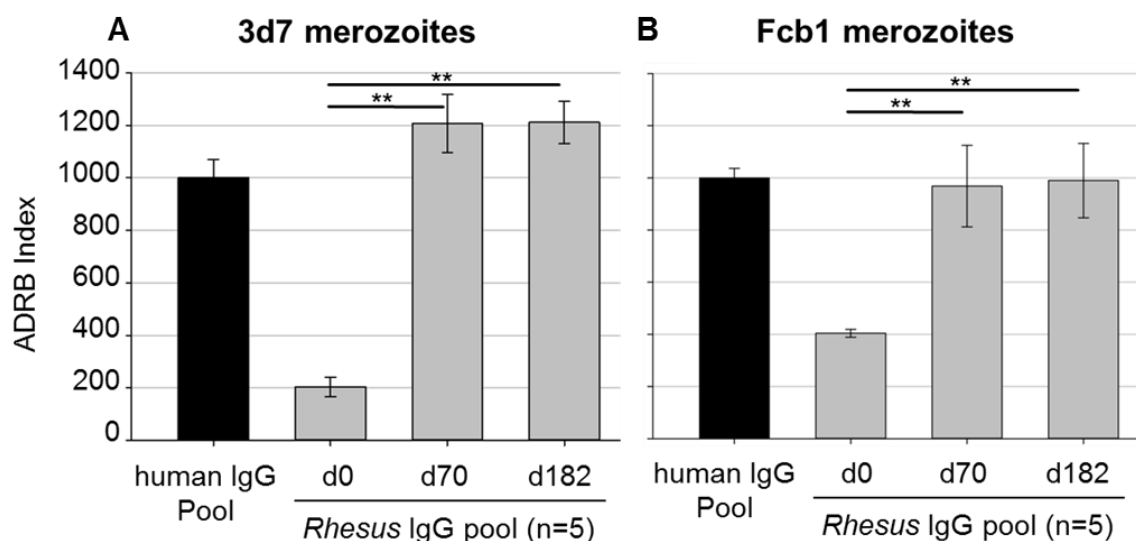
Figure 5.19: Immunization with MSP-1D elicits opsonizing antibodies in rhesus macaques. *Rhesus monkeys (n=5) were immunized three times (d0, d28, d56) with recombinant MSP-1D and adjuvant (CoVaccine HT). Blood was sampled at d0, d70 and d182. ADRB activity of Protein G purified IgG from blood was analyzed against P. falciparum 3d7 (A) and FCB1 (B) merozoites. d0: pre-immune control IgG. Human IgG Pool: Protein G purified IgG from 11 semi-immune individuals from Burkina Faso. Statistical differences were assessed by One Way RM Anova.*
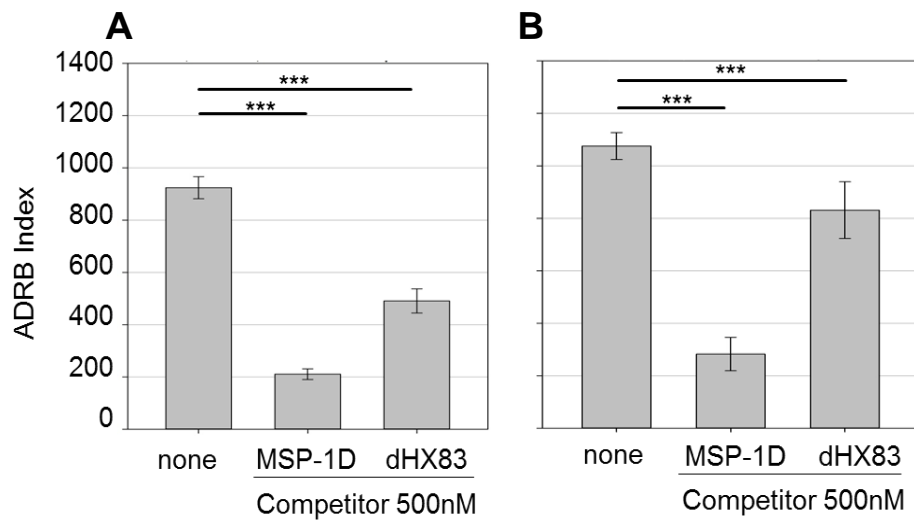
Figure 5.20: Antigen-Reversal ADRB with rhesus MSP-1D antibodies and MSP-1 competitors. Protein G purified IgG from rhesus monkeys (n=5) immunized 3x with MSP-1D (d70) were analyzed for opsonizing activity to 3d7 *(A)* and FCB1 *(B)* merozoites in presence of 500nM MSP-1D or MSP-1$_{83}$. Shown are the means of two independent experiments, each with duplicate measurements. Statistical differences were assessed using One Way Anova.

5.3. Cellular immune response against *Pf*MSP-1D in individuals with naturally acquired immunity

5.3.1. Identification of CD8+ T-cell epitopes within MSP-1D

Since MSP-1 is initially synthesized in infected hepatocytes at late liver stage (Szarfman et al., 1988), it can be a target of the cellular immune response. HLA-A0201 restricted CD8+ T-cell epitopes have already been identified within MSP-1D in the laboratory of Prof. H. Bujard (Carralot et al., 2008; Idler, 2004) (Table 5.2; Figure 5.21) using two different approaches: (i) *In silico* prediction of potential CD8+ T-cell epitopes within MSP-1D for the HLA haplotype A0201 by the computer program SYFPEITHI (Rammensee et al., 1999). The *in vivo* relevance of the predicted epitopes was analyzed in HHD mice which express the human HLA-A0201 receptor. These mice were vaccinated with MSP-1D and MSP-1 specific CD8+ T cells were discovered *via* tetramer technology. The strongest response was detected for epitope 291 (Idler, 2004). (ii) MSP-1D was tetracyclin-inducible expressed in a cell line positive for HLA-A0201; the CD8+ T-cell epitopes presented under physiological conditions were eluted and identified by MS. *Via* this strategy epitope 674 was discovered (Carralot et al., 2008). CD8+ T-cell epitopes with a high binding score were synthesized and the most promising peptides - 291, 374, 437 and 674 (according to (Idler, 2004; Jäschke, 2012) - were used in this thesis (Figure 5.21).

Epitope	Sequence	Aa position	Binding score	MW [Da]	Location
182 Y	YLLRAKLNDV	182	26	1204,7	p83/30
313	KLLDKINEI	313	30	1085,3	p83/30
162	YLIDGYEEI	162	27	1114,2	p83/30
674	KLKEFIPKV	674	30	1101,7	p83/30
291	GLHHLITEL	1180	28	1032,6	p38/42
374	SLLTELQQV	1263	28	1030,6	p38/42
437	VIYLKPLAGV	1326	27	1072,7	p38/42

Table 5.2: Overview of MSP-1 specific CD8+ T-cell epitopes for the HLA haplotype A0201. *The peptides were discovered previously (Carralot et al., 2008; Idler, 2004); four epitopes are located within MSP-1$_{83/30}$ (light grey) and three within MSP-1$_{38/42}$ (dark grey). The binding score was derived from SYFPEITHI.*
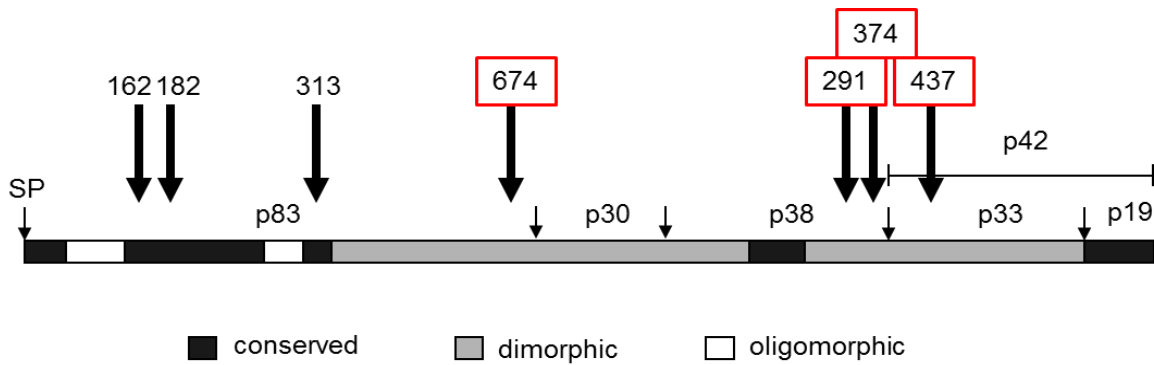
Figure 5.21: Location of MSP-1 specific CD8+ T-cell epitopes for the HLA haplotype A0201. Schematic depiction of MSP-1 with subunits, processing sites (small arrows) and regions of different degree of conservation. HLA-A0201 restricted CD8+ T-cell epitopes are indicated (large arrows), enframed peptides were used in this study.

5.3.2. MSP-1 specific CD8+ T-cell response in semi-immune individuals

The identified CD8+ T-cell epitopes within MSP-1D – 291, 374, 437 and 674 (Figure 5.21) – were used to examine the CD8+ T-cell response of the semi-immune individuals from Nouna *via* IFNγ ELISPOT assay. Additionally, PBMCs from malaria-naive European donors were analyzed as controls. All individuals have the same HLA haplotype A0201.

Unfortunately, the quality of the cryopreserved PBMCs from the Nouna donors was quite bad: 60 % of the samples tested contained only dead cells, the apoptosis rate within 24 h after thawing was > 50 % and PBMCs from three donors were contaminated with bacteria. Nevertheless, PBMCs from three donors (Donor 2, 7 and 8) were of sufficient quality and could be analyzed by IFNγ ELISPOT assay after stimulation with the respective MSP-1 specific peptides. Interestingly, all of them recognized the CD8+ T-cell epitopes from MSP-1 (Figure 5.22 A). Although one of four malaria-naïve individuals also reacted to peptide 291 (Figure 5.22 B), the overall MSP-1 specific CD8+ T-cell response of the semi-immune individuals from Nouna (n=3) was significantly higher compared to malaria-naive individuals (n=4) for the peptide pool 291+374+437 and for peptide 674 (P=0.023 and P=0.000135, respectively) (Figure 5.22 C and D). Thus, CD8+ T-cell epitopes within MSP-1D were recognized by individuals with naturally acquired immunity.
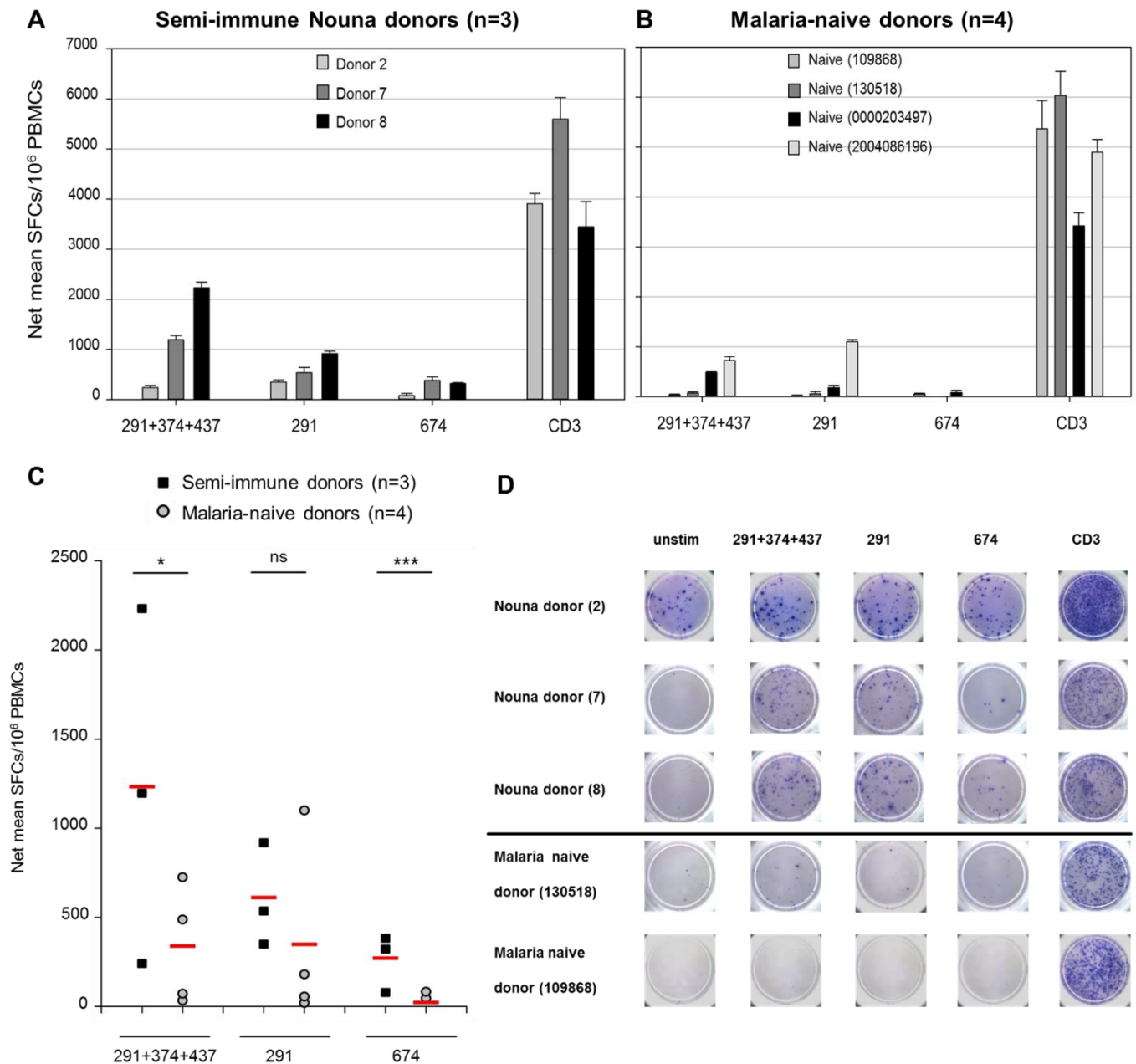
Figure 5.22: MSP-1 specific CD8+ T-cell response in semi-immune and malaria-naive individuals. *CD8+ T-cell response of 3 semi-immune individuals **(A)** and 4 malaria-naive individuals **(B)** to the MSP-1 specific epitopes 291, 374, 437 and 674; a monoclonal antibody against CD3 was used as positive control. Cryopreserved PBMCs from each individual were stimulated with MSP-1 derived peptides (10 µg/ml) and their activation was measured by 24h pre-cultered IFNγ-ELISPOT assay. The net number of Spot Forming Cells (SFCs) in one million PBMCs is shown +/- SEM. **(C)** Comparison of CD8+ T-cell activation between semi-immune (n=3) and malaria-naïve (n=4) individuals following stimulation of PBMCs with the indicated MSP-1 specific peptides. Statistical analysis was performed via t-test. **(D)** Selected ELISPOT wells from 3 semi-immune and 2 malaria-naïve individuals showing the CD8+ T-cell response against MSP-1 derived peptides or the positive control CD3. Each blue spot represents a cell secreting IFNγ.*

5.3.3. HLA-independent analysis of the MSP-1 specific T-cell response using peptide pools

Since we aimed to examine the MSP-1 specific CD8+ T-cell response in larger cohorts which are not HLA-typed, HLA independent methods were investigated. One possibility is the use of MSP-1 specific peptide pools covering many important HLA-types. Thus, potential HLA binding epitopes within MSP-1D were predicted to the most frequent HLA-types by the Artificial Neural Networks (ANN) algorithm (Honeyman et al., 1998). 161 CD8+ T-cell epitopes (nonamers) within MSP-1D were predicted to the most frequent HLA-types in Mali; their location is distributed quite equally throughout the whole MSP-1 molecule (Figure 5.23 A). No region with especially high prevalence of CD8+ T-cell epitopes could be detected – a disadvantage for creating peptide pools since considering the whole MSP-1 protein for peptide synthesis would be very expansive.

