Malaria and relapsing fever *Borrelia*
-Interactions and potential therapy

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To my family!

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Abstract

Infectious diseases such as malaria and relapsing fever borreliosis (RF), cause severe human mortality and morbidity in developing countries. Malaria, caused by *Plasmodium spp.* parasites, is estimated by the World Health Organization to cause 1.5-2.7 million deaths annually. RF, caused by *Borrelia* spirochetes, has the highest prevalence described for any bacterial disease in Africa, with infection outcomes ranging from asymptomatic to fatal. RF borreliosis manifests in humans as a recurring fever and with other symptoms very similar to those of malaria.

RF borreliosis has been regarded as a transient infection of the blood. However, *B. duttonii* exploits the brain as an immunoprivileged site escaping the host immune response while spirochetes in the blood are cleared. To investigate whether residual bacteria are dormant or actively dividing, mice with residual brain infection were administered ceftriaxone, a β-lactam antibiotic interfering with cell wall synthesis. Hence, it only affects actively dividing bacteria. Ceftriaxone eradicated brain RF infection in all treated mice, demonstrating that the bacteria are actively multiplying rather than in a dormant state. The findings support the therapeutic use of ceftriaxone for RF neuroborreliosis since penetration into cerebrospinal fluid is greater for ceftriaxone than for the often recommended doxycycline.

The clinical features of malaria and RF are similar and diagnosis is further complicated by the frequently occurring concomitant malaria-RF infections. Therefore, we established a mouse model to study the pathogenesis and immunological response to *Plasmodium/Borrelia* mixed infection. Interestingly, malaria was suppressed in the co-infected animals whereas spirochete numbers were elevated 21-fold. The immune response in the concomitantly infected mice was polarized towards malaria leaving the spirochetes unharmed. Mice with co-infections also exhibited severe anemia and internal damages, probably attributed to escalating spirochete numbers. A secondary malaria infection reactivated the residual brain RF infection in 60% of the mice. This highlights the importance of co-infections as diagnostic pitfalls as well as the need for novel treatment strategies.

Currently there is no commercial malaria vaccine and increasing drug resistance presents an urgent need for new malaria chemotherapeutics. Blood-stage malaria parasites are rapidly growing with high metabolic and biosynthetic activity, making them highly sensitive to limitations in polyamine supply. Disrupting polyamine synthesis *in vivo* with *trans*-4-methylcyclohexylamine (4MCHA) eradicated the malaria infection gradually, resulting in protective immunity. This leads the way for further biochemical and pharmacological development of the polyamine inhibitor 4MCHA and similar compounds as antimalarial drugs.

Abbreviations

IFN-γ	Interferon gamma
TNF-α	Tumour necrosis factor alpha
CS	Circumsporozoite protein
MSP-1	Merozoite surface protein-1
NK	Natural killer cell
DC	Dendritic cell
RF	Relapsing fever
PfEMP1	*Plasmodium falciparum* erythrocyte membrane protein-1
ICAM1	Intercellular adhesion molecule 1
CSA	Chondroitin sulphate A
Vmp	Variable membrane protein
Ig	Immunoglobulin
T_H	T helper cell
T_H1	Cell-mediated T_H response
T_H2	Humoral T_H response
IL	Interleukin
ODC	Ornithine decarboxylase
AdoMetDC	*S*-adenosylmethionine decarboxylase
SPDS	Spermidine synthase
SPS	Spermine synthase
SSAT	Spermidine/spermine N1-acetyltransferase
DFMO	α-difluoromethylornithine inhibitor of ODC
CSKO	CS protein knock out parasite
T1	T cell epitope in CS protein
T*	universal T cell epitope in CS protein
CS(*Pf*)	*P. berghei* parasite with the *CS* gene from *P. falciparum*
DHFR-TS	Selectable marker dihydrofolate reductase-thymidylate synthase
hDHFR	Selectable marker ,the human dihydrofolate reductase
Hb	Hemoglobin
SMA	Severe malaria anemia
AdoDATO	*S*-adenosyl-1,8-diamino-3-thiooctane, an inhibitor of SPDS
4MCHA	*trans*-4-methylcyclohexylamine, an inhibitor of SPDS

Papers in this thesis

This thesis is based on the following papers, which will be referred to by their roman numbers I-III.

I. **Larsson, C., Lundqvist, J. & Bergström, S.** Residual brain infection in murine relapsing fever borreliosis can be successfully treated with ceftriaxone. *Microbial Pathogenesis* 44 (2008) 262–264.

II. **Lundqvist, J., Larsson, C., Nelson, M., Bergström, S., & Persson, C.** Mixed infection decrease malaria burden and escalate relapsing fever. *Manuscript.*

III. **Lundqvist, J., Nelson, M., Plym-Forshell, T., Nilsson, J., & Persson, C.** An *in vivo* study of the antimalarial effect of polyamine synthesis inhibitors in *Plasmodium berghei*. *Manuscript.*

Introduction

Infectious disease is a major public health problem in the developing world and in many areas of Africa the main reason for hospitalization, especially among young children (Reyburn et al., 2004). Several pathogens cause fever, which might be due to anything from a trivial viral infection to more severe, even fatal infectious diseases such as malaria or typhoid fever. In developing countries where the resources for diagnosis are limited, most infectious diseases are identified by their clinical symptoms only. Febrile illness is generally assumed to be malaria, without taking into consideration the diagnostic overlap with bacterial infections, viral infections or other parasites (Amexo et al., 2004). Since fever is such a general symptom, presumptive diagnostics often result in misdiagnosis and inappropriate medication. This thesis focuses on persistence, novel treatment strategies and co-infection of two severe tropical febrile diseases, malaria and relapsing fever borreliosis.

1. Malaria epidemiology

Malaria is by far the most devastating acute febrile illness of the developing world. The disease is estimated by the World Health Organization (WHO) to be responsible for 1.5-2.7 million deaths annually, with about 1 million of these deaths among children under the age of five (Snow et al., 1999; WHO, 2008). Malaria is endemic in over 109 countries and about half the world's population lives in areas with risk of exposure (WHO, 2008).

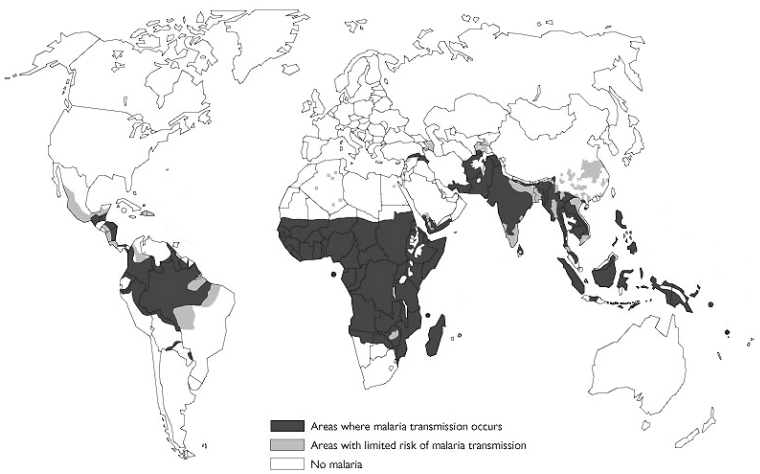


Figure 1. Geographical distribution of malaria, (WHO, 2008).

The highest malarial incidence is predominately found in sub-Saharan Africa where about 90% of all clinical cases and deaths occur (Fig. 1). Apart from the human toll, the disease has a huge economic impact estimated to decrease GDP (gross domestic product) five-fold in malaria endemic countries and the disease is estimated to cost the African continent about 12 billion USD every year (Gallup and Sachs, 2001).

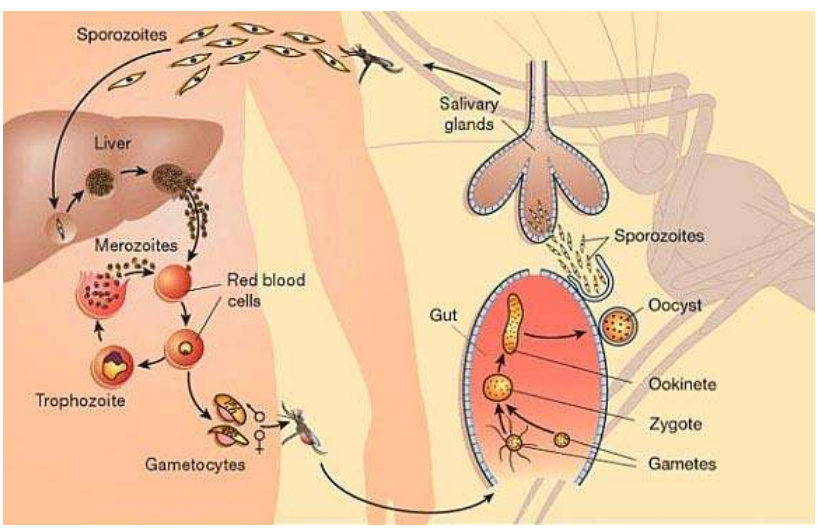
2. The malaria parasite

Malaria is caused by protozoan parasites from the genus *Plasmodium,* with four species responsible for almost all human infections: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale*. Apart from these, there is the simian parasite *P. knowlesi* that occasionally can infect humans, in particular in Malaysia

(Chin et al., 1965; Singh et al., 2004). The majority of malaria infections are caused by *P. vivax* and *P. falciparum*. The former parasite has a wider geographical distribution and affects millions of people across Africa, Asia, the Middle East, and Central and South America. This worldwide spread is due to the ability of *P. vivax* to complete the mosquito developmental cycle at a lower minimum temperature than *P. falciparum*. Still, due to its greater pathogenicity, *P. falciparum* is responsible for the majority of fatal infections and is usually labeled as causing severe malaria.

3. The malaria life cycle

The life cycle of malaria parasites involves both a mammalian host and an anopheline mosquito, both equally important for the parasite's development. The life cycle begins with the blood meal of a female mosquito, which via the blood meal ingests female and male gametocytes, the sexual stages of the parasite (Fig. 2).



The picture is adapted from Menard (2005) and the reproduction of this material is granted by Nature publishing group (Menard, 2005).

Figure 2. Life cycle of the *Plasmodium* parasite. Both the mammalian host and the anopheline mosquito vector are essential in the parasites developmental cycle.

Within the mosquito, midgut gametogenisis is initiated where the ingested gametocytes transform into microgametes and macrogametes (Baton and Ranford-Cartwright, 2005). These gametocytes undergo fertilization inside the mosquito midgut and transform into motile ookinetes. These ookinetes transverse the midgut wall, become arrested beneath the basal lamina, and differentiate into the next developmental stage, the oocyst. Within 10-12 days of growth and maturation in the oocyst, thousands of sporozoites are released into the hemolymph of the mosquito. The parasite's own movements and the circulating hemolymph transport the sporozoites to the salivary glands, which they actively invade. During the mosquito blood meal, sporozoites are deposited in the dermis together with saliva.

Inside the dermis sporozoites move randomly until they come across a blood vessel where they enter and are rapidly carried away by the blood toward the liver (Vanderberg and Frevert, 2004). The sporozoites cross the liver endothelial barrier presumably through Kupffer cells, the macrophages of the liver, to access the underlying hepatocyte layer (Meis et al., 1983). Upon entrance into the target hepatocyte, the sporozoite forms a parasitophorous vacuole in which it can multiply and differentiate into thousands of merozoites. Merozoite-filled vesicles, called merosomes, bud off from the infected cell and are released into the blood. These vesicles are of host cell origin, concealing the parasites from the immune response and guaranteeing the merozoites a safe journey through the blood vessel (Sturm and Heussler, 2007; Thiberge et al., 2007).

In the blood, the merozoites quickly infect erythrocytes where the initial merozoite differentiates into a trophozoite that further matures into a schizont. This parasite stage multiplies into high numbers of new merozoites that finally burst the cell and thereby are ready to infect new erythrocytes. A fraction of the merozoites take a different path, developing into gametocytes that in turn are able to infect the mosquito, thereby completing the life cycle.

4. Clinical manifestations of malaria

Following the bite of an infected mosquito, the malaria incubation time is about 9-14 days. The first symptoms are non-specific and similar to those seen in, for instance, a minor systemic viral illness, such as headache, myalgia, weakness, vomiting, and diarrhea, followed by fever and chills, the classical hallmarks of malaria. The fever peaks are a direct result of the massive discharges of merozoites into the blood. Malaria also causes hepatosplenomegaly, anemia, thrombocytopenia, hypoglycemia, pulmonary and/or renal dysfunction, and neurologic changes. Patients suffering from severe malaria usually display one or more of the following symptoms: cerebral malaria with acute neurological symptoms, respiratory distress, acute renal failure and severe anemia. Severe malaria is usually fatal if left untreated. Still, the clinical symptoms vary substantially depending on the infecting *Plasmodium* species, the level of infection and the immunological status of the patient. For example, during pregnancy, a malaria infection can result in maternal anemia, fetal loss, premature delivery, intrauterine growth retardation and reduced birth weight (Guyatt and Snow, 2001; Murphy and Breman, 2001).

4.1 Anemia

There is a delicate balance between the destruction of erythrocytes and the influx of newly generated reticulocytes from hematopoietic tissue to maintain the numbers of circulating erythrocytes. When an infection increases destruction of erythrocytes, it disturbs this balance, resulting in anemia. During malaria infection, the destruction of erythrocytes is increased both by the rupture of infected blood cells, by a decreased production of new reticulocytes, and a shortened erythrocyte life span (Abdalla et al., 1980; Looareesuwan et al., 1991; Srichaikul et al., 1967). The shortened erythrocyte half-life has been suggested to be a direct result of a hyperactive phagocytic system, which could explain the consistently observed connection between relatively low parasite burden and severe anemia (Bojang et al., 1997; Evans et al., 2006).

5. Diagnostics and treatment of malaria

Malaria diagnosis is based on two components: clinical diagnosis and detection of parasites in the blood of the patient. Clinical diagnosis involves the identification of classical malaria symptoms such as recurring fever and hepatosplenomegaly. These are often used as a primary diagnosis of malaria infection in endemic countries, and WHO advises presumptive diagnosis as the basis for first-line treatment (Koram and Molyneux, 2007; WHO, 2006). As a confirmatory diagnosis, parasites can be identified in the blood by light microscopic examination of Giemsa stained blood smears. This technique is simple, inexpensive and yet efficient; it is the gold standard for laboratory diagnostics of malaria infection. However, the accuracy is dependent on quality of the reagents, the microscope, and on the experience of the laboratory personnel.

The treatment of choice is dependent on the infecting *Plasmodium* species, the severity of the disease and the patient's age. For instance in young children anti-malarial drugs are often combined with antibiotics since bacterial septicemia and severe malaria are associated and there is a risk for diagnostic overlap (Berkley et al., 1999). *P. ovale*, *P malariae*, and *P vivax* are usually sensitive to chloroquine and very susceptible to artemisinins. *P. falciparum* infections are usually treated using an artemisinin-based combination therapy, which results in rapid clearance of parasites and resolution of symptoms (WHO, 2006). However, a fast-spreading resistance to conventional anti-malarial drugs and an emerging tolerance to artemisinins have prompted the necessity of new and improved means to fight the malaria parasite by prophylaxis, drugs and vaccine (Gregson and Plowe, 2005; Talisuna et al., 2004; White et al., 1999; WHO, 2007).

6. Immune response to malaria

Immunity to malaria is complex and essentially both species and stage specific. The immune response can limit the infection, prevent severe pathology and reduce the load of circulating infected erythrocytes. However, the elicited response usually fails to completely eliminate the infection and the parasites can persist as a low-grade infection. Besides being protective, there is also evidence that the immune response is in part responsible for severe malarial pathogenesis, such as cerebral malaria particularly linked to elevated IFN-γ and TNF-α levels (Amani et al., 2000; Riley, 1999).

The complement cascade may be an important protective response towards a malaria infection but there is no consistent evidence of a complement evasion strategy in *Plasmodium*. Although in persistent infection parasitized erythrocytes can, in spite of their capacity to activate complement, be resistant to complement-mediated lysis (Kawamoto et al., 1997). This phenomenon is ascribed to the presence of complement-regulatory proteins on the parasitized erythrocytes mediated by the host cell itself (Wiesner et al., 1997).

Sporozoites, the first parasitic form entering the host, elicit antibodies targeting the major sporozoite surface protein, the circumsporozoite (CS) protein (Hoffman et al., 1986). Unfortunately, the extracellular journey of the sporozoite is short; to eradicate this parasitic stage, repeated re-infections that elicit high titers of antibodies in the circulation are needed (Sidjanski and Vanderberg, 1997; Yamauchi et al., 2007). In the pre-erythrocytic liver stages, the immune response mainly depends on cytotoxic $CD8^+$ T cells inhibiting parasite development in hepatocytes (Good and Doolan, 1999). Antibodies targeting merozoite protein 1 (MSP-1) inhibit the invasion of merozoites into erythrocytes (Miller et al., 1975). However, to kill merozoites, the time for antibodies to affect parasites is short since the exposure only occurs between the time of release from one erythrocyte until the invasion of another.

The intracellular erythrocytic stages of the parasites are initially restricted by the actions of the innate immune response via macrophages, natural killer (NK) cells and the complement system. Macrophages can, besides killing parasites by engulfment, also produce anti-parasitic molecules such as nitric oxide (Sherman, 1998). NK cells mediate cytotoxicity, essential for limiting

intracellular infection but also contribute to protective immunity by the production of IFN-γ (De Souza et al., 1997; Mohan et al., 1997). However, to restrict and eliminate the infection, activation of adaptive immunity is essential. Antigens are mainly presented to $CD4^+$ T cells by dendritic cells (DC) but also by macrophages and B cells (Stevenson and Riley, 2004). This activation predominantly occurs in a T_H1 manner, which subsequently results in the production of cytophilic IgG antibodies, mediating phagocytosis by macrophages (Sherman, 1998; Su and Stevenson, 2002). The preservation of protective immunity requires continuous re-infections. This immunity is often semi-protective, resulting in milder symptoms but is seldom able to eradicate infection.

7. Virulence factors of malaria

7.1 Antigenic variation

To stay a step ahead of the host immune defense, some pathogens have the capacity to alter their outer appearance and thereby escape the mounted response. This immune evasion mechanism, called antigenic variation, is found in eukaryotic parasites such as the malaria parasite and trypanosomes, as well as in bacteria such as relapsing fever (RF) *Borrelia* and *Neisseria gonorrhoeae* (Borst and Rudenko, 1994; Brown and Brown, 1965; Meyer et al., 1982; Stoenner et al., 1982). The changes of outer membrane proteins are achieved almost without exception by rearrangement of repeated genes or gene segments. The *Plasmodium* parasite undergoes antigenic variation in merozoite, latent trophozoite, and schizont stages of the infection. The major antigenic variable protein is the *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), which has a switch rate of 10^{-2} per generation which also occurs in the absence of immune pressure (Roberts et al., 1992). The molecular mechanism is switched transcription among different members of the *var* gene family (Kyes et al., 2007).

7.2 Adherence

Upon invasion, the merozoite alters the surface properties of the erythrocyte by inserting parasite-derived proteins into its cell membrane. This modulation is a process that continues throughout the infection and the most prominent alteration is the formation of knobs. These parasite-derived protrusions are important in the parasitized erythrocyte's adhesion

to vascular endothelial cells (Trager et al., 1966). The ability to adhere is directly connected to severe pathology and is one of the key features distinguishing *P. falciparum* from the other human *Plasmodium* species. The protein-mediating adhesin in *P. falciparum* is PfEMP1, which is anchored in the knob structures (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). PfEMP1 can bind to a wide variety of host receptors including CD36, intercellular adhesion molecule 1 (ICAM1) and chondroitin sulphate A (CSA) (Baruch et al., 1997; Reeder et al., 1999; Smith et al., 2000). Parasite adhesion to ICAM1 in the brain is connected to cerebral malaria and adhesion to CSA expressed in the placenta is closely associated with malaria complications during pregnancy (Fried and Duffy, 1996; Turner et al., 1994). Whether PfEMP1 alone is responsible for cytoadhesion and how it can mediate attachment to such a variety of receptors is still not known.

Besides the ability to adhere to endothelial cells, malaria-parasitized erythrocytes have the capacity to aggregate uninfected erythrocytes, a phenomenon known as rosetting, that is involved in both host pathogenesis and parasite survival. Studies in malaria endemic areas have shown a strong correlation between rosetting capacity and severity of the malaria infection (Carlson et al., 1990; Rowe et al., 1995). In *P. falciparum* PfEMP1 is the parasite adhesin aggregating uninfected erythrocytes and it interacts with multiple erythrocyte surface receptors such as heparan sulfate, proteoglycan and ABO blood group antigens (Carlson and Wahlgren, 1992; Chen et al., 1998; Rowe et al., 1995). Besides binding uninfected erythrocytes, parasitized erythrocytes bind to each other in so-called autoagglutination. The parasite-induced rosettes also attach to the endothelium and obstruct blood flow in small blood vessels, resulting in microthrombosis and microhemorrhages. This is one of the major factors causing cerebral malaria.

7.3 Persistence strategies

One way of prolonging infection and thereby increasing transmission is to reach a state of equilibrium between the infecting pathogen and immune system. The parasites reside within erythrocytes which in combination with antigenic variation allow them to remain as an asymptomatic low-level infection which might persist for many months or years. Under such conditions, parasite titers frequently fall below the microscopic detection limit (Franks et al., 2001). This type of restricted infection is frequent; it is

estimated that up to 90% of children and 40% of adults in Senegal carry a persistent malaria infection (Trape et al., 1994).

Besides maintaining a low-grade infection, *P. vivax* and *P. ovale* are able to reside asymptomatically within hepatocytes as hypnozoites reemerging after the initial infection (Cogswell, 1992; Krotoski et al., 1980; Krotoski et al., 1982). This phenomenon results in what is often labeled relapsing malaria since the clinical symptoms reemerge after an apparent eradication of the initial infection. This parasite stage can remain dormant within the liver for several months and up to two years before the parasite relapses into the blood. Little is known about the mechanism behind hypozoite reactivation, which is mostly due to the lack of suitable animal models.

8. Animal models for malaria

With the exception of *P. knowlesi*, *Plasmodium* species are strictly host specific which has severely hampered malaria research. There are several *Plasmodium* species infecting animals with host specificity ranging from reptiles to monkeys, and these serve as valuable models in the study of human malaria. Predominately there are the four rodent-specific malaria species that have been exploited: *P. berghei*, *P. chaubadi*, *P. yoelii*, and *P. vinckei*. *P. berghei* was the first isolate causing rodent malaria and it is widely used as a genetic tool for studies of gene regulation in *Plasmodium*. *P. chaubadi* is often used in immunological and drug resistance studies and *P. yoelii* is a favored model in vaccine development due to the parasite's high infectivity in mice (Khan and Vanderberg, 1991). *P. vinckei* is the least utilized rodent malaria model but it is sometimes used in drug administration studies. Unfortunately, none of these rodent malaria species displays all the clinical features of severe malaria. However, merging information from the different models has increased our knowledge of human malaria tremendously. These rodent malaria models are invaluable tools especially in the study of pre-erythrocytic stages of malaria.

9. Epidemiology of relapsing fever *Borrelia*

The geographical distribution of RF *Borrelia* is similar to that of malaria, with both diseases being most prevalent in the sub-Saharan African region (Fig. 3).

Figure 3. Geographical distribution of RF *Borrelia*.

RF occurs sporadically on almost all continents but is endemic in many African countries (de Jong et al., 1995; McConnell, 2003). RF borreliosis has the highest described incidence of any bacterial disease in Africa with an incidence of 11 infected per 100 persons each year in a Senegalese clinical study (Vial et al., 2006). In light of the high prevalence in Senegal, its impact is probably greatly underestimated in many regions, mainly due to inadequate diagnostics and general unawareness of the disease. The severity of RF borreliosis is dependent on the infecting *Borrelia* species and strain, and ranges from fatal to asymptomatic with mortality rates generally below 5% but may be considerably higher among sensitive patient groups such as young children, pregnant women, and the elderly (Southern and Sanford, 1969). In epidemic RF borreliosis, the mortality rate might be as high as 40% if left untreated (Southern and Sanford, 1969). Altogether, the epidemiology of RF borreliosis is poorly understood compared to malaria.

10. The bacterium

RF borreliosis is caused by spirochetes of the genus *Borrelia*, which are transmitted to vertebrate hosts by the bite of soft-bodied ticks or lice. Unlike malaria, RF is not developmentally dependent on its arthropod vector although the vector is essential for transmission. A wide variety of *Borrelia* species cause RF borreliosis in tropic and subtropic areas worldwide. There are mainly three species responsible for human illness in Africa: *B. recurrentis*, *B. duttonii,* and *B. crocidurae* although recent genome sequencing has suggested *B. recurrentis* to be a subspecies of *B. duttonii* (Lescot et al., 2008).