Furthermore, HLA-frequencies show a wide variety between different countries, such as Mali and Germany (Figure 5.23 B and C). Only two HLA haplotypes are present in both populations (HLA-A0201 and C0401). Thus, separate peptide pools would have to be synthesized for individuals from different countries e.g. Germany and Mali. Since the approach with peptide pools covering full-length MSP-1 is expensive and time-consuming, the whole protein was tested *via* ELISPOT assay.
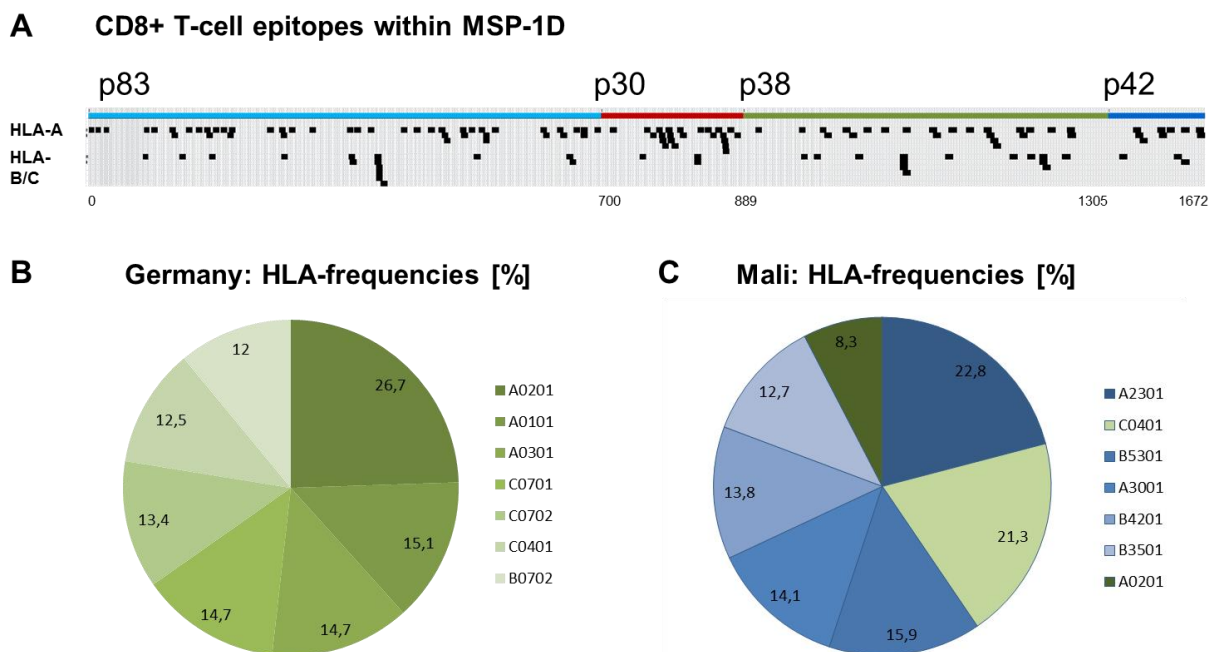
Figure 5.23: Distribution of CD8+ T-cell epitopes within MSP-1D and HLA-frequencies. (A) MSP-1 specific CD8+ T-cell epitopes to the most frequent HLA-types in Mali: 161 strong and weak binding CD8+ T-cell epitopes (nonamers) within MSP-1D were predicted by the Artifical Neural Networks (ANN) algorithm. Their location within MSP-1 is indicated (black bars). (B) HLA allele frequencies in Mali and Germany. HLA allele frequencies were derived from the "allele frequency net" database which used the bone marrow registry (n ≈ 40 000) for Germany and an anthropology study (n = 138; (Cao et al., 2004)) for Mali. Only HLA-A0201 and C0401 are present in both populations.

5.3.4. HLA-independent analysis of the MSP-1 specific T-cell response using recombinant MSP-1D

The use of recombinant proteins such as full-length MSP-1D for analysis of the MSP-1 specific T-cell response has several advantages: (i) it is independent from the HLA type of the blood donor, (ii) it is a much easier and cheaper approach compared to peptide pools and (iii) it is likely that the protocol can be easily adapted to other recombinant antigens such as MSP-1F, MSP-6 or MSP-7, if they are available in sufficient quality - e.g. without endotoxins - and quantity.
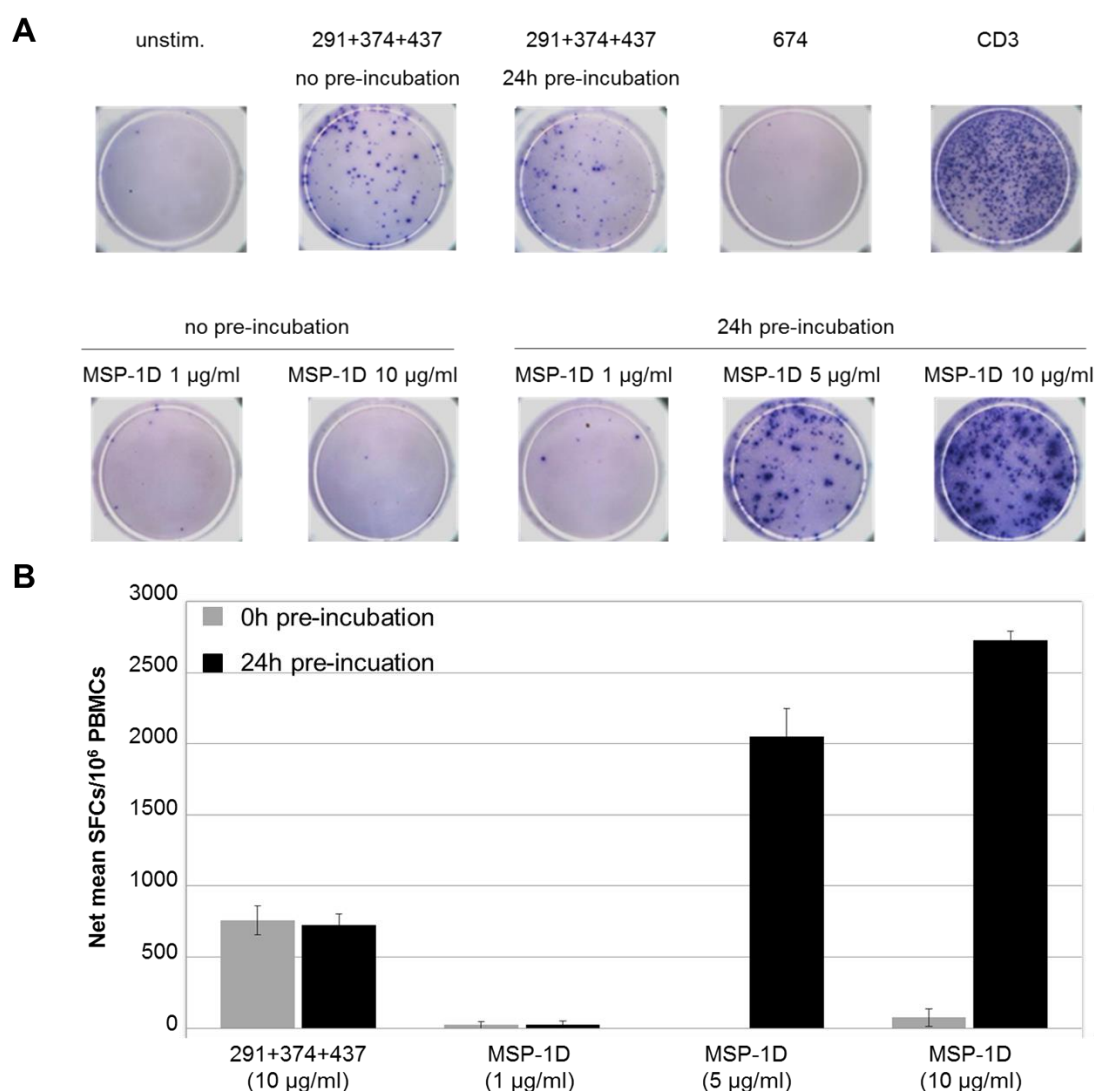
Figure 5.24: T-cell response against recombinant MSP-1D protein. (A) Cryopreserved PBMCs of the cross-reactive malaria-naïve donor were stimulated with the indicated peptides (10 µg/ml) or MSP-1D protein (1 µg/ml, 5 µg/ml, 10 µg/ml) either with or without a 24h pre-incubation step in presence of the respective stimuli and following IFNγ ELISPOT assay. Positive control: mAb CD3. Selected ELISPOT wells are shown; each blue spot represents a cell secreting IFNγ. (B) Diagram showing the net number of Spot Forming Cells (SFCs) in one million PBMCs +/- SEM. PBMCs were stimulated with either MSP-1 peptides or recombinant MSP-1D protein at the indicated concentrations and – partly following a 24h pre-incubation step in presence of the stimuli - analyzed via IFNγ ELISPOT assay.

I successfully adapted the IFNγ ELISPOT protocol for the use of full-length MSP-1D protein. PBMCs from the cross-reactive malaria-naïve donor were stimulated with either MSP-1 specific peptides (291, 374, 437 and 674; 10 µg/ml) or MSP-1D protein (1 µg/ml, 5 µg/ml or 10 µg/ml) and analyzed by IFNγ ELISPOT assay. Importantly, the approach with MSP-1D protein requires a pre-incubation step (24 h) in presence of MSP-1D prior to the ELISPOT assay while for MSP-1 specific peptides this step could be omitted (Figure 5.24). A strong response was detected with 5 µg/ml and 10 µg/ml MSP-1D if pre-incubated for 24 h.

The other three malaria-naïve individuals were also tested with MSP-1 specific peptides and MSP-1D protein but - as expected – showed no reaction. Due to insufficient quality of PBMCs from the Nouna donors, which contain no antigen presenting cells like monocytes or dendritic cells in contrast to the malaria-naïve PBMC preparations (Appendix, Figure 7.4), MSP-1D protein could not be processed and presented to T-cells and thus, no IFNγ response was detected.

5.3.5. Planned analysis of PBMCs from a large cohort in Mali

In cooperation with Prof. O. Doumbo and Prof. B. Kouriba (MRTC, University of Bamako) we aimed to investigate the MSP-1 specific T-cell response in a cohort of 210 children (2-15 years) from Samako, a malaria-hyperendemic village with strong seasonal malaria transmission in Mali, West-Africa. From each study participant several blood samples were available taken before, during and after transmission season over a two year period (2011-2013). Since the volunteers differed in age and detailed health records were available, we wanted to study a possible connection between MSP-1 specific immune response and acquired immunity against malaria.

Unfortunately, the Mali cohort could not be employed for analysis of the cellular immune response since all cryopreserved PBMCs which arrived in Heidelberg (~1400 tubes) were unusable. Samples from all available time-points and from different donors were tested directly after thawing for their viability by light microscopy (trypanblue staining) and by flow cytometry (staining with propidium iodide) (Figure 5.25). From eleven samples tested, nine contained no viable cells and two less than 20 %. In contrast, cryopreserved PBMCs from Heidelberg which were thawed in parallel and treated the same had a viability rate of about 99 % (Figure 5.25). Thus, so far a correlation between MSP-1 specific T-cell responses and protection against malaria could not be analyzed since appropriate PBMC samples at sufficient quality were not available.

A

	Malaria-naive donor, HD	Donor 12 P6	Donor 36 P4	Donor 121 P5
Representative images (trypanblue-st.)				
Living cells	$2,37 \times 10^7$	0	$5,5 \times 10^5$	0
Dead cells	2×10^5	$1,55 \times 10^6$	$2,3 \times 10^6$	$7,5 \times 10^6$
% viable cells	99,2	0	19,3	0

B

C
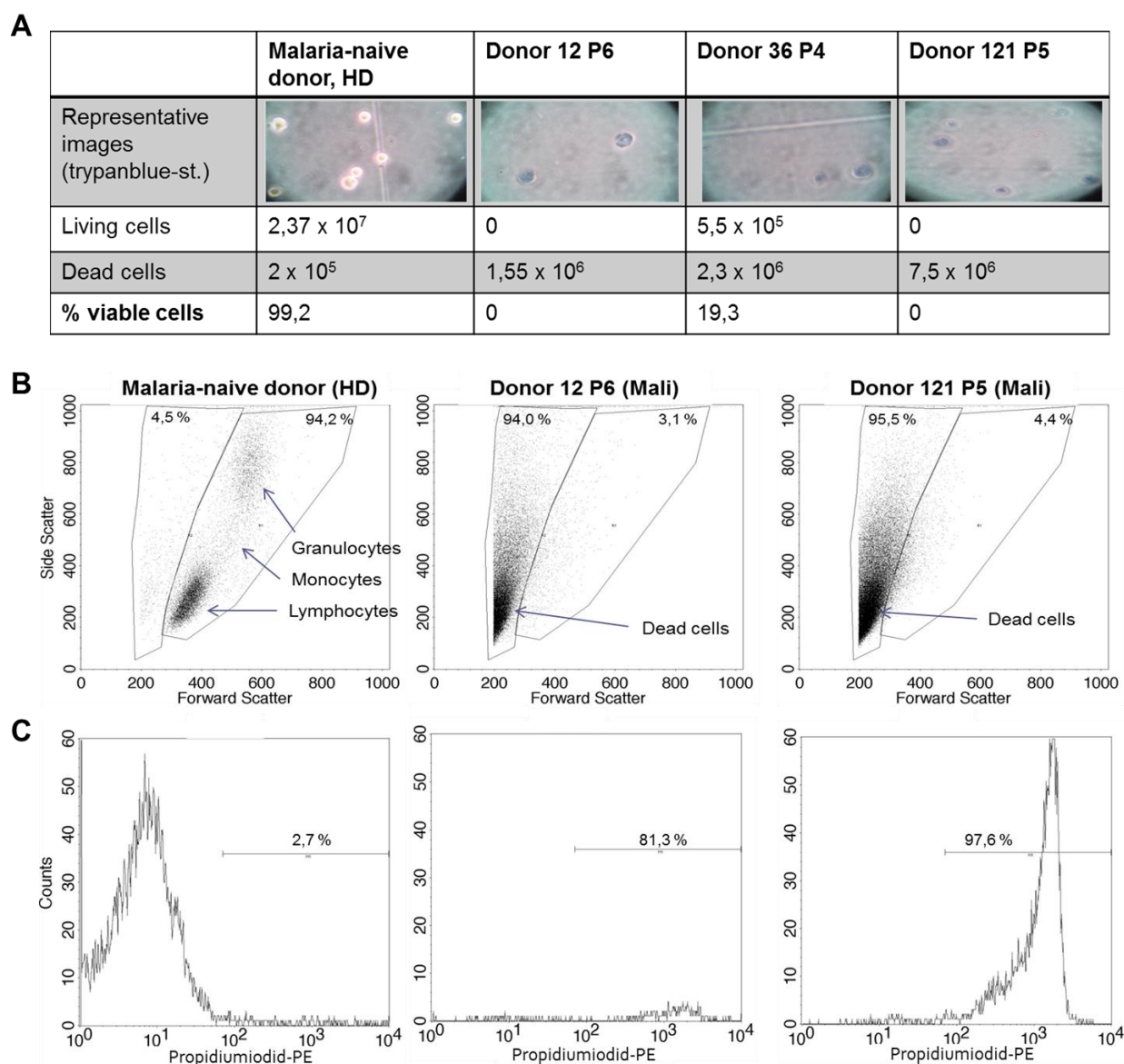
Figure 5.25: Viability of PBMCs from Mali determined by microscopy and flow cytometry. (A) *Cryopreserved PBMCs from one malaria-naïve donor from Heidelberg and three donors from Mali were thawed, stained with trypanblue and analyzed by light microscopy. Viable (white) and dead (blue) cells were counted using a Neubauer hemocytometer and the viability rate was calculated. Representative images are shown (40x magnification, 3x camera zoom).* *(B/C)* *Cryopreserved PBMCs from one malaria-naïve donor from Heidelberg and two donors from Mali were thawed, stained with Propidiumiodide-PE for 20 min and analyzed by FACS. Percentages are calculated from total events. In the forward / side scatter cells are sorted according to their size and granularity; living cell populations could only be detected for the malaria-naïve donor. The respective Propidiumiodide-PE staining is shown under each image. Propidiumiodide stains only dead cells.*

5.4. Immune response after controlled human malaria infection (TUECHMI I)

TUECHMI I, a controlled human malaria infection study in Tübingen, was carried out in 2011/2012 in order to establish a standardized malaria infection of humans. 30 malaria-naïve adult volunteers (18 - 55 years) were challenged with 50 to 3200 cryopreserved, GMP-graded *Pf*Spz (NF54 strain) from Sanaria by either intravenous or intradermal injection (Figure 5.26 B). Blood was sampled at different time points and – upon detection of blood stage parasites by microscopy (thick blood smear) - volunteers were treated immediately with ACT (Lumefantrine-Artemether) (Figure 5.26 A). In total, 22 out of 30 *Pf*Spz infected volunteers developed malaria with a pre-patency time period of 10 – 15 days while 6 non-infected individuals remained malaria-negative. 100 % of volunteers (9/9) in the highest dose group (3200 *Pf*Spz i.v.) were malaria-positive (Figure 5.26 B) (Mordmuller et al., 2015).