B. recurrentis was the first *Borrelia* described and it is the exception within the genus since it is transmitted by the human body louse rather than a tick. The actual transmission occurs not through the bite itself but when the louse is crushed and spirochete containing coelomic fluid is rubbed into the bite wound (Felsenfeld, 1971; Southern and Sanford, 1969). Louse-borne RF occurs in epidemics when people are forced to live under poor conditions, famine, war or during environmental disasters.

B. duttonii is presumably the spirochete responsible for the majority of the world's RF cases, primarily in children and pregnant women. *B. duttonii* is closely associated with fetal loss and neonatal death (McConnell, 2003). *B. crocidurae* causes less severe disease than *B. duttonii* and *B. recurrentis*. However, *B. crocidurae* has been shown to be the most common cause of disease in rural areas of Senegal, Mauritania, and Mali (Vial et al., 2006). The reservoirs for most *Borrelia* species are small rodents, although no natural reservoirs besides humans have been found for *B. duttonii* and *B. recurrentis*. Still, *B. duttonii* can cause infection in laboratory mice, which indicates that the lack of animal reservoirs could be ascribed to feeding preferences of the ticks.

11. Clinical manifestation of RF borreliosis

From the time the spirochetes are transmitted by a tick bite, it takes about seven days until a sudden high fever occurs which is accompanied by high titers of spirochetes in the blood. Non-febrile periods separate the recurring fever peaks, a clinical pattern for which the disease is named (Southern and Sanford, 1969). Besides this, there are various clinical manifestations such as weakness, general ache, often myalgia, headache, vomiting, and diarrhea. The RF infection often causes hepatosplenomegaly, anemia, thrombocytopenia, and respiratory dysfunction. These symptoms are very similar to those observed in, for instance, malaria, which can hide an RF infection (Nordstrand et al., 2007). RF spirochetes cross blood-tissue barriers like the blood-brain barrier resulting in neuroborreliosis, which is manifested as e.g. meningitis and facial palsy. In pregnant women, the bacterium frequently crosses the placental barrier resulting in pregnancy complications such as reduced birth weight, pre-term delivery and spontaneous abortion (Brasseur, 1985; Dupont et al., 1997; Goubau and Munyangeyo, 1983; Jongen et al., 1997; Melkert and Stel, 1991).

11.1 Anemia

As in malaria, anemia is a classical hallmark of RF and occurs in close connection to the spirochete peaks (Gebbia et al., 1999). Compared to malarial anemia, RF anemia is less severe and the hemoglobin concentration is quickly restored after the spirochetemic peaks (Paper II). In contrast to malaria, the spirochetes are not actively destroying erythrocytes, affecting the lifespan or production of the erythrocytes; therefore, it is tempting to assume that the anemia is a result of the spirochete rosetting abilities and those erythrocytes simply are removed from circulation when the rosettes are entrapped in microvascular tissue. Besides anemia, RF borreliosis results in thrombocytopenia, which unlike the anemia remains long after the infection is cleared (Gebbia et al., 1999; Southern and Sanford, 1969).

12. Diagnostics and treatment of RF

Even if RF prevalence is high in many areas, knowledge of this disease is poor and it is difficult to perform clinical diagnostics since several other diseases share its clinical symptoms. Laboratory diagnostics is based on visual detection of spirochetes in Giemsa stained blood smears, which is straightforward during the fever peaks when spirochete titers are high. Between peaks and during milder disease, spirochete titers are low and therefore difficult to diagnose. This in combination with the lack of awareness by medical personnel in endemic areas has lead to underdiagnosis of RF borreliosis which often is confused for drug-resistant malaria (Nordstrand et al., 2007; Vial et al., 2006). RF spirochetes are highly sensitive to several types of antibiotics, which makes treatment easily available, simple, and cheap. A single dose of antibiotics, such as tetracycline or preferably doxycycline, is often recommended (Perine et al., 1974). This treatment regime is usually sufficient to abolish the infection and may be justified in extreme circumstances such as epidemics or disasters when availability of antibiotics is limited.

However, this does not take into consideration spirochetes residing in tissues less accessible to antibiotics, for instance the brain (Cadavid and Barbour, 1998; Larsson et al., 2006). The treatment of bacterial meningitis has been revolutionized by the use of third generation cephalosporins, which have been found superior to the usually bacteriostatic chloramphenicol both in adult and childhood meningitis (Tunkel et al., 2004). Ceftriaxone is one such cephalosporin, with a long half-life, the capacity to penetrate the blood-brain barrier and accumulate at high concentrations in the cerebral spinal fluid (Yuk et al., 1989). Ceftriaxone is the antibiotic of choice in treatment of bacterial meningitis caused by *Haemophilus influenzae* and Lyme neuroborreliosis caused by *B. burgdorferi* (Dattwyler et al., 1997; Wormser et al., 2000; Yuk et al., 1989). Since Lyme neuroborreliosis shares several features with persistent RF infection, we suggest that the method of treatment should be similar. The recommended treatment for Lyme neuroborreliosis is daily intravenous injections of ceftriaxone or penicillin G for two to four weeks (Wormser et al., 2000). Applying this therapy for persistent RF *Borrelia* has shown to be highly efficient in abolishing brain infection (Larsson et al., 2008).

13. Immune response to RF *Borrelia*

The innate immune system is the first line of defense in combating spirochetes that enter the mammalian host. The complement system is a central part of this initial defense line and is crucial in protection against microbial infections. Complement components tag pathogens, making them recognizable to macrophages, and in addition forming a membranolytic complex on the pathogen surface. Like many other *Borrelia* species, *B. duttonii* and *B. recurrentis* can evade recognition and destruction of complement by binding complement regulators such as factor H, factor H-like protein and C4b-binding protein while maintaining their biochemical properties (Meri et al., 2006). Another part of the innate immune defense is phagocytic cells like macrophages and neutrophils, which recognize and destroy spirochetes. The importance of the macrophages is emphasized by the observation that depletion of macrophages by injection of clodronate liposomes resulted in escalated spirochete titers and death of the infected mice (Larsson, Lundqvist *et.al*, manuscript in preparation). There is also evidence that RF *Borrelia* enhance the infiltration of neutrophils at the site of infection by direct interaction with endothelial cells, which might result in increased spirochete destruction (Shamaei-Tousi et al., 2000).

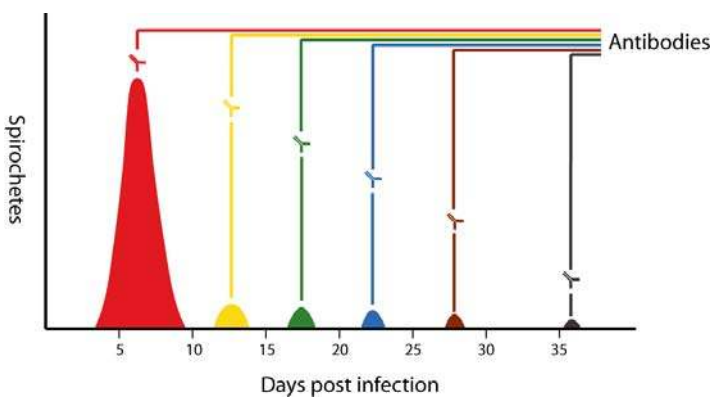


Figure 4. Antigenic variation. RF *Borrelia* is prolonging the blood infection by antigenic variation of the variable membrane proteins (Vmp). Elimination of the initial spirochetemic peak (red) is achieved by antibodies targeting that specific serotype. RF spirochetes of a different serotype are left unharmed and multiply to cause the first relapse (yellow). The bacterial infection continues to recur until the immune system can clear the blood infection.

In RF borreliosis, the production of protective antibodies is an important part of the defense to control and eliminate the infection. The first bacterial peak is abolished by a specific antibody response, which rapidly clears the blood of almost all spirochetes (Fig. 4). This elimination is mediated by complement independent bactericidal IgM which directly kills the spirochetes by inflicting damage to their outer membrane (Connolly and Benach, 2001; Connolly and Benach, 2005; Connolly et al., 2004). B cells from the peritoneal and pleural cavities are suggested to be responsible for the production of these protective IgM in a T cell independent manner (Alugupalli et al., 2003). Later in the infection a variety of protective IgG is produced which enhance protection against the infection (Yokota et al., 1997). The immune response mounted by RF usually results in complete elimination of the spirochetes in the blood. However, *B. duttonii* has strategies for immune evasion and persistence, which are discussed below.

14. Virulence factors of RF *Borrelia*

14.1 Antigenic variation

As mentioned previously, RF *Borrelia,* like *Plasmodium*, undergo antigenic variation as an important persistence mechanism to circumvent the antibody-mediated immune response. The relapsing pattern during RF infection is a reflection of the opposition between the protective immune response and the antigenically variable spirochetes (Fig. 4). At each peak, the majority of the spirochetes are of the same serotype, although spirochetes with new serotypes are present at much lower levels. When the spirochetes harboring the major serotype are eradicated by the mounted serotype-specific immune response, spirochetes with surface antigens with less abundant serotypes will replicate to cause a subsequent peak (Barbour et al., 1982; Stoenner et al., 1982). Antigenic variation in RF *Borrelia* is a result of DNA rearrangements of the *vmp* genes, whereas *Plasmodium* switches the expression between different *var* genes (Kitten and Barbour, 1990; Scherf et al., 1998).

During antigenic variation in RF *Borrelia*, the expression switches between different *vmp* genes by exchanging a copy of a transcriptionally silent *vmp* into the active expression site. These rearrangements occur at an estimated rate of approximately 10^{-4}-10^{-3} per cell and generation, and result in a new variable membrane protein (Vmp) on the surface of the bacterium (Dai et

al., 2006; Stoenner et al., 1982). The serotype switch is semi-predictable where each relapse contains a mixture of a few different serotypes, which minimizes the exposure of the full Vmp repertoire and thereby prolongs the infection (Stoenner et al., 1982).

14.2 Adherence

In contrast to malaria, adherence of RF spirochetes to host cells is relatively unexplored. However, the Lyme disease agent *B. burgdorferi* can adhere to numerous cell types *in vitro*, for instance endothelial cells, skin fibroblasts, and neuroglial cells (Garcia-Monco et al., 1989; Klempner et al., 1993; Szczepanski et al., 1990). Lyme *Borrelia* can bind to several molecules on the cell surface, e.g. heparin sulfate, chondroitin sulfate, and decorin (Guo et al., 1995; Parveen et al., 1999). There are several *B. burgdorferi* adhesins mediating cell attachment, for instance does both decorin binding protein A and B recognize decorin (Guo et al., 1995; Guo et al., 1998).
Some RF species such as *B. duttonii* and *B. crocidurae* have the ability to aggregate erythrocytes, similar to the malaria parasite (Burman et al., 1998; Mooser, 1958). Unlike erythrocyte rosetting in malaria, there is no evidence that RF rosettes adhere to endothelial cells. Still, they obstruct blood flow in a similar manner, which is likely due to steric hindrance when aggregated erythrocytes and spirochetes block small vessels (Shamaei-Tousi et al., 2001). Unlike in malaria where the rosetting adhesin PfEMP1 is known, neither a potential spirochete adhesin, nor an erythrocyte receptor for the adhesion have been identified in RF. Actually, the spirochete-erythrocyte contact seems to be a weak *interaction* rather than a stable *adhesion* since the bacteria easily swim back and forth within the rosette.

14.3 Persistence strategies

As described earlier, RF spirochetes undergo antigenic variation to prolong infection. This strategy has its limitations and the mounted antibody response will eventually clear the infection. However, infection of the central nervous system is a characteristic feature of RF *Borrelia* and it has been shown in mice that spirochetes can reside within the brain for an extended period of time (Cadavid and Barbour, 1998; Cadavid et al., 2006; Larsson et al., 2006). Behind the blood-brain barrier, RF spirochetes are protected from circulating antibodies and are unnoticed by the brain immune response (Larsson et al., 2006). These residual spirochetes are

slowly dividing and can occasionally reenter the blood and thereby increase the probability of transmission to a naïve vector (Larsson et al., 2008). Persistent brain infection has been suspected in humans but has not been proven. However, the observation that 11% of apparently healthy villagers in Tanzania carried low numbers of *B. duttonii* in their blood strengthens this theory (Cutler, 2006).

15. Animal models for RF

As described previously, most RF *Borrelia* species infect small mammals like mice and rats as principal hosts. The only exceptions are *B. duttonii* and *B. recurrentis* where the natural hosts are unknown, however *B. duttonii* readily infects laboratory mice. This is a huge advantage compared to human-specific malaria since the use of a natural host as an animal model is a tremendous strength for research. Besides rodent infection models, a few studies, mainly using *B. recurrentis*, have been performed in primates (Judge et al., 1974a; Judge et al., 1974b; Judge et al., 1974c).

16. Immune defense

16.1 Innate immunity

The first line of defense is the innate immune system, which is activated directly by the infecting microorganism although incapable of generating a lasting protective immunity. The initial detection and response to infection is dependent on a family of pattern recognition receptors, such as the Toll-like receptors, which recognize a number of microbial molecules shared by several pathogens such as lipopolysaccharides and peptidoglycans (Janeway and Medzhitov, 2002). Several cell types such as DCs and macrophages, which are continually circulating in the body, carry these receptors. When a pathogen enters tissue, it encounters phagocytic macrophages, which engulf and kill the intruder. Macrophages initiate the inflammatory response involving the release of cytokines and chemokines which attract additional phagocytic cells like neutrophils, NK and DC cells. The innate immune response also includes the complement system, which is a biochemical cascade of plasma proteins that can opsonize pathogens to enhance phagocytosis, induce lysis of pathogens by introducing a pore-forming membrane attack complex, and induce inflammatory response (Janeway et al., 2005). If the innate immune system fails to clear infection, the next line of defense is the adaptive immunity.

16.2 Adaptive immunity

To be successful, the adaptive immune response, depends upon innate immunity, with DCs as the crucial link between the two arms (Steinman, 2003). The adaptive response is required to generate specific antibodies against the infectious agent resulting in immunological memory. The adaptive immune response is composed of B and T lymphocytes that recognize diverse antigens that are presented to them. These immature lymphocytes circulate in the blood and the peripheral lymphatic system and reside in lymphatic tissue such as lymph nodes and spleen. In these locations, the naïve lymphocytes encounter antigen-presenting cells such as macrophages and DCs. When encountering antigen or aided by T cells, the B cells mature into plasma cells which produce antibodies specific for the

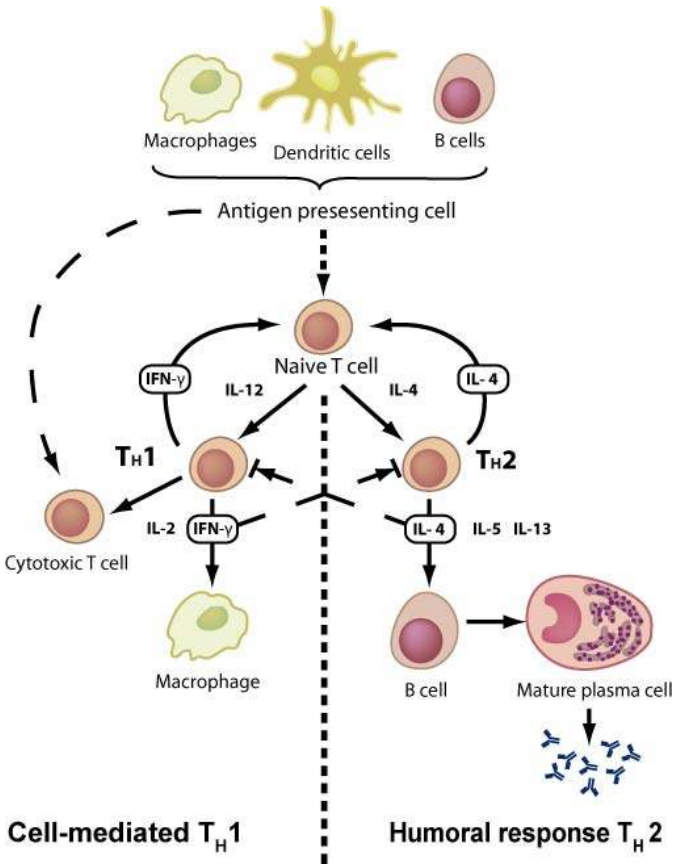


Figure 5. A schematic picture of the adaptive immune response. Antigens are presented to the naïve T cell by antigen presenting cells such as dendritic cells. The nature of this interaction is important since it polarizes the T cells against a cell-mediated T_H1 or a humoral T_H2 response. A T_H1 polarization is characterized by the production of IFN-γ, IL-12 and IL-2 while a T_H2 response is associated with IL-4, IL-5 and IL-13.

foreign antigen. T cells can roughly be divided into two subclasses, the $CD4^+$ and $CD8^+$ T cells. $CD4^+$ cells serve primarily as T helper (T_H) cells whereas $CD8^+$ cells are cytotoxic T cells providing a defense against intracellular pathogens and tumors by destroying infected or transformed cells (Fig. 5). T cells are activated to become distinct effector cells dependent on the recognition of antigens, along with co-stimulatory molecules induced by the pathogen (Janeway et al., 2005).

16.2. 1 The Th1/Th2 concept

The key cell of the adaptive immune system is the T_H cell, which recognizes foreign antigens by the ligation of a T cell receptor to pathogen-derived peptides presented by the major histocompatibility complex. This antigen presentation is carried out by phagocytic cells such as DCs or macrophages (Janeway et al., 2005). Depending on this interaction and surrounding cytokine signals induced by the infecting pathogen, the T_H cell is polarized into an effector cell mediating immune responses (Fig. 5). This polarization can differentiate the T_H cell into either a T_H1 cell characterized by the production of e.g. IFN-γ, IL-12 and IL-2 or T_H2 cells accompanied by the expression of IL-4, IL-5 and IL-13, among other cytokines (Liew, 2002; Mosmann et al., 1986). T_H1 cells activate macrophages, NK cells and cytotoxic $CD8^+$ T cells in a response called "the cell-mediated immune response", which is active mainly against viruses and intracellular pathogens such as malaria (Gazzinelli et al., 1993). In contrast, the T_H2 cells are important for the production of antibodies termed "humoral immunity", which controls extracellular infections like helminth infection (Urban et al., 1991). The two pathways can also inhibit each other by the antagonistic effects that IFN-γ and IL-4 have on the opposing T_H subset (Fernandez-Botran et al., 1988; Gajewski et al., 1988).

This dichotomous T_H1/T_H2 model is useful, although it cannot explain all complicated immunological situations. New T_H cell functions and subsets are constantly discovered, including regulatory T_{reg} cell orchestrators of the overall immune response and T_H17, which is involved in defensive mechanisms against extracellular bacterial infections, and is involved in the pathogenesis of many autoimmune diseases (Ouyang et al., 2008).

17. Concomitant infections

Concomitant infections are recognized with increasing frequency and often result in acute and chronic diseases (Brogden et al., 2005). Depending on the nature of the infectious agents involved and the host immune response, the organisms can have a synergistic or inhibitory effect on each other, thereby often forming a different clinical outcome than just the symptoms of the two diseases combined. Synergistic effects occur when the presence of one microorganism makes the host more susceptible to another infection. The mechanisms behind these types of interactions vary, but generally the primary infection somehow creates a favorable niche for the secondary infection, which is seen e.g., when respiratory tract viruses destroy the epithelium and thereby increase bacterial adhesion and colonization (Bakaletz, 1995). These respiratory tract viruses play a key role in enhancing the susceptibility of the middle ear to bacteria such as *Streptococcus pneumonia* and *H. influenzae,* resulting in otitis media e.g., inflammation of the middle ear (Heikkinen et al., 1999).

An inhibitory effect is when one microorganism suppresses the other by direct microbial interference (Regev-Yochay et al., 2004). This is achieved when one pathogen or commensal occupies or generates a niche that prevents the colonization of other microorganisms (Brogden et al., 2005). The normal flora is an important line of defense protecting its host from additional and potentially harmful infections by competing with invading pathogens for nutrients and attachment sites (Janeway et al., 2005). In addition, some commensal bacteria like *Escherichia coli* can produce antimicrobial agents such as colicins which inhibit colonization of other bacteria (Riley and Wertz, 2002). Another example of microbial interference occurs between *S. pneumonia* and *Staphylococcus aureus.* In upper respiratory tract infections, *S. pneumonia* produces H_2O_2 that is bactericidal for *S. aureus,* thereby inhibiting colonization of additional bacteria (Regev-Yochay et al., 2006; Regev-Yochay et al., 2004).

Other means to influence the secondary infection is by altering the immune response towards that particular microorganism. The model of polarization of a T_H response into either T_H1 or T_H2 has been useful in explaining interactions between microorganisms and the immune system and the effect on disease. For instance, during measles and *Listeria monocytogenes* co-infection, the virus suppresses the innate immunity but also affects the

adaptive response by reducing the IFN-γ expressing T_H1 cells. Since *L. monocytogenes* is an intracellular pathogen, this loss of T_H1 response impairs clearance of the bacterial infection (Slifka et al., 2003). There are numerous examples of how mixed infections alter the outcome of disease and in this thesis the focus is on malaria concomitant infections where some of the most important are presented in following section.

17.1 Malaria and viral infection

HIV has a unique position when discussing concomitant infections since the infection has an immunosuppressive effect due to the progressive destruction of $CD4^+$ T cells. By destroying T_H cells, HIV undermines the adaptive immunity, which could impair the immune response during malaria infection. With constantly increasing numbers of HIV-infected people living in malaria endemic areas, this type of concomitant infection is becoming more and more common (Whitworth et al., 2000).

In adults with semi-immunity to malaria, an additional HIV infection increases the risk of clinical malaria and in adults without immunity, a concomitant malaria/HIV infection enhances the incidence of severe malaria and increases morality rates (Grimwade et al., 2004; Whitworth et al., 2000). During pregnancy, mixed malaria and HIV infection increases the risk of anemia, preterm birth and intrauterine growth retardation (Brentlinger et al., 2006). Less is known about the effect of malaria on HIV infection but there are indications that the viral load increases during acute malarial episodes but resolves after antimalarial treatment (Kublin et al., 2005).

Epstein-Barr virus is a B cell infecting herpes virus that shares geographical distribution with malaria and is usually established in early childhood (Henle and Henle, 1970). It is frequently sustained as a lifelong, asymptomatic infection depending on the delicate balance between viral replication and host immune response (Thorley-Lawson and Babcock, 1999). A secondary *Plasmodium* infection might disrupt this balance, resulting in Burkitt's lymphoma which is the most frequent form of pediatric cancer in sub-Saharan Africa (Burkitt, 1983). Increased viral loads are observed in children with acute malaria, titers that are diminished after receiving efficient antimalarial treatment (Donati et al., 2006). There are two main theories which might explain how malaria can induce such a reactivation. One is that

the secondary malaria infection suppresses the Epstein-Barr virus specific T cells, thereby escalating the viral infection. Another explanation is that the expansion of B cells in response to malaria provides the virus with more cells to infect (Thorley-Lawson and Allday, 2008). Whether Epstein-Barr virus alters the pathology of malaria infection is not yet known.

17.2 Malaria and helminthes

In developing countries, the prevalence of co-infection between malaria and helminthes is high (Brooker et al., 2007). During a helminth infection, the T_H response is polarized towards T_H2 with high serum levels of e.g., IgE, whereas malaria initially induces a strong T_H1 response (Loukas and Prociv, 2001; Sherman, 1998; Su and Stevenson, 2002). The T_H2 profile of a preexisting helminth infection might impair the T_H1 response needed to control the *Plasmodium* infection or vice versa. Besides a direct immune modulating effect, a helminth infection is commonly associated with malnutrition which could make the worm-infected patient more susceptible to additional infections, for instance malaria (Stephenson et al., 2000).

Studies in mice have shown that nematode infections increase susceptibility to malaria by impairing the T_H1 immune response against *P. chabaudi* infection (Su et al., 2005). A similar result was seen with *Schistosoma mansoni* and *P. chabaudi* co-infected mice, which also presented with increased mortality and anemia (Helmby et al., 1998). In humans, there is increased susceptibility to malaria and more severe malaria pathology in patients with underlying helminth infection (Sokhna et al., 2004; Spiegel et al., 2003). However, there are conflicting results indicating that a low-grade schistosome infection might generate modest protection against severe malaria (Lyke et al., 2005). Further, others have found that an *Ascaris lumbricoides* infection is associated with protection against cerebral malaria while others report contradictory results (Murray et al., 1978; Nacher et al., 2002). Summarizing, even if there are conflicting reports, most evidence concerning helminth and malaria mixed infections indicate that a helminth infection enhances pathology of the *Plasmodium* infection (Nacher et al., 2002; Sokhna et al., 2004; Spiegel et al., 2003). Likewise, malaria and helminth infections are known to generate anemia, and the extent to which a concomitant infection further enhances the risk of anemia should be taken under consideration, especially among vulnerable groups such as children and pregnant women (Brooker et al., 2007).