This standardized clinical study allowed us to investigate the human immune response induced by experimental *P. falciparum* wildtype sporozoite infection. In cooperation with Dr. Kirsten Heiss and Dr. Ann-Kristin Müller, we examined the CD8+ T-cell response to the liver stage antigens LSA-1 and CSP and the cross-stage antigen MSP-1 *via* IFNγ ELISPOT assay. Additionally, I analyzed the antibody levels to the merozoite surface antigens MSP-1, MSP-6 and MSP-7 *via* ELISA. Moreover, a cytokine profile was created *via* cytokine bead array by our cooperation partner in Munich (Dr. Michael Pritsch). The malaria antigens examined in this study and their expression throughout the *Plasmodium* life cycle are shown below (Figure 5.26 C).

5.4.1. Antibody response after single *Pf* sporozoite infection

Serum antibody titer against MSP-1, MSP-6 and MSP-7 were analyzed by ELISA at different time points after sporozoite infection. MSP-1 antibodies could be detected in 12 out of 19 malaria positive volunteers at day 28 post infection (Figure 5.27 A) and they were still present at day 84 and even at day 168 (Figure 5.27 A and E), thus the elicited MSP-1 antibodies were quite long-lived. However, compared to the MSP-1 antibody levels of the semi-immune individuals from Nouna, the antibody titer after single sporozoite infection is much lower (average titer of 6500 versus highest titer of 136, respectively).

Antibody levels against MSP-6 and MSP-7 were measured in the 3200 *Pf*Spz group (n=9) at the day before challenge (C-1), day 11 and day 28 post infection. Despite a "background titer" present already at C-1 for both MSP-6 and MSP-7 - probably due to cross-recognition of other antigens - there was a significant increase in antibody levels against both antigens at day 28 (Figure 5.27 B, C and D). Thus, a single infection with *Pf*Spz elicits antibodies against MSP-1, MSP-6 and MSP-7 with MSP-1 being more immunogenic as MSP-6 and MSP-7 (Figure 5.27 D).

A

Blood sampling: C-1, d5, d7, d9, d11, d12, d13, d15, d17, d28, d84

Liver stage	Blood stage Parasitemia

	Treatment (Lumefantrine-Artemether)
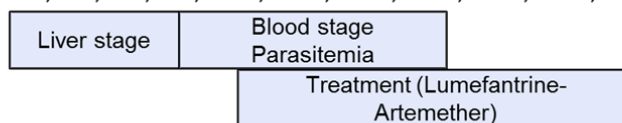
B

Sporozoites i.v.	Malaria-Positive	Pre-Patency
50	1 / 3	d13
200	1 / 3	d14
800	7 / 9	d11-12
3200	9 / 9	d10-12
2500 i.d.	4 / 6	d12-15

C
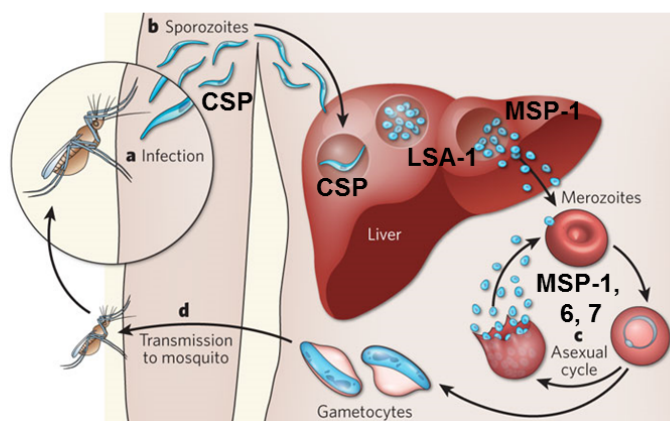
Figure 5.26: Study design of TUECHMI I and malaria antigen-specific immune responses investigated. (A) 30 volunteers (18-55y) were challenged with cryopreserved P. falciparum NF54 sporozoites. Blood samples were taken one day prior infection (C-1) and on several time points after infection (d5 to d84). Upon detection of blood stage parasites by microscopy, volunteers were treated with Lumefantrine-Artemether. (B) Malaria-naïve volunteers received different doses of cryopreserved Pf NF54 sporozoites i.v. (50, 200, 800, 3200) or one dose i.d. (2500). The number of volunteers who showed blood stage parasites in the blood and their pre-patency time is indicated and depends on the Pf sporozoite dose. (C) Malaria antigens analyzed in this study and their expression in different stages of parasite infection. CSP is present on the surface of sporozoites and can be detected in early liver stages; the cross-stage antigen MSP-1 is expressed in late liver stages and blood stages. LSA-1 expression is restricted to liver stages. Using PBMCs and sera from the volunteers of this study, the cellular immune response against CSP, LSA-1 and MSP-1 as well as the humoral immune response against MSP-1/-6/-7 was analyzed. Picture modified from (Michalakis and Renaud, 2009).
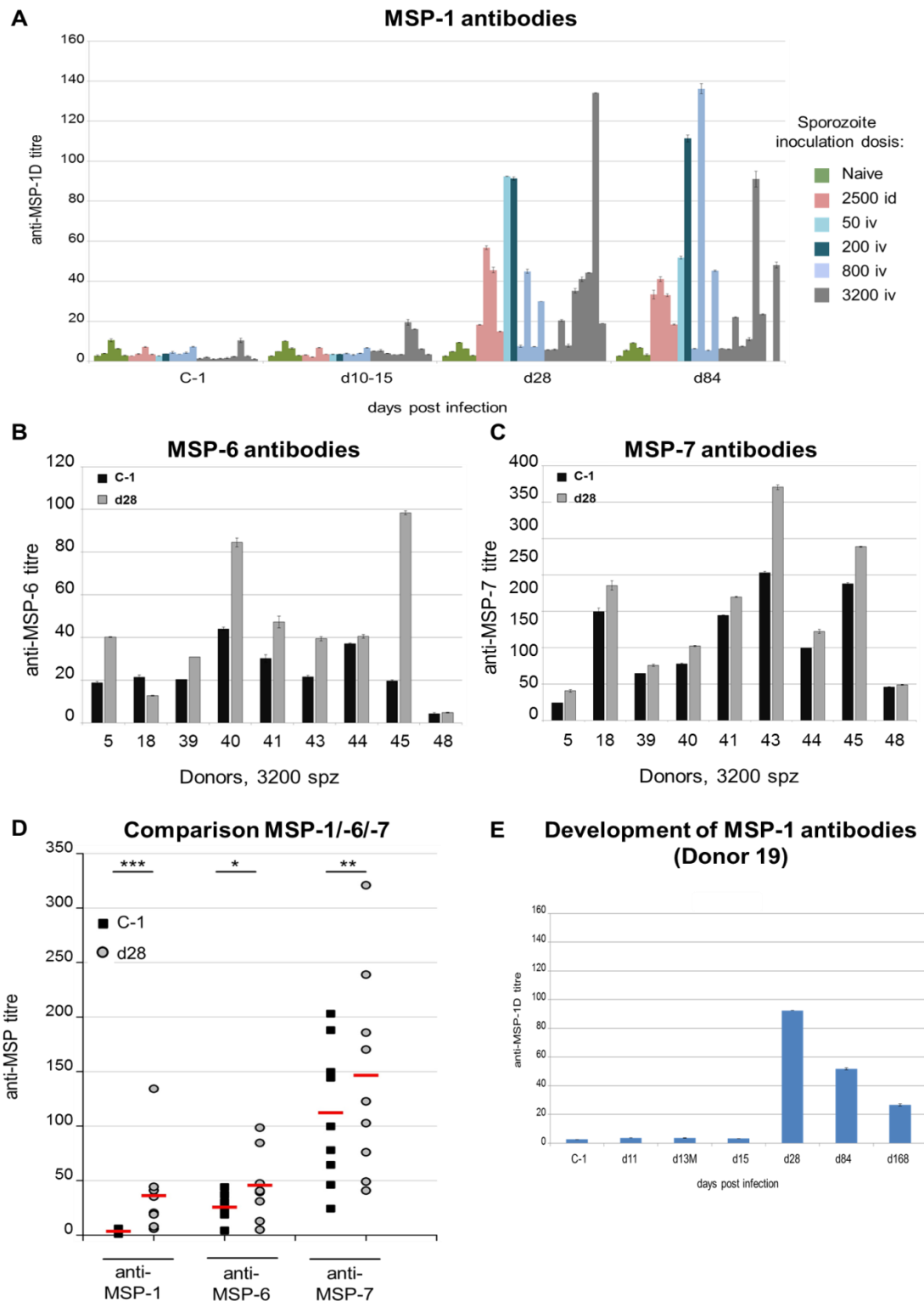
Figure 5.27: Antibody levels to MSP-1, -6 and -7 of TUECHMI volunteers (A) *Anti-MSP-1 antibody titer determined by ELISA to recombinant MSP-1D for malaria positive TUECHMI volunteers (n=19) and malaria-naïve controls (n=5) at four different time points. The individuals were grouped according to their Pf sporozoites dose received (naïve, 2500 id, 50 iv, 200 iv, 800 iv, 3200 iv) **(B/C)** Anti-MSP-6 and -7 antibody titer. The malaria-positive 3200 sporozoite group (9 volunteers) was tested by ELISA to recombinant MSP-6$_{36}$ and MSP-7$_{22}$ at time point C-1 and d28. **(D)** Comparison of antibody titers to MSP-1/-6/-7 before (C-1) and after (d28) the Pf sporozoite injection in the 3200 sporozoite i.v. group (n=9). There was a significant increase in mean titers for all MSPs tested using paired t-test. **(E)** Development of the MSP-1 antibody titer over time from the day before challenge (C-1) until day 168 for one malaria-positive TUECHMI volunteer. The antibody titer peaks at day 28.*

5.4.2. CD8+ T-cell response after single *Pf* sporozoite infection

PBMCs from the malaria-positive 3200 *Pf*Spz group (n=9) were analyzed at different time-points for the presence of CD8+ T-cells targeting MSP-1, LSA-1 and CSP *via* IFNγ ELISPOT assay. The antigen-specific peptide pools used for this experiment are described in detail in the method section (chapter 4.11.2.); for MSP-1 we used the HLA-A0201 restricted peptides 291, 374, 437 and 674.

Antigen-specific CD8+ T-cells could be detected against MSP-1, CSP and LSA-1 in three out of nine individuals (Volunteer 5, 40 and 44; Figure 5.28). Importantly, MSP-1 shows a comparable CD8+ T-cell response as the known liver stage candidate antigens LSA-1 and CSP (Figure 5.28). However, the CD8+ T-cell response detected after single *Pf*Spz infection was much lower compared to the response of the semi-immune individuals from Nouna (maximum 300 versus ~2200 net SFUs per 10^6 PBMCs, respectively). MSP-1 and LSA-1 specific stimulation of CD8+ T-cells occurs later than for CSP, according to their expression in late liver stages (highest response on day 10-12 versus day 7 post infection, respectively; Figure 5.28). Cytokine bead array analysis showed a balanced cytokine profile in the serum of volunteers infected with 3200 *Pf*Spz (n=9). The concentration of the pro-inflammatory cytokine IFNγ was below the detection limit (Appendix, Figure 7.5).
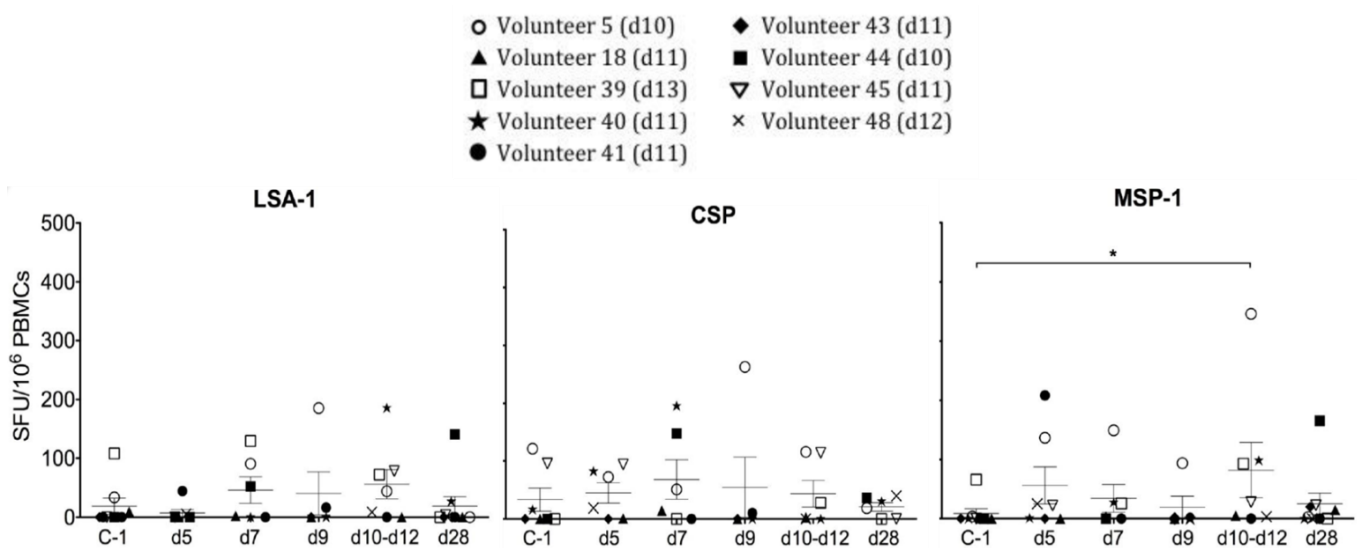
Figure 5.28: Antigen-specific CD8+ T-cell response to LSA-1, CSP and MSP-1. *Cryopreserved PBMCs from 9 malaria-positive individuals who received 3200 PfSpz i.v. were stimulated with peptide pools derived from LSA-1, CSP and MSP-1 and analyzed by 24h pre-cultured IFNγ ELISPOT assay. The net number of IFNγ-secreting cells (SFU: spot forming units) in 10^6 PBMCs is shown for each individual at different time points (C-1, day 5/7/9/10-12/28). For statistical analysis Mann-Whitney test was applied.*

6. Discussion

Since around thirty years the merozoite surface protein MSP-1 has been under consideration as malaria vaccine candidate. Its essential role in *Plasmodium* blood stages (O'Donnell et al., 2001) for erythrocyte invasion (Baldwin et al., 2015; Blackman et al., 1990; Woehlbier et al., 2006) as well as erythrocyte rupture and merozoite egress (Das et al., 2015) support the candidacy of this protein. Furthermore, a protective potential of MSP-1 has been suggested by various epidemiological studies in malaria endemic regions (al-Yaman et al., 1996; Conway et al., 2000; Egan et al., 1996; Tolle et al., 1993) as well as by immunization experiments in mice (Holder and Freeman, 1981) and monkeys (Etlinger et al., 1991; Kumar et al., 1995; Perrin et al., 1984; Siddiqui et al., 1987; Singh et al., 2006). Even though MSP-1$_{42}$ could not convince so far in clinical trials assessing protective efficacy of vaccination (Ogutu et al., 2009; Sheehy et al., 2012), several studies show that other regions within MSP-1 might be important for protection (Carralot et al., 2008; Cavanagh et al., 2004; Conway et al., 2000; Früh et al., 1991; Galamo et al., 2009; Idler, 2004; Muller et al., 1991; Siddiqui et al., 1987; Tolle et al., 1993).

The recent successful production of recombinant MSP-1D under GMP conditions allows us to study the potential of full-length MSP-1 as vaccine candidate against malaria. However, the epitopes within MSP-1 eliciting protective antibodies or T-cells and their immune mechanisms have not been clearly identified. For a vaccine candidate currently in clinical phase I trials, it is important to know how the protein is targeted by the human immune response and also to have suitable *in vitro* assays available to measure functional activity. Thus, the focus of this work was to analyze the human antibody and T-cell response against MSP-1D in individuals with naturally acquired immunity against malaria and in malaria-naïve volunteers experimentally infected with *Pf* wildtype sporozoites. Furthermore, appropriate functional *in vitro* assays have been established in our laboratory which can be used for immunological investigations during the clinical trials.

6.1. Humoral immune response against MSP-1D

The importance of antibodies for protection against malaria was already established in 1961 by passive transfer experiments of immunoglobulins (Cohen et al., 1961). However, their antigenic targets and protective mechanisms remain unclear but are urgently needed for malaria vaccine development. Correlations between direct growth inhibition of *Plasmodium* blood stages *in vitro* - as measured in the GIA – and protection against malaria have been weak and inconsistent (reviewed in (Duncan et al., 2012)), suggesting that protective antibodies may act *via* other functional mechanisms.