17.3 Malaria and bacterial infections

Bacterial infections causing fevers are common in malaria endemic areas and constitute a diagnostic pitfall for medical personnel. This has lead to the recommendation to administer broad-spectrum antibiotics together with antimalarial drugs to young children (Berkley et al., 1999). In addition to misdiagnosis, a mixed malaria and bacterial infection might affect the outcome of each infection and make correct diagnosis even more difficult. Depending on whether the bacteria have an intracellular or extracellular lifestyle, the T_H response differs (Janeway et al., 2005). Bacteria like RF *Borrelia* and *S. pneumoniae* usually remain extracellular and induce a T_H2 response while others like *Mycobacterium tuberculosis* elicit a T_H1 response in the host (Liew, 2002).

In clinical studies, non-typhoid *Salmonella* species seem to particularly affect malaria. Co-infection with *Salmonella* is often associated with low parasitemia and increased incidence of severe malarial anemia (Mabey et al., 1987). However, if a *S. pneumoniae* infection were established during an episode of acute, severe malaria, this leads to enhanced pathology while parasite titers remain low (Berkley et al., 1999). In animal studies, an established *M. tuberculosis* infection reduces the pathology of a secondary *Plasmodium* infection, by induction of IFN-γ expression, which results in a T_H1 response against the parasites (Page et al., 2005). Taken together, there are surprisingly few studies on the effect of malaria and bacterial co-infections despite their high prevalence and potential impact on clinical outcome.

17.3.1 Malaria and syphilis

There is a well-documented success story within the field of co-infection. During the early 20th century before the discovery of penicillin, syphilis infections were common and caused severe mental disorders if untreated. Lacking antibiotics, the disease was treated with fever therapy where the increase in body temperature is assumed to abolish the temperature-sensitive spirochete *Treponema palladium*. Predominantly *P. vivax*, which could be cured with quinine, was used, but also other fever-inducing pathogens such as the transient RF *Borrelia*. This crude method for prevention of neurosyphilis became obsolete with the discovery of penicillin, although malaria fever therapy was used throughout Europe and the United States until the early 1950s (Frankenburg and Baldessarini, 2008).

18. Microbial persistence strategies

If a pathogen overcomes the elicited immune response and can attain equilibrium with the host immune defense, the infection can continue as a lifelong infection more or less asymptomatically. This type of infection is often named a persistent infection and is described by the Oxford Medical Concise Dictionary as a constantly repeated or an enduring obstinate infection. During chronic infections, the pathogen is actively dividing but restrained by the immune system to be maintained as a low-grade infection. This type of continuous low-grade infection is adopted by *Helicobacter pylori* and in long-term RF brain infections (Everhart, 2000; Franks et al., 2001; Larsson et al., 2008). When a persistent infection endures obstinately, the pathogen is truly dormant without prominent biological activity such as seen during chronic *M. tuberculosis* infection (Young et al., 2002).

To sustain a persistent infection the pathogen must be able to create or invade an immunoprivileged niche, protecting it from the surrounding immune response. This could be achieved by invading host cells, or remaining protected within a niche with little or no access by the immune system, such as an abscess (Nataro et al., 2000). Within this immunoprivileged site, the pathogen will reside until the immunological balance for some reason is disrupted. If this happens an asymptomatic infection can be reactivated into an acute disease. Persistent infection occurs with several pathogens but is especially important for vector-borne diseases since a prolonged infection enhances the possibility of transmission to a naïve vector.

18.1 Antigenic variation

Through antigenic variation, pathogens such as trypanosomes, *Neisseria*, RF *Borrelia* and *Plasmodium* avoid the humoral immune defense and establish recurring infections as described above. This approach prolongs the course of infection but eventually antibodies are formed against a non-variable antigen and the adaptive immune response will clear infection. However, pathogens that employ antigenic variation usually have an additional means to prolong infection (Nataro et al., 2000).

18.2 Residing in immunoprivileged niche

Another strategy for persistence is to inhabit immunoprivileged niches created by the microorganism or exploit preexisting niches where the immune system's access is limited, such as residing intracellularly or within the brain. Microorganisms like *Pseudomonas aeruginosa* or *S. aureus* can create their own immunoprivileged site by the formation of a biofilm on a prosthetic device or directly on tissue. Within a biofilm the access of circulating leucocytes and penetration of antibiotics is limited, which allows the microorganisms to persistently infect (Costerton et al., 1999). Another example is *H. pylori* which resides mainly in the gastric mucosa where it can persist for life, usually without clinical symptoms (Everhart, 2000).

Invading host cells and residing intracellularly to avoid the immune response are strategies favored by many pathogens. This protective intracellular lifestyle is employed by *M. tuberculosis,* the causative agent of tuberculosis, which can reside asymptomatically within its host for a lifetime. *M. tuberculosis* invades macrophages and actively recruits additional macrophages and other immune cells to form a granuloma, protecting the bacterium from the immune system (Cosma et al., 2003). *Toxoplasma gondii* is another example of a parasite that exploits an intracellular niche. Inside the tissue, the parasite can form cysts, which like tuberculosis, can remain dormant lifelong. *Toxoplasma* increases the success rate of the persistent infection by remaining intracellular in immunoprivileged tissue such as the brain. Similar to RF *Borrelia,* the parasite can penetrate blood-tissue barriers to avoid the mounted immune response and acquire access to immunoprivileged tissue like the brain (Cadavid and Barbour, 1998; Larsson et al., 2006; Luft et al., 1984).

19. Reactivation of infection

Pathogens that are forced into a persistent state by the immune response can be reactivated and cause acute disease if the immune response is attenuated. Immune suppression can be caused by numerous factors impairing or regulating the immune system which create conditions favorable for the pathogen. A decline in T cell immunity during immune senescence in the elderly can result in reactivation of latent tuberculosis and herpes infection (Schmader, 2001; Young et al., 2002). Immunosuppressive medication given as treatment for autoimmune disease or after organ

transplantation can also activate persistent infection. Reactivation of tuberculosis is frequently observed in Crohn's disease and rheumatoid arthritis patients receiving anti-TNF-α treatment (Keane et al., 2001; Schmader, 2001). Similarly, latent *Toxoplasma* and herpes infections are frequently reactivated by immunosuppressive therapy (Montoya and Liesenfeld, 2004; Thomas and Hall, 2004). Persistent RF *Borrelia* brain infection in mice can be reactivated by immunosuppressive cortisone injections (Larsson et al., 2006). Reactivation can also be a result of a secondary infection. For instance, *Toxoplasma* and tuberculosis are often reactivated as a direct consequence of secondary immunosuppressive HIV infections (Montoya and Liesenfeld, 2004; Young et al., 1986).

20. Polyamines

Polyamines are small organic, aliphatic cations derived from amino acids which are ubiquitous in all living organisms (Gerner and Meyskens, 2004). The most important polyamines are spermidine, spermine and their precursor, putrescine. They have essential roles in critical cellular processes such as cell growth, differentiation, and macromolecular biosynthesis (Casero and Marton, 2007). Polyamines are ionically bound to anions within the cells, including anions of DNA, RNA, proteins, and phospholipids. Although clearly essential, the molecular function of the polyamines is not fully understood, mainly because of the reversible nature of their ionic interactions (Casero and Marton, 2007). Since polyamines are essential during cell growth, intervening with polyamine biosynthesis has been an anti-cancer strategy but also a means to treat infectious diseases.

20.1 Polyamine biosynthesis in mammals

In mammalian cells, polyamine synthesis is initiated by ornithine decarboxylase (ODC), generating putrescine from ornithine (Fig. 6). Spermidine is formed by the donation of aminopropyl from *S*-adenosylmethionine decarboxylase (AdoMetDC) which is transferred to putrescine by spermidine synthase (SPDS). The final step of polyamine biosynthesis is catalyzed by spermine synthase (SPS) aided by AdoMetDC, transferring an aminopropyl group to spermidine to generate spermine. This procedure is tightly regulated, primarily through the short-lived ODC and AdoMetDC (Seely et al., 1982; Shirahata and Pegg, 1985).

Besides *de novo* synthesis, mammalian cells are actively importing exogenous polyamines as a direct response to reduced intracellular polyamine pools (Casero and Marton, 2007). It is important for the cell to maintain polyamine homeostasis since excessive levels of polyamines result in hyperproliferation and tumorigenesis, whereas limitations in the polyamine supply are associated with growth arrest and eventually cell death (Casero and Marton, 2007). Equilibrium is accomplished by balancing polyamine synthesis with catabolism, where the latter involves the catabolic enzymes spermidine/spermine N1-acetyltransferase (SSAT) (Fig. 6) (Bolkenius and Seiler, 1981; Casero and Pegg, 1993).

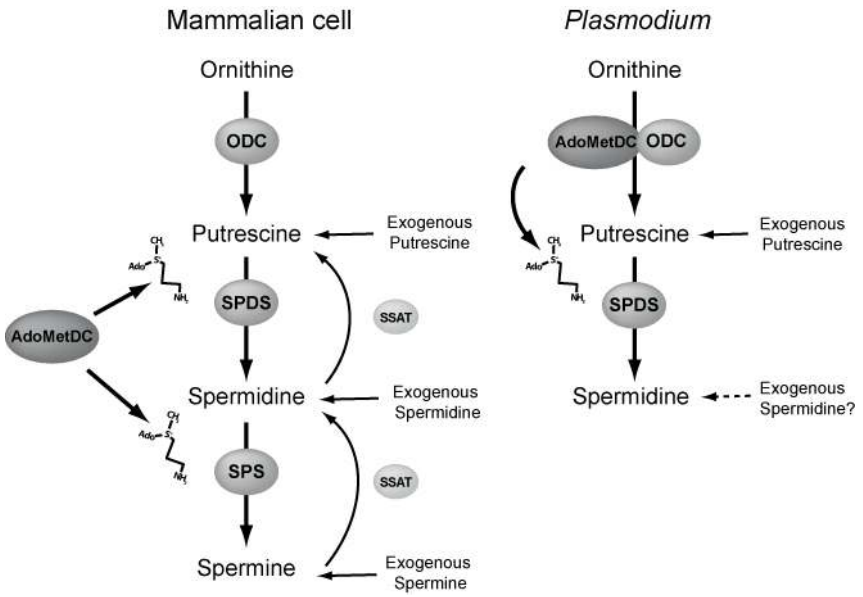


Figure 6. Polyamine synthesis of mammalian cells and *Plasmodium*. The polyamine metabolic pathway in mammals involves the enzymes ornithine decarboxylase (ODC), *S*-adenosylmethionine decarboxylase (AdoMetDC), spermidine synthase (SPDS) and spermine synthase (SPS). In addition, mammalian cells have spermidine/spermine N1-acetyltransferase (SSAT) enzymes crucial for maintaining polyamine homeostasis. *P. falciparum* have a bifunctional ODC/AdoMetDC protein and SPDS. No SPMS and SSAT have been identified in *Plasmodium*.

20.2 Polyamine biosynthesis in protozoa

Protozoans like *Trypanosoma*, *Leishmania* and *Plasmodium* have, in comparison to mammals, a very simple polyamine biosynthesis pathway that primarily differs in the lack of the SPS enzyme (Muller et al., 2001). Further, there are some species specificities separating the protozoan parasites. For instance, in *T. cruzi* both SPS and ODC are absent which implies that this parasite is entirely dependent on AdoMetDC and SPDS for spermidine synthesis (Persson et al., 1998). Other peculiarities are found in *P. falciparum* where ODC and AdoMetDC form of a bifunctional protein in which catalytic activity is affected by each subunit, their substrate, and products (Fig. 6) (Muller et al., 2000).

Unlike the mammalian cell, the protozoans are generally more restricted in their ability to transport exogenous polyamines. In *Leishmania* and *T. cruzi,* there are both spermidine and putrescine transporters (Basselin et al., 2000; Carrillo et al., 2006; Hasne and Ullman, 2005). Studies in *P. knowlesi* have revealed the existence of a putrescine transporter, and the presence of a spermidine transporter in the erythrocytes might provide the parasite with exogenous spermidine (Fukumoto and Byus, 1996; Singh et al., 1997). In contrast, in *T. brucei* there are still no identified polyamine transporters, which make the parasites entirely dependent on their *de novo* synthesis. Unlike in mammalian cells, there is no polyamine catabolism pathway regulating the polyamine homeostasis identified in these protozoans (Muller et al., 2001).

20.3 Inhibiting polyamine biosynthesis

Since increased polyamine levels were found to be closely associated with tumors, the disruption of polyamine biosynthesis has been an important anti-cancer strategy (Casero and Marton, 2007; Russell and Snyder, 1968). One of the most widely studied inhibitors of polyamine synthesis is α-difluoromethylornithine (DFMO), which targets and irreversibly inhibits ODC by binding to the active site of the enzyme (Metcalf et al., 1978). In cancer therapy, DFMO has failed in multiple clinical trials despite promising preclinical studies, although there is a promising use of DFMO as chemoprevention against colon cancer (Gerner and Meyskens, 2004).

Since polyamines are especially important in rapidly dividing cells such as protozoan parasites, and these organisms have a slightly different biosynthetic machinery, inhibitors of these pathways have been investigated as anti-parasitic agents (Muller et al., 2001). The half-life of both ODC and AdoMetDC in protozoans is longer than in their mammalian counterparts, which should enhance the effect of polyamine inhibitors as anti-parasitic drugs (Ghoda et al., 1990; Muller et al., 2001). Inhibiting ODC with DFMO has remarkable therapeutic efficacy in treating African sleeping sickness caused by *T. brucei gambiense* (Bacchi et al., 1992; Pepin et al., 1987; Van Nieuwenhove et al., 1985). Apart from this success story, DFMO has shown a moderate effect on *Leishmania* promastigotes *in vitro*, is more or less ineffective on erythrocytic stages of *P. berghei* and ineffective against *T. cruzi* since this parasite lacks ODC (Fairlamb et al., 1985; Gillet et al., 1982; Persson et al., 1998). Inhibition of other enzymes within the polyamine

biosynthesis pathway is less well explored and can potentially be more successful than DFMO in curing *Plasmodium*, *Leishmania* and *T. cruzi* infections.

21. The circumsporozoite protein of *Plasmodium*

The sporozoite surface is covered by the circumsporozoite protein (CS), which also is the most abundant protein synthesized by the sporozoites (Yoshida et al., 1981). CS is a highly immunogenic protein and antibodies targeting it prevent sporozoite infection in mice (Potocnjak et al., 1980). CS antibodies have an important role in the acquired immunity generated by continuous exposure to malaria (Hoffman et al., 1986).

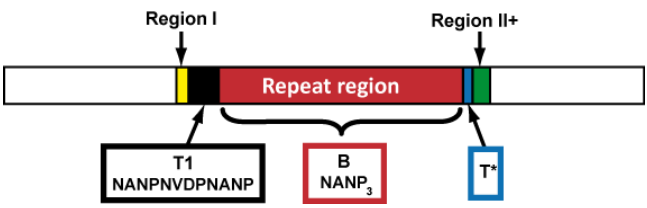


Figure 7. The circumsporozoite protein. The protein has two adhesive domains, region I and II$^+$, separated by the repeat region. The N-terminal part of the repeats is predominantly edified by the amino acids NANPNVPNANP whereas the central part consists of recurrent blocks of $NANP_3$. The former reiterating sequence is a T cell epitope T1 while the later is a B cell epitope. The CS protein also contains a universal T* epitope attracting both $CD8^+$ and $CD4^+$ T cell .

CS is a multifunctional protein with a crucial role at various points of the sporozoite developmental cycle. It is essential during sporozoite development in the mosquito; a CS knock out (CSKO) parasite is unable to form sporozoites and if CS levels are reduced, sporozoites are deformed and unable to invade the salivary glands (Menard et al., 1997; Thathy et al., 2002). Sporozoites can move by gliding motility on many surfaces. During this process CS is constantly translocated from the posterior pole to be shed at the anterior end of the parasite, leaving trails of CS behind as the parasite glides (Stewart and Vanderberg, 1988; Stewart and Vanderberg, 1991).

Sporozoite adherence is dependent on CS, more precisely the two domain regions I and II$^+$ (Fig. 7). According to *in vitro* experiments, region I is important in adherence to both the mosquito salivary glands and hepatocytes, whereas region II$^+$ mediates adherence to hepatocytes (Frevert

et al., 1993; Sidjanski et al., 1997). These two adhesive domains are separated by the proline-rich and highly repetitive repeat region with unknown function. The CS repeat region is conserved among *Plasmodium* species and is the region targeted by protective antibodies (Stewart and Vanderberg, 1988).

21.1 The biological function of the repeats

There are several theories on the function of the repetitive, immunogenic part of CS, although the biological relevance for this domain has not been demonstrated. One of our theories is that it is a flexible hinge, separating the two adhesive domains. In ongoing work, we want to test this hypothesis by exchanging the CS protein repeats with proline/alanine-rich or threonine-rich repeats from MSP-1 or simply by reducing the number of repetitive blocks (Fig. 7). Another of our ideas is that the repeats act as a protective shield, sheltering the sporozoite from degrading proteases. This type of protective mechanism is exploited by *T. brucei* to escape proteolytic degradation within the gut of the tsetse fly vector (Acosta-Serrano et al., 2001), a theory that is further strengthened by the lack of protease sites in the repeats. To investigate this theory we want to introduce a protease site within the repeats and perform *in vivo* and *in vitro* survival studies. A third yet unexplored theory is that the repeats have a function in parasite movement since antibodies targeting the repeats disrupt sporozoite gliding (Stewart and Vanderberg, 1988).

21.2 Developing hybrid parasites as tools to evaluate vaccines and study immune responses

The major focus of malaria vaccine development has been the CS protein since it is a target of protective immune responses and targeting this protein would block the initial stage of infection (Nussenzweig and Nussenzweig, 1989). Unfortunately, development of a malaria vaccine has been severely hampered by the lack of simple and efficient evaluation methods. However, using genetically modified *P. berghei* parasites expressing *P. falciparum* CS protein (CS(*Pf*))can provide a simple means to evaluate vaccines as well as to investigate the immune response (Persson et al., 2002). The repeat region is predominantly a B cell epitope which is flanked by a T_H cell (T1) and a universal T^* cell epitope (Fig. 7) (Calvo-Calle et al., 1997; Nardin et al., 1989). These three regions are the cornerstones of the most promising

malaria vaccine to date (Aponte et al., 2007; Gregson et al., 2008; Nardin et al., 2000). To gain specific information on each of these three regions, hybrid parasites have been constructed combining a functional or nonfunctional universal T^* cell epitope from *P. falciparum* isolates joined to the repeat region from *P. falciparum* or *P. berghei* (Fig. 6) (Nardin et al., 2001).

Antibodies targeting CS protein can either be elicited by B cells recognizing the B cell epitope or by T_H cells identifying the T1 epitope. By creating hybrid parasites with these two epitopes separated, it might be possible to define the specificity of the neutralizing anti-repeat antibodies in sporozoite-infected mice and vaccinated humans.

21.3 Creating hybrid CS parasites

When Menard *et al.* created the CSKO parasite, they disrupted the *CS* gene by introducing the selectable marker dihydrofolate reductase-thymidylate synthase (*DHFR-TS*) into the *CS* gene. The CSKO parasites developed like wild type *P. berghei* parasites in the erythrocytic stages, although when infecting mosquitoes they developed normal numbers of oocysts but were unable to form sporozoites, which demonstrated that CS is essential for sporozoite formation (Menard et al., 1997). The inability of CSKO parasites to generate sporozoites was later exploited when selecting for the CS(*Pf*) hybrid parasite, since only parasites with an introduced construct complementing the disrupted *CS* gene would form sporozoites (Persson et al., 2002). This strategy reduced the time spent on selection since the right parasites were enriched for in the infectious cycle in the mosquito.

When creating our CS hybrids, new and unique restriction enzyme sites had to be introduced into the wild type *P. berghei CS* gene to facilitate introduction of the different mutations. These constructs were sub-cloned into the transfection plasmid previously used when creating the CS(*Pf*) hybrid (Persson et al., 2002). As a selectable marker the transfection plasmid contains the human dihydrofolate reductase gene (*hDHFR*) which conveys resistance to both pyrimethamine and WR99210 (de Koning-Ward et al., 2000). Our *CS* gene hybrid constructs were to be introduced into the genome of the CSKO parasite by a single crossover event (Fig. 8). The anti-folate drug WR99210 was used for selection since the CSKO parasite already possesses resistance to pyrimethamine (Menard et al., 1997). As

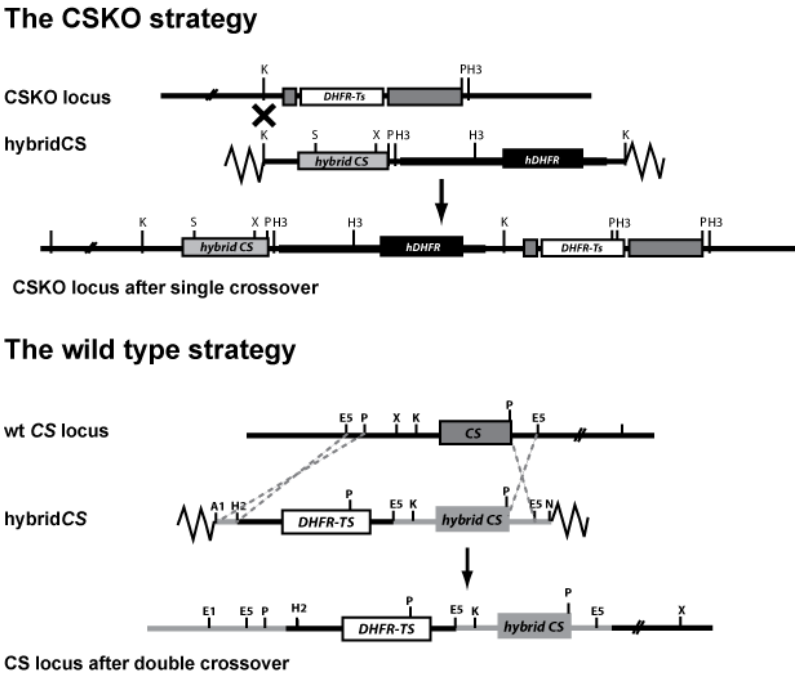


Figure 8. Two strategies to create *P. berghei* CS hybrid parasites. In the CSKO strategy, the mutated *CS* gene is to be introduced with a single recombination event into the *P. berghei* CSKO locus. In the new wild type strategy, the mutated *CS* gene is introduced with a double crossover event into the wild type *CS* locus.

described above, this strategy would minimize the time spent on selection. Unfortunately, all attempts to introduce these hybrid constructs into *P. berghei* CSKO parasites have so far been unsuccessful.

In retrospect, this strategy is applicable but has its shortcomings. Using the CSKO parasite necessitates employing WR99210, a drug with a narrow selection window where higher drug concentrations promote selection for parasites with multiple *hDHFR* genes and vice versa (de Koning-Ward et al., 2000). Further, *Plasmodium* parasites have a high frequency of gene conversion and spontaneous recombination (Su et al., 1999). Therefore, it can be disadvantageous to use a CSKO parasite as recipient, since its inability to fulfill the life cycle requires that the parasite is maintained only as

an erythrocyte culture with high risk of losing genes crucial for parasite development within the mosquito.

One way of circumventing these potential problems is to change transfection strategy and introduce the *CS* gene hybrid constructs into the wild type *P. berghei* genome through a double crossover event (Fig. 8). Unlike in the case of the CSKO parasites, "a virulence competent" genome can be maintained by having wild type parasites go through the full infectious life cycle. This new strategy would also allow the employment of *DHFR-TS* as a selectable marker, which confers resistance to pyrimethamine which would significantly increase selection since pyrimethamine, unlike WR99210, has a fairly large selection window. In addition, introducing the *CS* gene hybrid constructs by a double crossover event would, in comparison to the CSKO strategy, decrease the size of the integrated DNA since only the mutated *CS* gene and the selectable marker *DHFR-TS* would be integrated into the parasite's genome (Fig. 8).

This new strategy involves creation of a new transfection vector which now is completed. The hybrid constructs are adapted and on their way to being sub-cloned. All of these mutant constructs will be separately introduced into the wild type *P. berghei* genome, followed by selection of parasite populations, cloning of the different hybrid parasite lines and verification of the mutations by Southern blot. The phenotypes of the different hybrid parasite lines will be investigated by studying parasite development within the mosquito, infectivity and invasion of *in vitro* cell cultures, and finally development and infectivity in mice.

Aims of this thesis

Malaria and RF borreliosis share many biological as well as clinical features, such as recurring high fever, vector transmission and erythrocyte rosetting. These similarities are extremely important since the pathogens share geographical distribution and cause diseases frequently occurring as concomitant infections. However, the two pathogens also have many differences, most apparent the fact that malaria is caused by an intracellular parasite, whereas RF *Borrelia* is an extracellular bacterium. Consequently, this affects their infection maintenance, the immune responses induced and how the secondary infection in a concomitant infection is managed by the host. The aims of this thesis have been to describe these differences and similarities, with the primary focus on co-infections, persistent infection, and the potential treatment regimes of malaria and RF borreliosis.