Indeed, recent studies strongly implicate the importance of opsonizing antibodies for naturally acquired immunity (Boyle et al., 2015; Chiu et al., 2015; Hill et al., 2013; Hill et al., 2016; Joos et al., 2010; Osier et al., 2014a; Tiendrebeogo et al., 2015). In this work, serum antibodies of semi-immune adults from Nouna, Burkina Faso, were analyzed for their ability to directly inhibit the growth of *P. falciparum* blood stages *in vitro* and also to opsonize *Plasmodium* merozoites and elicit neutrophil respiratory burst. Additionally, the contribution of MSP-1 specific antibodies to GIA and ADRB activity was determined and compared to other merozoite surface proteins.

6.1.1. Growth inhibitory potential of MSP-1 specific antibodies

Most studies so far have analyzed the direct growth inhibitory potential of MSP-1 specific antibodies *in vitro* although a correlation between this mechanism and protective immunity remains elusive. Furthermore, it is controversial whether or under which circumstances MSP-1 contributes to direct growth inhibition of *Plasmodium* blood stages since some publications reported MSP-1 specific GI activity (Bergmann-Leitner et al., 2009; Bergmann-Leitner et al., 2006; Blackman et al., 1990; Blackman et al., 1994; Egan et al., 1999; O'Donnell et al., 2000; Singh et al., 2006; Woehlbier et al., 2006) and others presented contradicting data (Bouharoun-Tayoun et al., 1990; Kumar et al., 1995; Malkin et al., 2007; McCallum et al., 2008; Wilson et al., 2011).

In this study, serum antibodies from four out of eleven semi-immune African adults showed growth inhibitory activity *in vitro* but only for the individual with the highest MSP-1 antibody titer (Donor 6) a contribution of MSP-1 specific antibodies of about 50 % to total GI activity could be detected. Interestingly, affinity-purified antibodies against MSP-1$_{83}$ of the same individual efficiently inhibited growth of *P. falciparum* blood stages *in vitro* (Woehlbier et al., 2006) supporting my results gained *via* antigen-reversal GIA. The latter was established during this work in our laboratory as an effective and quite easy approach how to measure the contribution of antigen-specific antibodies to total growth inhibitory activity *in vitro*. Since only a small amount of serum and recombinant antigen is required, it could be used to examine also other merozoite surface proteins.

The different outcomes regarding MSP-1 specific GI activity both in the previous studies as well as in this work could have several reasons: Firstly, there could be different IgG isotypes or affinities of antibodies targeting MSP-1 which partly inhibit parasite growth *in vitro*. Secondly, antibodies may target various MSP-1 epitopes and only some may have growth inhibitory potential. In rabbits immunized with MSP-1D it appears that about half of the antibodies active in the GIA are cross-reactive and about 30 % target MSP-1$_{42}$ - as shown *via* antigen-reversal GIA. However, no remarkable differences could be detected between the semi-immune African donors regarding the relative distribution of their antibodies to the MSP-1 subunits. Thirdly, the MSP-1 antibody titer may be too

low for detectable GI activity in the *in vitro* assay. The latter is a very reasonable possibility since high antibody levels are necessary for direct growth inhibitory activity *in vitro* (Wilson et al., 2011), although this might not reflect the *in vivo* situation. However, a threshold level of antibodies targeting merozoite proteins is also required for clinical immunity against malaria (Murungi et al., 2013).

6.1.2. Search for new antigens eliciting growth inhibitory antibodies by MS

Several merozoite proteins inducing growth inhibitory activity against *P. falciparum* blood stages *in vitro* are known so far, such as MSP-1_{19} (Blackman et al., 1990; Egan et al., 1999; O'Donnell et al., 2001), MSP-1_{83} (Woehlbier et al., 2006), AMA-1 (Dutta et al., 2003), *Pf*Rh5 (Douglas et al., 2014), EBA-175 (Badiane et al., 2013) GLURP (Pratt-Riccio et al., 2011), MSPDBL-1 (Chiu et al., 2015; Sakamoto et al., 2012) and MSPDBL-2 (Chiu et al., 2015). Most of these results were obtained using recombinant proteins and are thus restricted to certain antigens. An unbiased approach to identify potentially unknown antigenic targets of naturally acquired antibodies would include the separation of *P. falciparum* schizont lysate via SDS-PAGE, followed by western blot analysis using serum antibodies from semi-immune individuals and identification of recognized antigens by mass spectrometry (Eisenhut, 2007).

Since the observed *in vitro* growth inhibitory activity of two semi-immune adults from Nouna (Donor 2 & 8) was definitely independent from MSP-1 specific antibodies, we aimed to identify potentially unknown target antigens which might be responsible for the detected GI activity using the unbiased method described above. However, due to the presence of erythrocytes in the *P. falciparum* schizont lysate preparation, most of the proteins detected by MS belong to human RBCs e.g. spectrin, flotillin or band 3 anion transport protein. Although antibodies against the latter are naturally occurring and supposedly play a role in clearance of *Plasmodium* infected erythrocytes (Pantaleo et al., 2008), no bands were detected using lysed erythrocytes and the same antibody preparations from the two semi-immune donors. Thus, all human proteins detected by MS are not targeted by these antibodies. Instead, it is likely that *Plasmodium* proteins running at about the same position could not be detected by mass spectrometry since they were much less abundant compared to the erythrocyte proteins.

However, two *P. falciparum* proteins were revealed by MS analysis as potential targets of growth inhibitory antibodies: MSP-2 and MSP-9. MSP-2 is an approximately 27 kDa GPI-anchored protein, which is highly abundant on the merozoite surface and essential for *Plasmodium* blood stages (Beeson et al., 2016). Antibodies against MSP-2 have been associated with naturally acquired protection (al-Yaman et al., 1994) and can act *via* opsonic phagocytosis (Osier et al., 2014a), ADCI

(McCarthy et al., 2011), in cooperation with complement factors (Boyle et al., 2015) and high antibody levels have also been associated with growth inhibitory activity *in vitro* (Courtin et al., 2009). In a Phase IIb clinical trial in children from PNG in combination with MSP-1 and RESA (Combination B vaccine) it showed protective efficacy against the parasite, albeit only strain-specific (Genton et al., 2003; Genton et al., 2002). A major obstacle to MSP-2 based vaccine design appears to be its high polymorphism (Fenton et al., 1991).

MSP-9 (ABRA), a 101 kDa protein on the surface of merozoites and the parasitophorous vacuole, binds in a complex with MSP-1_{42} (Li et al., 2004) to band 3 on human erythrocytes and is involved in RBC invasion (Beeson et al., 2016). Although MSP-9 is considered as vaccine candidate against malaria, no reports are available about growth inhibitory antibodies targeting this protein. Furthermore, there is no vaccine development at clinical stage for MSP-9 (Beeson et al., 2016).

For future research, a better separation of the proteins from *P. falciparum* schizont lysate or merozoites using 2D electrophoresis would probably benefit the identification of *Plasmodium* proteins by MS. However, this step would also require more material (*Plasmodium* schizont lysate or merozoites) and special equipment. Furthermore, the erythrocytes present in the parasite schizont preparation should be completely removed in order to enable MS identification of low-abundant *Plasmodium* proteins.

6.1.3. Correlation between neutrophil respiratory burst and protection

Besides direct growth inhibition of *Plasmodium* blood stages we were interested whether MSP-1 specific antibodies may be able to opsonize *Plasmodium* merozoites and recruit immune effector cells. Antibodies which opsonize *P. falciparum* merozoites and mediate the release of reactive oxygen species (ROS) from neutrophils can be measured in the antibody-dependent respiratory burst (ADRB) assay (Joos et al., 2010) (Figure 5.7). Oxidant damage mediated by ROS can kill the parasite (Allison and Eugui, 1983; Brown and Smalley, 1981; Clark and Hunt, 1983; Golenser et al., 1992; Gopalakrishnan and Kumar, 2015) and is associated with protection against malaria (Friedman, 1979; Greve et al., 1999; Joos et al., 2010). Interestingly, the antimalarial drugs mefloquine and artesunate cause parasite death by generation of ROS (Gopalakrishnan and Kumar, 2015; Gunjan et al., 2016) and ROS also protects against severe malaria in sickle and fetal erythrocytes (Cyrklaff et al., 2016) as well as in thalassemic and G6PD deficient red blood cells (Friedman, 1979). However, if high ROS secretion might also play a role in malaria complications such as anemia (Greve et al., 2000) requires further investigation.

ROS secretion from neutrophils can be induced by merozoite-specific antibodies (Joos et al., 2010; Kumaratilake et al., 1992; Salmon et al., 1986). Since neutrophils constitute the largest group of blood leukocytes in mammals and are highly mobile phagocytes, they represent possible immune effector cells. Furthermore, ROS production and release by neutrophils happens very fast – the peak of chemiluminescence activity measuring ROS was detected after approximately 2 minutes (Joos et al., 2010) – and thus, it is plausible that ROS can kill *Plasmodium* merozoites, which *in vitro* remain free after schizont rupture for several minutes before invading a new erythrocyte (Boyle et al., 2010b). Additionally, reactive oxygen species can probably target *Plasmodium* parasites also inside erythrocytes (Allison and Eugui, 1983; Clark and Hunt, 1983; Greve et al., 1999).

Interestingly, in malaria endemic regions higher age (Perraut et al., 2014) and a more diverse anti-merozoite antibody repertoire (Murungi et al., 2016) promote ADRB activity suggesting that it plays a role in naturally acquired immunity. Indeed, ADRB activity correlates with protection from clinical malaria in meso- and holoendemic areas in Senegal (Joos et al., 2010) as well as with protection against severe malaria in Kenyan children within the first 6 month of life (Murungi et al., 2017). Furthermore, in 28 Kenyan volunteers experimentally infected with *Pf* sporozoites, only the individual showing the highest ADRB activity could remarkably reduce parasite multiplication and parasites were not detectable in the blood by microscopy up to day 21 (Hodgson et al., 2016). Despite a poor correlation between ADRB and GIA activity (Murungi et al., 2016; Osier et al., 2014a), total IgG active in both assays is associated with protection against severe malaria in Kenyan children (Murungi et al., 2016). Thus, protective immunity may result from a combination of antibody-mediated mechanisms.

6.1.4. Establishment of the ADRB and antigen-reversal ADRB assay

In this work, the ADRB assay (Joos et al., 2010) was successfully established in our laboratory and a much higher activity was observed with serum antibodies derived from semi-immune African individuals compared to malaria-naïve volunteers. Furthermore, the assay showed a good reproducibility, even with different *P. falciparum* merozoite and neutrophil preparations. For detection of neutrophil respiratory burst we employed the chemiluminescent reagent isoluminol which particularly detects extracellular ROS (Dahlgren and Karlsson, 1999); the latter is considered to be especially toxic for *Plasmodium* parasites (Allison and Eugui, 1983; Clark and Hunt, 1983; Greve et al., 1999).

Interestingly, remarkably lower antibody concentrations were required (about 40-fold) compared to the GIA assay. The antibody subtypes IgG1 and IgG3 are considered as the main actors against *Plasmodium* merozoites (Reiling et al., 2010; Richards et al., 2010) and - by interaction with the Fc

receptor of immune cells or with complement factors - they are supposed to be responsible for opsonic phagocytosis by monocytes (Osier et al., 2014a), complement-dependent inhibition (Boyle et al., 2015) and ADRB activity (Joos et al., 2010). However, comparable ADRB activity was obtained with Protein A or Protein G affinity-purified antibodies although human IgG3 is only purified *via* Protein G chromatography. Thus, IgG1 seems to be most important for ADRB activity. Alternatively, the opsonizing capacity of IgG3 might be replaced by the lower opsonizing potential of IgA and/or IgM for sera purified *via* Protein A (Figure 5.11 B). The induction of neutrophil respiratory burst by IgA has been reported (Shi et al., 2011).

Limitations of the ADRB assay include: (i) Neutrophils are quite short-lived with a half-life of 6-8 hours *in vivo* and thus, need to be purified freshly from human volunteers and used within one hour after purification for the ADRB assay. (ii) Since antibodies have to match human Fcγ receptors present on neutrophils, antibodies generated by immunization in most animals, such as mice or rabbits, cannot be tested. However, this work shows that antibodies from *rhesus* monkeys are compatible with human Fcγ receptors (details in 6.1.7). (iii) Neutrophil donors may vary in their expression of Fcγ RII A or B (= CD32) (Llewellyn et al., 2015), an important Fcγ receptor on human neutrophils responsible for respiratory burst and phagocytosis (Huizinga et al., 1989; Mitchell et al., 1994). Interestingly, different polymorphisms in Fcγ RII A or B associate either with protection against malaria (Cherif et al., 2015; Ouma et al., 2012; Shi et al., 2001) or with an increased susceptibility to severe malaria (Cooke et al., 2003; Omi et al., 2002). In order to improve reproducibility of the ADRB assay performed with various neutrophil donors, we pooled the neutrophils from three donors for each assay and normalized the data to a standard, an IgG pool made from the eleven semi-immune African donors.

Several approaches can be used in order to quantify the contribution of antigen-specific antibodies to opsonizing activity. Firstly, antibodies targeting the antigen of interest could be generated by immunization of animals and tested for their opsonizing capacity (Boyle et al., 2015). Secondly, transgenic parasites could be produced where the antigen of interest is either replaced (Joos et al., 2015) or knocked out (Hill et al., 2016). And thirdly, affinity purified antibodies against merozoite antigens can be assessed for their opsonizing potential e.g. *via* ADCI (Oeuvray et al., 1994; Singh et al., 2009) or OPA (Chiu et al., 2015; Osier et al., 2014a). However, antigen-specific affinity purification of antibodies is quite time-consuming, requires special equipment for immuno-affinity chromatography and uses up a relatively large amount of serum and antigen.

Here, two experimental approaches were employed to quantify the contribution of single antigens to ADRB activity, affinity purification of MSP-1 specific antibodies and antigen-reversal ADRB. In the latter, pre-incubation of serum antibodies with recombinant antigens allows us to quantify the

contribution of certain antigens to ADRB activity. Since this relatively easy approach requires only a small volume of serum and recombinant antigen it could be readily adapted to other merozoite antigens. Prior to their use in antigen-reversal ADRB experiments, all recombinant proteins were pre-tested in absence of merozoites. Importantly, all showed only background chemiluminescence activity. Furthermore, the control protein BSA did not cause a reduction in ADRB activity, indicating that the results gained *via* antigen-reversal ADRB assay using merozoite surface proteins are probably specific. Remarkably, the antigen-reversal ADRB requires very low protein concentrations: a large decrease in ADRB activity was already observed with 2 nM recombinant MSP-1D using human IgG from semi-immune individuals; the minimum was reached at approximately 50 nM MSP-1D (Figure 5.16). An excess protein concentration of 500 nM was used for all experiments and thus, it can be assumed that all antigen-specific antibodies are bound to the respective protein.

6.1.5. ADRB activity of MSP-1 specific antibodies in semi-immune individuals

In this work, we provide further support for opsonizing antibodies mediating respiratory burst of neutrophils in individuals with naturally acquired immunity against malaria and identified PfMSP-1_{83} as major target antigen of ADRB activity. We show that the antibody titer to MSP-1D correlates with ADRB activity in the eleven semi-immune individuals from Burkina Faso. Furthermore, MSP-1 specific antibodies purified *via* affinity chromatography to recombinant MSP-1D from two African donors (Donor 2 and 8) are able to opsonize *P. falciparum* merozoites and elicit neutrophil respiratory burst while the removal of MSP-1 specific antibodies from total IgG causes a substantial decrease in ADRB activity. Interestingly, the same IgG fractions from the two African donors could not directly inhibit the growth of *P. falciparum* blood stages *in vitro* – as tested *via* GIA assay. Moreover, by using serum antibodies from the eleven African volunteers in the antigen-reversal ADRB and MSP-1D as competitor protein, ADRB activity observed against the heterologous strain *P. falciparum* (FCB1) was reduced almost as strongly as against the homologous strain (3d7). Thus, opsonizing antibodies eliciting neutrophil respiratory burst seem to be mainly cross-reactive. Importantly, we identified MSP-1 and especially its partially conserved subunit MSP-1_{83} as an important target of opsonizing antibodies (the mean reductions observed in the antigen-reversal ADRB were 58.6 % and 50.2 %, respectively).

So far, only one antigenic target of ADRB activity has been identified – the conserved C-terminal part of PfMSP-1, MSP-1_{19} (Joos et al., 2015). Antibodies to MSP-1_{19} were shown to contribute about 33 % to total neutrophil respiratory burst activity in two Senegalese villages using isogenic *P. falciparum* parasites with the non-cross-reactive MSP-1_{19} orthologue from *P. chabaudi* and sera after removal of MSP-1_{19} antibodies (Joos et al., 2015). Since we used alternative experimental approaches, an ADRB protocol, which differs in certain aspects such as the use of purified IgG instead of sera and

individuals from another African country, a direct comparison of our results with the previous study is not possible. However, we show that antibodies acquired by natural exposure to malaria opsonize *P. falciparum* merozoites of different strains equally well and ADRB activity against the *P. falciparum* FCB1 strain can be efficiently reduced with MSP-1 from the 3d7 strain (MSP-1D). Thus, cross-reactive regions within MSP-1 – present in both MSP-1$_{19}$ and MSP-1$_{83}$ - appear to be the main targets of opsonizing antibodies which elicit neutrophil respiratory burst.