Specific aims of this thesis

- **Investigate the biological state of a persistent RF infection and evaluate ceftriaxone as potential therapy**
- **Examine the outcome of a concomitant murine infection involving *P. berghei* and RF *Borrelia.***
- **Evaluate the potential antimalarial capacity of polyamine synthesis inhibitors in an *in vivo P. berghei* infection**

Results and discussion

Paper I Residual brain infection in murine relapsing fever borreliosis can be successfully treated with ceftriaxone

RF *Borrelia* is generally considered an acute infection of the blood, manifested by recurring peaks of spirochetes. Elimination of the spirochetemic peak is achieved by bactericidal antibodies which eventually clear the blood of bacteria (Connolly and Benach, 2001). However, RF spirochetes are capable of traversing the blood-brain barrier and gaining access to the brain, which the bacterium exploits as an immunoprivileged niche to avoid contact with circulating antibodies (Cadavid and Barbour, 1998; Cadavid et al., 2006; Larsson et al., 2006). This brain infection can gradually develop into a persistent infection, where spirochetes remain in the brain at low densities without evoking an immune response (Larsson et al., 2006). Persistent RF infection in humans is suspected to exist. Although it has not been clinically demonstrated, there have been observations of apparently healthy individuals with low numbers of spirochetes in their blood, which supports this hypothesis (Cutler, 2006).

Persistent RF infection frequently occurs in mice and has been utilized as a method to preserve RF *Borrelia* before cryopreservation came into use (Cadavid and Barbour, 1998; Felsenfeld, 1971). However, little is known about the nature of this persistent infection. Are the spirochetes actively dividing or have they entered a latent state with no metabolic activity? An actively growing RF infection, with dissemination of bacteria back into the blood, would be beneficial for transmission, whereas a latent infection would be a dead end for the bacteria. To answer this question, mice with persistent RF were treated with an antibiotic effective only against actively dividing bacteria. The antibiotic used was ceftriaxone, a third generation cephalosporin interfering with cell wall formation by disrupting peptidoglycan synthesis, and thereby only effective against actively growing, cell wall synthesizing spirochetes. In addition, ceftriaxone penetrates the blood-brain barrier and accumulates at high concentrations in the cerebrospinal fluid and thereby gains access to sites where the RF infection persists (Yuk et al., 1989).

Ceftriaxone clears persistent RF brain infection

In paper I, the therapeutic potential of ceftriaxone on a persistent RF infection was evaluated in *in vivo* experiments. Mice were infected with *B. duttonii*, and 80 days later, when the spirochetes were in a persistent state, they received daily intravenous injections of 100 mg/kg of ceftriaxone for two weeks, an administration strategy comparable to the treatment of disseminating Lyme borreliosis (Wormser et al., 2000). Control mice received daily injections of saline. After two weeks, the mice were sacrificed and half of each brain was homogenized and administrated as an intraperitoneal injection into a naïve mouse to test for the presence of spirochetes. All mice receiving ceftriaxone treatment had eradicated the brain infection (0/20) while 40% of the control mice still maintained a persistent infection (4/10). These data support that intravenous ceftriaxone injection is a successful treatment regime against persistent murine RF infection and support the clinical therapeutic use of ceftriaxone in treating residual, persistent RF infections.

Persistent RF infection is actively growing

The experiments also show that *B. duttonii* is actively growing rather than in a latent state within the brain, since ceftriaxone interferes with cell wall synthesis and thereby is bactericidal only for actively dividing bacteria. We wanted to verify these results, by evaluating the effect of ceftriaxone on non-dividing spirochetes. To mimic latency in spirochete cultures, *B. duttonii* division was inhibited *in vitro* with the bacteriostatic antibiotic chloramphenicol, which effectively inhibits bacterial cell division by interfering with the bacterial 50S ribosomal subunit, thereby arresting protein synthesis. A 24-hour-long pretreatment with 40 μg/ml chloramphenicol efficiently prevented cell division and left the majority of the spirochetes viable. When the arrested "latent" spirochetes were subsequently exposed to ceftriaxone, they were protected from the bactericidal effect while actively growing spirochetes were killed. These data validate the *in vivo* experimental set-up. However, these data do not address whether the RF spirochetes are growing slowly or just have an active cell wall turnover.

For vector-borne pathogens such as RF *Borrelia,* it is beneficial to the bacterium to prolong infection since this enhances the possibility of

transmission. Transmission would be further enhanced if the infection is active and occasionally "leaks" out into circulation. Previously, it has been shown that RF spirochetes isolated from brain are sensitive to serum from the animal from which they were obtained, indicating that the spirochetes are restricted to the brain by the circulating antibodies (Steiner and Steinfeld, 1925). Therefore, to successfully re-infect the blood the spirochetes probably undergo antigenic variation within the brain and emerge in the blood as a new serotype, not recognized by the circulating antibodies. The reactivation could also come as a result of an immunosuppressive event, during immunosuppressive therapy, or a secondary infection as shown in Paper II (Larsson et al., 2006).

Paper II Mixed infection decrease malaria burden and escalate relapsing fever

Establishing accurate diagnosis and correct treatment is crucial for patient well-being. In developing countries, diagnosis is often crude and principally based on clinical symptoms, which occasionally is verified by laboratory diagnostics. In the case of malaria, the recommendation from WHO for endemic areas is presumptive diagnostics solely based on clinical features such as fever (WHO, 2006). This approach can decrease malaria morbidity but sometimes is inappropriate since there are many infectious diseases with symptoms resembling *Plasmodium* infection (Amexo et al., 2004). RF borreliosis is one of them (Nordstrand et al., 2007).

In a field study in West African Togo, 10% of the fever patients receiving malarial treatment were later diagnosed as having RF borreliosis. Even if these RF cases presumably could have been identified by Giemsa staining and microscopy, the medical personnel generally are unaware of RF existence. Even more alarming is that 4.5% of the fever patients carried a concomitant RF *Borrelia* and malaria infection (Nordstrand et al., 2007). Similar frequencies of malaria/RF *Borrelia* co-infections were observed in Senegal (Vial et al., 2006). Considering, the frequency of malaria/RF *Borrelia* concomitant infections, it is surprising that nothing is known about the clinical picture of such an infection.

Concomitant infection decreases parasite burden but escalates spirochete titers

In Paper II, a mouse model for concomitant malaria/RF *Borrelia* infection was established to evaluate the outcome of such a co-infection, using the rodent malaria *P. berghei* parasite together with *B. duttonii* in BALB/c mice. The parasite infection was assessed by calculating the percentage of infected erythrocytes (parasitemia) and RF infection was monitored by counting spirochetes in blood.

Interestingly, concomitantly infected mice had substantially reduced parasitemia, never exceeding 3.2%, in comparison to a parasitemia of 49.6% in animals infected with only *P. berghei*. The contrary was observed with spirochete titer, which in concomitantly infected animals was elevated 21-fold, with individuals displaying up to 1.95×10^9 spirochetes/ml, while

animals infected with only *B. duttonii* never exceeded 9.3×10^8 spirochetes/ml. Concomitantly infected animals died unexpectedly early, most likely due to sepsis caused by the escalated spirochete titers.

The immune response in concomitantly infected mice is directed to the malaria parasite

T cells have a key role in the adaptive immune response and depending on signals from antigen-presenting cells, they can be polarized into mediating either a cell-mediated T_H1 or a humoral T_H2 response. These two pathways are characterized by the expressed cytokines, which in the case of T_H1 is predominantly IFN-γ and IL-2, whereas the T_H2 response is associated with expression of IL-4, IL-5 and IL-13 (Liew, 2002; Mosmann et al., 1986). Malaria is an obligate intracellular parasite, which evokes a T_H1 response while RF *Borrelia* predominantly proliferates extracellularly in the blood and thereby induces a T_H2 response (Connolly and Benach, 2005; Connolly et al., 2004; De Souza et al., 1997; Langhorne et al., 2002).

To examine the T_H response in concomitant *P. berghei*/*B. duttonii* infection, sera was collected daily throughout infection and IFN-γ and IL-4 concentration was analyzed using ELISA. Serum concentrations of IFN-γ in concomitantly infected animals were indistinguishable from IFN-γ levels in *P. berghei* infection, and 4-fold higher than in *B. duttonii*-infected mice. On the other hand, *B. duttonii*-infected mice displayed increased serum concentrations of IL-4 around the spirochete peaks at days 5 and 11, a pattern not seen in *P. berghei*/*B. duttonii*-infected mice. In summary, the serum concentrations of both IFN-γ and IL-4 in the *P. berghei*/*B. duttonii*-infected mice were similar to those of mice infected with *P. berghei* alone. This indicates that the immune response is polarized against the malaria infection rather than the RF infection, which can explain the escalating spirochete titers observed.

The progression of concomitant infections may be dependent on which pathogen establishes infection first, since the initial infection determines the direction of the adaptive response. Hypothetically, such a polarization should be disadvantageous to the primary infection but beneficial for the secondary infection (Cox, 2001). To investigate this, mice were initially infected with *P. berghei* and five days later with *B. duttonii*, or given *B. duttonii* and after five days *P. berghei*. Parasitemia and spirochete titers of these

infections were similar to those of simultaneous *P. berghei*/*B. duttonii* infection. This demonstrates that the immune response in *P. berghei*/*B. duttonii* concomitant infections primarily is towards the malaria parasite, rather than towards RF *Borrelia* independently, when the concomitant infection is established.

A secondary malaria infection reactivates persistent RF infection

B. duttonii persists in murine brain as a residual infection for an extensive period of time after the bacteria have disappeared from the blood (Cadavid and Barbour, 1998; Cadavid et al., 2006; Larsson et al., 2006). Behind the blood-brain barrier, spirochetes are protected from circulating antibodies and reside as a silent infection unnoticed by the brain immune response (Larsson et al., 2006). It has been shown earlier that persistent RF infection can be reactivated by immunosuppressive cortisone injections, with spirochetes reentering the blood after months of absence (Larsson et al., 2006). Besides immunosuppressive therapy, a secondary infection is known to reactivate other persistent infections, such as a HIV infection which can reactivate both persistent *Toxoplasma* and tuberculosis (Montoya and Liesenfeld, 2004; Young et al., 1986). Could the "immunomodulated" environment generated by a secondary malaria infection reactivate a persistent RF *Borrelia* infection?

To test this hypothesis, mice were infected with *B. duttonii* and 80 days later when the RF infection was in its persistent state, the animals were infected with *P. berghei*. The malaria infection reactivated the RF infection in 60% (6/10) of the mice, a significantly higher reactivation rate than achieved by cortisone injections (Larsson et al., 2006). Furthermore, the first spirochetes were observed at day 13 post-malaria infection which was 19 days earlier than when cortisone treatment was administered (Larsson et al., 2006). Besides reactivation of persistent RF infection, animals with residual RF displayed a reduction in malaria burden which never exceeded 5.7% during the first 17 days of infection. However, this data must be confirmed with additional experiments and is not yet included in the manuscript. This is the first finding of a biologically and clinically relevant approach to reactivating a persistent RF *Borrelia* infection.

Severe anemia in concomitantly infected animals despite low parasitemia

When analyzing blood smears from *P. berghei*/*B. duttonii*-infected mice, it became clear that the animals, despite their low parasitemia, had reduced erythrocyte counts. Moreover, since anemia is a mutual hallmark for malaria and RF borreliosis, we wanted to investigate the effect of concomitant infections on hemoglobin (Hb) levels (Abdalla et al., 1980; Gebbia et al., 1999). In BALB/c mice, severe anemia is defined as ≤55% of Hb concentrations of the baseline value for uninfected control animals (Lamikanra et al., 2007). According to this definition, a *B. duttonii* infection never induced severe anemia while the contrary was seen in both *P. berghei*/*B. duttonii*- and *P. berghei*-infected mice. It was surprising that *P. berghei*/*B. duttonii* infection was considerably lower in parasitemia than *P. berghei* infection alone. The correlation of low parasitemia with severe anemia is frequently observed in humans where the anemia has been suggested to be connected to hyperactive phagocytic cells that destroy both infected and uninfected erythrocytes (Bojang et al., 1997; Evans et al., 2006). Keeping that in mind, it is possible that the high spirochete titers activate erythrocyte destruction, thereby increasing the anemia although the parasite count is low.