In contrast, it was reported that antibodies against MSP-1$_{block2}$ - located within MSP-1$_{83}$ - show activity against *P. falciparum* by monocyte-dependent inhibition detected *via* ADCI (Galamo et al., 2009) as well as by fixation of complement factors (Boyle et al., 2015). Due to the high cross-reactivity observed in the ADRB assay it seems unlikely that antibodies targeting the oligomorphic region MSP-1$_{block2}$ play a major role in neutrophil respiratory burst. Interestingly, opsonizing antibodies which recruit monocytes for merozoite phagocytosis - as measured in the OPA - are effective against 15 different parasite strains indicating a high strain-transcendence (Hill et al., 2016). Thus, opsonizing antibodies functional in ADRB or OPA seem to mainly target cross-reactive regions within merozoite antigens, which would be beneficial for vaccine development.

6.1.6. Comparison of MSP-1 specific ADRB activity to other MSPs

Having identified MSP-1 as important target of opsonizing antibodies eliciting neutrophil respiratory burst, we aimed to compare the contribution of MSP-1 to ADRB activity with other merozoite surface proteins. The recombinant proteins MSP-3, MSP-9 and MSPDBL-1 were kindly provided from Prof. A. Cowman & Dr. C. Lin (Walter and Eliza Hall Institute of Medical Research, Melbourne). All are peripheral MSPs which interact with MSP-1 on the merozoite surface and are shed following invasion (Li et al., 2004; Lin et al., 2016); for MSPDBL-1 also an interaction with erythrocytes has been reported (Lin et al., 2016; Sakamoto et al., 2012). Importantly, MSP-3 and MSPDBL-1 belong to the same protein family - the MSP-3 multigene family (MSP-3.1 – MSP-3.8) – and both contain the C-terminal SPAM domain, which is conserved among different *P. falciparum* isolates and induces cross-reactive antibodies with opsonizing activity (Oeuvray et al., 1994; Singh et al., 2009). Thus, both MSP-3 and MSPDBL-1 have been described as targets of opsonizing antibodies effective *via* ADCI (Druilhe et al., 2005; Lundquist et al., 2006; Oeuvray et al., 1994; Singh et al., 2009) as well as *via* OPA (Chiu et al., 2015; Osier et al., 2014a). ADRB activity has not been reported so far for the two proteins. Interestingly, a correlation between antibody titers to MSP-3 (Osier et al., 2014a) and MSPDBL-1 (Chiu et al., 2015; Tetteh et al., 2013) with protection against malaria has been reported. Furthermore, vaccination of children from Burkina Faso with the conserved part of MSP-3 not only induced antibodies active *via* ADCI (Druilhe et al., 2005) but also reduced malaria episodes significantly (Sirima et al., 2011).

Regarding MSP-9, which has not yet progressed to clinical trials, nothing has been reported yet about its ability to elicit opsonizing antibodies. In the antigen-reversal ADRB performed with the respective competitor proteins and antibodies from the eleven semi-immune African individuals, the average ADRB activity could be reduced almost 50 % using 500 nM recombinant MSP-9, around 60 % with MSP-1D and almost 90 % using MSP-3 or MSPDBL-1, respectively. For the latter two proteins, likely the same cross-reactive opsonizing antibodies, which target the conserved SPAM domain, are responsible for ADRB activity. Interestingly, the reductions observed with these four proteins add up to more than 100 %, even though antibodies to other merozoite proteins may also contribute to neutrophil respiratory burst activity. Thus, it seems that the results of the antigen-reversal ADRB assay should be interpreted in a qualitative rather than quantitative manner.

Besides the complexity of the antigen-reversal ADRB assay which may make an exact quantification regarding the contribution of single antigens to ADRB activity impossible, other explanations may be possible: (i) there could be cross-reactive antibodies with opsonizing potential not only between MSP-3 and MSPDBL-1 but between all four proteins tested and thus, upon addition of one of these proteins in excess all antibodies will bind. However, a blast-p alignment between MSP-1, MSP-3, MSP-9 and MSPDBL-1 showed no conserved or similar regions except the SPAM domain present in MSP-3 and MSPDBL-1. (ii) Since MSP-3, MSP-9 and MSPDBL-1 can all bind to MSP-1 (Li et al., 2004; Lin et al., 2016) and we add one of these proteins in excess to the antigen-reversal ADRB, it might be possible that not all protein molecules are covered with antibodies and could bind to free MSP-1 on the merozoite surface. Thus, MSP-3/-9/DBL-1 may sterically hinder access of opsonizing antibodies, e.g. against MSP-1, to the merozoite surface and cause a higher reduction of neutrophil activation. (iii) Antibodies bound to the competitor protein might still be able to bind to the neutrophil *via* their Fc part. However, this might not result in cross-linking of neutrophil Fc receptors which is crucial for their activation but maybe spatially block the access for opsonized merozoites.

In order to exclude the possibilities listed above which may disturb neutrophil respiratory burst activity in presence of the peripheral merozoite surface proteins MSP-3/-9/-DBL-1, ideally the protein-antibody complexes should be removed prior to performance of the ADRB. This approach was recently described for antibodies targeting MSP-1$_{19}$ (Joos et al., 2015): by using hexa-histidine-tagged MSP-1$_{19}$, antigen-antibody complexes could be removed by binding of the his-tag to metal-chelate affinity resin and thus, sera were depleted from antigen-specific antibodies prior to ADRB experiments. Additionally, a *Plasmodium* non-merozoite surface protein - such as AMA-1 or an intracellular protein - could be included as further control for antigen-reversal ADRB experiments. In conclusion, the results indicate that antibodies to several merozoite surface proteins - including MSP-1, MSP-3, MSP-9 and MSPDBL-1 - can opsonize merozoites and induce neutrophil respiratory burst.

6.1.7. Induction of ADRB activity in *rhesus* monkeys by immunization with MSP-1

Importantly, opsonizing antibodies which cause neutrophil respiratory burst could be induced in *rhesus* monkeys by immunization with recombinant MSP-1D and adjuvant CoVaccine HT (Mahdi Abdel Hamid et al., 2011). Antibody titers against MSP-1D and ADRB activity were comparable between an IgG pool obtained from the eleven semi-immune African individuals and the immunized *rhesus* monkeys. This finding has several major implications. Firstly, we demonstrate here for the first time that antibodies raised in non-human primates are compatible with human Fcγ receptors on neutrophils. So far, this has been one of the major limitations of the ADRB assay since no animal species was known in which compatible antibodies can be generated and neutrophils from other species such as mice differ substantially from human neutrophils regarding their Fcγ receptor expression and binding abilities (Bruhns, 2012). Secondly, we show for the first time that antibodies functional in ADRB can be induced by immunization with a recombinant protein. In a recent study, ADRB effective antibodies could not be induced by vaccination of mice with AdHu5-*Py*MSP-1$_{42}$ but by primary infection with *P. yoelii* (Llewellyn et al., 2014). Furthermore, a single infection with cryopreserved radiation-attenuated *Pf* sporozoites did not elicit ADRB activity in malaria-naïve individuals (Hodgson et al., 2016). Opsonizing activity of antibodies raised in mice or rabbits by immunization with different merozoite antigens has been recently reported for opsonic phagocytosis (Quintana Mdel et al., 2016) and complement-dependent inhibition (Boyle et al., 2015). However, this is the first report on opsonizing antibodies induced in primates. Thirdly, we reveal that immunization with recombinant MSP-1D generates antibodies which are (i) long-lasting with undiminished ADRB activity more than four month after last immunization, (ii) mainly cross-reactive showing almost comparable activity against *P. falciparum* 3d7 and FCB1 parasites, (iii) targeting primarily conserved and dimorphic regions within MSP-1$_{83}$, and (iv) active *via* the same functional mechanisms as naturally acquired antibodies, thus supporting full-length MSP-1 as promising vaccine candidate.

6.1.8. Antibodies targeting MSP-1 act *via* different mechanisms

Besides direct growth inhibition of *P. falciparum* blood stages *in vitro*, MSP-1 also elicits opsonizing antibodies which can act at much lower antibody levels compared to GIA. MSP-1$_{19}$ (Blackman et al., 1990; Egan et al., 1999; O'Donnell et al., 2001), MSP-1$_{83}$ (Woehlbier et al., 2006) and epitopes distributed within the complete MSP-1 protein (Woehlbier et al., 2006) have been described as targets of growth inhibitory antibodies. Interestingly, antibodies effective *via* direct growth inhibition *in vitro* seem to decline with increasing age in malaria highly endemic regions (Courtin et al., 2009; Dent et al., 2008; McCallum et al., 2008) suggesting that these antibodies may be involved in protection against severe malaria during childhood. In contrast, opsonizing antibodies active in ADCI

(Tiendrebeogo et al., 2015), OPA (Osier et al., 2014a), complement-dependent inhibition (Boyle et al., 2015) and ADRB (Joos et al., 2010; Perraut et al., 2014) increase with age and thus, may be important for naturally acquired immunity against malaria. The activity of naturally acquired antibodies in both GIA and ADRB is weakly correlated (this work) or poorly correlated (Murungi et al., 2016), indicating that these functional mechanisms may be mediated by various antibody subsets.

Additionally, for MSP-1 specific opsonizing antibodies also different immune mechanisms have been described: (i) the recruitment of monocytes by MSP-1$_{block 2}$ antibodies measured *via* ADCI (Galamo et al., 2009), (ii) the fixation of complement factors by antibodies directed against MSP-1$_{19}$ and MSP-1$_{block 2}$ as detected *via* antibody-mediated complement dependent inhibition (Boyle et al., 2015), (iii) the induction of neutrophil respiratory burst analyzed *via* ADRB assay and reported for antibodies to MSP-1$_{19}$ (Joos et al., 2015) and MSP-1$_{83}$ (this work). However, MSP-1$_{19}$ antibodies did not seem to play a role in monocyte opsonic phagocytosis since the OPA response for transgenic *P. falciparum* merozoites with the MSP-1$_{19}$ orthologue from *P. chabaudi* did not differ from wildtype parasites (Hill et al., 2016). Furthermore, we observed a high cross-reactivity between different parasite strains in the ADRB suggesting that antibodies to the highly polymorphic block 2 of MSP-1 which is located within MSP-1$_{83}$ likely plays no role for neutrophil respiratory burst activity. Thus, opsonizing antibodies elicited by different regions within MSP-1 appear to act by various independent immune mechanisms. Interestingly, higher antibody levels and their action *via* multiple mechanisms is associated with naturally acquired immunity (Murungi et al., 2013; Rono et al., 2013) suggesting that several antibody-mediated mechanisms contribute to protection against malaria. Since the antigenic epitopes within MSP-1 are distributed over the whole protein and elicit a variety of immune responses, we propose full-length MSP-1 as malaria vaccine candidate.

6.2. Cellular immune response against MSP-1D

Many studies within the last 30 years indicate the crucial role of cytotoxic CD8+ T-cells targeting *Plasmodium* liver stages in infected hepatocytes for protection against malaria (reviewed e.g. in (Frevert and Krzych, 2015)). Although CD8+ T-cells can kill intracellular parasites directly *via* cytolytic activity (White et al., 1996), their release of the cytokine IFNγ appears to be more important against *Plasmodium*. IFNγ released by certain immune cells such as CD4+ Th1 cells and CD8+ cytotoxic T-cells inhibits the development of pre-erythrocytic *Plasmodium* stages in hepatocytes and is absolutely required for sterile immunity to sporozoite challenge (Ferreira et al., 1986; Imai et al., 2010; Mellouk et al., 1987; Schofield et al., 1987). Importantly, in all whole sporozoite vaccination approaches – RAS, GAP and CPS – sterile protection appears mostly mediated by IFNγ-producing CD8+ T-cells (Brando et al., 2014; Epstein et al., 2011; Jobe et al., 2007; Lewis et al., 2015; Mueller et al., 2007; Nganou-Makamdop et al., 2012; Tarun et al., 2007; Weiss and Jiang, 2012).

Priming of parasite-specific CD8+ T-cells can occur already in the lymph nodes by CD8α+ dendritic cells presenting antigens from migratory *Plasmodium* sporozoites *via* their HLA-I receptor (Cockburn and Zavala, 2016; Radtke et al., 2015). Furthermore, priming also takes place in the liver where it is mediated by CD8α+ dendritic cells (Cockburn and Zavala, 2016; Jobe et al., 2009) or infected hepatocytes (Balam et al., 2012; Cockburn et al., 2014; Frevert and Krzych, 2015). However, direct intravenous injection of *Plasmodium* sporozoites – as done currently in most whole parasite vaccination approaches – probably does not pass through the lymph nodes and thus, protective T-cells may only be induced in the liver. Interestingly, imaging analysis have revealed that CD8+ T-cells cluster around infected hepatocytes which present parasite-derived antigens *via* their HLA-I receptors (Cockburn et al., 2013) and contribute to protection (Huang et al., 2015). Since MSP-1 is already expressed by *Plasmodium* late liver-stages in infected hepatocytes (Haussig et al., 2011; Szarfman et al., 1988) it potentially elicits a protective T-cell response in the liver.

6.2.1. MSP-1 specific T-cell response: what is known so far?

A MSP-1 specific T-cell proliferation has been observed in volunteers immunized with irradiated *P. falciparum* sporozoites (Krzych et al., 1995): three out of four vaccinated individuals recognized both pre-erythrocytic (CSP and LSA-1) and blood stage antigens (MSP-1, MSP-2, SERA-1) – as detected *via* proliferation assay using ^{3}H-thymidine. Regarding MSP-1, four recombinant fragments from the *Pf* CAMP strain, which is very similar to the 3d7 strain, were used for stimulation of PBMCs.

Up to date, several studies have analyzed the T-cell response against mostly MSP-1$_{19}$ or MSP-1$_{42}$ in African individuals with naturally acquired immunity against malaria, focusing on CD4+ T-cells. Lymphocyte preparations from African adults living in malaria-endemic regions recognize MSP-1$_{19}$

(Bisseye et al., 2011) and MSP-1$_{42}$ (Dent et al., 2009; Moormann et al., 2013) recombinant proteins or CD4+ T-cell epitopes thereof. Additionally, a response of African children to several recombinant MSP-1 derived fragments (Riley et al., 1992a) and to a CD4+ T-cell epitope located within MSP-1$_{83}$ (aa 20-39) (Chelimo et al., 2003; Quakyi et al., 1994) has been reported. Importantly, lymphocytes from African individuals secrete IFNγ upon stimulation with MSP-1$_{19}$ (Bisseye et al., 2011) or MSP-1$_{42}$ (Bowman et al., 2016; Chelimo et al., 2011; Dent et al., 2009; Moormann et al., 2013). Furthermore, MSP-1 specific IFNγ production increases with age in children from Kenya (Chelimo et al., 2011; Chelimo et al., 2003) and also in individuals from Gambia (Riley et al., 1992a). Importantly, a correlation with protection against malaria has been reported for the T-cell response against MSP-1$_{42}$ in Kenyan children and adults (Bowman et al., 2016; Moormann et al., 2013).

In contrast, the MSP-1$_{19}$ fragment is supposed to be a poor T-cell target due to its disulfide bridges hindering protein processing and presentation (Egan et al., 1997). Thus, MSP-1$_{19}$-based vaccinations - even *via* viral vectors (Douglas et al., 2010) - could not elicit a CD8+ T-cell response. Interestingly, in mice immunized with MSP-1$_{42}$ using viral vectors (AdHu5-MVA), CD4+ and CD8+ T-cells targeting MSP-1$_{33}$ were induced. For vaccinated mice increased serum IFNγ levels and a reduced *Plasmodium* liver burden were detected, which was associated with superior protection upon sporozoite challenge (Draper et al., 2009).

Even though CD4+ T-cells seem to play a role in controlling *Plasmodium* blood stages (Perlmann and Troye-Blomberg, 2000; Quakyi et al., 1994; Riley et al., 1992b; Troye-Blomberg et al., 1994), CD8+ T-cells are considered as the key players against *Plasmodium* liver stages and crucial for protection against malaria. For example, depletion of CD8+ but not CD4+ T-cells *in vivo* reverses RAS-induced sterile protection in mice (Schofield et al., 1987; Weiss et al., 1988) and primates (Weiss and Jiang, 2012). Furthermore, RAS-immunized mice with a defect HLA-I receptor are not protected against parasite challenge (White et al., 1996). Interestingly, naïve recipient mice could be protected against parasite infection by transfer of CD8+ T-cells from immune mice (Imai et al., 2010; Lewis et al., 2015; Schofield et al., 1987). Thus, CD8+ T-cells appear to be crucially required for sterile protection against malaria infection while CD4+ T-cells may contribute to protective immunity e.g. by inducing B-cells to produce protective antibodies (Brando et al., 2014).

Importantly, CD8+ T-cell epitopes for the HLA haplotype A0201 have been identified in full-length MSP-1D (Carralot, 2008; Idler, 2004). Peptide 291 dominated the CD8+ T-cell response in HHD mice immunized with MSP-1D while peptide 674 was identified under physiological conditions by mass spectrometry (details in 5.3.1). Both CD8+ T-cell epitopes, 291 and 674, are located in dimorphic regions within MSP-1$_{38}$ and MSP-1$_{83}$, respectively. Thus, they were not included in previous studies reported so far.

6.2.2. HLA-A0201 restricted MSP-1 specific CD8+ T-cell response

In this work, peripheral blood mononuclear cells (PBMCs) from semi-immune African and malaria-naïve European individuals were used to analyze the MSP-1 specific CD8+ T-cell response. Thus, monocytes, B-cells and dendritic cells present in peripheral blood were employed as antigen-presenting cells. If MSP-1 derived epitopes are presented by these cells *via* their HLA-I receptors to cytotoxic CD8+ T-cells leading to production of IFNγ, it will be most likely that MSP-1 can also induce a CD8+ T-cell response in the liver. Recently, it was even reported that in RAS-immunized mice memory CD8+ T-cells, which are essential for protection against sporozoite challenge, remain constantly in the liver (Fernandez-Ruiz et al., 2016). Interestingly, in nonhuman primates immunized with RAS, *P. falciparum*-specific CD8+ T-cells secreting IFNγ showed an approximately 100 fold higher frequency in the liver compared to blood (Ishizuka et al., 2016). Thus, we can assume that if we detect a MSP-1 specific T-cell response using human blood samples, the actual immune reaction in the liver of these individuals might be much higher.

Since *Plasmodium*-specific CD8+ T-cells are known to be present at very low frequency in peripheral blood from individuals with naturally acquired immunity (Lalvani et al., 1996; Plebanski et al., 1997), we had to choose a highly sensitive detection method – the IFNγ ELISPOT assay. The latter shows the lowest detection limit regarding all *in vitro* assays available so far to measure cytokine producing T-cells, such as lymphoproliferation, cytotoxicity assays or intracellular cytokine staining combined with flow cytometry. Strikingly, its sensitivity is about 400 fold higher compared to conventional ELISA. Since the ELISPOT method is quite robust without using radioactivity, it probably can be also used in tropical regions for field studies.

In this part, HLA-A0201 restricted CD8+ T-cell epitopes located within the complete MSP-1D protein (Figure 5.21) and identified previously in the laboratory of Prof. Bujard (Carralot et al., 2008; Idler, 2004) were used for stimulation of PBMCs from semi-immune African adults from Burkina Faso and from malaria-naïve European controls. Importantly, PBMCs from all African individuals, which were available in sufficient quantity and quality and thus, could be tested *via* IFNγ ELISPOT assay, reacted against the MSP-1 epitopes 291, 674 and 374 and/or 437 (Figure 5.22 A). In contrast, PBMCs from only one out of four tested malaria-naïve individuals cross-reacted against peptide 291. The amino acid sequence of 291 was examined by blast-p analysis indicating that this peptide is not only present in *P. falciparum* MSP-1 but also in certain bacteria such as *Streptomyces*, *Kribella or Enterococcus faecium* (see Appendix 7.7), which may explain the observed cross-reaction. However, despite the limited sample size a significant difference could be detected for the CD8+ T-cell response of semi-immune compared to malaria-naïve individuals to the MSP-1 specific peptide 674 and to a peptide pool consisting of 291+374+437 (Figure 5.22 C/D).

Interestingly, a ChAd63-MVA-vectored MSP-1 vaccine construct - consisting of conserved regions and MSP-1$_{42}$ - could induce a T-cell response in vaccinated volunteers but no protection against mosquito bite challenge (Sheehy et al., 2012). However, their vaccine construct did not contain the potentially protective CD8+ T-cell epitopes 291, 374 and 674 which are all located in dimorphic regions.

There are several limitations of the results obtained using HLA-A0201 restricted CD8+ T-cell epitopes and the PBMCs tested: (i) the sample size was quite small - PBMCs with sufficient quality were available from 3 semi-immune and 4 malaria-naïve individuals, (ii) the quality of the African PBMC preparations was poor due to interruptions of the cold chain in Burkina Faso or during transportation, thus the quite fragile monocyte and dendritic cell populations were missing in the PBMCs from semi-immune individuals (see Appendix 7.5) and a high cell death rate was observed during cultivation. We can assume that the T-cell response might be even higher with intact PBMC preparations. (iii) We do not know if individuals with different – possibly even closely related – HLA subtypes can present the identified HLA-A0201 restricted MSP-1 specific epitopes to CD8+ T-cells since each HLA-type may present different antigenic epitopes (Barouch et al., 1995). (iv) The identified CD8+ T-cell epitopes within MSP-1D – 291, 374, 437 and 674 – are all located in dimorphic regions and their amino acid sequence may differ between *P. falciparum* parasite strains. Aligning the amino acid sequence of MSP-1D for the respective peptides between 3d7 and FCB1 strains shows, that there is one amino acid difference for 291 and 437 and two for 674; 374 differs in almost all amino acids (Figure 6.1). Thus, it is unknown whether these MSP-1 specific epitopes may elicit a CD8+ T-cell response only against the homologous strain 3d7 or also against other parasite strains. However, all these limitations can be overcome by analyzing a larger cohort of semi-immune African as well as malaria-naïve control individuals using an HLA-independent approach and including many potential MSP-1 specific T-cell epitopes.

Figure 6.1: Comparison of the amino acid sequence within MSP-1 between P. falciparum 3d7 and FCB1. Both MSP-1 specific CD8+ T-cell epitopes 674 and 291 are located in dimorphic regions within MSP-1$_{83}$ (block 7) and MSP-1$_{38}$ (block 13), respectively. There are two (674) or one (291) amino acid differences detected between Pf 3d7 or FCB1 strains. The program Clustal 2.1. was used for alignment.

6.2.3. HLA-independent analysis of the MSP-1 specific T-cell response

One possibility for an HLA-independent approach to investigate the MSP-1 specific T-cell response would be the use of comprehensive peptide pools covering all frequent HLA-types for stimulation of PBMCs and following analysis *via* IFNγ ELISPOT assay. However, potential CD8+ T-cell epitopes within MSP-1D - predicted by the artifical neural networks (ANN)-algorithm (Honeyman et al., 1998) to several HLA-types - are distributed throughout the whole protein (Figure 5.23 A), suggesting that the complete MSP-1D sequence should be taken into account. Furthermore, a diverse pattern of frequent HLA-types can be detected in Africa and Europe e.g. in Mali (Cao et al., 2004) and Germany (Source: Bone marrow registry, n ≈ 40 000) (Figure 5.23 B/C). In order to cover about 80 % of HLA-alleles in Germany, 5 peptide pools would be required but at most two of them (for HLA-A0201 and C0401) could be also used for individuals from Mali. Thus, separate peptide pools spanning the complete MSP-1 sequence would have to be synthesized in order to analyze the T-cell response from semi-immune African individuals as well as malaria-naïve European controls.

Another HLA-independent approach – the use of complete proteins such as MSP-1D as stimulus for PBMCs – shows several advantages: (i) it is a much easier and cheaper method compared to the use of large peptide pools, (ii) since the complete proteins contains both CD4+ and CD8+ T-cell epitopes, a more comprehensive picture of the MSP-1 specific T-cell response will be obtained and (iii) the protocol may be adapted to test further antigens such as MSP-1F, MSP-6 or MSP-7. The application of protein antigens, e.g. tetanus toxoid, in IFNγ ELISPOT assay has been reported previously (Schmittel et al., 2001) and seems to require a pre-incubation period in presence of the protein stimulus as well as monocytes as antigen-presenting cells.

In this work, the IFNγ ELISPOT assay was successfully modified for the use of complete MSP-1D protein. A pre-incubation period (24 h) of PBMCs in presence of MSP-1D appears to be crucial for protein processing and presentation, presumably by monocytes (Schmittel et al., 2001), while this step could be omitted using short peptides (Figure 5.24). A MSP-1D concentration of 5 µg/ml was found to be optimal. However, these results were obtained using PBMCs from the cross-reactive malaria-naïve individual. PBMCs from the semi-immune African adults from Burkina Faso were also tested but showed no reaction to MSP-1D protein since professional antigen-presenting cells (monocytes and dendritic cells), which are crucial for protein processing and presentation (Schmittel et al., 2001), were missing in their PBMC preparations (Appendix 7.5). The three malaria-naïve individuals not cross-reacting to MSP-1 specific peptides showed also no response to MSP-1D protein indicating that the detected T-cell response to MSP-1D may be specific. However, these results should be verified using intact PBMC preparations from malaria-exposed individuals.

Originally, we aimed to apply the HLA-independent MSP-1 protein approach to analyze the MSP-1 specific T-cell response of 210 children from Samako, Mali, for which blood samples were taken at different time points before, during and after malaria transmission season and detailed health records were available. Thus, it would have been possible to investigate a potential correlation between MSP-1 specific T-cell response and protection against malaria. Unfortunately, the PBMCs which arrived in Heidelberg (~ 1400 cryotubes) were all dead and could not be used (Figure 5.25). Thus, these questions may be addressed in the future as soon as intact PBMCs from a suitable cohort of malaria-exposed individuals are available.

6.3. TUECHMI I

Besides the MSP-1 specific immune response in individuals with naturally acquired immunity against malaria, we were also interested in immune responses elicited in malaria-naïve volunteers by experimental *P. falciparum* sporozoite infection. In TUECHMI I – a controlled human malaria infection study performed in Tübingen in 2011/12 – thirty malaria-naïve volunteers were infected intravenously or intradermally with different doses of cryopreserved *Pf* sporozoites (NF54 strain) from Sanaria. Results of this dose-finding trial have already been published (Mordmuller et al., 2015) showing safety and tolerability of *Pf* sporozoite challenge and the detected parasitemia for each individual but not the induced immune responses. In 22 out of 30 infected malaria-naïve volunteers parasites could be detected in the blood by microscopy 10-15 days after infection. Importantly, in the highest dose group – 3200 *Pf* sporozoites by intravenous injection – 100 % of individuals (9/9) developed malaria (Figure 5.26 B) (Mordmuller et al., 2015). This finding laid the foundations for TUECHMI II (Mordmuller et al., 2017), in which malaria-naïve volunteers were immunized 3x intravenously with non-attenuated cryopreserved *Pf* sporozoites under chloroquine cover and challenged ten weeks after last immunization. In TUECHMI II, 3200 *Pf* sporozoites was the lowest dose used and protected 33 % (3/9) of vaccinated volunteers against homologous *Pf* sporozoite challenge while the highest dose, 51 200 *Pf* sporozoites, protected 100 % (9/9) of individuals.

In this work, we aimed to characterize the human immune response induced by experimental non-attenuated *P. falciparum* sporozoite infection. In detail, serum antibody levels against the merozoite surface proteins MSP-1/-6/-7 were analyzed by ELISA, and – in cooperation with Dr. Kirsten Heiss and Dr. Ann-Kristin Müller – the CD8+ T-cell response against LSA-1, CSP, malS and MSP-1 was examined *via* IFNγ ELISPOT assay. Antibodies to MSP-1/-6/-7 could be induced by experimental *Pf* sporozoite infection (Figure 5.27) even though infected volunteers were immediately treated with ACT (Lumefantrine-Arthemeter) upon detection of parasites in the thick blood smear. Since it is assumed

that the parasite can undergo 2 - 2.5 blood stage cycles, corresponding to 4-5 days following release of merozoites from liver schizonts, before it is detectable by blood film microscopy (Bejon et al., 2005), this short exposition time of the merozoite to the human immune system seems to be enough to produce merozoite-specific antibodies. Strikingly, these antibodies decrease quite slowly since they can still be measured 84 and even 168 days after infection. Interestingly, MSP-1 is more immunogenic than MSP-6 or MSP-7. However, in comparison to the MSP-1 specific antibody level measured in semi-immune adults from Burkina Faso, the antibody titer against MSP-1 induced by experimental *Pf* sporozoite infection is approximately 100-fold lower.

Recently, the antibody response induced by experimental intravenous injection of cryopreserved *Pf* sporozoites in 18 malaria-naïve volunteers from the UK as well as 28 malaria-exposed individuals from Kenya was analyzed by ELISA, GIA and ADRB assay (Hodgson et al., 2016). Interestingly, antibodies to MSP-1$_{19}$, AMA-1 and *Pf* schizont lysate could also be elicited by *Pf* sporozoite infection in both groups. However, since they applied a different ELISA protocol and showed antibody levels only for one time point after infection (day 35), a direct comparison with my data is not possible.

Additionally, we aimed to investigate the CD8+ T-cell response against the cross-stage antigen MSP-1 and the known pre-erythrocytic antigens CSP and LSA-1 following experimental challenge with *P. falciparum* sporozoites. Since the parasites are injected intravenously, a potential immune response in the skin (Mac-Daniel et al., 2014) and lymph nodes – such as priming of naïve CD8+ T-cells by lymph node-resident dendritic cells (Cockburn and Zavala, 2016) – is omitted. However, T-cell priming is also possible in the liver (Balam et al., 2012; Cockburn and Zavala, 2016; Jobe et al., 2009) and, according to numerous whole sporozoite vaccine approaches administered intravenously (reviewed in (Hollingdale and Sedegah, 2017)), can induce sterile immunity against malaria.

CSP and LSA-1 are both known targets of IFNγ producing T-cells - not only in individuals with naturally acquired immunity against malaria (Jagannathan et al., 2015; Luty et al., 1999; Lyke et al., 2005) but also in RAS-immunized volunteers (Krzych et al., 1995). Furthermore, it was shown in mice that hepatocytes can present CSP-derived peptides to CD8+ T-cells resulting in protection against *Plasmodium* infection (Balam et al., 2012). Importantly, we detected a MSP-1 specific CD8+ T-cell response comparable to LSA-1 and CSP by analyzing PBMCs before and at several time points after *Pf* sporozoite infection *via* IFNγ ELISPOT assay (Figure 5.28). Interestingly, the highest MSP-1 and LSA-1 specific CD8+ T-cell stimulation was detected on day 10-12 post infection while the highest response against CSP was observed on day 7 – according to their expression in late or early liver stages, respectively. However, compared to the MSP-1 specific CD8+ T-cell response of semi-immune adults from Burkina Faso, the activation of MSP-1 / LSA-1 / CSP-specific CD8+ T-cells after a single *Pf* sporozoite infection is much lower (~2200 versus maximum 300 net SFUs per 10^6 PBMCs,

respectively). Furthermore, levels of several cytokines were determined in sera from malaria-positive individuals by cytokine bead array (performed by Dr. Michael Pritsch in Munich, Appendix 7.6). However, the concentration of IFNγ was below the detection limit while the levels of IL-4, IL-6, IL-10 and TNFα showed no significant differences over the course of the study.

Interestingly, cellular immune responses against *Pf* sporozoites, *Pf* RBCs (schizont stage) and against several *Pf* proteins such as CSP or LSA-1 were also assessed in 5 malaria-naïve individuals experimentally infected with *Pf* sporozoites *via* mosquito bite (Teirlinck et al., 2011). By overnight stimulation followed by intracellular cytokine staining and flow cytometry, an IFN γ response against *Pf* sporozoites and *Pf* RBCs was detected 35 days after challenge and strikingly, this response did not decrease up to 14 month post infection. However, they could not detect a response against any of the proteins tested, supposedly because the cytokine concentrations were below the detection limit of flow cytometry analysis. According to their results, about 1 % of total lymphocytes reacted to *Pf* RBCs with IFNγ secretion at day 35 post infection while only approximately 0.05 % reacted against *Pf* sporozoites. Thus, antigens present in blood stage schizonts, such as MSP-1, might be stronger elicitors of lymphocyte-specific IFNγ secretion than sporozoites. T-cell responses against MSP-1 have – to my knowledge – not been investigated following CHMI.