Severe internal damage in concomitant malaria/RF Borrelia *infection*

Besides generating recurring fevers and anemia, malaria and RF borreliosis share other clinical features such as hepatosplenomegaly. Pathological examination of co-infected animals revealed that the spleen was enlarged, misshapen and presented massive necrotic lesions. The spleen is important for initiating the adaptive immune response against infecting pathogens (Janeway et al., 2005). Therefore, immunohistochemical and pathological analyses were preformed on 8 μm spleen sections. Spleens from concomitantly infected mice had necrotizing splenitis with massive infiltration of bacteria in the necrotic areas. The numbers of infiltrating macrophages and lymphocytes were reduced and the majority of the leukocytes were pyknotic or already degenerated. None of these features where seen in animals that had received single infections of either *P. berghei* or *B. duttonii*. Co-infected animals also displayed substantially enlarged and hemorrhagic Peyers patches. Since Peyers patches, like the spleen, are

secondary lymphoid tissue, it is tempting to assume that these pathological changes are correlated with escalated bacterial titers.

Paper III An *in vivo* study on the antimalarial effect of polyamine synthesis inhibitors in *Plasmodium berghei*

The malaria parasite undergoes extensive multiplication throughout several stages of its life cycle, both in the mosquito vector and its mammalian host. Considering this, polyamine synthesis must be very important for the parasite and therefore a potential target for new antimalarial drugs. The approach has been successful for treatment of African sleeping sickness where the irreversible inhibition of ornithine decarboxylase (ODC) by α-difluoromethylornithine (DFMO) is a well-tolerated and approved treatment against *T. brucei gambiense* (Bacchi et al., 1993; Milord et al., 1992; Van Nieuwenhove et al., 1985).

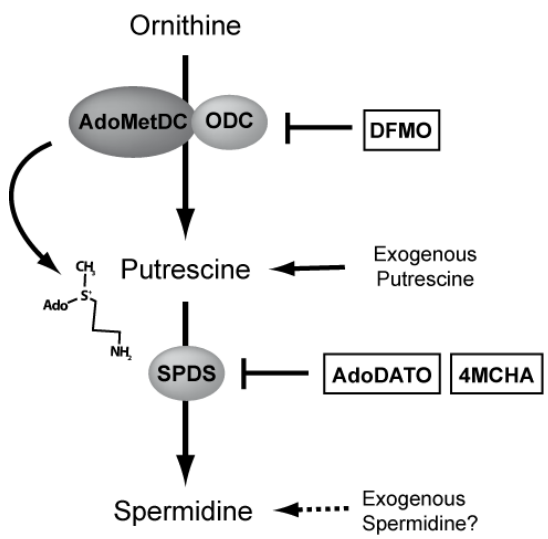


Figure 9. Inhibitors of the polyamine biosynthesis in *Plasmodium*. The polyamine inhibitor α-difluoromethylornithine (DFMO) binds irreversibly to ornithine decarboxylase (ODC) while both *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) and *trans*-4-methyl-cyclohexylamine (4MCHA) bind and inhibit spermidine synthase (SPDS)

.

In *Plasmodium,* DFMO has an inhibitory effect on sporozoite development within the mosquito and on the exoerythrocytic forms, the parasite developmental stages within the hepatocyte, since mice treated with DFMO are protected against sporozoite infection (Gillet et al., 1983; Lowa et al., 1986). This inhibition was confirmed in hepatocyte cultures where the drug blocks transformation from the exoerythrocytic form into merozoites (Hollingdale et al., 1985). Unfortunately, DFMO has a limited effect on erythrocytic stages of *P. berghei* parasites *in vivo* (Gillet et al., 1982).

In *Plasmodium,* there are two additional enzymes involved in polyamine biosynthesis besides ODC: *S*-adenosylmethionine decarboxylase (AdoMetDC) and spermidine synthase (SPDS) (Muller et al., 2001). Almost all the focus has been on ODC and AdoMetDC as targets for inhibitors (Muller et al., 2001). However, the recent discovery of a putrescine transporter in *P. knowlesi* has switched some of the focus towards SPDS as a potential target for antimalarial drugs (Singh et al., 1997). Two inhibitors, *S*-adenosyl-1,8-diamino-3-thiooctane (AdoDATO) and *trans*-4-methyl-cyclohexylamine (4MCHA) have been shown to bind to the active pocket of *P. falciparum* SPDS in crystallization studies (Fig. 9) (Dufe et al., 2007). 4MCHA inhibitory capacity was confirmed in *in vitro P. falciparum* cultures, although the effect of AdoDATO is unexplored (Haider et al., 2005). Still, the therapeutic value of these polyamine synthesis inhibitors has not been validated *in vivo*.

The polyamine synthesis inhibitor 4MCHA abolished P. berghei *infection* in vivo *while AdoDATO was ineffective*

In paper III, the inhibitory effect of AdoDATO, 4MCHA but also a combinatory treatment with both 4MCHA and DFMO were investigated as antimalarial drugs against *P. berghei* infection *in vivo*. The antimalarial effect was evaluated by monitoring the percentage of infected erythrocytes (parasitemia) by analyzing Giemsa stained blood smears. Despite the promising crystallization results reported by Dufe *et al*., 2007, animals that received AdoDATO displayed similar parasitemia compared to untreated animals. The failure of AdoDATO to eradicate or even reduce parasite numbers might be explained by the short plasma half-life of the drug, or simply that the bulky inhibitor is incapable of passing through the plasma membrane of the erythrocyte into the intracellular parasite. In contrast, animals treated with 4MCHA displayed a 4.8-fold decrease in parasitemia

compared to the untreated animals. The 4MCHA-treated animals also showed a lowered peak parasitemia, never exceeding 21.3% compared to 57.8 % in untreated animals. Surprisingly, animals administered 4MCHA abolished the infection within 24 days of treatment.

4MCHA restricts the parasites rather than eradicating infection

Despite the fact that 4MCHA abolished overall infection, "cured" mice showed sporadically recurring parasites, implying that parasites could survive the treatment but as a low-grade infection below the detection limits of microscopy. A potential reason for the moderate effect of 4MCHA could be that the parasite to some extent can exploit the erythrocyte's own spermidine transporter, limiting the curative effect of the inhibitor or that the parasite has a yet unidentified spermidine transporter (Fukumoto and Byus, 1996). An additional explanation is that 4MCHA has a short half-life, which is supported by observations of Kobayashi *et al.* (Kobayashi et al., 2005). Still, 4MCHA was administered *ad libitum* throughout the experiments, which would maintain 4MCHA at a constant level.

Inhibiting ODC together with SPDS did not enhance the curative effect of 4MCHA

Targeting multiple steps in the polyamine metabolism pathway has been shown to enhance the inhibitory effect of DFMO when combined with the polyamine analogs bis(benzyl)polyamines in *in vivo P. berghei* infection (Bitonti et al., 1989). A similar strategy, combining DFMO with the irreversible inhibition of AdoMetDC, 5'-([(Z)-4-amino-2-butenyl]-methylamino)-5'-deoxyadenosine had a better curative effect on mice infected with *T. brucei rhodesiense* than DFMO alone (Bacchi et al., 1992). Presumably, the effect of 4MCHA could be enhanced by combining it with DFMO and thereby affecting both ODC and SPDS. However, infected animals treated with both 4MCHA and DFMO did not display a lowered peak parasitemia, nor did they clear the infection earlier compared to animals treated with 4MCHA alone. It is possible that the presence of a putrescine transporter overrides the potential synergistic effect of a 4MCHA/DFMO treatment. A future strategy to overcome this problem is to complement the 4MCHA/DFMO treatment with an inhibitor of putrescine transport (Singh et al., 1997).

4MCHA-cured mice generated immunity to P. berghei *infection*

It has been shown that suppression of malaria infection with low doses of chloroquine results in protective immunity in the treated patient (Pombo et al., 2002). Could a 4MCHA-suppressed *P. berghei* infection result in protective immunity in mice? To pursue this, animals that cleared infection by 4MCHA treatment were re-infected four months after initial infection. Surprisingly, all animals cleared the second infection within 13 days. To investigate the nature of this protective response, serum was transferred into naïve mice that were subsequently infected with *P. berghei*. However, none of them were able to clear the infection, indicating that this immunity is coupled to a strong cell-mediated response rather than parasite-specific antibodies. Taken together, these results suggest that 4MCHA suppressed the infection long enough to induce a protective cell-mediated immune response, which points towards a biological and potentially clinical relevance for 4MCHA and similar compounds interfering with *Plasmodium* polyamine metabolism.

Conclusion

I. **Persistent *B. duttonii* bacteria in the brain are actively dividing rather than being in a latent none growing state since the cell wall disrupting antibiotic ceftriaxone can clear the infection.**

These results supports the clinical use of ceftriaxone in treating residual, persistent RF infections.

II. **Concomitant *P. berghei*/*B. duttonii* infected mice display lower parasite burden but escalated spirochete titers.**

The immune response is polarized towards the parasite infection.

A persistent RF brain infection can be reactivated by a secondary malaria infection.

Co-infected animals display severe anemia and major damage of internal organs.

III. **The polyamine inhibitor 4MCHA can clear a *P. berghei* infection and as a consequence mediate the induction of protective immunity preventing re-infection.**

Sammanfattning på Svenska

Swedish summary

Malariaparasiter som tillhör släktet *Plasmodium* och bakterien *Borrelia* som orsakar återfallsfeber har en snarlik geografisk utbredning. Dessutom, har de ett flertal gemensamma symtom som till exempel hög feber, anemi och förstoring av lever och mjälte. Därför är det ofta svårt att med hjälp av kliniska symtom skilja dessa två sjukdomar åt. Båda organismerna kan även ge ihållande infektion som inte immunförsvaret rå på. Bakterien som orsakar återfallsfeber kan passera över blod-hjärnbarriären och etablera en persistent hjärninfektion medan malariaparasiten kan bibehålla en infektion i blodet.

I **artikel I** behandlades möss som fått en ihållande återfallsfeberinfektion i hjärnan med antibiotikan ceftriaxone. Denna hämmar bakteriernas cellväggssyntes vilket innebär att ceftriaxone endast är effektivt mot växande bakterier. Behandlingen med ceftriaxone dödade alla bakterier vilket bevisade att bakterierna i hjärnan var aktivt växande och inte inaktiva, latenta. Borreliainfektionen begränsas till hjärnan eftersom immunförsvaret är lägre eller iallafall annorlunda än i resten av kroppen. En aktivt delande ihållande hjärninfektion som ibland kan återinfektera blodet borde vara fördelaktigt för återfallsfeber då detta ökar chansen att infektionen sprids vidare till den blodsugande fästingen.

Malaria är en vanlig infektionssjukdom i de tropiska delarna av Afrika. I dessa områden används oftast enbart de kliniska symtomen för att fastsälla diagnos för att snabbt kunna ge rätt behandling och därmed minska antalet dödfall orsakade av malaria. Eftersom återfallsfeber ger upphov till liknande symtom som malaria är risken stor att denna infektion antas vara malaria med felaktig behandling som följd. Det är också vanligt att dessa två infektioner förekommer samtidigt men hur detta påverkar patienten vet man inte. I **artikel II** skapar vi ett modellsystem för denna typ av dubbelinfektion för att undersöka påverkan på infektionsförlopp och immunförsvar. Vi visar att när malaria och återfallsfeber förekommer samtidigt blir parasitinfektionen mycket mildare men bakterienivåerna i blodet blir däremot 21 gånger högre än med enbart återfallsfeber. Troligtvis bero detta på att immunförsvaret fokuserar på malariainfektionen vilket gör att återfallsfeberbakterierna kan tillväxa mer eller mindre okontrollerat.

Möss med dubbelinfektion var också mer anemiska och hade allvarliga interna skador, framför allt på den för immunförsvaret viktiga mjälten. Vi visar också att malaria kan reaktivera en ihållande hjärninfektion av *Borrelia* så att bakterierna lämnar hjärnan och åter finns fritt i blodet.

Malariaparasiterna världen över blir mer och mer resistenta mot de tillgängliga malariamedicinerna vilket gör att behovet att hitta nya substanser mot malaria är stort. I **artikel III** undersöks om molekyler som förhindrar polyaminsyntesen kan användas som behandling för malaria. Polyaminer är viktiga för att celler ska kunna växa. Speciellt för snabbväxande parasiter är det viktigt att tillgången på polyaminer är konstant hög. Genom att blockera polyaminsyntes med hjälp av drogen 4MCHA kunde malariainfektionen kontrolleras och reduceras. Detta gjorde att immunförsvaret fick tillräkligt med tid för att kunna aktivera en immunitet mot malariaparasiterna, vilket resulterade att immunitet mot malaria byggdes upp.

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