However, several factors limit the significance of the gained results regarding the T-cell response: (i) the HLA-types of the tested individuals are unknown and it is not allowed to determine them because genetic analysis was not included in the informed consent sheet of the clinical study. Thus, we cannot verify whether the malaria-positive volunteers showing a specific CD8+ T-cell response against MSP-1, LSA-1 and CSP (3 out of 9) have the compatible HLA-A0201 subtype. (ii) The T-cell responses are generally quite low after a single *Pf* infection. (iii) Since the malaria-naïve volunteers were only infected once, it is not possible to investigate a potential correlation between measured immune responses and protection against experimental malaria infection. Thus, a study with several parasite infections of human volunteers e.g. during CPS immunization followed by *Pf* challenge, would provide more interesting information.

6.4. Summary & Outlook

6.4.1. Summary

The objective of this work was to characterize the human humoral and cellular immune response against the malaria vaccine candidate *Pf*MSP-1D in semi-immune African adults as well as in malaria-naïve individuals experimentally infected with *P. falciparum* wildtype sporozoites. By using sera from eleven individuals from Burkina Faso with naturally acquired immunity against malaria it was demonstrated that (i) antibodies against MSP-1 partially contribute to direct growth inhibition of *P. falciparum* blood stages *in vitro*, (ii) opsonizing antibodies which induce neutrophil respiratory burst detected *via* ADRB assay, correlate with antibody levels against MSP-1 and are mainly cross-reactive, (iii) opsonizing antibodies target MSP-1 and its fragment MSP-1$_{83}$ and contribute strongly to ADRB activity. Moreover, we show that immunization with recombinant MSP-1D elicits opsonizing antibodies in *rhesus* monkeys, suggesting that the induction of ADRB-effective antibodies may be an important immune mechanism for a vaccine based on MSP-1. Regarding the T-cell response after natural exposure to *P. falciparum*, we detected MSP-1-specific IFNγ-secreting CD8+ T-cells in PBMCs from semi-immune African adults from Burkina Faso.

Additionally, the immune response following experimental infection of malaria-naïve volunteers with *P. falciparum* sporozoites (TUECHMI I) is investigated, indicating that (i) antibodies to MSP-1, MSP-6 and MSP-7 are induced by *Pf* sporozoite challenge and (ii) the cross-stage antigen MSP-1 elicits a CD8+ T-cell response comparable to the known pre-erythrocytic antigens LSA-1 and CSP.

6.4.2. Outlook

This study is limited in its ability to examine a potential correlation between MSP-1 specific immune responses and protection against malaria since the sample size is quite small (eleven African individuals) and the degree of immunity against malaria between different samples is comparable (same transmission area and similar age). Furthermore, the malaria-naïve volunteers from the TUECHMI trial were only once experimentally infected with *Pf* sporozoites without prior vaccinations. Thus, investigating a potential correlation between detected immune responses and either naturally acquired immunity against malaria or protection against experimental malaria infection is not possible. For this aim, it would be crucial to investigate the MSP-1 specific immune response in a large cohort of individuals living in malaria-endemic regions with optimally different age groups and available health records. Additionally, it will be of prime importance for MSP-1 as vaccine candidate to investigate whether functional antibodies and T-cells can be induced in humans by immunizations during clinical trials and if these MSP-1 specific immune mechanisms can protect against malaria.

Regarding the humoral immune response, sera from malaria-naïve volunteers, immunized with recombinant *Pf*MSP-1D and adjuvant IDRI-SE + GLA during a Phase 1 clinical study in Heidelberg, will be analyzed this year. Recent results indicate that antibodies against MSP-1D are considerably induced by immunization and the functionality of these antibodies will soon be examined *via* GIA and ADRB assay. In a potential Phase II study it can be additionally analyzed if the elicited antibodies contribute to protection against malaria. Moreover, it would be interesting to investigate if opsonizing antibodies targeting MSP-1 may also act *via* different mechanisms than neutrophil respiratory burst, such as opsonic phagocytosis (Osier et al., 2014a), complement-dependent inhibition (Boyle et al., 2015) or monocyte-dependent inhibition (Bouharoun-Tayoun et al., 1990).

Regarding the cellular immune response, PBMCs with sufficient quality will be hopefully obtained from individuals with naturally acquired immunity against malaria – optimally from regions with different malaria transmission intensity or from varying age groups, thus the individuals should differ in their acquired immunity. The following questions will be interesting to investigate using these samples: (i) Can we validate the use of recombinant MSP-1D protein as stimulus for PBMCs and following IFNγ ELISPOT analysis? (ii) Can we detect MSP-1 specific T-cells in this cohort? (iii) Do we observe an age dependency in developing MSP-1 specific T-cells? (iv) Can we detect a correlation between MSP-1 specific T-cell responses and naturally acquired immunity against malaria? (v) Does the MSP-1 specific T-cell response acquired by natural exposure differ from the T-cell response experimentally induced by whole sporozoite vaccinations e.g. *via* CPS immunization during the TUECHMI II study? Importantly, PBMCs from several malaria-naïve volunteers – e.g. from the clinical phase I trial with *Pf*MSP-1D before immunization (day 0) – should be analyzed in parallel in order to examine potential cross-reactions to amino acid sequences within MSP-1 (as observed for one malaria-naïve volunteer).

Furthermore, by using PBMCs from the clinical Phase I study with *Pf*MSP-1D currently running in Heidelberg, it will be analyzed if MSP-1 specific T-cells can be induced by immunization with recombinant MSP-1D. However, usually T-cell responses elicited by vaccination with proteins are quite low, thus viral vectors – such as MVA or adenoviruses – might be included in a potential Phase II trial with MSP-1 in order to stimulate T-cell responses. Indeed, high-capacity adenoviral vectors expressing full-length *msp-1* are already available and the induction of antibodies and high levels of IFNγ-producing CD8+ T-cells in mice has been shown (Zong et al., 2011). Thus, it might be interesting to compare the MSP-1 specific T-cell response elicited by different immunization strategies during clinical trials. Additionally, PBMCs derived from a possible Phase II study with MSP-1 could be employed to investigate whether the elicited T-cell response contributes to protection against malaria.

6.4.3. MSP-1 as malaria vaccine candidate

Although several malaria vaccine candidates have been tested in clinical trials, so far none of them showed protective efficacy in malaria-endemic countries. The most progressed vaccine candidate up to date, RTS.S/AS01, recently demonstrated no efficacy in African children within a 7 year period (Olotu et al., 2016). Even though whole sporozoite vaccinations are able to confer sterile immunity against malaria at least against the homologous strain and for a short time-period (e.g. (Mordmuller et al., 2017; Seder et al., 2013)), many major obstacles and difficulties have to be overcome to potentially realize vaccinations in malaria-endemic regions (see 1.3.2 for details). Thus, an effective malaria vaccine as well as robust *in vitro* assays measuring correlates of protective immunity are still urgently required.

Although immunization with native *Pf*MSP-1 could protect *Saimiri* and *Aotus* monkeys against a lethal *P. falciparum* infection (Etlinger et al., 1991; Perrin et al., 1984; Siddiqui et al., 1987), clinical vaccination trials in humans – performed with recombinant *Pf*MSP-1$_{19}$ or *Pf*MSP-1$_{42}$ – showed no protective efficacy against malaria so far (Ogutu et al., 2009; Sheehy et al., 2012). Thus, possibly different MSP-1 regions besides MSP-1$_{42}$ may be important for protection. Indeed it was shown recently, that MSP-1 serves as platform for several peripheral MSPs and the formation of these complexes on the merozoite surface can be inhibited by targeting MSP-1$_{83}$ resulting in reduced parasite growth *in vitro* (Lin et al., 2016). Furthermore, antibody levels against MSP-1$_{83}$ (Tolle et al., 1993) or a part of it, namely MSP-1$_{block 2}$ (Cavanagh et al., 2004; Conway et al., 2000), have been correlated with naturally acquired immunity against malaria. Importantly, antibodies against MSP-1$_{83}$ seem to act *via* several immune mechanisms such as direct growth inhibition of *Plasmodium* blood stages (Woehlbier et al., 2006), monocyte-dependent ADCI activity (Galamo et al., 2009), complement-dependent parasite inhibition (Boyle et al., 2015) and by induction of neutrophil respiratory burst (this work). Furthermore, many potential CD8+ T-cell epitopes are distributed throughout the complete MSP-1D protein and for some epitopes located in dimorphic regions – 674 within MSP-1$_{83}$ as well as 291 and 374 within MSP-1$_{38}$ – a stimulation of IFNγ-secreting CD8+ T-cells in PBMCs from semi-immune African individuals has been shown (this work).

Since multiple immune mechanisms probably contribute to naturally acquired immunity against malaria (Murungi et al., 2016; Rono et al., 2013), it is advantageous for a malaria vaccine candidate to contain both CD4+ and CD8+ T-cell epitopes and to induce a variety of antibody- and cell-mediated immune mechanisms. A malaria vaccine providing several relevant epitopes may also impede the development of resistance by the parasite and may reduce the effect of genetic diversity between individuals, such as the HLA-subtype, for developing a protective immunity.

Additionally, most of the difficulties and challenges described for whole parasite vaccinations will not apply for a malaria vaccine based on a recombinant protein. Thus, we suggest *Pf*MSP-1D as malaria vaccine candidate, possibly in combination with other *Plasmodium* antigens.

This work provides useful information about the human immune response against *Pf*MSP-1D, acquired by natural exposure to *P. falciparum* or by experimental challenge with *Pf* sporozoites. Furthermore, the immunological assays established in our laboratory can be applied to characterize the humoral and cellular immune response elicited by immunization with MSP-1 during clinical trials. Finally, our results further support the ADRB as robust *in vitro* assay to investigate the function of merozoite-specific antibodies and strengthen the candidacy of MSP-1 as promising malaria vaccine antigen.

7. Appendices

7.1. Protein sequence of recombinant *Pf*MSP-1D

MVTHESYQELVKKLEALEDAVLTGYSLFQKEKMVLNEEEITTKGASAQSGASAQSGASAQGASAQSGASAQSGASAQSGTSGPSGPSGTSPSS
RSNTLPRSNTSSGASPPADASDSDAKSYADLKHRVRNYLFTIKELKYPELFDLTNHMLTLCDNIHGFKYLIDGYEEINELLYKLNFYFDLLRAKLND
VCANDYCQIPFNLKIRANELDVLKKLVFGYRKPLDNIKDNVGKMEDYIKKNKTTIANINELIEGSKKTIDQNKNADNEEGKKKLYQAQYDLSIYNK
QLEEAHNLISVLEKRIDTLKKNENIKKLLDKINEIKNPPPANSGNTPNTLLDKNKKIEEHEEKIKEIAKTIKFNIDSLFTDPLELEYYLREKNKKVDVTP
KSQDPTKSVQIPKVPYPNGIVYPLPLTDIHNSLAADNDKNSYGDLMNPHTKEKINEKIITDNKERKIFINNIKKKIDLEEKNINHTKEQNKKLLEDYE
KSKKDYEELLEKFYEMKFNNNFNKDVVDKIFSARYTYNVEKQRYNNKFSSSNNSVYNVQKLKKALSYLEDYSLRKGISEKDFNHYYTLKTGLEADI
KKLTEEIKSSENKILEKNFKGLTHSANGSLEVSDIVKLQVQKVLLIKKIEDLRKIELFLKNAQLKDSIHVPNIYKPQNKPEPYYLIVLKKEVDKLKEFIPK
VKDMLKKEQAVLSSITQPLVAASETTEDGGHSTHTLSQSGETEVTEETEETEETVGHTTTVTITLPPTQPSPPKEVKVVNSIEHKSNDNSQALTKT
VYLKKLDEFLTKSYICHKYLVSNSSMDQKLLEVYNLTPEEENELKSCDPLDLLFNIQNNIPAMYSLYDSMNNDLQHLFFELYQKEMIYYLHKLKEE
NHIKKLLEEQKQITGTMSSTSSPGNTTVNTAQSATHSNSQNQQSNASSTNTQNGVAVSSGPAVVEESHDPLTVLSISNDLKGIVSLLNLGNKTK
VPNPLTISTTEMEKFYENILKNNDTYFNDDIKQFVKSNSKVITGLTETQKNALNDEIKKLKDTLQLSFDLYNKYKLKLDRLFNKKKELGQDKMQIKK
LTLLKEQLESKLNSLNNPHNVLQNFSVFFNKKKEAEIAETENTLENTKILLKHYKGLVKYYNGESSPLKTLSEVSIQTEDNYANLEKFRVLSKIDGKL
NDNLHLGKKKLSFLSSGLHHLITELKEVIKNKNYTGNSPSENNKKVNEALKSYENFLPEAKVTTVVTPPQPDVTPSPLSVRVSGSSGSTKEETQIPT
SGSLLTELQQVVQLQNYDEEDDSLVVLPIFGESEDNDEYLDQVVTGEAISVTMDNILSGFENEYDVIYLKPLAGVYRSLKKQIEKNIFTFNLNLNDI
LNSRLKKRKYFLDVLESDLMQFKHISSNEYIIEDSFKLLNSEQKNTLLKSYKYIKESVENDIKFAQEGISYYEKVLAKYKDDLESIKKVIKEEKEKFPSSP
PTTPPSPAKTDEQKKESKFLPFLTNIETLYNNLVNKIDDYLINLKAKINDCNVEKDEAHVKITKLSDLKAIDDKIDLFKNPYDFEAIKKLINDDTKKD
MLGKLLSTGLVQNFPNTIISKLIEGKFQDMLNISQHQCVKKQCPENSGCFRHLDEREECKCLLNYKQEGDKCVENPNPTCNENNGGCDADATC
TEEDSGSSRKKITCECTKPDSYPLFDGIFCSSSN

Figure 7.1: Protein sequence of recombinant PfMSP-1D. ...

7.2. Antibody titers against MSP-1
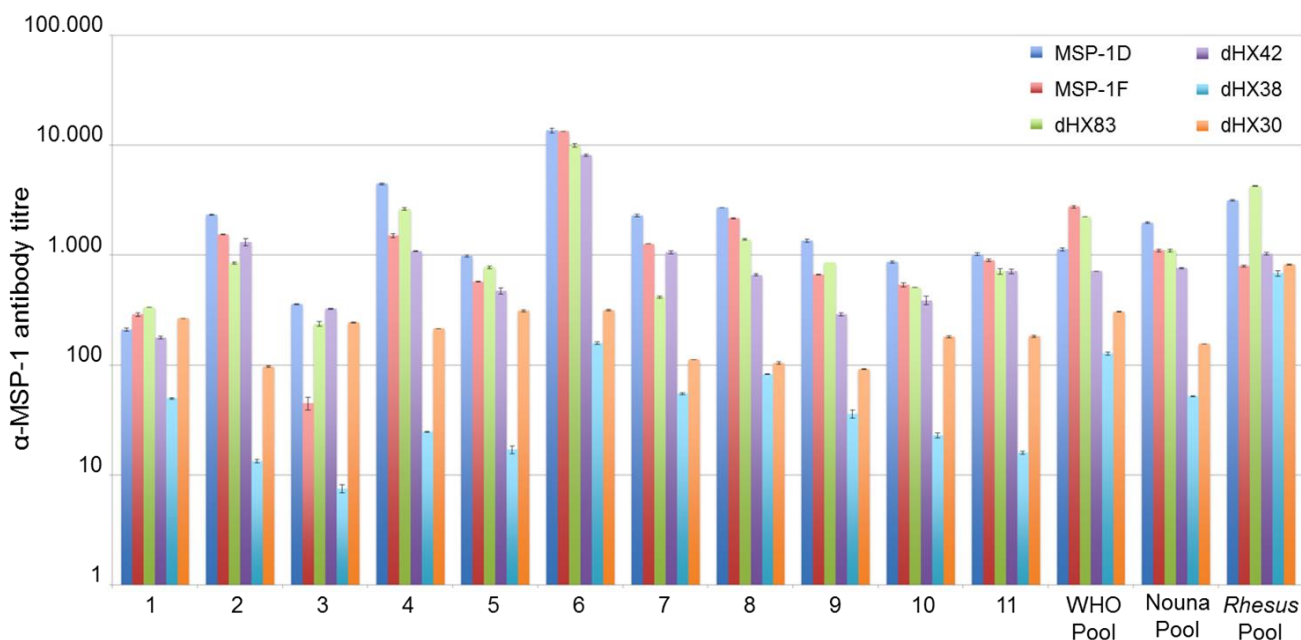
Figure 7.2: Antibody levels to MSP-1 in semi-immune individuals and immunized rhesus monkeys. ...

7.3. Mass Spec analysis of *Pf* schizont lysate
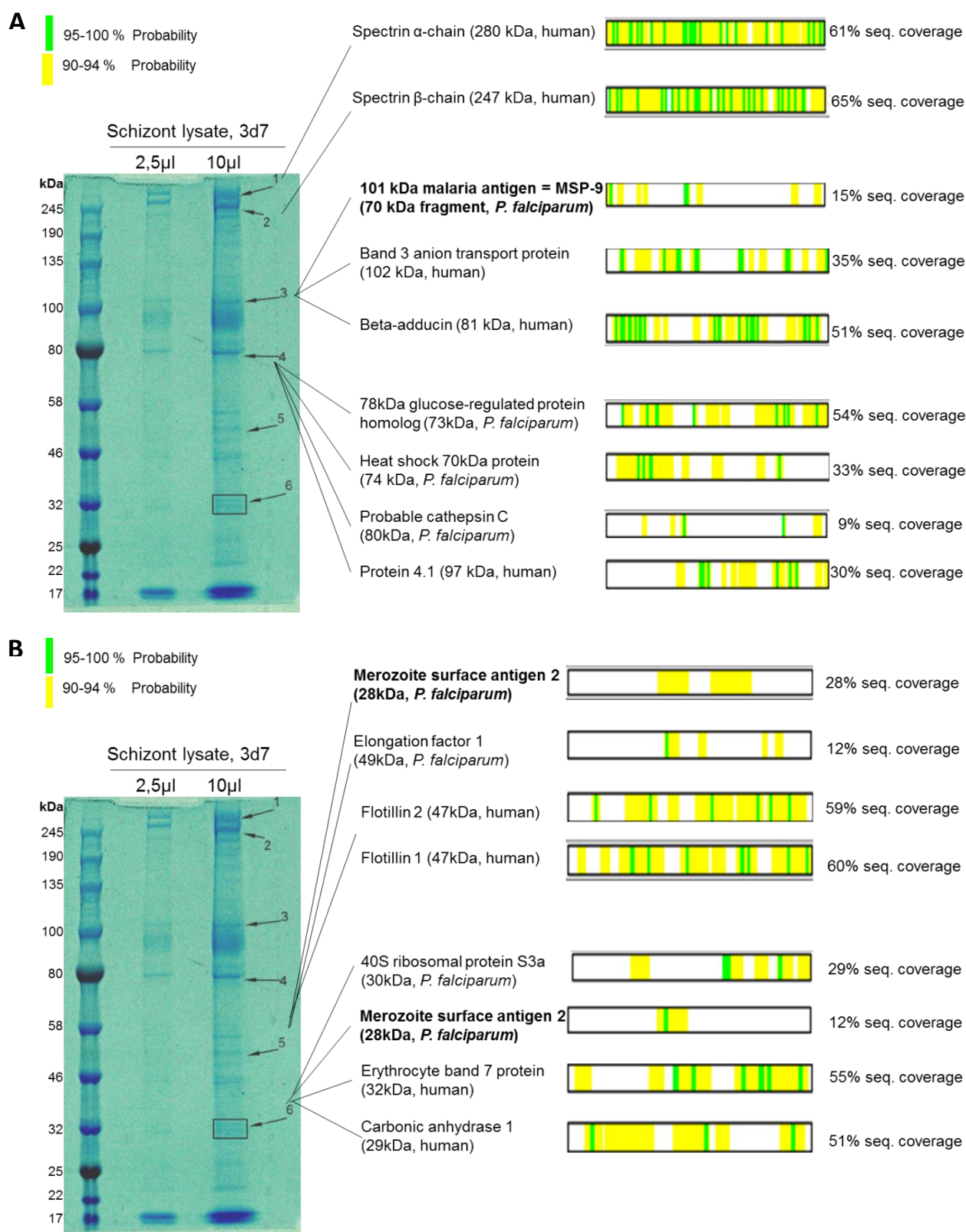
Figure 7.3: Mass Spec results. *P. falciparum (3d7) schizont lysate was separated on a gel via SDS-PAGE and stained with Coomassie brilliant blue. At the MS facility in the ZMBH (Heidelberg) six bands were cut out, digested with Trypsin and compared to the Swiss-Prot protein sequence database. The best results for human and P. falciparum proteins for band 1-4 (A) and 5-6 (B) are shown. The sequence coverage for each protein is indicated with yellow (90-94 % probability) and green (95-100 % probability) color.*

7.4. Correlations between antibody levels and GIA/ADRB activity

ELISA	Growth inhibition(3d7)		ADRB activity (3d7)	
	r	P	r	P
α-SZL (3d7)	0,755	0,00336 (**)	0,767	0,000695 (***)
α-Merozoites (3d7)	0,720	0,00707 (**)	0,855	0,0000002 (***)
α-MSP-1D	0,769	0,00228 (**)	0,758	0,00102 (**)
α-MSP-1F	0,791	0,00211 (**)	0,615	0,0237 (*)

Table 7.1: Spearman`s rank correlation coefficients between antibody levels to MSP-1 or P. falciparum blood stages and GIA or ADRB activity. Antibody levels to P. falciparum 3d7 schizont lysate or merozoites or the recombinant proteins MSP-1D/F were determined via ELISA in 11 semi-immune individuals from Burkina Faso and correlated to their GIA and ADRB activity against P. falciparum 3d7. All correlations are significant, the P value is indicated.

7.5. Quality of Nouna PBMCs
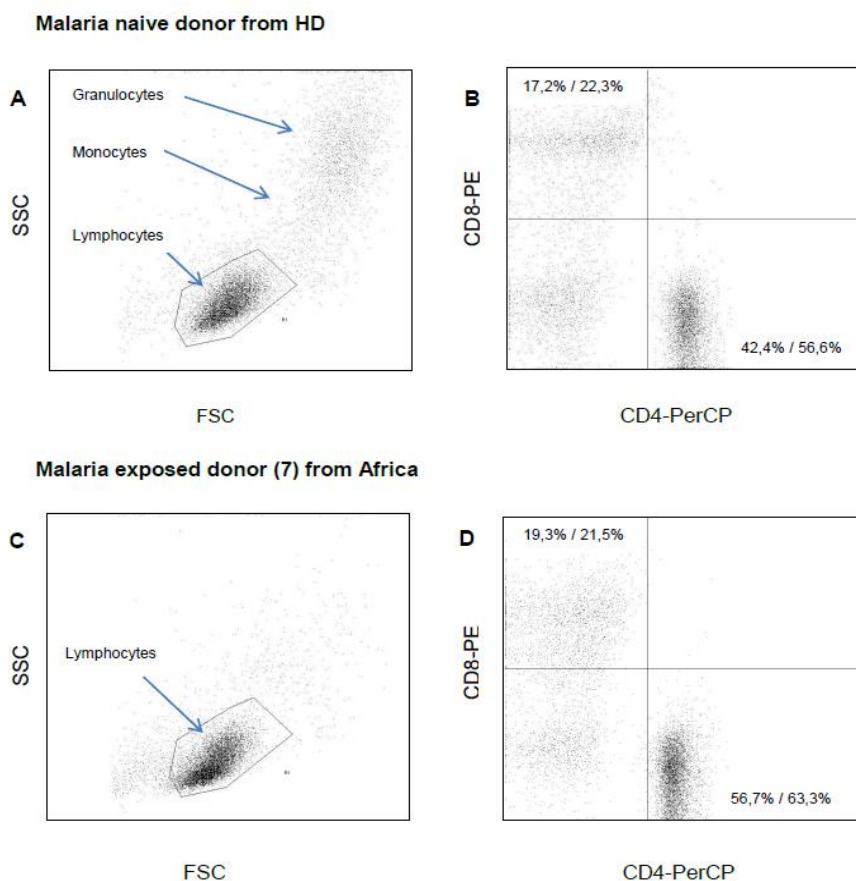
Figure 7.4: Quality control of PBMCs from a malaria-naïve donor (Heidelberg) and a semi-immune donor (Nouna). PBMCs were thawed, stained with CD4-PerCP and CD8-PE antibodies for 20 min, fixed with 1 % PFA and analyzed by flow cytometry. (A/C) FSC-SSC diagram showing different cell populations; lymphocytes were gated. (B/D) CD4-PerCP and CD8-PE staining of lymphocytes; percentages of stained cells from total / gated cells are indicated in the respective field. This experiment was performed by myself during my master thesis.

7.6.   TUECHMI I, Cytokine analysis (Munich)
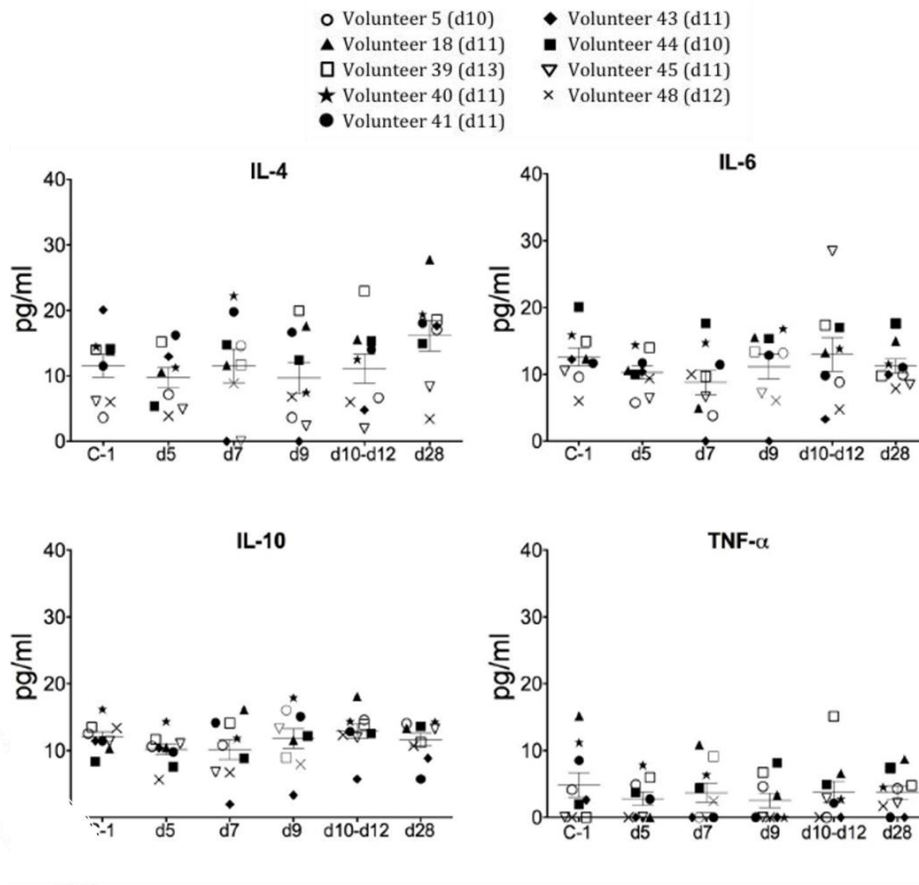
Figure 7.5: Cytokine profile of 9 malaria-positive individuals after challenge with 3200 cryopreserved PfSpz. *This experiment was done by our cooperation partner in Munich (Dr. Michael Pritsch). Sera of the indicated volunteers were analyzed by cytokine bead array using flow cytometry for cytokine levels to IL-4, IL-6, IL-10 and TNFα at different time points starting at the day before challenge (C-1) up to day 28. Mann-Whitney test was used for statistical analysis.*

7.7 Taxonomy report of peptide 291 (BLAST-p search)

```
root .................................................... 194 hits    62 orgs
. cellular organisms ................................... 192 hits    60 orgs
. . Eukaryota .......................................... 105 hits    20 orgs
. . . Alveolata ........................................  77 hits    11 orgs
. . . . Plasmodium (Laverania) ........................  75 hits     9 orgs
. . . . . Plasmodium falciparum .......................  75 hits     9 orgs
. . . . . . Plasmodium falciparum CAMP/Malaysia ......    1 hits     1 orgs
. . . . . . Plasmodium falciparum 3D7 ................    2 hits     1 orgs
. . . . . . Plasmodium falciparum Mad20/Papua New Guinea . 1 hits    1 orgs
. . . . . . Plasmodium falciparum FC27/Papua New Guinea ..  1 hits   1 orgs
. . . . . . Plasmodium falciparum RO-33 ..............    1 hits     1 orgs
. . . . . . Plasmodium falciparum Palo Alto/Uganda ........1 hits    1 orgs
. . . . . . Plasmodium falciparum isolate WELLCOME ......   1 hits    1 orgs
. . . . . . Plasmodium falciparum K1 ..................    1 hits     1 orgs
. . . . Paramecium ....................................    2 hits     2 orgs
. . . . . Paramecium tetraurelia ......................    2 hits     2 orgs
. . . . . . Paramecium tetraurelia strain d4-2 ........    1 hits     1 orgs
. . . Opisthokonta ....................................   18 hits     6 orgs
. . . . Coelomata .....................................   16 hits     4 orgs
. . . . . Neoptera ....................................    2 hits     2 orgs
. . . . . . Acyrthosiphon pisum .......................    1 hits     1 orgs
. . . . . . Solenopsis invicta ........................    1 hits     1 orgs
. . . . . Eutheria ....................................   14 hits     2 orgs
. . . . . . Cricetulus griseus ........................    1 hits     1 orgs
. . . . . . Bos taurus ................................   13 hits     1 orgs
. . . . mitosporic Nakaseomyces .......................    2 hits     2 orgs
. . . . . Candida glabrata ............................    2 hits     2 orgs
. . . . . . Candida glabrata CBS 138 ..................    1 hits     1 orgs
. . . Populus trichocarpa .............................    4 hits     1 orgs
. . . Dictyostelium purpureum .........................    2 hits     1 orgs
. . . Trichomonas vaginalis G3 ........................    4 hits     1 orgs
. . Bacteria ..........................................   85 hits    39 orgs
. . . Actinomycetales .................................   20 hits     7 orgs
. . . . Micrococcineae ................................    4 hits     2 orgs
. . . . . Renibacterium salmoninarum ATCC 33209 .......    2 hits     1 orgs
. . . . . Xylanimonas cellulosilytica DSM 15894 .......    2 hits     1 orgs
. . . . Streptomyces ..................................   14 hits     3 orgs
. . . . . Streptomyces cyaneogriseus subsp. noncyanogenus 10 hits    1 orgs
. . . . . Streptomyces sp. e14 ........................    2 hits     1 orgs
. . . . . Streptomyces caeruleus ......................    2 hits     1 orgs
. . . . Kribbella .....................................    2 hits     2 orgs
. . . . . Kribbella catacumbae ........................    1 hits     1 orgs
. . . . . Kribbella sancticallisti ....................    1 hits     1 orgs
. . . Proteobacteria ..................................    8 hits     3 orgs
. . . . Marinomonas mediterranea MMB-1 ................    2 hits     1 orgs
. . . . Methylobacterium sp. 4-46 .....................    4 hits     1 orgs
. . . . Neisseria cinerea ATCC 14685 ..................    2 hits     1 orgs
. . . Firmicutes ......................................   55 hits    28 orgs
. . . . Clostridia ....................................    4 hits     2 orgs
. . . . . Clostridium nexile DSM 1787 .................    2 hits     1 orgs
. . . . . Halanaerobium hydrogeniformans ..............    2 hits     1 orgs
. . . . Enterococcus faecium ..........................   51 hits    26 orgs
. . . . . Enterococcus faecium E980 ...................    2 hits     1 orgs
. . . . . Enterococcus faecium Com15 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium 1,231,501 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium TC 6 ...................    2 hits     1 orgs
. . . . . Enterococcus faecium D344SRF ................    2 hits     1 orgs
. . . . . Enterococcus faecium E1636 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium TX1330 .................    2 hits     1 orgs
. . . . . Enterococcus faecium 1,141,733 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium 1,231,408 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium Com12 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium PC4.1 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium DO .....................    3 hits     1 orgs
. . . . . Enterococcus faecium 1,230,933 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium 1,231,502 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium 1,231,410 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium E1162 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium E1071 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium U0317 ..................    2 hits     1 orgs
. . . . . Enterococcus faecium TX0133a04 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium TX0133C ................    2 hits     1 orgs
. . . . . Enterococcus faecium TX0082 .................    2 hits     1 orgs
. . . . . Enterococcus faecium TX0133A ................    2 hits     1 orgs
. . . . . Enterococcus faecium TX0133B ................    2 hits     1 orgs
. . . . . Enterococcus faecium TX0133a01 ..............    2 hits     1 orgs
. . . . . Enterococcus faecium E4452 ..................    1 hits     1 orgs
. . . . . Enterococcus faecium E4453 ..................    1 hits     1 orgs
. . . Pedobacter heparinus DSM 2366 ...................    2 hits     1 orgs
. . Pyrococcus sp. NA2 ................................    2 hits     1 orgs
. artificial sequences ................................    2 hits     2 orgs
. . synthetic construct ...............................    1 hits     1 orgs
. . Cloning vector pBSK*-MSP-1s/FCB ...................    1 hits     1 orgs
```

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